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IMPROVED METHODS FOR DIAGNOSIS OF MELIOIDOSIS

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ABSTRACT

Melioidosis is a tropical and subtropical bacterial infection caused by Burkholderia pseudomallei, *a gram-negative bacterium previously known as* Pseudomonas pseudomallei. *The disease is found predominantly in Southeast Asian countries and the northern part of Australia. In Thailand, the disease is reported more frequently from the Northeast. It has a broad clinical spectrum, varying from highly fatal acute septicemia to asymtomatic subclinical infection. One of the major clinical problems with this disease is the lack of an appropriate diagnostic method. Such a method should be relatively cheap and simple to perform but highly reliable and accurate. It should also yield results quickly, so that proper management can be implemented as soon as possible. During the past 5 years, our groups at the Faculty of Science, Mahidol University and Chulabhorn Research Institute have been working on the immunology of melioidosis with particular emphasis on development of an appropriate diagnostic method. In this communication, we describe our approach in improving both antibody-detection and antigen-detection methods, and discuss our ideas as to how they would be readily applicable for use, not only in the endemic area of Thailand, but in other endemic regions as well.*

BACKGROUND

The bacterial infection caused by *Burkholderia (Pseudomonas) pseudomallei* (commonly referred to as melioidosis) is a tropical and subtropical disease occurring in the region 20°N and 20°S of the equator (Fig. 1).[1,2] Practically all of the reported cases from outside endemic areas can be traced back to origins from the endemic regions. The disease, once considered a rarity in man, is now recognized as an important cause of morbidity and mortality among farmers in Southeast Asian countries including Thailand.[3,4] It was first reported to have occurred in a Thai national in 1955 (for review, see ref. 5). Because of its geographical feature, the disease and its etiological agent historically received little attention in medical circles outside the endemic areas of infection. However, this situation changed dramatically during the Vietnam war when a

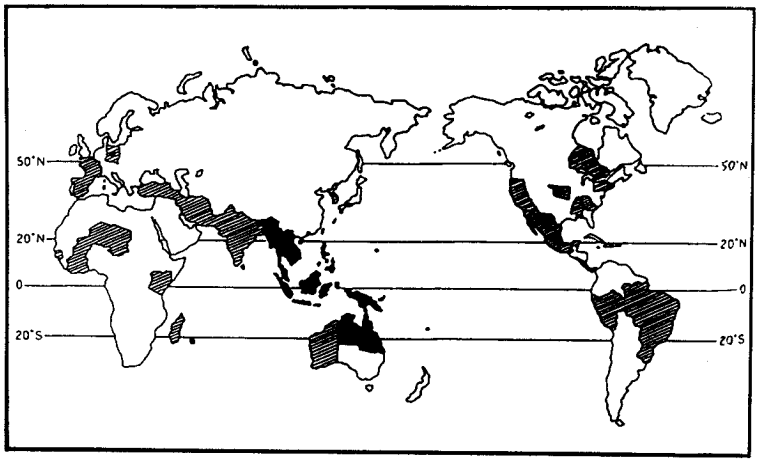

Fig. 1 Distribution of melioidosis in human and animals. ■ - Major endemic areas; ▨ - Areas where sporadic cases were reported [From Yabuuchi and Arakawa (1993) *Microbiol. Immunol.* **37**, 823].

large number of American soldiers, particularly helicopter crewmen, came down with pulmonary melioidosis (Vietnam tuberculosis). Moreover, upon returning home, a number of these apparently healthy veterans developed severe melioidosis several years later (Time-bomb disease). This phenomenon most likely resulted from reactivation of subclinical infections acquired during the time of stationing in endemic Vietnam, since the organism can stay dormant in the host for a long period. A high proportion of soldiers from outside the endemic area of infection were found to have positive seroconversion within 1 year after entering the endemic area.[5]

According to Leelarasamee and Bovornkitti,[4] there were only 3 cases of melioidosis reported in Thailand before 1955, but there were over 800 cases reported in 1986. This increase was most likely related to better diagnosis and increased awareness by the health profession than to any change in epidemiology. It was reported from one hospital in the endemic northeastern part of Thailand that the disease accounted for as high as 19% of cases and 40% of deaths from community-acquired septicemia.[6] The disease is, therefore, an important public health problem of the country and it deserves much more attention from not only the medical and scientific communities but also from public health administrators.

Melioidosis is a disease affecting all age groups, but it predominates at ages between 40 and 50 years.[1,2,7,8] Farmers comprise the group most likely to be infected. Other high risk professions include all those coming into contact with soil and dust. Males are infected more than females. The disease is seasonal, with a higher incidence in the rainy season, and it varies with the amount of rainfall. Thus, in Thailand the disease is more frequently reported between June and November. Although the disease occurs in healthy individuals, it is known to be associated more often with a number of predisposing conditions including diabetes and renal failure.[2,4] Moreover, those who have blood diseases or are immunosuppressed are said to be more prone to infection. It may be in the future, that there will be an increase of incidence among those with HIV infection and full-blown AIDS.

The disease has a broad clinical spectrum and can present itself in different clinical forms, ranging from a fulminating fatal acute septicemic illness to a milder indolent localized infection.[2,4,5] Any organ in the body can be affected, and thus, it is difficult to clinically diagnose. This feature has led to it being named the Great Imitator. Relapse is relatively common, particularly in those with severe primary infection and in those receiving inappropriate antibiotic therapy.[9] These features are consistent with the idea of a latent infection and its reactivation as mentioned above. However, reinfection is also known to occur, thus adding some doubt as to the value of protective immunity following natural infection and any benefit that might come from a vaccine, if one were to be developed. Seroepidemiological surveys of individuals in endemic areas of infection suggest that a large proportion of people exposed to *B. pseudomallei* develop subclinical or asymptomatic infections.[7,8] Although clinicians and investigators with special interest in this disease estimate the number of acute septicemic cases caused by *B. pseudomallei* to be only 2,000-5,000 per year in Thailand, this should not be taken lightly. The real figure is probably much higher than this. Moreover, the case fatality rate in this severe form of the disease is very high. Even with proper diagnosis and appropriate antibiotic treatment, a considerable proportion of patients in this group die. That fact makes this bacterial infection one of the most severe infectious diseases in the tropics.

The disease is transmitted largely through the skin (e.g., wounds and skin abrasions) and the respiratory tract. Sexual transmission has been documented. On the other hand, it is still controversial whether or not it can occur *via* the gastrointestinal tract, at least in the humans. Infection *via* laboratory accidents by less virulent laboratory strains has been reported on a few occasions. The iatrogenic route of infection is also known to occur. Although animal infections do exist, animal-to-man transmission has never been document with any degree of certainty.

The etiological agents of melioidosis are gram-negative bacilli belonging to a newly classified genus *Burkholderia*.[1] Medically important species in this genus are *B. pseudomallei, B. cepacia* and *B. mallei. B. pseudomallei* is a free-living saprophyte found in water and soil all over the country.[10,11] During the dry season, the organism can be found under the soil surface as deep as 40 cm or more. It is very curious that disease prevalence does not seem to closely follow the distribution pattern of the organism, *i.e.* melioidosis is reported to occur predominantly in the Northeast, while *B. pseudomallei* is found throughout the country. Does this discrepancy reflect differences in virulence of the organism in different geographical areas? This question can be answered, at least in part, when one applies new typing methods (e.g. ribotyping) and compares the results for organisms isolated from different geographical areas and from patients. Indeed, preliminary studies using RNA typing and pulsed-field gel electrophoresis very recently reported by Trakulsomboon have suggested that nucleic acid differences may correlate with differences in virulence

(personal communication). Perhaps the different ribotypes found in different clinical forms of the disease can be extrapolated to mean that different types of endotoxin are produced. These endotoxins are thought to be one of the virulence factors for this organism. This speculation is consistent with the recent report that different *B. pseudomallei* isolates may possess chemically different forms of endotoxin, based on structural analysis.[12] On the other hand, one should keep in mind the fact that the higher incidence of melioidosis reported from the Northeast may reflect a greater awareness on the part of clinicians, and the fact that there are now more alert and efficient supportive laboratory facilities in the endemic areas, particularly in Khon Kaen and Ubonratchatani provinces.

CURRENT DIAGNOSTIC METHODS

Although clinical diagnosis, based on experience accumulated by clinicians in the endemic areas of infection, can be made with varying degrees of accuracy, it is not easy, because the melioidosis mimics a number of other diseases. Because of this problem, definitive diagnosis must always be confirmed by laboratory diagnosis. Currently, comparison with a positive bacterial culture remains the only acceptable gold standard for definitive diagnosis. However, the culture method is not always possible, as most diagnoses currently performed must be based on other methods, such as serology. Unfortunately, unless improvements can be made, the serodiagnosis now in use gives only presumptive results. Fairly reliable methods for the identification of *B. pseudomallei* directly from clinical specimens (e.g., pus, urine and sputum) that can be readily collected have been described in recent years and can be useful for rapid diagnosis. Of the many other methods currently in use, each has advantages and disadvantages, but none is completely satisfactory. The main drawback for the current diagnostic methods is that they are time-consuming and not practical for routine use by remote health centers in endemic areas. In general, the diagnostic methods can be categorized into 3 groups as follows.

1. Culture method

This remains the gold standard, and it is the most reliable diagnostic method for *B. pseudomallei* infection.[13,14] Its main drawback is that it is time-consuming, as it generally takes at least 4-7 days for bacterial identification. Although this time frame is acceptable for milder localized forms of the disease, it is too slow for the acute septicemic form. A large proportion of such patients die during the first 48 hours after admission. Different approaches have been used in attempting to shorten the identification protocol from positive hemoculture. These include the use of highly specific monoclonal antibodies and DNA probes or polymerase chain reaction (PCR) technology.[15-19] These approaches are highly specific for *B. pseudomallei* and can therefore reduce problems of misidentification of bacteria by inexperience personnel outside endemic areas.

2. Molecular biology methods

Very recently, DNA hybridization and genetic engineering have played an important role in the diagnosis of infectious and non-infectious diseases. For infectious diseases, these techniques are valuable not only for rapid diagnosis but also for confirmation of bacterial culture. Melioidosis is not an exception. When one surveys the literature, one finds that within the past few years there have been several publications describing the development and use of nucleic acid hybridization technology for the detection and identification of *B. pseudomallei*.[19-25] A specific DNA probe has been constructed by a group of investigators in Khon Kaen and its diagnostic potential has been described.[19] Reports on the use of ribotyping and PCR technology have also given new insights into the heterogeniety of *B. pseudomallei* and have played an important role in helping us to understand the epidemiology of the disease.[20-25] Invaluable information regarding the pathogenesis of relapse also begins to emerge from the ribotype data of *B. pseudomallei* isolates from primary and current infection.[26,27]

3. Immunological methods

Provided that one has appropriate reagents, methods based on immunological reagents are highly specific. They can be readily performed with minimal laboratory equipment. A variety of approaches have been made for the detection of either antigens or antibodies, depending on the circumstances and the availability of clinical specimens. For melioidosis, the most common approach is antibody detection, as it is very simple to do and requires minimal laboratory equipment. A number of serological tests have been developed. These include bacterial agglutination, indirect hemagglutination (IHA), complement fixation,

ELISA and immunofluorescence, to name just a few.[28] One of the requirements for a reliable antibody test is a specific antigen(s). Sometimes these are produced in only trace quantities by bacteria and this can lead to a problem of mass production for routine diagnostic work. To get around this, some antigens can be produced by using genetic engineering techniques, but this approach is not applicable to all antigens. One major drawback to the antibody detection approach is interpretation of the results, because it is very difficult to distinguish ongoing from past infections in many situations. As with many other infectious diseases, paired acute and convalescence serum specimens can be used to distinguish these situations, but, as we will see later, this is not always possible with melioidosis.

More recently, the antigen-detection approach, based on the use of specific polyclonal or monoclonal antibodies, has received considerable attention. These biologicals are highly valuable reagents, not only for rapid diagnosis, but also for culture identification. Monoclonal antibodies (MAbs) specific for a number of infectious agents are now commercially available at prices that can be afforded. If not available (e.g. in the case of *B. pseudomallei*) one can readily produce them, as hybridoma technology has become almost a routine procedure in several laboratories. On the other hand, one should keep in mind that the propagation of specific clones or keeping them to continuously secreting the antibodies is not a fool-proof procedure and one may encounter a number of different problems.

Approaches for development of appropriate method(s) to diagnose melioidosis

Because of our expertise in immunology, we have been working for the past several years on different aspects of the immunology of tropical diseases. With melioidosis, our main objective was to develop appropriate and/or improved immunodiagnostic method(s) with an ultimate goal of providing better care for patients. Although the antigen-detection approach was the most desirable since it could readily detect ongoing infections, practically all the diagnostic methods available in endemic areas of infection were aimed at detecting antibodies to *B. pseudomallei*.[28] These serological methods varied from simple IHA tests to more elaborate and expensive, equipment-dependent immunofluorescence tests. Because of its simplicity, the IHA tests are currently the most popular, and they are very well adapted for use by all health centers in endemic areas of infection. However, all the IHA and other serological tests currently in use have one major drawback. That is, interpretation of the results. This is particularly serious for melioidosis in Thailand where asymptomatic infection is fairly common. In such cases, the presence of elevated background antibody is not uncommon to begin with, and this makes it difficult to distinguish present from past infections. The cut-off point cannot be readily standardized and it varies from one laboratory to another. In order to alleviate this problem, a number of investigators have developed methods for detecting specific IgM antibody rather than IgG antibody.[28] The methods for IgM antibody detection also vary from IgM-ELISA to IgM-IFA. Reports based on these methods have been made from a relatively small number of patients. Therefore, additional analysis and a more critical evaluation of the methods are needed. Very recently, diagnosis based on antigen detection has been developed and the results reported thus far are highly encouraging. Our investigations have covered development of method(s) for the detection of either antibody or antigen, based on the use of purified antigen and monoclonal antibody, respectively.

1. Characterization, identification and purification of *B. pseudomallei* antigens

The rational behind this part of our investigation is that if one had purified antigen specific for *B. pseudomallei*, one might be able to improve the antibody-detection method to the point where reliable interpretation of the results could be achieved. In the past, only crude bacterial extracts or culture filtrates were used and therefore cross-reactivity was unavoidable. Crude extracts of *B. pseudomallei* are highly complex and cannot be readily distinguished from those of other gram-negative bacteria.[29-31] That is, there is no protein component unique to *B. pseudomallei* that can be singled out from the SDS-PAGE protein profile (Fig. 2).[30] Our group was however able to demonstrate by immunoblot that a 19.5 kDa specific antigen was present in *B. pseudomallei* and also in trace quantity also in *B. cepacia* (Fig. 3).[30] It was not present in all other bacteria tested. This antigen was found to be highly immunogenic, and an anti-19.5 kDa was found in a large majority of patients with septicemic melioidosis by either ELISA or immunoblot (Fig. 4).[30] Cross-reactivity with *B. cepacia* (Fig. 3) should not pose much problem as in Thailand the infection by this bacterium is rather rare and is mostly encountered in conjunction with other disorders, e.g. cystic fibrosis.

The efficacy of this antigen in the diagnosis of melioidosis was subsequently evaluated by ELISA. The purified 19.5 kDa antigen used for antibody detection was prepared by separating it from other *B. pseudomallei* components using SDS-PAGE. Then, the portion of the gel containing it was cut out, the antigen

was electroeluted, and SDS was removed.[30] Using this antigen, highly promising results were obtained: 82% sensitivity, 96% specificity, 94% positive predictive value and 87% negative predictive value based on a background value obtained with normal sera from individuals in the endemic areas of infection. However, purification of this antigen was time-consuming and not too practical for mass production. We felt that having a MAb specific for the antigen would allow us to use an immunoaffinity purification procedure that would be more simple and more efficient. Unfortunately, using the purified 19.5 kDa as an immunogen to immunize BALB/c mice, we have so far been unable to produce any MAb that could satisfactorily react with the native 19.5 kDa antigen (Unpublished data).

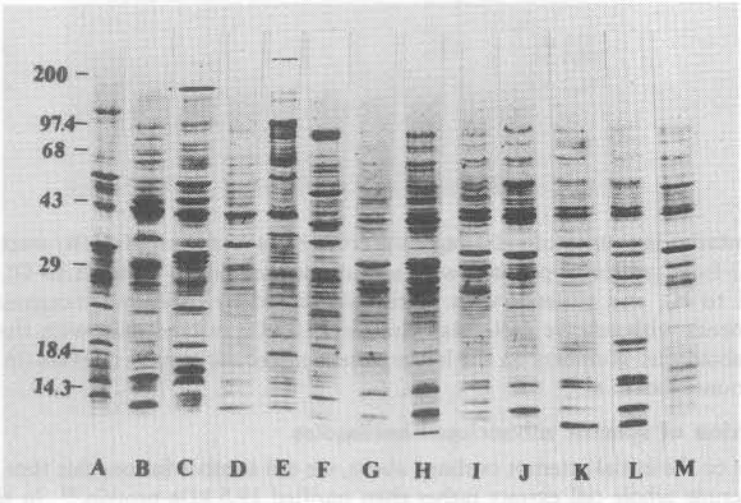

Fig. 2 SDS-PAGE profiles of crude extracts from *B. pseudomallei* (lane A), *P. aeruginosa* (lane B), *B. cepacia* (lane C), *P. putida* (lane D), *Xanthomonas maltophilia* (lane E), *P. alcaligenes* (lane F), *K. pneumoniae* (lane G), *E. cloacae* (lane H), *E. coli* (lane I), *S. typhi* (lane J), *S. enteritidis* (lane K), *S. krefeld* (lane L), and *Proteus mirabilis* (lane M). A total of approximately 100 μg of protein was applied to each lane. Numbers on the left represent molecular weight markers (in thousands). Taken from Anuntagool *et al.*[30]

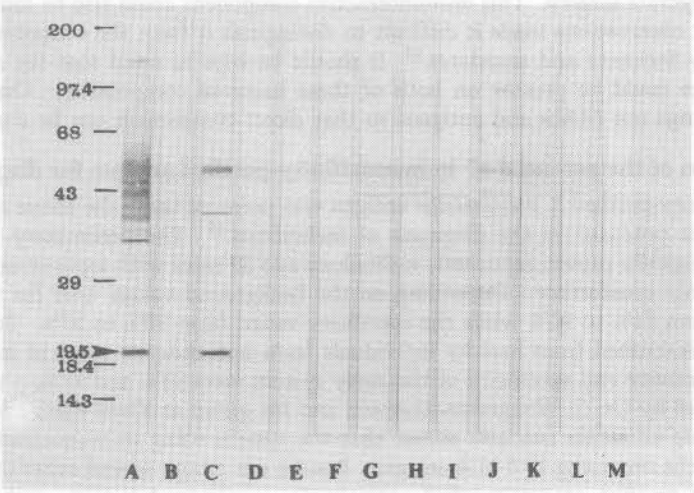

Fig. 3 Immunoblot patterns of the crude extracts (100 μg of protein) probed with pooled serum (1:500 dilution) from patients with bacteriologically proven septicemic melioidosis. The position of each extract is the same as in Fig. 2. Numbers on the left represent molecular weight markers (in thousands). Taken from Anuntagool *et al.*[30]

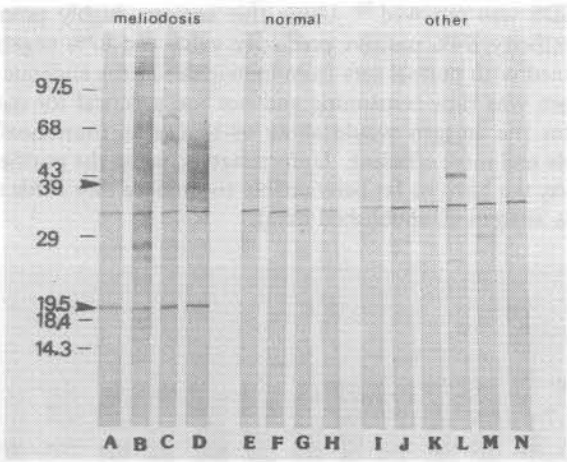

Fig. 4 Representative immunoblot patterns of the crude extract of *B. pseudomallei* reacting with sera (1:500 dilution) from groups of patients with septicemic melioidosis (lanes A to D), normal individuals (lanes E to H), and patients with septicemia caused by other micro-organisms (lanes I to N). Components with relative molecular mass of 19.5 kDa reacted only with the sera from patients with melioidosis. Numbers on the left represent molecular weight markers (in thousands). Taken from Anuntagool *et al.*[30]

2. Production of specific monoclonal antibodies

Subsequent to the initial attempt outlined above, we did another fusion, this time, using BALB/c mice immunized with crude whole cell extract rather than purified 19.5 kDa protein.[18] In this fusion, we were able to clone a hybrid producing IgM antibody that was highly specific for *B. pseudomallei* by ELISA screening. This MAb was shown to react with the surface envelope of *B. pseudomallei* by immunofluorescence tests and by its ability to agglutinate the bacterium. This MAb reacted with all 56 *B. pseudomallei* isolates collected from both the patients and the environment. Different lines of evidence suggested that the MAb reacted most probably with lipopolysaccharide (LPS) components of the cell wall. However, immunoblotting profiles showed a smearing only in the high molecular weight region, instead of a ladder-like pattern generally expected for LPS. On the other hand, the reactive component could be stained with lectin indicating the presence of a carbohydrate moiety. This immunoreactive component could also be found in bacterial culture filtrates. The latter observations made it difficult to distinguish it from the exopolysaccharide component recently reported by Steimetz and associates.[32] It should be kept in mind that the same immunoreactive carbohydrate epitope could be present on both of these bacterial components. One way to resolve the problem is to exchange our MAbs and antigens so that direct comparison can be done.

3. Evaluation of the potential of immunoaffinity-purified antigen for diagnosis of melioidosis

Immunoaffinity-purified *B. pseudomallei* antigen was prepared using the above MAb and evaluated by indirect ELISA for it potential in the diagnosis of melioidosis.[18] The preliminary IgG-ELISA data from 27 cases of bacteriologically proven septicemic melioidosis and 35 cases with septicemia due to other bacterial infections were highly satisfactory. Depending on the background values used for the cut-off point, the sensitivity varied from 81% to 93% while the specificity varied from 90% to 97%. For example, when the cut-off point was established from healthy individuals from non-endemic areas of infection (e.g. Bangkok metropolis) the sensitivity and specificity of the assay system were 93% and 97%, respectively. This assay system has been used by Dr. S. Wongratanacheewin and his group at Khon Kaen and the results are now being analyzed. Data currently available shows that the system using immunoaffinity-purified antigen is slightly better than the one using 19.5 kDa antigen. Besides our group, several other investigators have been trying to use other purified or semipurified antigens for antibody detection, and they have met with varying degrees of success. These antigens include endotoxin,[33] exotoxin,[34] flagellin,[35] glycolipid[36] and bacterial culture filtrates.[37]

As mentioned earlier, one major drawback of the antibody detection approach for the diagnosis of melioidosis is the presence of background antibody in healthy individuals in endemic areas of infection. This somewhat complicates interpretation of the results. It was suggested a few years ago that a better correlation

with clinical disease might be achieved if one measured IgM instead of IgG antibodies. Several laboratories have pursued this idea further and obtained results that look supportive (see review in ref. 28). However, the conclusion reached was based on data from a relatively small number of patients. Therefore, very recently, we conducted an experiment, using a large group of patients, all of whom had their clinical courses and other supportive laboratory data monitored very closely, in the hope of settling this question once and for all.[38] In this study, more than 100 patients with signs and symptoms compatible with acute melioidosis were admitted to Sappasitprasong Hospital in the endemic northeastern part of Thailand. Approximately half of these patients were subsequently proven to be bacteriologically positive for *B. pseudomallei*. The remaining patients were hemoculture negative for *B. pseudomallei*. IgG and not IgM antibody was detected in a majority of patients with culture positive for *B. pseudomallei*. The specific IgG antibody was found to be better than the specific IgM in identifying acute melioidosis in the first 5 days after admission. These results indicate that detection of specific IgG antibody is clinically useful for the diagnosis of acute melioidosis in endemic areas.

Cummulative data on the specific IgG-ELISA test in 189 patients with signs and symptoms compatible with acute melioidosis who were admitted to Srinagarind (Khon Kaen) and Sappasitprasong (Ubonratchathani) Hospitals are shown in Fig. 5 and Table 1. The mean antibody level in patients with culture proven *B. pseudomallei* infection shown in the figure was considerably and significantly higher than in the group with culture negative for *B. pseudomallei*. The mean antibody level in the latter group was nevertheless significantly higher than in septicemic patients with hemoculture positive for other micro-organisms. When the mean O.D. value + 3 S.D. of blood donors from the endemic area of infection (Khon Kaen) was used as a cut-off level, it was found that of the 189 patients, 106 were labelled as antibody positive by our test while 101 was bacteriologically positive for *B. pseudomallei* (Tables 1 and 2). The correlation between the culture method and the antibody test was as high as 80% (Table 2). The existence of a small number of *B. pseudomallei* positive but antibody negative patients can be readily explained based on the kinetics of antibody production. On the other hand, there can be a number of acceptable explanations for the existence of a group of 21 *B. pseudomallei* negative patients with positive antibody test. Using the above criterion, the IgG-ELISA test developed by our group exhibits highly satisfactory results, with 84% specificity, 95% sensitivity, 90% accuracy, 93% positive predictive value and 87% negative predictive value.

TABLE 1 Diagnostic potential of IgG-ELISA test in clinical melioidosis

Source of serum	No.	Mean ± S.D. (O.D. at 490 nm)	No. positive (%)*
Clinical melioidosis	189	0.422 ± 0.402	106 (56.99)
Positive culture for *B. pseudomallei*	101	0.617 ± 0.387	85 (84.16)
Negative culture for *B. pseudomallei*	88	0.197 ± 0.228	21 (23.86)
Non-melioidosis (culture positive for other micro-organisms)	34	0.056 ± 0.047	2 (5.88)
Endemic	25	0.064 ± 0.051	2 (8.00)
Non-endamic	9	0.036 ± 0.030	0 (0)
Blood donors	225	0.030 ± 0.034	4 (1.78)
Endemic	89	0.042 ± 0.043	4 (4.49)
Non-endemic	136	0.022 ± 0.023	0 (0)

*The cut-off O.D. value for antibody positivity was 0.171 [mean + 3 S.D. of normal (blood donors) from the endemic areas of infection]. The immunoaffinity-purified *B. pseudomallei* antigen was used in the analysis. All serum specimens were used at a 1:2,000 dilution.

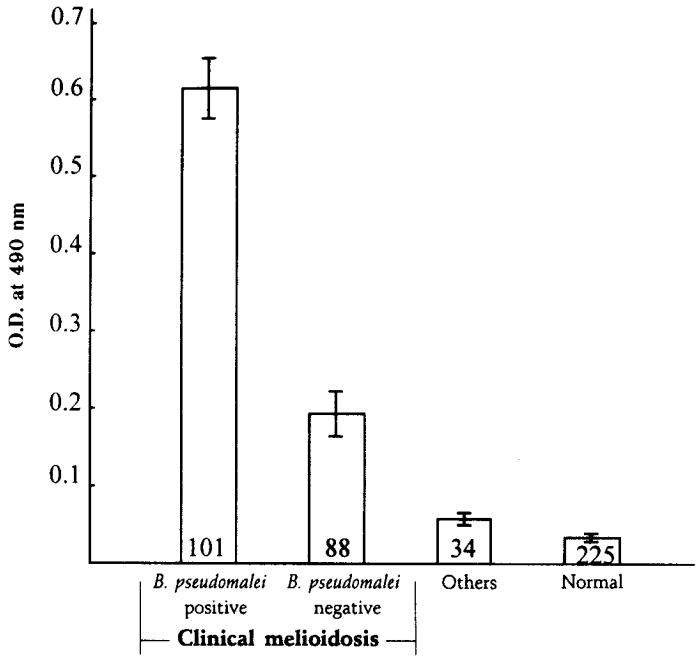

Fig. 5 Immunoreactivity of sera from patients with suspected acute melioidosis. The antigen used in this IgG-ELISA analysis was immunoaffinity-purified and used at a concentration of 0.5 μg carbohydrate per ml. All sera were diluted at 1:2,000 and the ELISA results were expressed as absorbance value at 490 nm. The melioidosis patients were divided into *B. pseudomallei* positive and negative groups based on hemoculture results and/or presence of *B. pseudomallei* in other clinical specimens. Septicemic patients whose hemoculture showed growth of other micro-organisms and healthy blood donors were included for comparison. Bars and lines represent mean and standard error of the mean. Numbers at the base of the bar are the number of subjects in each group.

TABLE 2 Summary of results of the antibody test **antigen** in patients with acute melioidosis, using affinity-purified *B. pseudomallei* compared with those of the culture method

		IgG antibody		
		+	−	Total
Culture	+	85	16	101
	−	21	67	88
	Total	106	83	189

The potential of this antibody test for epidemiological study has also been examined on a limited scale in man and animals. Data in the humans, as presented in Table 3, clearly showed that with the exception of individuals in the northeastern part of Thailand, all the remaining Thais and those from other countries were all antibody negative. The data demonstrated further that within the endemic area of infection, the antibody levels in people residing in the rural were higher than in those in the urban. This is not entirely unexpected in view of the fact that residents of the villages in the rural area should be more readily exposed to *B. pseudomallei* present in the soil and water. Consistent with this expectation is the fact that higher

number of colony forming units of *B. pseudomallei* were found in the soil from the endemic rice fields than in those from the non-endemic central region.[39] All subjects in the rural area in our study were farmers while a majority of those in the urban area were not.

TABLE 3 Immunoreactivity of normal human sera collected from different regions of Thailand and other countries

Specimens from	No.	Mean ± S.D. (O.D. at 490 nm)	% Antibody positive
Thailand			
Bangkok	136	0.022 ± 0.023	0
North	30	0.020 ± 0.012	0
South	53	0.042 ± 0.060	1.9
Northeast			
Blood donors	89	0.042 ± 0.043	4.50
Rural (villagers)	104	0.175 ± 0.164	30.80
Urban (villagers)	52	0.059 ± 0.051	5.80
Malaysia	38	0.025 ± 0.024	0
Taiwan	5	0.018 ± 0.012	0
Korea	50	0.013 ± 0.019	0
Nepal			
Blood donors	45	0.028 ± 0.015	0
Patients with fever of unknown origin	31	0.035 ± 0.023	0
India	202	N.A.	0

N.A., raw data not available for calculation and different cut-off O.D. value was used for the calculation of positivity (Dharakul, T., personal communication).

See footnote of Table 1 for all other explanations.

Serum specimens from healthy cattle, buffaloes, horses and goats were found to be antibody negative by this test (Table 4). Although the O.D. values from different species of animals could not be directly compared because different types of conjugated secondary antibodies were used, all readings were nevertheless not noticeably different from the corresponding background values reported for normal humans. On the other hand, further analysis of the serum specimens from sick goats showed the presence of antibody that could be readily detected by our test system (Fig. 6). Although in this small group of animals, the clinical manifestions were not well defined and the data on bacteriology were not available, it is tempting to conclude that the illness was probably caused by *B. pseudomallei*. It should be cautioned however that the involvement of *B. mallei* could not be ruled out at this time because the latter was not available for testing.

All data available till now clearly indicate to us that the antibody detection method herein developed by our group gives the results that are just as reliable as the culture method in the diagnosis of acute melioidosis. Moreover, our method has one distinct advantage over the culture method in that the results can be made available very shortly, *i.e.* within a few hours after specimens collection. On the contrary, it takes at least a few days for definitive diagnosis by culture. The ELISA method described herein is also simple to do and does not require special skill or equipment and can be readily performed in any laboratory setting in peripheral health centers.

TABLE 4 Immunoreactivity of sera from healthy animals with affinity-purified *B. pseudomallei* antigen

Serum from	Number analyzed	O.D. (490 nm)	
		Mean	S.D.
Cattle	58	0.006	0.008
Buffaloes	34	0.005	0.006
Horses	230	0.014	0.014
Goats	89	0.186	0.198

Immunoaffinity-purified *B. pseudomallei* antigen was used for the detection of specific IgG antibody by ELISA essentially as described for the human system. Different horseradish peroxidase conjugated rabbit antibodies were used as secondary antibody: anti-bovine IgG (Zymed Laboratories, Inc., U.S.A.) for cow and buffalo; anti-equine IgG (Sigma, U.S.A.) for horse and anti-goat IgG (Zymed) for goat. Optimal dilution for all these conjugated antisera were found to be 1:1,000 by checkerboard titration. All serum specimens were tested at a 1:2,000 dilution.

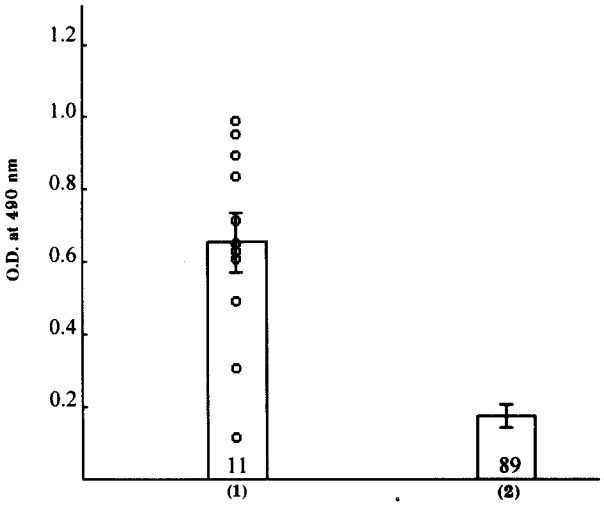

Fig. 6 Scattergram of antibody to *B. pseudomallei* in unhealthy and sick goats (Group 1) compared with healthy normal goats (Group 2). Dots represent antibody levels of individual serum specimens. See legend to Fig. 5 for all other explanations.

4. The use of monoclonal antibody for antigen detection and rapid identification of bacteria

Both specific polyclonal and monoclonal antibodies have been used for antigen detection or identification of micro-organisms in clinical specimens, and the results currently available from different laboratories are encouraging. The Welcome group, in collaboration with Thai investigators, used polyclonal antibodies prepared by immunizing rabbits with crude bacterial extract to detect *B. pseudomallei* antigen in the urine of patients with melioidosis.[40,41] The results looked extremely good, depending however, on the methods used. For example, when an amplified sandwich ELISA was used, highly satisfactory results were obtained, although problems regarding cross-reactivity still persisted.[40] On the other hand, latex agglutination gave rather poor sensitivity, and it could not be used with any degree of reliability.[41] This polyclonal antibody was also used for bacterial identification by immunofluorescence, again giving a high degree of accuracy.[16] A Thai group at the National Institute of Health in Nonthaburi, in collaboration with Japanese scientists, found their polyclonal anti-endotoxin to be highly specific and useful for bacterial identification.[15]

It should be noted that with both studies an expensive immuno-fluorescent microscope must be available, a condition that rarely exists in developing countries like Thailand. A few years ago, Wongratanacheewin and his associates at Khon Kaen developed an antigen detection assay system employing polyclonal rabbit antiserum in an avidin-biotin-enzyme-linked immunosorbent assay and found it to be highly sensitive for antigen detection.[42] However, to our knowledge this method has not yet been tested in a clinical setting.

We previously showed that our IgM monoclonal antibody was highly specific for *B. pseudomallei* envelope antigen.[18] Thus, it was successfully used in an indirect immunofluorescence test to identify the bacterium in clinical specimens (unpublished data). This was highly specific and sensitive, and could be particularly valuable in situations where the bacterium cannot be cultured, e.g. where it is present in small numbers in specimens heavily contaminated by normal bacterial flora. One highly desirable property of this surface-reactive MAb is that it is an IgM antibody with strong agglutinating activity.[18] Bacterial suspensions prepared from colonies subcultured from primary isolates can be readily agglutinated so that identification can be obtained within a few minutes. We are now in the process of trying to develop an assay system to accelerate identification using early hemoculture from patients suspected of having acute septicemic melioidosis. If successful, this approach, could shorten the hemoculture procedure to within 24-48 h. This, in turn, would allow clinicians to provide proper management and cut down the unnecessary use of expensive antibiotics in patients negative for *B. pseudomallei*.

More recently, we have reported the use of this MAb to directly identify *B. pseudomallei* in different clinical specimens, e.g., urine, pus, sputum and pleural fluid, etc.[43] This was carried out by a sandwich ELISA using the MAb as a capturing antibody. Biotinylated polyclonal rabbit IgG anti-*B. pseudomallei* was used as the secondary antibody in this assay method. Sensitivity and specificity of the method were 75% and 98% respectively. A modified dot ELISA that we are now working on gives results that are similar to the sandwich ELISA system (Unpublished observations). Using either of these 2 assay systems, it is now possible to have a reliable diagnostic result within a few hours in 3 out of 4 patients with suspected melioidosis. It is possible that a simple and inexpensive commercial kit could be developed and made available for use in any health center in the region.

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