Slow growth of *Burkholderia pseudomallei* compared to other pathogens in an adapted blood culture system in Phnom Penh, Cambodia

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**Abstract**

**Purpose.** *Burkholderia pseudomallei* is a key pathogen causing bloodstream infections at Sihanouk Hospital Center of Hope, Phnom Penh, Cambodia. Here, visual instead of automated detection of growth of commercial blood culture bottles is done. The present study assessed the performance of this system.

**Methodology.** Blood culture sets, consisting of paired adult aerobic and anaerobic bottles (bioMérieux, FA FAN 259791 and FN FAN 252793) were incubated in a standard incubator for 7 days after reception. Each day, the bottle growth indicator was visually inspected for colour change indicating growth. Blind subculture was performed from the aerobic bottle at day 3.

**Results.** From 2010 to 2015, 11,671 sets representing 10,389 suspected bloodstream infection episodes were documented. In 1058 (10.2%) episodes, pathogens grew; they comprised *Escherichia coli* (31.7%), *Salmonella Paratyphi* A (13.9%), *B. pseudomallei* (8.5%), *Staphylococcus aureus* (7.8%) and *Klebsiella pneumoniae* (7.0%). Blind subculture yielded 72 (4.1%) pathogens, mostly (55/72, 76.4%) *B. pseudomallei*. Cumulative proportions of growth at day 2 were as follows: *E. coli*: 85.0%, *Salmonella Paratyphi* A: 85.0%, *K. pneumoniae*: 76.3% and *S. aureus*: 52.2%; for *B. pseudomallei*, this was only 4.0%, which increased to 70.1% (70/99) at day 4 mainly by detection on blind subculture (55/99). Compared to the anaerobic bottles, aerobic bottles had a higher yield and a shorter time-to-detection, particularly for *B. pseudomallei*.

**Conclusions.** Visual inspection for growth of commercial blood culture bottles in a low-resource setting provided satisfactory yield and time-to-detection. However, *B. pseudomallei* grew slowly and was mainly detected by blind subculture. The aerobic bottle outperformed the anaerobic bottle.

**INTRODUCTION**

Bloodstream infections (BSI) are an important public health concern worldwide, particularly considering the increasing antimicrobial resistance, which disproportionally affects low-resource settings [1]. State-of-the-art diagnosis of BSI relies on the culture of blood into blood culture bottles, which are incubated into automated systems that continuously monitor growth during a 5 day incubation time [2, 3]. In low-resource settings however, such systems are not suited due to high cost of procurement and maintenance and vulnerability to high temperature, humidity, power fluctuations and dust [4]. Clinical laboratories in low-resource settings therefore rely on manual, i.e. equipment-free, often homemade, bottles, which are incubated in a regular incubator and visually assessed for signs of growth such as turbidity, haemolysis or pellicle formation on the broth's surface, but this requires experience from the laboratory staff [4].

In Sihanouk Hospital Centre of Hope (SHCH), Phnom Penh, Cambodia, an alternative system was chosen: BacT/ALERT...
aerobic and anaerobic bottles (bioMérieux, Marcy-L’Etoile, France, product codes FA FAN 259791 and FN FAN 252793) manufactured for an automated system are daily monitored for growth by visual assessment of the chromogenic growth indicator. A so-called blind subculture is performed after 3 nights of incubation of all aerobic bottles appearing negative [5]. Blood cultures were implemented in SHCH in 2007, and data have been recorded in a laboratory information system since July 2010. At implementation, the system of visual inspection of BacT/ALERT bottles was validated by terminal (i.e. at the end of the 7 day incubation) subculture of a 5% subset of bottles. In this validation, 3172 bottles were sampled at the end of the incubation period. Only 7 of these bottles (0.2%) showed growth of a clinically significant organism. A similar approach (visual detection of growth in BacT/ALERT bottles) has also been described by Andrews et al. in 2013 for the diagnosis of typhoid fever, showing satisfactory results when compared to automated blood culture [6].

To evaluate and optimize this system, retrospective data (2010–2015) were compiled and analysed to determine (i) the yield of the blood culture system, (ii) time-to-detection of pathogens according to aerobic versus anaerobic bottle and (iii) the yield of te blind subculture.

METHODS

Study site

SHCH is a 30-bed non-governmental organization hospital for adults providing healthcare services at a limited cost. In 2016, care was given to 30 500 outpatients and 800 hospitalized patients. Since 2007, microbiological surveillance is conducted by collection of blood cultures in patients presenting with presumed BSI according to criteria previously described [7].

Study design

The performance of the blood culture system was retrospectively assessed for the period July 2010 to December 2015. Basic patient demographic and clinical information, including use of antibiotics in the 14 days before sampling, as well as detailed microbiological data (e.g. day of growth per bottle type) were extracted from the laboratory information system (Structured Query Language, SQL) into Excel (Microsoft Corporation, Redmond, WA, USA). Incomplete and doubtful results were verified with the laboratory notebooks. Only paired aerobic-anaerobic bottles were considered; solitary and homemade bottles were excluded from analysis. For definitions and criteria, see Table 1.

| Definitions | Explanation |
|-------------|-------------|
| Blood culture set | One blood culture set in adult patients consisted of one aerobic and one anaerobic BacT/ALERT bottle. In some patients, additional blood culture set(s) were sampled, see definition of a BSI episode. |
| Solitary bottle | Only one bottle instead of two bottles collected in a blood culture set |
| Suspected Bloodstream infection (BSI) episode | A suspected BSI episode was defined as a 14 day interval since the first sample unless growth (see below). |
| Culture-confirmed BSI episode | A BSI episode was defined as [1] the initial recovery of a pathogen [2], the recovery of a pathogen different from the initial pathogen ≥48 h after the recovery of the initial pathogen, or [3] the recovery of the same pathogen after at least a 14 day interval since the previous grown culture with this pathogen [8]. |
| Blind subculture (BS) | A subculture performed in the absence of any visual signs of growth (in this case, change in colour of the growth indicator). |
| Rate of contaminants | Skin and environmental bacteria (coagulase-negative staphylococci, Corynebacterium species, Cutibacterium (former Propionibacterium) acnes and Bacillus species) were categorized as blood culture contaminants [9]. The rate of contaminants was defined as the number of bottles grown with contaminants divided by the total number of bottles collected (as each bottle was sampled by a separate venipuncture) and expressed as a percentage. |
| Yield of pathogens | The yield or growth rate of pathogens was defined as the number of BSI grown with pathogens divided by the total number of suspected BSI episodes and expressed as a percentage. |
| Volume of blood sampled in blood culture bottles | A correct blood volume sampled in adult blood culture bottles was considered 8–12 ml (adequately filled). Bottles with less than 8 ml of blood or more than 12 ml of blood inoculated were considered respectively as underfilled or overfilled. |
| Day of incubation | Days of incubation were mentioned to indicate for instance the time-to-detection. They were defined as follows: day 0=reception in the laboratory day 1=after 1 night of incubation day 2=after 2 nights of incubation day 3=..... |
| Community-acquired and healthcare-associated BSI episodes | Community-acquired and healthcare-associated BSI were defined according to the day of sampling, i.e. at ≤2 days and >2 days of hospital admission respectively [10]. |
Blood culture methods and processing
Blood cultures consisting of 2×10 ml of blood were sampled from separate venipunctures in paired aerobic (FA FAN) and anaerobic (FN FAN) BacT/ALERT bottles and were incubated at 35 °C for 7 days. Inspection for colour change of the chromogenic growth indicator at the bottom of the bottle was performed once daily. Of the bottles with detected growth, a subculture was made on specific culture media according to the Gram-stain results. Colonies were identified with conventional phenotypical tests [7, 8]. At day 3, blind subculture on chocolate agar (Oxoid, Waltham, MA, USA) was performed for all aerobic bottles appearing negative.

Blood volume sampled
At reception in the laboratory, bottles were weighed and the volume of blood inoculated was calculated by subtracting the average empty weight of the bottle (measured average anaerobic bottle=69.55 g ±0.16, measured average aerobic bottle=59.16 g ±0.31) and next dividing the result by the density of blood (=1.06 g ml⁻¹ [9]). Bottles sampled from children less than 15 years old (n=162 bottles) were not included in the analysis of the blood culture volume.

Statistical analysis
Numbers of sets and BSI episodes were calculated using R (R Foundation for Statistical Computing, Vienna, Austria) and statistical analysis was done with the Vassarstats software (http://vassarstats.net/). Differences in proportions and median values were assessed for statistical significance using chi-square analysis and the Wilcoxon rank-sum test respectively.

RESULTS
A total of 11 671 sets from 10 389 suspected BSI episodes were sampled (Fig. 1). They were obtained from 8717 patients with median age 48 years (0–101 years), including 77 (0.9 %) children (<15 years old); 56.8 % were women. Most (90.9 %) suspected BSI episodes were community-acquired (Table 2).

In 10.2 % of suspected BSI episodes, a pathogen was detected; with E. coli, Salmonella Paratyphi A and B. pseudomallei ranking first, second and third in frequency (Fig. 1). Blind subculture yielded 117 isolates, of which 38.5 % (45/117) contaminants and 61.5 % (72/117) pathogens, representing

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**Fig. 1.** Breakdown of blood cultures sampled as part of the surveillance study (2010–2015). BCB: blood culture bottle, BSI: bloodstream infection. *From 25 and 2 BSI episodes, two and three different pathogens were retrieved respectively within a ≤48 hours’ delay between the sampling of the different blood culture sets.*
4.1 % (72/1768) of all pathogens recovered. *B. pseudomallei* accounted for the majority [76.4 % (55/72)] of pathogens on blind subculture; 55.6 % (55/99) of sets grown with *B. pseudomallei* were detected by blind subculture.

The contamination rate expressed per bottle, set and BSI episode was 2.9 % (688/23 342), 5.6 % (655/11 671) and 6.3 % (655/10 389) respectively (Table 3). Contaminants were most often recovered from the aerobic bottle [4.9 % (569/11 671)], versus 1.0 % (120/11 671) in the anaerobic bottle (*P*<0.001). In only 27 (6.2 %) out of 434 blood culture sets contaminated with coagulase-negative staphylococci, growth was observed in both bottles.

Table 2. Demographic data of the patients with suspected and confirmed BSI episodes. Data represent number (%) unless otherwise stated

|                      | All suspected BSI episodes | Culture confirmed BSI episodes |
|----------------------|---------------------------|--------------------------------|
| No. of patients      | 8717                      | 847                            |
| Female               | 4955 (56.8 %)             | 478 (56.4 %)                   |
| Median age in years (range) | 48 (0–101)               | 50 (5–90)                      |
| Total no. of BSI episodes | 10 389                  | 1058                           |
| Healthcare versus community | Community-acquired         | 8528 (92.9 %)                 |
|                      | Healthcare-associated     | 647 (7.1 %)                    |
|                      | No data                   | 1214                           |
| Antibiotic treatment | Recent*                   | 4800 (47.3 %)                  |
|                      | None                      | 5347 (52.7 %)                  |
|                      | Unknown                   | 242                            |

*refers to administration of antibiotics within 2 weeks prior to sampling.

Time-to-detection was shortest for *E. coli*, with cumulative growth at day 1, 2 and 3 of 54.8, 85.0 and 91.8 % among 341 blood culture sets respectively. Other pathogens grew slower (Fig. 2): at day 2, proportions of cumulative growth were 85.1 % (126/148) for *Salmonella Paratyphi A*, 76.3 % (58/76) for *K. pneumoniae* and 52.2 % (47/90) for *S. aureus*. Time-to-detection of *B. pseudomallei* was slow, with cumulative growth of 4.0 and 18.2 % at day 2 and 3 respectively and 88.9 % (88/99) at day 4, of which 55 sets detected by blind subculture. Other aerobic, non-fermenting organisms such as *Pseudomonas* sp. and *Acinetobacter* sp., although few in numbers, tended to grow faster than *B. pseudomallei* [cumulative growth

Table 3. Numbers of pathogens and contaminants grown according to bottle type and numbers of blood culture sets. For each blood culture set, the first bottle (aerobic or anaerobic) that showed growth is displayed. Growth on BS is considered separately

| Pathogen                        | Aerobic bottle only | Anaerobic bottle only | Both bottles | Total no. of sets with growth |
|---------------------------------|---------------------|-----------------------|--------------|------------------------------|
| *Escherichia coli*              | 85+0                | 33                    | 223+0        | 341                          |
| *Salmonella Paratyphi A*        | 17+1                | 8                     | 122+0        | 148                          |
| *Burkholderia pseudomallei*     | 21+49               | 1*                    | 22+6         | 99                           |
| *Staphylococcus aureus*         | 28+2                | 14                    | 46+0         | 90                           |
| *Klebsiella pneumoniae*         | 16+2                | 6                     | 52+0         | 76                           |
| Anaerobic organisms             | 0                   | 34                    | 0            | 34                           |
| All pathogens                   | 267+66              | 135                   | 644+6        | 1118                         |
| Coagulase negative staphylococci| 325+37              | 45                    | 27+0         | 434                          |
| *Bacillus* spp.                 | 157+8               | 41                    | 7+0          | 213                          |
| *Corynebacterium* species       | 8                   | 0                     | 0+0          | 8                            |
| All contaminants                | 490+45              | 86                    | 34+0         | 655                          |

*The complementary aerobic bottle was overgrown by a contaminant (*Bacillus* sp.).
on day 3 was 18% (18/99) for *B. pseudomallei*, compared to 60% (9/15) for *Acinetobacter* sp. (*P*=0.001) and 36.4% (8/22) for *Pseudomonas* sp. (*P*=0.11). Excluding *B. pseudomallei*, 70.6% (719/1019) of blood culture sets showed growth at day 2 of incubation.

The aerobic bottle outperformed the anaerobic bottle in both yield and time-to-detection. For nearly 60% of blood culture sets (650/1118, 58.1%), growth occurred in both bottles; among sets with growth in only one bottle (*n*=468), the aerobic bottle outnumbered the anaerobic bottle for all pathogens except for the strict anaerobes (Table 3). Strict anaerobic organisms were found in only 34 blood culture sets (3.0% of total number of grown sets).

*B. pseudomallei* grew from both bottles or from the aerobic bottle only (28.3 and 70.7% of 99 grown sets). For all pathogens excluding *B. pseudomallei*, growth was simultaneously detected in both bottles for 55.0% (560/1019) of blood culture sets. When there was a difference in time-to-detection between the aerobic and the anaerobic bottle, it was mostly the aerobic bottle that grew first (*n*=45, 73.8%). For *B. pseudomallei*, first growth was consistently observed in the aerobic bottle, although mostly on the account of the blind subculture (Fig. 2).

The median (IQR) volume of blood sampled per bottle was 8.3 ml (7.0–9.3 ml) and more than one third of bottles was underfilled. Although significant, differences between aerobic and anaerobic bottles were small (0.3 ml, Table 4). Yield was similar between adequately filled (7.3%, 1009/13 778) and underfilled and overfilled bottles [7.8% (690/8892) and 6.8% (32/468) respectively (*P*=0.41).

Antibiotic treatment prior to blood collection was noted in 4800 suspected BSI episodes (47.3%), proportions ranged from 40.1% (59/141) for *S. Paratyphi A* to 68.9% (62/90) for *B. pseudomallei* respectively (*P*<0.001). Growth rates among suspected BSI episodes with and without prior antibiotic use were 9.5% (455/4800) versus 10.7% (571/5347), *P*=0.045.

**DISCUSSION**

The 5 years retrospective analysis of the adapted blood culture system in a low-resource setting provided satisfactory yield and time-to-detection for most key pathogens. However,
**Table 4.** Median volume of blood sampled per type of bottle (aerobic and anaerobic) and proportions of bottles adequately filled, underfilled and overfilled for 23,138 bottles for which data were available. IQR=interquartile range. The P-value expresses significance between the aerobic and anaerobic bottles.

|                     | Median volume (IQR) | Adequately filled n (%) | Underfilled n (%) | Overfilled n (%) |
|---------------------|---------------------|-------------------------|-------------------|-----------------|
| Aerobic bottles     | 8.3 (7.4–9.3)       | 7104 (61.3 %)           | 4153 (35.9 %)     | 323 (2.8 %)     |
| (n=11,580)          |                     |                         |                   |                 |
| Anaerobic bottles   | 8.0 (7.0–9.0)       | 6674 (57.7 %)           | 4739 (41.0 %)     | 145 (1.3 %)     |
| (n=11,558)          |                     |                         |                   |                 |
| All bottles         | 8.3 (7.0–9.3)       | 13,778 (59.5 %)         | 8,892 (38.4 %)    | 468 (2.0 %)     |
| (n=23,138)          |                     |                         |                   |                 |
| P-value             | <0.001              | <0.001                  | <0.001            | <0.001          |

*B. pseudomallei* grew slowly and its detection was mainly dependent on blind subculture. The aerobic bottle outperformed the anaerobic bottle in yield and time-to-detection, particularly for *B. pseudomallei*.

*B. pseudomallei*, a Gram-negative soil-dwelling bacterium and the causative agent of melioidosis [10], is endemic to southeast Asia and northern Australia [11]. It is one of the key pathogens in SHCH [7]. The case fatality rate of BSI infections caused by *B. pseudomallei* in SHCH is still high but is decreasing over the recent years (53 % in 2012 to 24 % in 2014) [7, 12]. Because of this high mortality rate, and the since empiric treatment differs for melioidosis compared to other common causes of sepsis [13], accurate and fast detection of *B. pseudomallei* is of utmost importance. Laboratories still rely on blood culture as the gold standard for detection of *B. pseudomallei*, despite its moderate sensitivity (60 %) [14], since no validated *in-vitro* diagnostic tests are available to detect melioidosis in the acute phase on direct specimen [15].

The pathogen yield of the blood culture system analysed (10.2 % expressed per BSI episodes) was within the expected 6–12 % range [5]. This was achieved by joined trainings of all involved staff as well as by consistent support of the SHCH management– which are both pivotal to successful implementation [16]. In addition, consumables and equipment were provided by project funding so that blood cultures were free of charge or at limited cost for the patients, precluding biases. Of note, this overall yield was achieved in a patient population of whom nearly half were on antibiotics upon presentation and yields among those on antibiotics was significantly lower than those with no recent antibiotic treatment. However, still 9.5 % growth was recorded among patients under antibiotics at the time of administration. This might be explained by the antibiotic-binding properties of the charcoal in the bottles [13].

The time-to-detection of pathogens, excluding *B. pseudomallei*, is in line with those previously reported for manual blood culture systems [17, 18], but obviously longer than provided by automated systems (89 % of growth within 24 h) [19]. Its long time-to-detection contrasts with a previous study from Thailand showing a mean (±sd) time-to-detection of 23.9 h±14.9 h and a cumulative growth of 93.1 % at day 2 of incubation for the BacT/ALERT automated system [20]. Differences with our study may be explained by agitation of the bottles in the BacT/ALERT equipment, known to increase speed of growth [21]. The effect of automated versus visual monitoring could further explain the differences seen. In a recent study from Thailand, detection of *B. pseudomallei* was significantly higher in homemade bottles compared to automated BacT/ALERT bottles, suggesting suboptimal growth of *B. pseudomallei* in BacT/ALERT bottles. However, the BacT/ALERT bottles were significantly faster in detection of growth [22]. The authors hypothesize that the nutrient composition of the blood culture medium may be a factor influencing the growth of *B. pseudomallei* but refer to the need of further studies to confirm this.

The aerobic bottles showed better performance than anaerobic bottles, both in terms of yield and of speed of growth. Of 1118 positive culture sets, only 3.0 % strictly anaerobic pathogens grew and were only detected in the anaerobic bottle, which suggests that growth of these pathogens is as good or better in the aerobic than the anaerobic bottle. These results incited to replace the anaerobic botte by a second aerobic bottle. This decision was further supported by the fact that work-up of anaerobic organisms is difficult in low-resource settings [23]. In addition, their antibiotic susceptibility patterns are often predictable, and the presence of anaerobic infections can often be derived from the clinical picture [24].

The overall contamination rate (2.9 %) in the present study was below the 3 % norm [5] and within the 0.6 to 6 % real-life estimate in high-resource settings [25]. A higher recovery of coagulase-negative Staphylococci from the aerobic bottle has been shown before [26], but many blood culture sets might *de facto* have been sampled from a single venepuncture (instead of two), with the aerobic bottle sampled first, thereby capturing the skin contaminants.

Blind subculture yielded few additional pathogens (6.1 %) at the cost of many contaminants, a known drawback of the procedure [27]. However, it was the first sign of growth of
more than half of the cultures grown with \textit{B. pseudomallei}. In the present study, blind subculture was done at day 3 as a back-up for growth missed by visual inspection [5, 28] and as used previously for detection of melioidosis by a manual blood culture system [20].

Among the limitations of the present study, there are the retrospective nature of the study and the few clinical data available (precluding, for instance, to link time-to-detection to clinical presentation in the case of \textit{B. pseudomallei}). In addition, terminal subculture was not systematically performed during the study period and stock ruptures requiring use of homemade bottles occurred during several weeks. A particular problem was the reluctance of patients and healthcare workers to sample high volumes of blood [4] and which may entail a lower yield (only 65–70 % compared to recommended blood culture volumes) [29]. Further, the present study focused on adults and did not assess the spectrum of pathogens and blood culture performance in children. As to the strengths, there are the large sample size with consistent sampling over the years as well as the free-of-charge system, bypassing any selection bias, e.g. towards financially capable patients or delayed sampling. Furthermore, the stringent daily recording of laboratory data in a paper logbook allowed for double-check and completion of data.

In conclusion, this study describes the successful implementation of a blood culture system in a low-resource setting. Apart from demonstrating the feasibility of equipment-free visual assessment of a commercial blood culture bottle, the study also demonstrated strengths and weaknesses of this blood culture system. It pointed to low-cost improvements, which are currently considered such as (i) replacement of the anaerobic bottle by a second aerobic bottle, with the expectation to increase yield in particular of \textit{B. pseudomallei}; (ii) advancing the day of blind subculture to shorten time-to-detection of \textit{B. pseudomallei} and (iii) increasing the frequency of blood culture bottle inspection during the first days of incubation in order to decrease time-to-detection. Finally, this study demonstrates that with close follow-up and training of dedicated clinicians and nursing staff, performance of a blood culture system in a low-resource setting can be monitored with satisfying results.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Ethical approval was granted as part of the study: ‘Surveillance of antimicrobial resistance among consecutive blood culture isolates in tropical settings, V3.0’ [IRB ITM 613/08, NECHR original protocol 009 and subsequent amendments 021, 0313 and 020]. Access requests for ITM research data can be made to ITM’s central point for research data access by means of submitting the completed Data Access Request Form. These requests will be reviewed for approval by ITMs Data Access Committee (https://www.itg.be/E/data-sharing-open-access).

Laboratory data were analysed retrospectively using a coded database. Patient information was coded with restricted access to the code key.

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Author contributions
Marjan Peeters: methodology, formal analysis, data curation, visualization, writing original draft. Sien Ombelet: methodology, formal analysis, data curation, writing review and editing. Panha Chung: resources, data curation. Achilles Tsoumanis: methodology, validation, formal analysis. Kruy Lim: resources. Leng Long: resources. Birgit De Smet: resources, writing review and editing. Chum Kham: resources. Syna Teav: resources, supervision. Erika Vlieghe: writing review and editing. Thong Phe: resources, supervision. Jan Jacobs: conceptualization, supervision, writing review and editing.
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