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Short Report: Monoclonal Antibody-Based Immunofluorescence Microscopy for the Rapid Identification of *Burkholderia pseudomallei* in Clinical Specimens

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Abstract. The diagnosis of melioidosis depends on the culture of *Burkholderia pseudomallei,* which takes at least 48 hours. We used a polyclonal-FITC-based immunofluorescence microscopic assay (Pab-IFA) on clinical samples to provide a rapid presumptive diagnosis. This has limitations including photobleaching and batch-to-batch variability. This study evaluated an IFA based on a monoclonal antibody specific to *B. pseudomallei* (Mab-IFA) and Alexa Fluor 488. A diagnostic evaluation was performed on a prospective cohort of 951 consecutive patients with suspected melioidosis. A total of 1,407 samples were tested. Test accuracy was defined against culture as the gold standard, and was also compared against Pab-IFA. A total of 88 samples from 64 patients were culture positive for *B. pseudomallei*. The diagnostic sensitivity and specificity of the Mab-IFA was comparable to the Pab-IFA (48.4% versus 45.3% for sensitivity, and 99.8% versus 98.8% for specificity). We have incorporated the Mab-IFA into our routine practice.

Melioidosis is an infectious disease caused by the environmental Gram-negative bacterium, *Burkholderia pseudomallei.* Most cases are reported from northern Australia and southeast Asia, and infection results from bacterial inoculation, inhalation, or ingestion.[1] In northeast Thailand, melioidosis is the third most common cause of death from an infectious disease, after human immunodeficiency virus (HIV) infection and tuberculosis.[2] Clinical features of melioidosis are diverse, but the most common manifestations are septicemia (50% of cases), pneumonia, and abscesses in internal organs.[3] Mortality in Thailand is 40%, rising to 90% in those with severe sepsis. Rapid diagnosis and administration of effective antimicrobial therapy is life saving, because *B. pseudomallei* is inherently resistant to a range of antibiotic classes, and patients require ceftazidime or a carbapenem drug.[1,3,4] Laboratory diagnosis is culture-based, which takes at least 48 hours from sample receipt to confirmed identification. Molecular methods including real-time polymerase chain reaction (PCR) and loop-isothermal amplification have been described, which can be applied to DNA extracted directly from the clinical sample, and these can provide a more rapid diagnosis but have a lower diagnostic sensitivity than culture.[5,6]

Our clinical research laboratory in Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand has used an in-house immunofluorescence microscopy assay (IFA) for the rapid detection of *B. pseudomallei* in clinical specimens since 1993.[7] This uses a fluorescein isothiocyanate (FITC)-labeled rabbit polyclonal antibody (Pab) against formalin-killed *B. pseudomallei*, two clinical evaluations have reported a sensitivity and specificity of 66–73% and 99%, respectively.[7,8] Used as an adjunct to culture, this can provide a very rapid presumptive diagnosis of melioidosis, pending culture confirmation. This assay has several limitations, however, including photobleaching of FITC[9–11] and the potential for higher batch-to-batch variation with polyclonal compared with monoclonal antibody (Mab) preparations.[12,13] We have produced an in-house Mab that recognizes *B. pseudomallei* exopolysaccharide,[14] and the aim of this study was to re-evaluate the IFA in the laboratory and clinical settings after replacing the Pab with this Mab and replacing FITC with a photo-stable dye.

The Mab-IFA was developed as an indirect assay. The primary detection antibody was unlabelled Mab 4B11 (IgG2b subclass) specific to *B. pseudomallei* exopolysaccharide,[14] and the secondary antibody was Alexa Fluor 488 conjugated-goat anti-mouse immunoglobulin G (IgG) (Molecular Probes, Carlsbad, CA). Mab was prepared from culture supernatant of hybridroma clone 4B11, as described previously.[14] The Mab-IFA was optimized for ease of use by preparing a single mixture of primary and secondary antibody, which was added to the slide in a single step. The Mab-IFA detection reagent contained 5 μg/mL of Mab and 20 μg/mL of secondary antibody in phosphate-buffered saline (PBS). The limit of detection was defined using a 10-fold dilution series ranging from 2×10^{10} to 20 colony-forming units (CFU)/mL of *B. pseudomallei* K96243. Ten microliters of each bacterial dilution was mixed with an equal volume of Mab-IFA and incubated at room temperature for 5 min before observing for the presence of green fluorescent bacteria using a fluorescent microscope at 1,000× magnification (Olympus BH-2, Tokyo, Japan). *Burkholderia pseudomallei* appeared as individual, uniformly stained bacilli (Figure 1A and B). The limit of detection of the assay, defined as the lowest number of bacteria that gave a positive result for Mab-IFA, was 2×10^3 CFU/mL.

The assay sensitivity of the Mab-IFA was defined using 20 clinical *B. pseudomallei* isolates. The Mab-IFA assay specificity was defined by testing 160 microorganisms representing a wide range of species. These were 20 Gram-positive bacteria (*Staphylococcus aureus* [16], unknown species of α-hemolytic *Streptococcus* [1], *Streptococcus pneumoniae*, [2], *Nocardia* spp. [1]), 136 Gram-negative bacteria (*Acinetobacter* spp. [5], *A. baumannii* [5], *Burkholderia thailandensis* [10]), *Chromobacterium violaceum* [1], *Haemophilus influenzae* [1], *Achromobacter xylosoxidans* [15]), *Aeromonas* spp. [22], *Burkholderia*

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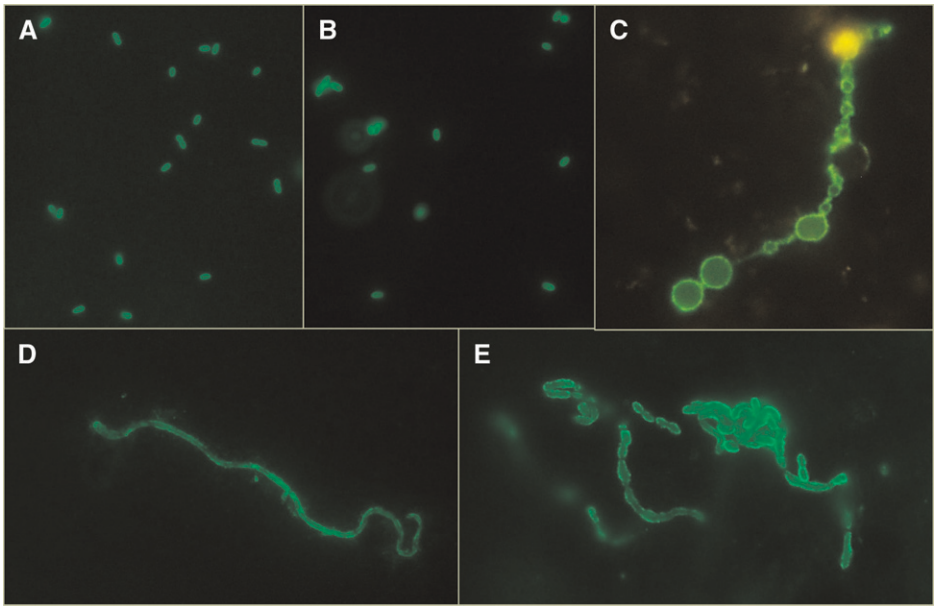

FIGURE 1. Fluorescent microscopy of *Burkholderia pseudomallei* stained with Mab-IFA reagent. The bacteria shown were from laboratory cultures on Columbia agar (**A**) or LB broth (**B**), or from clinical samples (urine [**C**], pus [**D**], or sputum [**E**]) from patients with melioidosis. The atypical appearance of the bacterial morphology including bacterial elongation (**D** and **E**) and swollen cells (**C**) was not uncommon.

cepacia [7], *Burkholderia pickettii* [2], *Citrobacter* spp. [1], *C. freundii* [2], *Enterobacter* spp. [5], *E. cloacae* [1], *Escherichia coli* [1], *E. fergusonii* [1), *Flavobacterium* spp. [1], *Hafnia alvei* [1), *Klebsiella* spp. [2], *K. aerogenes* [2], *K. oxytoca* [3], *K. ozaenae* [1], *K. pneumoniae* [7], *Morganella morganii* [2], *Proteus mirabilis* [3], *Pseudomonas* spp. [1], *P. acidovorans* [3], *P. aeruginosa* [8], *P. alcaligenes* [2], *P. fluorescens* [2], *P. mesophilia* [1], *P. pseudoalcaligenes* [2], *P. putida* [3], *P. stuartii* [1], *P. stutzeri* [4], *Ralstonia paucula* [1], *Salmonella enterica* serovar Paratyphi A [1], *S. enterica* serovar Typhi [1], *Serratia marcescens* [1], *Sphingomonas paucimobilis* [1], *Stenotrophomonas maltophilia* [3]), and 4 fungi (*Candida* spp. [1], *Cryptococcus neoformans* [3]). Microorganisms were sub-cultured on Columbia agar and incubated overnight at 37°C in air. Fastidious bacteria were sub-cultured on chocolate agar and incubated overnight at 37°C in 5% CO_2. The assay sensitivity was 100% (20 of 20 *B. pseudomallei* positive), and specificity was 90.0% (144 of 160 other species negative). The 16 false positive tests were all *S. aureus*, which on immunofluroresence appeared as weakly staining cocci in clusters. The false positive reaction occurred in both the presence and absence of Mab, indicating direct binding of the goat anti-mouse IgG conjugated with Alexa Fluor 488 to the organism. This is likely to be caused by antibody binding to the surface-expressed *S. aureus* immunoglobulin binding protein, Spa.

A diagnostic evaluation of the Mab-IFA was performed on a prospective cohort of 951 patients recruited at Sappasithiprasong Hospital, Ubon Rachanthani, northeast Thailand. These were consecutive patients presenting with suspected melioidosis between May and September 2012, from whom a total of 1,407 samples were taken for culture (respiratory secretions, $N = 406$; urine, $N = 937$; pus, $N = 21$; other body fluids, $N = 43$). Blood cultures were also taken from the cohort, but these are not included here because the bacterial load in blood is too low for direct microscopic detection. Ethical approval for the study was obtained from the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University.

Clinical samples were prepared for examination by Mab-IFA as follows. Up to 10 mL of urine or other body fluid (or the available sample volume if less was collected) was centrifuged at 3,000 rpm and the supernatant discarded before the sediment was used in the IFA. Pus or respiratory secretions were tested without a sample preparation step. Ten microliters of working IFA reagent containing the primary and secondary antibody probe was placed on a glass slide, to which 10 μL of sediment was added and mixed and a coverslip applied. Slides were left at room temperature for 5 min, and then examined, as before, using a fluorescent microscope.

Our current polyclonal antibody-based IFA (Pab-IFA) was performed in parallel on every sample, and was performed as described previously[8]; in brief, Pab-IFA reagent (FITC conjugated-rabbit antibody to formalin-killed *B. pseudomallei*) was diluted 1:200 in 5% skimmed milk. Ten microliters of reagent was placed onto a glass slide, mixed with 10 μL of sample prepared as described previously, and a coverslip applied. The slide was observed as for the Mab-IFA. All slides were read by two independent technicians. Heat-killed *B. pseudomallei* and *B. thailandensis* (1×10^6 CFU/mL in PBS) were used in both assays as positive and negative controls, respectively.

Statistical analyses were performed by using STATA/SE version 11.1 (StataCorp., College Station, TX). Performance of the two assays was compared using the exact McNemar test. Diagnostic sensitivity, specificity, positive and negative predictive values of the Mab-IFA, and Pab-IFA assays were defined against the result for bacterial culture and identification.

A total of 88 samples from 64 patients were culture positive for *B. pseudomallei*. The diagnostic sensitivity of the Mab-IFA (48.4%; 31 of 64 patients) was comparable to the Pab-IFA (45.3%; 29 of 64 patients) ($P = 0.69$). Assay sensitivity varied between specimen types, as shown in Table 1. The diagnostic specificity of the Mab-IFA (99.8%, 885 of 887 patients) was significantly higher than the Pab-IFA (98.8%, 876 of 887 patients) ($P = 0.02$). The Mab-IFA was false positive for

TABLE 1

IFA results compared with *Burkholderia pseudomallei* culture by specimen type*

Specimen type	*B. pseudomallei* isolated	Mab-IFA positive (%)	Pab-IFA positive (%)
Respiratory secretions	49	16 (32.7)	16 (32.7)
Urine	28	13 (46.4)	12 (42.9)
Pus	6	3 (50.0)	3 (50.0)
Other body fluids	5	2 (40.0)	1 (20.0)
Total	88	34 (38.6)	32 (36.4)

*IFA = immunofluorescence assay; Mab = monoclonal antibody; Pab = polyclonal antibody.

1 sputum and 1 urine sample, the culture results of which later showed unidentified normal flora and *E. coli*, respectively. The Pab-IFA was false positive for 11 clinical samples, the culture results of which were respiratory secretions: *Acinetobacter* spp. ($N = 1$), mixed flora ($N = 2$); and urine: *E. coli* ($N = 1$), *K. pneumoniae* ($N = 1$), and unidentified mixed flora ($N = 6$). None of the clinical specimens were false positive for both Mab-IFA and Pab-IFA. The positive predictive values of the Mab-IFA and Pab-IFA were 93.9% (31 of 33 patients) and 72.5% (29 of 40 patients), respectively, and the negative predictive values of the Mab-IFA and Pab-IFA were 96.4% (885 of 918 patients) and 96.2% (876 of 911 patients), respectively.

The appearances of bacteria seen by fluorescent microscopy were recorded for the last 60 positive samples. *B. pseudomallei* often had a filamentous appearance in urine (14 of 19 samples), respiratory secretions (16 of 35), pus (2 of 4), and other body fluids (1 of 2) (Figure 1C–E). Some *B. pseudomallei* cells in urine (5 of 19) and respiratory secretions (2 of 35) were elongated with focal areas of swelling, suggesting cell damage (Figure 1C). The change in cell morphology observed was similar in both the Mab-IFA and Pab-IFA.

The Mab-IFA developed and evaluated in this study was at least as good as our current Pab-IFA, and we intend to adopt the Mab-IFA into our routine practice for the rapid identification of patients with presumptive melioidosis. The specificity was high but not perfect and culture confirmation and bacterial identification is still required to confirm the presence of *B. pseudomallei*, as well as for susceptibility testing. The sensitivity was considerably lower and detected less than half of the patients with melioidosis. Although this makes a valuable contribution to the diagnosis of some patients, we will continue to seek ways to improve the diagnostic sensitivity. The likely explanation for false negative samples is that the numbers of bacteria present fall below the limit of detection. This may be because the patient has received effective antimicrobial drugs by the time the sample was taken. This possibility is consistent with the observation in this study that bacterial cells frequently looked damaged on microscopy. We recommend that samples be collected before antibiotic administration wherever possible, although this should not be done at the risk of delaying antimicrobial therapy. Using a higher volume of sample might improve the sensitivity of the Mab-IFA, although there are technical limitations to the final volume that can be applied to a glass slide. Sensitivity could be increased by using a pre-enrichment step in which the sample is incubated in culture media or a further development of a method to concentrate the bacteria in the specimens before the IFA. This could be performed on those samples that are IFA negative on immediate testing but when the clinical suspicion for melioidosis is high, and if completed within the same working day could still reduce the time to diagnosis. An important disadvantage of the IFA is that it is not suitable for direct testing of blood cultures, however testing after a pre-incubation step may increase the sensitivity sufficiently to give it clinical use. This is the subject of further investigation.

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REFERENCES

1. Wiersinga WJ, Currie BJ, Peacock SJ, 2012. Melioidosis. *N Engl J Med 367:* 1035–1044.
2. Limmathurotsakul D, Wongratanacheewin S, Teerawattanasook N, Wongsuvan G, Chaisuksant S, Chetchotisakd P, Chaowagul W, Day NP, Peacock SJ, 2010. Increasing incidence of human melioidosis in northeast Thailand. *Am J Trop Med Hyg 82:* 1113–1117.
3. Limmathurotsakul D, Peacock SJ, 2011. Melioidosis: a clinical overview. *Br Med Bull 99:* 125–139.
4. Cheng AC, Currie BJ, 2005. Melioidosis: epidemiology, pathophysiology, and management. *Clin Microbiol Rev 18:* 383–416.
5. Chantratita N, Wuthiekanun V, Limmathurotsakul D, Thanwisai A, Chantratita W, Day NP, Peacock SJ, 2007. Prospective clinical evaluation of the accuracy of 16S rRNA real-time PCR assay for the diagnosis of melioidosis. *Am J Trop Med Hyg 77:* 814–817.
6. Chantratita N, Meumann E, Thanwisai A, Limmathurotsakul D, Wuthiekanun V, Wannapasni S, Tumapa S, Day NP, Peacock SJ, 2008. Loop-mediated isothermal amplification method targeting the TTS1 gene cluster for detection of *Burkholderia pseudomallei* and diagnosis of melioidosis. *J Clin Microbiol 46:* 568–573.
7. Walsh AL, Smith MD, Wuthiekanun V, Suputtamongkol Y, Desakorn V, Chaowagul W, White NJ, 1994. Immunofluorescence

microscopy for the rapid diagnosis of melioidosis. *J Clin Pathol 47:* 377–379.
8. Wuthiekanun V, Desakorn V, Wongsuvan G, Amornchai P, Cheng AC, Maharjan B, Limmathurotsakul D, Chierakul W, White NJ, Day NP, Peacock SJ, 2005. Rapid immunofluorescence microscopy for diagnosis of melioidosis. *Clin Diagn Lab Immunol 12:* 555–556.
9. Benchaib M, Delorme R, Pluvinage M, Bryon PA, Souchier C, 1996. Evaluation of five green fluorescence-emitting streptavidin-conjugated fluorochromes for use in immunofluorescence microscopy. *Histochem Cell Biol 106:* 253–256.
10. Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung WY, 1999. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem 47:* 1179–1188.
11. Mahmoudian J, Hadavi R, Jeddi-Tehrani M, Mahmoudi AR, Bayat AA, Shaban E, Vafakhah M, Darzi M, Tarahomi M, Ghods R, 2011. Comparison of the photobleaching and photostability traits of Alexa Fluor 568- and fluorescein isothiocyanate-conjugated antibody. *Cell J 13:* 169–172.
12. Irani AM, Bradford TR, Kepley CL, Schechter NM, Schwartz LB, 1989. Detection of MCT and MCTC types of human mast cells by immunohistochemistry using new monoclonal anti-tryptase and anti-chymase antibodies. *J Histochem Cytochem 37:* 1509–1515.
13. Haines DM, Clark EG, Dubovi EJ, 1992. Monoclonal antibody-based immunohistochemical detection of bovine viral diarrhea virus in formalin-fixed, paraffin-embedded tissues. *Vet Pathol 29:* 27–32.
14. Anuntagool N, Naigowit P, Petkanchanapong V, Aramsri P, Panichakul T, Sirisinha S, 2000. Monoclonal antibody-based rapid identification of *Burkholderia pseudomallei* in blood culture fluid from patients with community-acquired septicaemia. *J Med Microbiol 49:* 1075–1078.