Comparison of Diagnostic Laboratory Methods for Identification of *Burkholderia pseudomallei*

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Limited experience and a lack of validated diagnostic reagents make *Burkholderia pseudomallei*, the cause of melioidosis, difficult to recognize in the diagnostic microbiology laboratory. We compared three methods of confirming the identity of presumptive *B. pseudomallei* strains using a collection of *Burkholderia* species drawn from diverse geographic, clinical, and environmental sources. The 95 isolates studied included 71 *B. pseudomallei* and 3 *B. thailandensis* isolates. The API 20NE method identified only 37% of the *B. pseudomallei* isolates. The agglutinating antibody test identified 82% at first the attempt and 90% including results of a repeat test with previously negative isolates. Gas-liquid chromatography analysis of bacterial fatty acid methyl esters (GLC-FAME) identified 98% of the *B. pseudomallei* isolates. The agglutination test produced four false positive results, one *B. cepacia*, one *B. multivorans*, and two *B. thailandensis*. API produced three false positive results, one positive *B. cepacia* and two positive *B. thailandensis*. GLC-FAME analysis was positive for one *B. cepacia* isolate. On the basis of these results, the most robust *B. pseudomallei* discovery pathway combines the previously recommended isolate screening tests (Gram stain, oxidase test, gentamicin and polymyxin susceptibility) with monoclonal antibody agglutination on primary culture, followed by a repeat after 24 h incubation on agglutination-negative isolates and GLC-FAME analysis. Incorporation of PCR-based identification within this schema may improve percentages of recognition further but requires more detailed evaluation.

*Burkholderia pseudomallei*, the cause of melioidosis, can be difficult to reliably identify in the clinical microbiology laboratory. Many diagnostic laboratories have no experience of this species. Even in locations such as Southeast Asia and northern Australia where melioidosis is endemic, a preponderance of septicemic cases during the wet season results in a low expectation of *B. pseudomallei* in clinical specimens at other times of the year (1). Practical difficulties for the diagnostic laboratory include the presence of closely related *Burkholderia* species in specimens from nonsterile sites and atypical colony morphology of some *B. pseudomallei* strains (4). Despite clear recommendations for screening suspect *B. pseudomallei* colonies (3), there is wide variation in the approaches used by diagnostic laboratories. Moreover, a lack of properly validated diagnostic test reagents means that laboratories have to rely on biochemical tests for definitive identification. The substrate utilization test panels in current diagnostic use can generate misleading identification profiles (6).

These problems and the rarity of melioidosis outside of northern Australia and Southeast Asia highlight the need for a more standardized culture-based diagnostic pathway. In the present study we evaluated phenotypic identification methods used in Australia to develop a laboratory case definition of melioidosis. This approach aims to integrate previously recommended screening tests with one or more confirmatory tests to complete the identification of suspect isolates. Some centers have reported the development of an in-house *B. pseudomallei* agglutination test (10, 11, 12). An alternative approach uses bacterial fatty acid methyl ester (FAME) profile analysis by gas-liquid chromatography (GLC) to detect a cellular fatty acid profile that distinguished *B. pseudomallei* from *B. thailandensis* (5). In the current study we compared the performance of a proprietary *B. pseudomallei* monoclonal antibody agglutination test, FAME profile analysis, and a widely used substrate utilization panel (API 20NE) with the PCR-based identification technique used in this center since 1998 to confirm presumptive *B. pseudomallei* identification.

**METHODS AND MATERIALS**

*Bacterial strains.* *Burkholderia* species isolated by the Western Australian Centre for Pathology and Medical Research have been stored in the Western Australian Culture Collection for the last 7 years. The *B. pseudomallei*-specific PCR status of all isolates was confirmed prior to their entry in the *Burkholderia* Culture Collection (BCC). The BCC contains reference strains, other imported *Burkholderia* species, and clinical and environmental isolates. These strains were stored at −80°C in 20% glycerol brain heart infusion broth (Excel Laboratory Products, Bentley, WA) under a unique reference number without other indication of their identity on the storage vial. The strains used in the present study are given in Table 1.

**Safety.** All aerosol-generating procedures were performed in a class II biological safety cabinet by gowned and gloved personnel who were subject to periodic melioidosis serology checks.

**Resuscitation.** Bacteria were resuscitated by subculture onto 5% horse blood agar and incubation for 24 h at 37°C. A single blood agar plate was used for each isolate, and stock cultures were spread to produce single-colony growth in the third or fourth quadrant. A single colony was then subcultured onto 5% horse blood agar and incubated at 37°C for 24 h to produce pure growth for all subsequent identification tests. Plates were labeled with the relevant culture collection reference code and no other identifying mark.

**Identification procedures.** Each of the identification procedures used was performed by a single operator for consistency, without reference to the
culture collection accession list. Results were recorded in laboratory diaries or worksheets, and the completed work was entered into a master list.

**Initial screening investigations.** Preliminary screening investigations were used to screen bacterial isolates as previously recommended (3). Gram stains were performed on a thin-smear preparation of fresh culture emulsified in sterile 0.89% NaCl solution (ELP, Bentley, WA). Crystal violet, Lugol’s iodine, acetone, and dilute carbol fuchsin were used. Gram reaction, bacterial shape, and the presence or absence of intracellular granules were noted. Oxidase tests were performed by spreading a linear smear from the single colony on filter paper impregnated with oxidase reagent. To be considered positive, a strong purple reaction was required in the paper before 10 s had elapsed. *Pseudomonas aeruginosa* was used as a positive control. Gentamicin and colistin susceptibilities were determined by demonstration of a zone of inhibition around a 10-μl disk. Positive-control isolates and all negative isolates were retested after a 24 h incubation. All agglutination reactions were conducted in a class II biological safety cabinet by gowned and gloved staff.

**API 20NE.** Substrate utilization tests were performed using the API 20NE panel (Biomerieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. The suspension of each isolate was subcultured onto a 5% horse blood agar plate on completion of API inoculation, and gentamicin and colistin disks were added to ensure that there had been no contamination. API test panels were incubated in air in a temperature-controlled incubator at 30°C ± 1°C. The first three tests in each panel were read after a timed 24-h incubation period. The assimilation tests were read after a timed 48 h. All tests were scored against the interpretive chart in the instruction sheet using the manufacturer’s interpretive color chart. Results were entered into the current version of the API interpretive software to obtain the final profile. No panel test results were altered to obtain a more meaningful or acceptable identification. Substrate utilization tests were repeated when purity plates showed more than one colony type, suggesting contamination or mixed growth.

**Cellular fatty acid analysis.** FAME profile analysis was performed on the fatty acid methyl ester derivative of bacterial suspensions using a fine capillary column gas chromatograph (MIDI Systems Inc., Wilmington, DE), according to the manufacturer’s instructions, and a previously reported protocol (5). Repeat determinations were performed on all negative results with twice the recommended quantity of bacteria. The method used tryptic soy broth agar (ELP, Bentley, WA). The manufacturer’s interpretive software was used to recognize retention time peaks consistent with 2-hydroxymyristic acid (2HMA).

**B. pseudomallei** PCR. A single colony of *B. pseudomallei* grown on blood agar was resuspended in deionized water, treated with diethylenetriaminepentaacetate to remove nucleases. The suspension was heated at 100°C for 15 min and centrifuged at 9,000 × g to pellet the cell debris. The supernatant was used as the template for all subsequent seminested PCR assays. The PCR primers used for identification were as described previously (7). Briefly, the first-round primers were bp1 (5′-CGATGATGTTGGCGCTT) and bp4 (5′-CGTGTGCGTGATTCAAT), and the seminested second-round primers were bp1 and bp3 (5′-ATTAGAGTCGAACT). The first-round mix consisted of 0.5 units of *Taq* polymerase (Applied Biosystems, Foster City, CA), 2 μl of buffer, 0.2 mM of pooled deoxynucleoside triphosphates, 1.5 mM of MgCl₂, and 0.2 μM of each of primers bp1 and bp4 (product = 302 bp). To this mix 8 μl of template DNA was added, giving a total volume of 20 μl. The second-round mix was identical to the first round but for use of primers bp1 and bp3 (product = 285 bp). To this mix 0.4 μl of first-round product was added, giving a total volume of 20.4 μl. The first-round cycling program consisted of a pre-PCR of 5 min at 94°C to fully denature the template DNA followed by 45 cycles of 30 s at 94°C for denaturation, 30 s at 55°C annealing, and 45 s at 72°C extension. The samples were maintained at 72°C for a further 7 min following the final cycle. After inoculation of first-round product into the second-round tubes, cycling was carried out under the same conditions as for the first round but with a 50°C annealing temperature. Second-round PCR products were demonstrated by ethidium bromide gel electrophoresis on 2.5% agar gels. Digital gel images were captured and optimized for brightness and intensity using a UVIdoc capture system (Cambridge, UK).

**Sequencing.** Sequencing was carried out using the previously mentioned first-round product and primers bp1 and bp4 for *B. pseudomallei* NCTC 13177 (second-round PCR product positive control) and *B. thailandensis BCC 89* (second-round PCR product negative control). The PCR products were treated with presequencing clean-up enzyme (ExoSap-IT USB Corp., Cleveland, Ohio) and then used as the template in a sequencing mix (Applied Biosystems, BigDye terminator v3.1). The now labeled products of the sequencing reaction were then purified using Microcon PCR filters (Amicon Millipore, North Ryde, Australia) and sequenced in an Applied Biosystems 3100 Avant genetic analyzer.

**Statistical methods.** The statistical method and graphing functions used were the Fisher’s exact test and the cumulative trend curves (Prism v2.01; GraphPad, San Diego, CA).

**RESULTS**

**Preliminary screening tests.** The combination of oxidase-positive, gram-negative bacilli with gentamicin and colistin resistance was inclusive of all but one isolate tested, a clinical isolate from Northern Australia which was gentamicin sensi-

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**TABLE 1. Bacteria used in this study**

| Bacterial species | No. of isolates | Features | Location | Source |
|-------------------|-----------------|----------|----------|--------|
| *B. pseudomallei* | 1               | NCTC 13177 | WA       | PathCentre |
|                   | 55              | Clinical  | Australia | PathCentre, RPH, RDH |
|                   | 4               | Veterinary | Australia | Dept. of Agriculture, WA |
|                   | 10              | Environmental | WA | PathCentre, Dept. of Agriculture, WA |
|                   | 1               | Clinical strain | Canada | University of Calgary |
|                   | 71              |           |          |        |
| *B. cepacia*      | 19              | Clinical | WA       | PathCentre |
|                   |                | Environmental |      | PathCentre |
| *B. multivorans*  | 1               | Clinical | WA       | PathCentre |
| *B. thailandensis*| 3               | Environmental | Thailand | Mahidol University |
| *B. vietnamiensis*| 1               | Environmental | United Kingdom | University of Hull |

Total 95

* a RPH, Royal Perth Hospital, Western Australia.
  * b RDH, Royal Darwin Hospital, Northern Territory, Australia.
The Gram stain appearance of bacilli with densely staining ends and a pale middle was common to all isolates tested.

**Monoclonal antibody agglutination test.** The strain used as a positive control (*B. pseudomallei* NCTC 13177) gave clear positive results visible behind safety cabinet glass with 4-μl volumes of reconstituted monoclonal antibody and bacterial suspension. The recommended volumes of 10 μl each produced too large a drop to safely handle on the glass microscope slide. A smaller volume of 2 μl each made the positive result difficult to read. A total of 86% of the *B. pseudomallei* isolates were positive on first agglutination test, and a further 8% were positive after another 24 h of incubation. Overall, 67 (94%) *B. pseudomallei* isolates were positive by agglutination test, and four other *Burkholderia* spp. were agglutination positive (Table 2). There were four persistently negative *B. pseudomallei* isolates: one was a mucoid environmental strain, two were clinical strains from sputa, and one was from a blood culture in three epidemiologically unrelated patients. One *B. cepacia* isolate found to be consistently agglutination positive had other features strongly suggesting incorrect identification by PCR (wrinkled colony surface, 2HMA positive). Repetition of *B. pseudomallei*-specific PCR produced a consistently negative result. The minimum time to completion of an agglutination test result was 5 min, including preparatory stages.

**API 20NE.** Substrate utilization performed according to manufacturer’s instructions indicated the possibility of the presence of *B. pseudomallei* in 37% of the PCR-positive isolates (Table 2). The remainder of proposed identifications for known *B. pseudomallei* were a wide range of non-*Burkholderia* gram-negative bacterial species (Table 3). The rate of correct identification was higher (50%) for *Burkholderia cepacia* isolates. In reactions in the first three tests of the API panel, particularly ADH, were often weak when they were read after the recommended 24 h. Reactions in the assimilation half of the panel were often weak or borderline after the recommended 48-h incubation. Two of the three *B. thailandensis* isolates were identified as *B. pseudomallei* by API (Table 3). One isolate (BCC 20) produced two distinct colony types, both of which were *B. pseudomallei* PCR positive. The corresponding API 20NE result (no identifiable species) was not included in further API analysis. The minimum time to result was 2 days due to the 48-h incubation time required. Of those *B. pseudomallei* isolates not identified by antibody agglutination, none were subsequently identified by API 20NE.

**Cellular fatty acid analysis.** Cellular fatty acid analysis detected the presence of the 2-hydroxymyristic acid marker for *B. pseudomallei* in 70 (98%) PCR-positive isolates and 4 other *Burkholderia* species isolates (Table 2). One negative isolate was the one (noted above) that possessed other features (wrinkled colony surface, agglutination test positive) consistent with *B. pseudomallei*. A total of 4% of the *B. pseudomallei* isolates were 2HMA positive only after a second extraction-analysis cycle using twice the amount of bacteria. The analytical process was lengthy and more suited to processing batches than single isolates. The minimum time to obtain a result was 28 h, including 24 h of incubation on suitable solid medium (tryptic soy agar) and 4 h for extraction, derivatization, and GLC batch analysis. Of those *B. pseudomallei* isolates not identified by antibody agglutination or by API 20NE, six were found to be 2HMA positive.

**Identification tests in combination.** The cumulative performance of the combination of *B. pseudomallei* identification tests was evaluated in this study. The best and most timely approach was the combination of agglutination performed on two consecutive days and FAME-GLC (98%) (Table 4). The addition of the API 20NE result added nothing to the combination of agglutination test and GLC. When performed as the mainstay of identification the API 20NE both delayed the result and required the addition of an agglutination test to compare favorably with the alternative. The first combination of these methods approached the sensitivity of the PCR-based method, and it is notable that both the agglutination test and the FAME profile analysis identified one PCR-negative isolate as a possible *B. pseudomallei* isolate.

### DISCUSSION

Reliable confirmation of a presumptive identification of *B. pseudomallei* requires a combination of the initial recognition that the species might be present, a well-appointed clinical microbiology testing facility, and a high level of scientific skill. Recognition of the possibility of *B. pseudomallei* outside the endemic area or peak septicemic disease season is a challenge conventional laboratory discovery pathways make no allowance for, particularly when the potential *B. pseudomallei* isolate is from a nonsterile body site. A missed identification of *B. pseudomallei* can result in the inadvertent exposure of laboratory personnel (2).
The preliminary identification methods used in this study were chosen to be inclusive of other *Burkholderia* species and closely related bacteria (3) and would have excluded only one isolate housed in our culture collection. Inclusion of polymyxin susceptibility results in the screening process, though not evaluated in this study, may help alert laboratory staff to the need to consider confirmatory identification procedures.

Given the greater gravity of a missed diagnosis of melioidosis, we placed more emphasis on the sensitivity of *B. pseudomallei* identification methods than on their specificity. We proceeded to confirm a presumptive identification of possible *B. pseudomallei* by a more definitive method. The conventional approach has been to use a substrate utilization panel such as the API 20NE, despite mixed reports on its performance (3, 6, 8). After an earlier report questioning the reliability of such test systems (6), the identification data bank appears to have expanded to include API panel results from additional *B. pseudomallei* strains. Some centers continued to use the API 20NE on the understanding that it had been improved or used the API 20NE alongside the API 20E as a reference point for other biochemical identification systems (8). However, the results of this study show that the API 20NE fails to reliably identify *B. pseudomallei* when used precisely according to manufacturer’s instructions to identify an unknown *Burkholderia* sp. isolate from a diverse range of sources.

In our hands, the probability of *B. pseudomallei* being given an incorrect bacterial identity was unacceptably high. We no longer consider the API 20NE to have a major part in preliminary *B. pseudomallei* identification. One study claiming a much higher sensitivity and specificity for the API 20NE used 48-h-old *B. pseudomallei* cultures (8), further extending the time taken to produce a definitive result. The higher specificity of the API 20NE substrate utilization panel suggests that it may be more suited to a supportive role in *B. pseudomallei* identification, once the possibility of the species has already been seriously considered. Modifications could then be made to the manufacturer’s recommended procedure to improve the prospect of an accurate confirmatory result, supported by complementary confirmatory procedures.

By contrast, the rapid agglutination test correctly identified the majority of *B. pseudomallei* isolates. This test was quick to perform, producing a useful positive result on the same day as

### TABLE 3. Comparison of test results

| PCR result       | API20NE result       | Remark or probability of stated API20NE result | No. of isolates |
|------------------|----------------------|-----------------------------------------------|----------------|
| *B. cepacia*     | *B. cepacia*         | Good, very good, or excellent                  | 8              |
| *B. cepacia*     | Acceptable, unacceptable, doubtful | 3                                                      |
| Not *B. cepacia* | Acceptable, unacceptable, doubtful | 4                                                      |
| *Comamonas acidovorans* | Good or better          | 2                                                      |
| *Alcaligenes denitrificans* | Good or better          | 1                                                      |
| *Pseudomonas fluorescens* | Good or better          | 1                                                      |
| *B. pseudomallei* | Presumptive identification | 2b                                                     |
| *B. pseudomallei* | Mentioned as possible result | 24c                                                    |
| *Pseudomonas aeruginosa* | Good or better          | 10d                                                     |
| *Pseudomonas fluorescens* | Good or better          | 3e                                                     |
| *Comamonas testonii* | Good or better          | 3f                                                     |
| No mention of *B. pseudomallei*, other results | 28g                                                    |
| No API result due to mixed PCR positive | 1                                                      |
| *B. multivorans* | *B. cepacia*         | Very good                                       | 1              |
| *B. thailandensis* | *B. pseudomallei*     | Doubtful                                        | 2              |
| *Pseudomonas aeruginosa* | Very good             | 1                                                      |
| *B. vietnamiensis* | Not *B. pseudomallei* or *B. cepacia* | Low discrimination | 1              |
| **Total**        |                      |                                               | 95             |

*a* API20NE profiles for isolates expected to be identified as *B. pseudomallei* are listed in footnotes b through g.

**TABLE 4. Identification test performance**

| Test            | Expected result | MTTPa | Sensitivity (%) | Specificity (%) | Pb   |
|-----------------|-----------------|-------|-----------------|-----------------|------|
| Agglutination   | Positive        | 5 min | 94              | 83              | <0.0001 |
| API20NE         | *B. pseudomallei* | 2.5 days | 37          | 92              | 0.0091 |
| GLC-FAME        | 2-HMA           | 3 days | 98              | 83              | <0.0001 |
| PCR             | Positive        | 1 day | 100             | 100             |      |

*a* MTTP, minimum time to positive (approximate).

*b* Fisher’s exact test; association between *B. pseudomallei* PCR and test outcome.
At present we use a PCR-based method to confirm one or another of the three phenotypic methods compared here, as indicated in the flow diagram (Fig. 1). In future it is possible that the greater reliability, shorter time to a result, simplicity, and lower cost of PCR may make it the preferred confirmatory method for *B. pseudomallei* identification. One method combination came close to the sensitivity but not the specificity of PCR. The observation of one PCR-negative, agglutination-positive, 2HMA-positive isolate indicates a need to approach PCR-based identification of *B. pseudomallei* with caution. Excessive reliance on specific PCR protocols for detection of human pathogens will leave a diagnostic laboratory vulnerable to unpredictable external factors such as genetic variation at the primer binding sites. Further work is needed on the use of PCR-based protocols to identify presumptive *B. pseudomallei* from clinical diagnostic media. As an interim measure, we propose that the *B. pseudomallei* discovery pathway depicted in Fig. 1 be used for identification of referred bacterial isolates that meet the recommended preliminary screening criteria of oxidase-positive, gentamicin- and polymyxin-resistant, Gram-negative bacilli.

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