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Performance Comparison of Francis Media with Other Methods in the Identification of *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is the causative agent for melioidosis, severe infection that is associated with high morbidity and mortality. Difficulties in laboratory diagnosis of melioidosis may delay the treatment and as a result, affect disease outcomes. Culture is the mainstay for laboratory diagnosis of the disease followed by further commercial biochemical testing for identification of organism. The whole process may take up to 72 hours. Furthermore, commercial identification system may fail to distinguish between *B. pseudomallei* and closely related species like *B. cepacia*. The objective of this study was to compare the efficacy of Francis media against commercial identification system such as API20NE and VITEK2GN for the identification of *B. pseudomallei*. This study showed that Francis media was 100% reliable in the identification of *B. pseudomallei* as compared to others. The media also offered fast identification of organism from clinical specimen with total incubation time required of only 18 hours. API20NE was able to correctly identify only 5 (12.5%) within 24 hours and 31 (78%) within 48 hours of incubation. VITEK2GN, identified 95% of the isolates correctly within 9 hours of incubation. However, both methods required an additional sub culturing prior to testing. Molecular method was used as the confirmatory method for identification of the isolate.

Keywords: Melioidosis; *B. pseudomallei*; Francis media

Introduction

Burkholderia pseudomallei is a facultative gram negative intracellular bacterium that is widely distributed in soil and stagnant waters. It causes melioidosis. The disease is prevalent in Southeast Asia, Northern Australia and South America in particularly Brazil [1-3]. In South East Asia, the endemic places includes paddy plantation areas. It is estimated to be 165,000 (95% credible interval 68,000-412,000) human melioidosis cases per year worldwide, of which 89,000 (36,000-227,000) die [4].

In Malaysia, a retrospective review of culture confirmed meliodosis from year 2005 to 2006 (by Medical Department, International Islamic University Malaysia) revealed incidence of 6.0 per 100,000 population per year of adult melioidosis in Pahang [5]. Another retrospective analysis of 145 confirmed cases extracted from a hospital-based Melioidosis Registry set up from 2005 to 2008 in Hospital Sultanah Bahiyah, Alor Setar, Kedah, showed remarkably high incidence at 16.35 per 100,000 population per year [6]. A profiling study involving five states hospitals in the state of Perak, from Aug 2013 till December 2014, successfully recorded 70 culture positive cases of melioidosis [7].

Various methods such as culture, serology and molecular detection are available for the diagnosis of melioidosis. However, culture remains the gold standard method for the diagnosis of *B. pseudomallei* infection. Various differential media such as Ashdown's selective agar (ASA) [8] and *Burkholderia pseudomallei* selective agar (BPSA) are being used to facilitate the selective isolation of this organism in the culturing process. The organism growing in these media produce differential features which help in the selective isolation especially of organisms from non-sterile sites. The distinctive purple, dry and wrinkled colony may be the characteristic morphology produced by the ASA. However, the agar is said to require prolong incubation time of at least 48 hours, sometimes up to 96 hours to produce sufficient growth [9]. The presence of crystal violet and gentamicin as selective agents to suppress the growth of other bacteria in the agar also produce slightly inhibitory effect on the growth of *B. pseudomallei*. Furthermore, ASA is unable to differentiate *B. pseudomallei* from *B. cepacia* [10]. BPSA is also able to support the growth of *B. pseudomallei*, however the agar is significantly less selective than ASA, producing mucoid colonies for *B. pseudomallei and Bukholderia cepacia*.

Materials and Methods

Sample selection

Forty confirmed *Bukholderia pseudomallei* isolates were selected for this study. The organisms were isolated from various clinical samples comprising of 33 from blood samples, 3 tissues sample, 2 knee aspirate fluid and tracheal and 2 pus samples. All blood samples were inoculated into BACTEC bottles and incubated in BACTEC 9240 Instrument Blood Culture System (Fluorescent series, Beckton Dickinson). Positive bottle from BACTEC were then cultured on Blood-sheep agar, Mac Conkey agar, ASA and Francis media and incubated for 24 hours at 37°C. All other samples were similarly sub-cultured on Blood-sheep agar, Mac Conkey agar, ASA and Francis Media. Plates were incubated at 37°C for 24 hours.

Identification procedures

After 18 to 24 hours incubation Francis media was observed for yellow haze along the 1st and 2nd streaking area. Single colonies extending from this streaking zone producing yellow haze were picked and further identify using API20NE (Biomerieux, France) brand, country of origin) and VITEX2GN (Biomerieux, France). Mixed colonies were first purified before proceeding with biochemical testing using similar methods.

API 20NE: The API 20NE panel consists of 20 microtubes containing dehydrated substrates utilization test. Inoculum suspension was prepared by picking up 1-4 colonies of identical morphology from the agar plate and emulsified into 2 ml API NaCl 0.85% medium.

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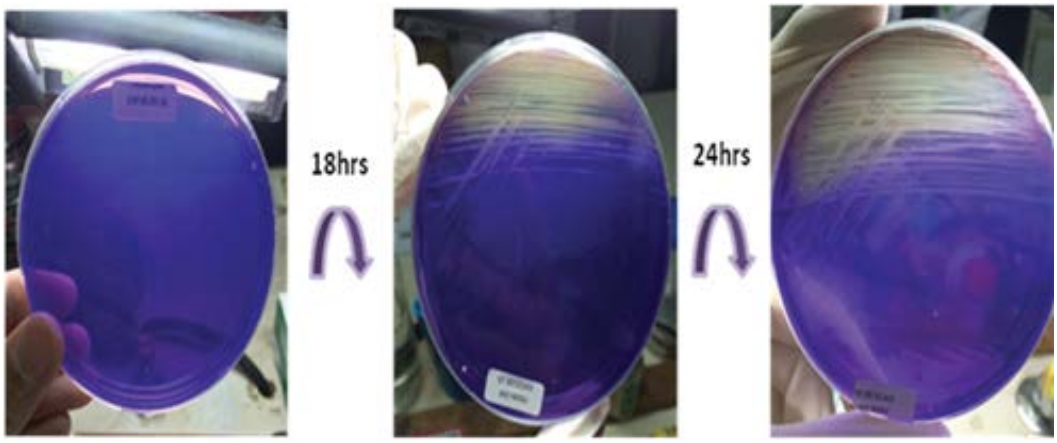

Figure 1: *B. pseudomallei* on Francis media showing yellow haze at primary streaking 18 hours and 24 hours.

Turbidity of the suspension was measured at equivalent reading of 0.5 McFarland. Inoculated test were incubated at 29°C ± 2°C for 24 hours (± 2 hours), and the suspension of each isolate was cultured onto 5% horse blood agar to check for purity of the culture used. The reactions of assimilation test were read after 24 and 48 hours using interpretive color chart and by giving score accordingly. Final profile from scored result was entered API interpretive software to get the most likely identification of the organism.

VITEK2GN: The reagent card for Vitek2GN consist 64 wells of individual test substrate that measured various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, and growth in the presence of inhibitory substances. Enough colonies from a pure culture plate was transferred into 3.0 mL sterile saline by using sterile applicator stick or swab. Suspension turbidity was adjusted to 0.5-0.63 McFarland. The inoculum test tube and reagent card were placed in vacuumed chamber in the vitek machine whereby suspension prepared will be injected into the test cassette. Following incubation period of 6 to 9 hours the results were recorded.

PCR and sequencing *B. pseudomallei*: Conventional PCR using a specific primer pair was performed to confirm identification of the organism. Total DNA from the cultured bacterial were prepared using crude boiling method and used as a template for PCR. In brief, five colonies of an overnight culture *B. pseudomallei* isolates were suspended in 100 µL of DNAse free distilled water. The suspension were heated at 90°C for 10 minute and later centrifuged at 15,000 × g for 15 minutes. The supernatant was discarded, and the pellet was resuspended in molecular biology-grade water (Eppendorf, Hamburg, Germany) and centrifuged again at 15,000 × g for 10 min. The pellet is used as a template for PCR. The isolate was amplified using Specific PCR primers PPM3 forward primers at position 452 to 472 (5' AATCATTCTGGCTAATACCCG 3') and PPM4 reverse primers at position 1023 to 1042 (5'CGGTTCTCTTTCGAGCTCG 3'). The primer amplify selected area from 16s rRNA region of *B. pseudomallei* [11]. Two microliters of total DNA was subjected to PCR amplification in a 50-µL reaction mixture. PCR reaction mix consisted of 1x PCR buffer, 10µmol/L of each primer and 10µM of Hot start Taq polymerase (Bioline, France) using appendorf thermocycler (Biomerieux, France). The cycling conditions were: initial DNA release and denaturation at 94°C for 30s, 52°C for 40s and 72°C for 50s followed by a single, final elongation step at 72°C for 5 minutes. PCR amplification product were analyzed by electrophoresis in a 5% agarose gel at 100 V for 45 minutes in 1 × TAE (40 mmol/LTris-HCl [pH8.3], 2 mmol/L acetat, 1 mmol/L EDTA) containing 0.05 mg/L red safe dye. The image is visualized using geldoc imager. Oligonucleotides primers were synthesized by an Applied Biosystems Step One 1 Plus DNA synthesizer. Sequencing process of the PCR product were outsourcing to First BASE Laboratories Sdn Bhd (Kuala Lumpur).

Results

All 40 isolates were confirmed as *B. pseudomallei* by PCR amplification. Sequencing results of the PCR products proved that all the isolates were 100% identical to *B. pseudomallei* nucleotide sequences.

Positive growth of *B. pseudomallei* culture on Francis media was determined by the ability of the organism to grow along with the production of with yellow haze (Figure 1). All 40 isolates tested produced the characteristic feature by 18 hours of incubation. After 24 hours incubation the haze has extended to secondary streaking area prominently. On Mac Conkey (Thermo Fisher Scientific Inc, USA) agar *B. pseudomallei* colonies produced pinkish growth and on blood agar (Thermo Fisher Scientific Inc, USA), a grayish colony with metallic sheen were observed after 24 hours incubation. The organism produced a purple color and dry and wrinkled colonies after 24 to 48 h of incubation on ASA.

The API20NE correctly identified 12.5% *B. pseudomallei* isolates within 24 hours and 82.5% within 48 hrs respectively. Profile numbers for API20NE as showed in Table 1. Four isolates were wrongly identified as *Burkholderia cepacian* (n=2), *Pseudomonas aeruginosa* (n=1) and *Pseudomonas fluorescents* (n=1) respectively. There was significant variation in API20NE results from 12.5% within 24 hours to 90.0% when incubation time extended to 48 hours.

Vitek2GN successfully identified 95% of *B. pseudomallei* within 6 to 9 hours of reaction from purified culture. However, using this method, two isolates (S24 and S37) were misidentified as *Bukholderia cepacia* (Table 2).

Comparison was made with all the three methods. Francis media was 100% reliable in the identification of *B. pseudomallei*, and hence, proved to be more superior to the other commercial methods for the purpose. The performance is comparable to the molecular method used for isolate conformation (Table 3).

API 20NE	Profile	%, T	Sample (n)	Identity>80%
Very good ID	1056574	81.7%, 0.46	2 (S3, S4)	12.5%
	1056576	81.7%, 0.46	1 (S9)	
Doubtful ID	1152576	99.4%, 0.38	1 (S12)	
Low discrimination	1154577	82.5%, 0.72	1 (S30)	
Low discrimination	1056574	57.3%, 0.27	4	78%
	054556	73.0%, 0.17	10	
	1056556	38.3%, 0.33	6	
	1052464	81.5%, 0.3	8	
Doubtful ID	4156577	77%, 0.45	3	
Very good ID	505777	99.9%, 0.64	1 (S37)	10%
	105755	99.6%, 0.8	1 (S23)	
Good ID	1054554	96.1%. 0.46	1 (S24)	
Acceptable ID	1056554	87.7%, 0.45	1 (S35)	

Table 1: API20NE profile number and identification results.

Vitek2GN	Sample (n)	Identity>90%
Excellent ID (96-99%)	33	82.5%
Very good ID (94-95%)	3	7.5%
Good ID (91-92%)	2	5%
Missed ID	2 (S24, S37)	5%

Table 2: Vitek2GN results.

Test	Result	Minimum time detection positive culture	Correct Identification (%)
Francis Media	Yellow haze	18-24 hours	100%
API 20NE	*B. pseudomallei*	48-72 hours	90%
Vitek2GN	*B. pseudomallei*	30-33 hours	95%
Molecular method	*B. pseudomallei*	27 hours	100%

Table 3: Comparison of sensitivity and specificity of *B. pseudomallei* identification.

Discussion

South East Asia is regarded as an endemic region for *B. pseudomallei* infection. Malaysia is one of the countries with high incidence rate as reported earlier hence clinicians managing the patient need to have high index of clinical suspicion when faced with the wide clinical spectrum that the infection manifest.

Transmission in humans occur through contact of skin lesions with soil or water containing the organism, inhalation of soil or water particles contaminated with the organism, resulting in a wide spectrum of clinical manifestations. The clinical disease spectrum ranges from subclinical infection or localized infection such as abscess, granuloma, pneumonia, meningoencephalitis, sepsis, chronic suppurative infection and disseminated fatal septicaemia due to septic shock [12]. Nevertheless, melioidosis in humans usually infects the lungs and causes abscess or pus cavity.

Being an intracellular organism, it is also capable of causing persistent infections in its human host. Due to incompletely clearance of the bacteria by human immune system, it is capable of causing recrudesce months or years after the initial infection [13]. Risk factors like diabetes mellitus, alcoholism, renal disease, chronic liver disease, steroid therapy and malignancy are considered responsible for reactivation and disease progression.

The gold standard diagnosis for melioidosis is culture. Therefore, the ability of laboratory services in hospitals in Malaysia to offer the test is very crucial. Although the incidence of melioidosis is high among the population, some laboratories personnel still do not have enough experience in culturing and identifying the organism making the possibility of misidentification of the isolates high. Lack of commercial kits in those laboratories further enhanced the problem. Furthermore, molecular identification is only offered by reference laboratory. Therefore, availability of Francis Media that acts as a reliable medium to support the growth and identify *B. pseudomellei* was very timely. Francis media was able to provide a preliminary diagnosis within 18-24 hours due to its unique differential and selective properties by production of yellow haze on the primary streaking of the plates. The unique yellow haze characteristic distinctly differentiates *B. pseudomallei* from *B. cepacia* and another non-fermenter [14]. Minimum detection time of 18 hours was very useful compared to commonly used ASA that require longer incubation time to produce enough growth on the agar. The use of other routine selective agar such as Mac Conkey also required an additional 24 to 48 hours for further identification process using biochemical tests as the agar alone does not definitively identify the organism.

A study by from Mindy et al., showed that *B. pseudomallei* was correctly identified in only 60% of the cases after 48 hours incubation by API20NE and the remaining cases was mainly misidentified as other non-fermenters [15]. Our study showed similar findings. When the incubation time of API20NE was prolonged to 48hrs, 33 isolates were correctly identified as *B. pseudomallei*. Vitek2GN showed a higher detection rate at 95% of *B. pseudomallei* identification within 6-9 hours from the inoculation of pure growth culture. In cases of mixed cultures obtained from non-sterile sites, identification could be further delayed for another 24 hours as pure culture need to be obtained first. Vitek2GN has more than 60 reaction substrates which offers a clear advantage for the detection of *B. pseudomallei* identification when compared to API20NE, hence making it more sensitive in the identification of *B. pseudomallei*. Two of the isolates which were not identified as *B. pseudomallei* on API20NE nor on VITEK20NE had shown similar morphology producing significant yellow haze on Francis media and was confirmed as *B. pseudomallei* by molecular method. Genome sequencing is a useful, alternative technique that may be used by laboratories for identification of bacteria [16]. However, the service was limited to certain laboratories only.

Conclusion

This study showed that Francis media was a useful medium that was able to provide fast presumptive identification of *B. pseudomallei*, hence shortened the time required by the laboratories in identifying the organism. The medium had similar performance as molecular method in the identification of the targeted organism. Although further study using a bigger sample size may be required to further validate our finding as well as to assess the sensitivity and specificity of the media. Nevertheless, the agar is proven useful especially for laboratory that lack additional commercial biochemical identification kit. The early detection and identification of *B. pseudomallei* using Francis media will significantly give positive impact on clinical management of patients.

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