Short Report: Quantitation of *B. Pseudomallei* in Clinical Samples

Vanaporn Wuthiekanun, Direk Limmathurotsakul, Gumphol Wongsuvan, Wirongrong Chierakul, Nittaya Teerawattanason, Prapat Teparrukkul, Nicholas P. Day, and Sharon J. Peacock*

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand; Medical Department, Sappasithiprasong Hospital, Ubon Ratchathani, Thailand; Center for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom

Abstract. We undertook a prospective study to quantitate *Burkholderia pseudomallei* in blood, pus, respiratory secretions, and urine obtained from 414 patients with melioidosis. The median was count $1.1 \times 10^6$, $1.1 \times 10^5$, and $1.1 \times 10^4$ CFU/mL in these sample types, respectively. This provides important insights into the likely feasibility of future studies such as expression microarray analysis using clinical material.

The gram-negative bacillus *Burkholderia pseudomallei* is a Category B biothreat agent and the cause of melioidosis. This organism is present in the environment across much of the Far East and northern Australia, and infection is most commonly acquired after inoculation or inhalation. Melioidosis accounts for 20% of all community-acquired septicemias in northeast Thailand, where it is associated with a mortality rate of ~50%. Defining the processes associated with disease pathogenesis is crucial to the development of improved treatment strategies, a major component of which is delineating bacterial gene expression. *B. pseudomallei* gene expression differs in the tissues of infected experimental animals compared with that after growth in broth, and direct examination of *B. pseudomallei* obtained from patients with melioidosis is essential if an accurate picture is to be obtained during human infection. Specimen types available from patients are limited to small volumes of blood, pus, and other body secretions and samples taken postmortem; the latter are not performed in many areas with the highest incidence of melioidosis, either because of a lack of facilities or cultural objections. The purpose of this study was to define whether the quantity of *B. pseudomallei* in clinical specimens from patients with melioidosis would be sufficient to support a future study of bacterial gene expression using microarray analysis.

A prospective study was performed between July 2004 and October 2006 at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand. Adult patients (≥ 14 years of age) with suspected melioidosis were identified during twice-daily rounds of the medical and intensive care wards as part of clinical and diagnostic trials approved by the Ministry of Public Health, Royal Government of Thailand. Multiple samples were taken from suspected cases, representing routine practice for the study of this patient group. A 15-mL blood sample was taken and divided between a BacT/ALERT FA bottle (BioMérieux, Durham, NC) for standard enrichment culture (5 mL) and an Isolator 10 lys centrifugation tube (Oxoid, Basingstoke, Hampshire, UK) for quantitative bacterial count (10 mL). Samples were taken for quantitative culture of sputum/tracheal aspirate (hereafter termed respiratory secretions), urine, pus, or other body fluids; additional samples including throat swabs and surface swabs from wounds and skin lesions were taken as part of the diagnostic process as appropriate or available but were not evaluated for quantitative count because we predicted that dry swabs were unlikely to contain very high numbers of *B. pseudomallei*. Specimens were carried to the on-site research laboratory at the end of each ward round. Quantitative blood and urine cultures were performed throughout the study, and quantitative sputum and pus cultures were started in June 2005.

Isolator tubes were processed as described previously: the pellet was divided between two Ashdown agar plates (ASH). The BacT/ALERT blood culture bottle was maintained at 37°C in air and sub-cultured onto horse blood agar after 1, 2, and 7 days and at additional time-points if cloudy. The lower and upper limits of detection were 0.1 CFU/mL blood (1 colony from 10 mL) and ≥ 100 CFU/mL blood (≥ 1,000 colonies from 10 mL), respectively. If blood culture was positive for *B. pseudomallei* from the standard bottle but negative from the Isolator tube, the colony count was considered to contain < 0.1 CFU/mL blood. Quantitative bacterial count in urine was performed using 1 μL of fresh urine plated onto one half each of a MacConkey agar plate and ASH plate. Colony counts of *B. pseudomallei* in neat urine were expressed as CFU per milliliter urine. The remaining urine sample was centrifuged at 3,000 rpm for 5 minutes, excess supernatant was removed, and the pellet was spread plated onto one half of an ASH plate. The lower and upper limits of detection were 10^5 CFU/mL urine (1 colony from 1 μL) and ≥ 10^6 CFU/mL urine (≥ 1,000 colonies from 1 μL), respectively. Positive *B. pseudomallei* culture from only the centrifuged pellet was considered to contain a colony count of < 10^5 CFU/mL. Quantitative bacterial counts in respiratory secretions and pus were performed using a serial dilution series that was based on positivity by direct immunofluorescent microscopy. Positive samples were serially diluted to 1:100, 1:1,000, 1:10,000, and 1:100,000, and 10 μL of each was spread onto an ASH. Samples found negative by immunofluorescence were plated onto ASHs in volumes of 1 and 10 μL. All pus and respiratory secretions were also subcultured using selective enrichment broth. Broths were incubated at 37°C in air for 2 days and subcultured onto an ASH. Colony counts were expressed as CFU per milliliter of fluid. The lower and upper limits of detection were 10^5 CFU/mL (1 colony from 10 μL of neat sample) and ≥ 10^6 CFU/mL (≥ 1,000 colonies from 10 μL of 1:10,000 dilution). Positive *B. pseudomallei* culture from enrichment broth alone was considered to contain a colony count of < 10^5 CFU/mL.

All ASH plates were incubated at 37°C in air for 4 days; plates were observed daily, and the *B. pseudomallei* count was performed on Day 4. MacConkey agar plates were incubated at 37°C in air for 2 days; plates were observed daily, and the

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* Address correspondence to Sharon J. Peacock, 420/6 Rajvithi Road, Bangkok 10400, Thailand. E-mail: Sharon@tropmedres.ac

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B. pseudomallei count was performed on day 2. Colonies of presumptive B. pseudomallei were initially identified on the basis of colony morphology. Colonies suspected to be B. pseudomallei were tested using the oxidase test, and oxidase-positive colonies were confirmed as B. pseudomallei using a highly specific latex agglutination test, as previously described. All colonies with colony morphology identical to those confirmed as B. pseudomallei on a given plate were assumed to be B. pseudomallei.

A total of 730 specimens taken on admission from 414 patients with culture-proven melioidosis were processed for quantitative culture (414 blood samples, 268 urines, 120 respiratory secretions, and 28 pus samples). Quantitative culture results are summarized in Figure 1. A total of 203 blood cultures were positive for B. pseudomallei, of which 22 (12%) were positive by standard blood culture but negative by isolate tube culture (< 0.1 CFU/mL). The median B. pseudomallei count in blood (including 22 cases positive by standard blood culture alone) was 1.1 CFU/mL (interquartile range [IQR], 0.2–7.7 CFU/mL); the highest count was > 100 CFU/mL (present in eight samples).

A total of 56 of 268 (21%) urine samples were positive for B. pseudomallei, of which 11 (20%) were positive from the pellet alone. The median count was 1.5 × 10^4 CFU/mL, and the highest count was ≥ 10^6 CFU/mL. A total of 94 of 120 (78%) respiratory secretions were positive for B. pseudomallei, of which 33 (35%) were positive from enrichment broth alone. The median count was 1.1 × 10^3 CFU/mL, and the highest count was 5.0 × 10^5 CFU/mL. A total of 23 of 28 (82%) pus samples were positive for B. pseudomallei, of which 2 were positive from enrichment broth alone. The median count was 1.1 × 10^3 CFU/mL, and the highest count was 2.3 × 10^4 CFU/mL (synovial fluid). Positive pus samples were synovial fluid (nine), liver aspirate (seven), soft tissue abscesses (six), and pleural fluid (one).

Our results indicate that blood and urine are unsuitable for microarray analysis. Although some patients had a very high B. pseudomallei count in respiratory secretions, it was not possible to predict these cases before culture, and the median count was too low in relation to pus. Sputum is also highly contaminated with other bacterial species, some of which could be genetically related to B. pseudomallei. The number of pus samples was small, but this group had the highest median count and has the added advantage that it usually contains B. pseudomallei in purity. This sample may prove suitable for microarray analysis provided sufficient pus can be obtained. Abscesses in the liver and spleen are often multiple and may be of a considerable size; some patients require spleenectomy as a result. Such samples could provide several milliliters of pus; efforts are now underway to obtain this clinical material.

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Authors’ addresses: Vanaporn Wuthiekanun, Direk Limmathurotsakul, Gunphol Wongsuvan, Wirongrong Chierakul, Nicholas P. J. Day, and Sharon J. Peacock, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand. Nittaya Teerawattanasook and Prapat Teparrukkul, Sappasitthiprasong Hospital, Ubon Ratchathani Province, Thailand.

Reprint requests: Sharon Peacock, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand. Telephone: 66-2-354-9172, Fax: 66-2-354-9169, E-mail: sharon@tropmedres.ac.

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Figure 1. Box plot of B. pseudomallei quantitative culture of four specimen types obtained from 414 patients with melioidosis. The vertical line within the box indicates the median, and the border of the box represents the IQR.