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Modified MacConkey agar: a simple selective medium for isolation of *Burkholderia pseudomallei* from soil

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Abstract

***Background and objectives**:* A selective medium is required for isolation of *Burkholderia pseudomallei* from soil. The present study aimed to develop an easy to prepare selective media by modifying MacConkey agar medium for improved isolation of *B. pseudomallei* from soil.

***Materials and methods**:* The media was prepared by using commercially available MacConkey agar as the basal medium and incorporating it with 4% glycerol and four antimicrobials namely vancomycin, amphotericin B, gentamicin and colistin at a concentration of 2.5 mg/L, 1 mg/L, 5 mg/L and 10 mg/L respectively. The media was initially optimized for growth of *B. pseudomallei* by addition of 100 organisms/plate of *B. pseudomallei* and ATCC strains of Gram negative and Gram positive bacteria. Sterile and unsterile soils were spiked with graded concentration ($1x10^6$ to $1x10^1$ CFU/gm of soil) of *B. pseudomallei* and other clinical and saprophytic Gram negative organisms and cultured on MacConkey, Ashdown and modified MacConkey media after enrichment in Ashdown broth. Growth of *B. pseudomallei* in the three media was compared. The newly devised media was termed as - Modified MacConkey agar for *Burkholderia* (MMB media).

***Results**:* Culture of supernatant from spiked sterile soil after enrichment showed equivalent isolation of *B. pseudomallei* on MMB and Ashdown's media and there was 100% inhibition of *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* on MMB medium. Almost similar inhibition of *Comamonas testosteroni, Aeromonas salmonicida* and *Burkholderia cepacia* was observed on both MMB and Ashdown's media. Culture of sterile soil seeded with different concentrations of *P. aeruginosa* showed no growth in MMB media. But there was growth of *P. aeruginosa* when sterile soil samples spiked with $1x10^6$ to $1x10^3$ CFU of *P. aeruginosa* were cultured in Ashdown media. When unsterile soil was seeded with graded concentration of *B. pseudomallei,* the colony count of this bacterium gradually declined in all three medium with decreased spiking concentrations. Growth of other soil organisms was less in MMB media compared to other two media.

***Conclusion**:* The newly devised MMB media is selective and easy to prepare for the detection of *B. pseudomallei* from soil.

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Introduction

Melioidosis, caused by a facultative β-proteobacterium, *Burkholderia pseudomallei,* is endemic in over 46 countries including Bangladesh [1-4]. *B. pseudomallei* is a saprophytic environmental organism found mainly in plant rhizosphere and distributed in many different environmental niches especially paddy field, stagnant surface water, water holes and sea water [5]. Detection of *B. pseudomallei* in clinical and environmental samples is fundamental to determine the source and geographical distribution of this organism [6]. The present standard for detection of *B. pseudomallei* in soil is culture. However, isolation of *B. pseudomallei* is difficult from soil samples due to the abundant presence of other non-fermentative Gram-negative species that morphologically resemble *B. pseudomallei*.

Although *B. pseudomallei* grows in many ordinary media including nutrient agar, blood agar or MacConkey agar, a selective media is required for its isolation from heavily contaminated unsterile environmental samples. Currently Ashdown selective agar is the favored media for isolation and identification of *B. pseudomallei* in areas where melioidosis is endemic [7]. Ashdown media performs well as a selective agar, but this media is not readily available in laboratories of many melioidosis endemic areas like Bangladesh. Apart from Ashdown media, *B. pseudomallei* selective agar (BPSA) medium, *B. cepacia* media were reported to yield improved recovery of *B. pseudomallei*; however, these media are not also commercially available. A clinical comparison of BPSA, Ashdown and *B. cepacia* media demonstrated equivalent sensitivity but lower selectivity of BPSA than the other two media [8,9]. Development of a selective, readily available and inexpensive culture media is very much essential for specific isolation of *B. pseudomallei* from various unsterile clinical and environmental samples.

Therefore, the present study was undertaken to develop a cheap and easy to prepare selective medium by modifying the easily available MacConkey agar medium for isolation of *B. pseudomallei* from spiked soil.

Materials and methods

MacConkey agar media was modified and compared with Ashdown agar medium for better isolation of the *B. pseudomallei* from spiked soil samples. The study was approved by the Institutional Review Board of Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) General Hospital, Dhaka, Bangladesh.

Bacterial strains used in the study: *B. pseudomallei* reference strain from Universiti Sains Malaysia (USM) and a total of ten local strains of *B. pseudomallei* from clinical specimens confirmed by colony characteristics, biochemical tests, monoclonal antibody based latex agglutination test (Melioidosis Research Center, Khon Kaen, Thailand) and polymerase chain reaction, were selected for the study [10,11]. One strain of *B. pseudomallei* from above mentioned local strains from clinical specimen was randomly selected for further laboratory work of this study. As control, the following strains were used in this experiment: *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* isolated from clinical specimens and *Comamonas testosteroni*, *Aeromonas salmonicida* and *Burkholderia cepacia* isolated from environmental samples.

Preparation of modified MacConkey agar medium: Modified MacConkey agar medium was prepared by adding glycerol and four antimicrobial agents to MacConkey agar medium and termed as 'Modified MacConkey agar for *Burkholderia* (MMB media)'. Four percent (4%) glycerol (40 mL/L) was added as previously described by Ashdown et al [7]. Four antimicrobial agents, namely vancomycin (2.5 mg/L), amphotericin B (1 mg/L), gentamicin (5 mg/L) and colistin (50 mg/L) were added into 51.5 gm/L of MacConkey agar medium. Vancomycin and amphotericin B were added to inhibit the growth of Gram positive bacterial and fungal species. The concentrations of gentamicin and colistin added were determined in accordance with minimum inhibitory concentrations (MIC) of selected *B. pseudomallei* strains. Following determination of MIC of the two antimicrobial drugs, two different concentrations of gentamicin and colistin were added to detect the optimum growth of *B. pseudomallei* in the MMB medium. The MIC of

gentamicin and colistin for all the test strains of *B. pseudomallei* were > 1024 µg/mL. Consequently, the two concentrations selected for colistin and gentamicin were well below the MIC, anticipating that when added together, they might have a synergistic inhibitory effect on the growth of *B. pseudomallei* [12].

Optimization of antimicrobial concentrations: MMB media was prepared with two different concentrations of gentamicin and colistin to determine the maximum growth of *B. pseudomallei* and maximum inhibition of other organisms. In one set of MMB media, gentamicin and colistin were added at a concentration of 5 mg/L and 50 mg/L respectively and in another set, 10 mg/L and 500 mg/L respectively. MMB media with the different concentrations of gentamicin and colistin were separately inoculated with 10 µl of 1×10^4 colony forming unit (CFU)/ mL (100 CFU) of *B. pseudomallei*, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 23853 and *Staphylococcus aureus* ATCC 25923. For each of the bacterial species, ten MMB plates were inoculated. The media were incubated at 37^0C aerobically for 72 hours. The number of colonies of each organism in MMB media with specific gentamicin and colistin concentrations was counted and expressed as the mean CFU.

Determination of inhibitory effect of antimicrobial agents in MMB media: To determine the inhibitory effect of multiple antimicrobials incorporated in MMB medium on *B. pseudomallei*, 10 µl of 1×10^4 colony forming unit (CFU)/ mL (100 CFU) of *B. pseudomallei* was inoculated in each of ten blood agar media, MacConkey agar, MMB and Ashdown agar media. All media were incubated at 37^0C aerobically for 72 hours. After 72 hours, the colonies of *B. pseudomallei* were counted on each set of three different media and the mean colony count recorded. The percentage of inhibition of bacterial growth was calculated by: [{(Total CFU in blood agar media – Total CFU in MMB media) ÷ Total CFU in blood agar media} x 100].

Evaluation of the MMB media using spiked soil samples: After initial trial, MMB medium having best combinations and concentrations of antimicrobials was selected to evaluate the culture of *B. pseudomallei* from spiked soil samples. The modified Ashdown broth as described previously, was used for the enrichment of all soil samples [9,13]. Soil samples were collected in two sterile plastic bags, sealed with rubber bands and transported to the laboratory. One set of soil samples was kept unsterile at room temperature and another set was sterilized by autoclaving at 121^0C for 15 minutes. Sterility of the soil was checked following enrichment in trypticase soy broth (TSB) for 48 hours and inoculating the soil samples in blood agar media. No growth was observed in the sterile soil samples.

Suspension of 1.5×10^8 CFU/mL of *B. pseudomallei* was prepared with sterile normal saline and serial 10-fold dilutions were made starting from 1×10^6 to 1×10^1 CFU/mL in 6 test tubes. One set of 6 tubes containing 3 gm of sterile soil and another set of 6 tubes containing 3 gm of unsterile soil were prepared. Six tubes of each set of soil were then spiked with *B. pseudomallei* with 1×10^6 CFU/gm to 1×10^1 CFU/gm of soil. Nine milliliter (9 mL) of modified Ashdown enrichment broth was added to each test tube containing 3 gm of soil. The tubes were vortexed for 30 seconds and incubated at 37^0C for 48 hours. After 48 hours, 20 µl of undisturbed supernatant from each dilution was inoculated in MMB, Ashdown and MacConkey agar media and incubated at 37^0C for 72 hours. As control, *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa* isolated from clinical specimens and *Comamonas testosteroni*, *Aeromonas salmonicida* and *Burkholderia cepacia* isolated from environmental samples were used. The organisms were diluted and spiked in tubes containing sterile soil in the same concentrations as that of *B. pseudomallei* as described previously. TSB (9 ml) was added to each test tube for enrichment and incubated at 37^0C for 48 hours. After 48 hours, 20 µl of undisturbed supernatant from each dilution was inoculated in MMB media, Ashdown agar and MacConkey agar media and incubated at 37^0C for 72 hours.

Results

Optimization of antimicrobial concentrations:

Optimized concentrations of gentamicin and colistin were determined to detect their ability to inhibit growth of both Gram positive and Gram

Table-1: *Effect of different concentrations of gentamicin and colistin on the growth of B. pseudomallei in MMB media*

Media	Antimicrobial concentrations	Number of *Bps* inoculated per plate	CFU of *Bps*/plate Mean ±SD (95% CI)	% inhibition of *Bps* Mean ±SD (95% CI)	Growth of *E. coli*, *S. aureus*, *P. aeruginosa*
MMB media	Gentamicin (5 mg/L) + colistin (50 mg/L) + amphotericin B (1 mg/L) + vancomycin (2.5 mg/L)	100 CFU	76.6 ± 1.2 (75.4-77.9)	24.7 ± 1.20 (23.4-25.9)	No growth
MMB Media	Gentamicin (10 mg/L) + colistin (500 mg/L) + amphotericin B (1 mg/L) + vancomycin (2.5 mg/L)	100 CFU	56.7 ± 0.6 (56.0-57.3)	43.3 ± 0.6 (42.7-43.9)	No growth
p value			p < 0.05	p< 0.05	

Note: p value determined by student's t test; CFU: colony forming unit; CI: confidence interval. The mean value was calculated from the growth of B. pseudomallei in 10 experimental culture plates. Bps: B. pseudomallei

negative bacteria as well as to support maximum growth of *B. pseudomallei*. Table-1 shows the effect of different concentrations of gentamicin and colistin on the growth of *B. pseudomallei* in our MMB medium. Growth of *B. pseudomallei* colonies was significantly (p<0.05) less in MMB media containing higher concentrations of gentamicin and colistin (Mean CFU/plate: 56.7 ± 0.6) compared to MMB media containing lower concentrations of gentamicin and colistin (Mean CFU/plate: 76.6 ± 1.2). Mean percentage inhibition of *B. pseudomallei* colonies was significantly (p<0.05) more in MMB containing higher concentration of gentamicin and colistin compared to media containing lower concentrations of gentamicin and colistin (43.3 ± 0.6 vs. 24.7 ± 1.20).

No growth of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 23853 and *S. aureus* ATCC 25923 was observed in MMB media containing either concentrations of gentamicin and colistin. Therefore, gentamicin 5 mg/L and colistin 50 mg/L plus vancomycin 2.5 mg/L and amphotericin B 1 mg/L concentration were selected for preparation of MMB media for subsequent use in this study.

Growth of B. pseudomallei in MMB medium: Table-2 shows the growth of *B. pseudomallei* in MacConkey, Ashdown and MMB media compared to blood agar media inoculated with (100 CFU/plate). The mean numbers of colony of *B. pseudomallei* in each of ten MacConkey (78.7 ± 1.5 CFU), Ashdown (77 ± 1 CFU) and MMB agar plates (76.7 ± 1.5 CFU) were significantly less (p< 0.05) than that in blood agar media (92.3 ± 2.5 CFU). However, there was no significant (p>0.05) difference of mean number of *B. pseudomallei* colonies in the MMB, MacConkey agar and Ashdown media. The colony morphology of *B. pseudomallei* after 48 hours of incubation at 42^0C aerobically was pink and centrally depressed in all three media in while colonies were dry and wrinkle in Ashdown media (Figure-1).

Table-2: *Comparison of growth of B. pseudomallei in blood, MacConkey agar, Ashdown and MMB media (100 CFU/plate)*

Media	CFU of *B. pseudomallei* Mean ± SD (95% CI)	MRT
Blood agar	92.3 ± 2.5 (89.5-95.2)	a
MacConkey agar	78.7 ± 1.5 (77.0-80.4)	b
Ashdown media	77 ± 1 (75.9-78.1)	b
MMB media	76.7 ± 1.5 (75.0-78.4)	b

Note: p value is determined by ANOVA; CFU: colony forming unit; MRT: multiple range test; Separate letters are assigned if the result differs significantly from each other; CI: confidence interval.

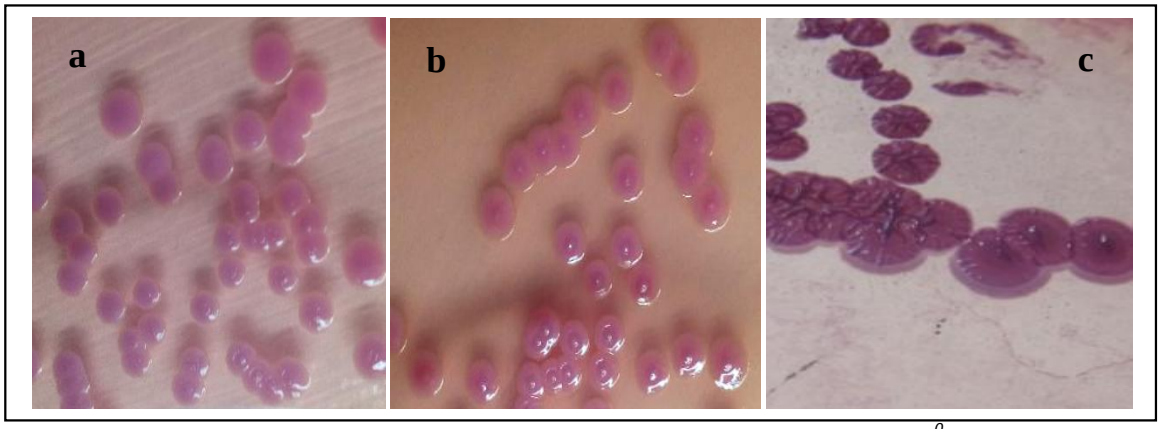

Figure-1: *Colony characteristics of B. pseudomallei after 48 hours of incubation at 42^{0}C aerobically on (a) MacConkey agar media, (b) MMB media and (c) Ashdown media.*

Evaluation of MMB media by culturing spiked sterile soil samples: Table-3 shows the results of culture of sterile soil spiked with graded concentration of *B. pseudomallei* and six other Gram negative bacilli in MMB media.

Table-3: *Comparison of growth of B. pseudomallei and other Gram negative bacteria from spiked sterile soil in MMB, MacConkey and Ashdown media*

Organisms	Sterile soil spiked with number of organism (CFU per gm)						
	Media	**1x10^6**	**1x10^5**	**1x10^4**	**1x10^3**	**1x10^2**	**1x10**
		Growth of organism (CFU/plate)					
B. pseudomallei	Mac	N	N	90	16	8	-
	MMB	N	100	84	12	5	-
	ASH	N	100	85	10	5	-
K. pneumonia	Mac	N	N	100	18	-	-
	MMB	-	-	-	-	-	-
	ASH	-	-	-	-	-	-
E. coli	Mac	N	N	98	17	-	-
	MMB	-	-	-	-	-	-
	ASH	-	-	-	-	-	-
P. aeruginosa	Mac	N	N	100	18	-	-
	MMB	-	-	-	-	-	-
	ASH	N	N	90	12	-	-
Comamonas tertosteroni	Mac	N	N	98	17	-	-
	MMB	N	N	95	15	-	-
	ASH	N	N	97	12	-	-
Aeromonas salmonicida	Mac	N	N	95	17	-	-
	MMB	N	N	89	15	-	-
	ASH	N	N	90	15	-	-
B. cepacia	Mac	N	N	100	18	-	-
	MMB	N	N	98	12	-	-
	ASH	N	N	97	15	-	-

Note: Mac- MacConkey agar; MMB- modified MacConkey for Burkholderia; ASH- Ashdown's medium; (N) indicates >100colonies; (-) indicates no growth

The growth of *B. pseudomallei* in MacConkey, Ashdown and MMB media was numerous from sterile soil spiked with *B. pseudomallei* with 1×10^6 CFU/gm of sterile soil. *B. pseudomallei* colony count was possible in Ashdown and MMB media when per gram of sterile soil was spiked with 1×10^5 to 1×10^2 CFU *B. pseudomallei*. None of the seven test bacteria grew in any of the 3 media when soil samples were spiked with 1×10^1 CFU of bacteria. There was 100% inhibition of *K. pneumoniae* and *E. coli* in both MMB and Ashdown media from culture of sterile soil seeded with all graded concentrations as compared to MacConkey agar media. Also, *P. aeruginosa* did not grow in MMB media at all bacterial concentration whereas in Ashdown media, growth of *P. aeruginosa* was observed at bacterial concentration from 1×10^6 to 1×10^3 CFU/gm of spiked soil. All other six types of bacteria grew in MacConkey agar media from culture of sterile soil seeded with all concentrations, except at spiking concentration of 1×10^2 and 1×10^1 CFU/gm of sterile soil. Growth of *C. testosteroni*, *A. salmonicida* and *B. cepacia* was found in all three media at bacterial concentration from 1×10^6 to 1×10^3 CFU/gm of sterile soil.

Evaluation of MMB media by culturing spiked unsterile soil samples: Table-4 shows the growth of bacteria from culture of unsterile soil samples seeded with graded concentration of *B. pseudomallei*. The growth of bacteria in MacConkey agar media, Ashdown agar and MMB media was numerous, uncountable and could not be differentiated into specific types of bacteria from unsterile soil seeded with *B. pseudomallei* with 1×10^6 CFU/gm of unsterile soil.

Table-4: *Comparison of growth of B. pseudomallei from unsterile soil seeded with graded concentration of B. pseudomallei*

Number of *B. pseudomallei* spiked/gm unsterile soil	**Growth of bacteria in**		
	MacConkey agar CFU/plate	**Ashdown media CFU/plate**	**MMB media CFU/plate**
1×10^6 CFU	Profuse mixed growth of organisms. Colonies could not be differentiated	Profuse mixed growth of organisms. Colonies could not be differentiated	Profuse mixed growth of organisms. Colonies could not be differentiated
1×10^5 CFU	Profuse mixed growth of organisms. Colonies could not be differentiated	*B. pseudomallei*- 98 Colonies of other organisms - 40	*B. pseudomallei*– 100 Colonies of other organisms - 27
1×10^4 CFU	*B. pseudomallei*– 90 Colonies of other organisms -70	*B. pseudomallei*- 85 Colonies of other organisms- 40	*B. pseudomallei*– 83 Colonies of other organisms - 27
1×10^3 CFU	*B. pseudomallei*– 18 Colonies of other organisms - 66	*B. pseudomallei*- 12 Colonies of other organisms- 40	*B. pseudomallei*– 15 Colonies of other organisms - 26
1×10^2 CFU	*B. pseudomallei*– 8 Colonies of other organisms - 70	*B. pseudomallei*- 5 Colonies of other organisms - 40	*B. pseudomallei*– 5 Colonies of other organisms - 30
1×10^1 CFU	*B. pseudomallei*– not detected Colonies of other organisms -66	*B. pseudomallei*- 0 Colonies of other organisms - 40	*B. pseudomallei*– 0 Colonies of other organisms - 30

Note: CFU: colony forming unit.

Colony count of *B. pseudomallei* and other types of bacteria was possible in Ashdown and MMB media from unsterile soil samples seeded with $1 \times 10^5 - 1 \times 10^2$ CFU of *B. pseudomallei*/gm soil. The colony counts of other types of bacteria was 26 – 30 CFU/plate in MMB media compared to 40 CFU/plate and 66 – 70 CFU/plate in Ashdown and MacConkey agar media respectively. The colony count of *B. pseudomallei* gradually declined in all three media with decrease of spiking concentrations of *B. pseudomallei* in unsterile soil. *B. pseudomallei* did not grow from unsterile soil seeded with *B. pseudomallei* with 1×10^1 CFU/gm of unsterile soil.

Discussion

Culture is the gold standard for diagnosis of melioidosis. Ashdown media is the currently used selective medium for isolation of *B. pseudomallei* from environmental and clinical samples [7]. However, overgrowth of other soil bacteria and fungi on Ashdown agar plates is common [9]. The media is also not readily available in prepared form in melioidosis endemic area like Bangladesh. So, a readily available selective media is needed for culture and isolation of *B. pseudomallei* for environmental survey.

In this study, commercially available MacConkey agar media was modified by addition of specific antimicrobials and glycerol to suppress the growth of soil flora while still allowing the growth of *B. pseudomallei*. The modified MacConkey media was termed as 'Modified MacConkey agar for *Burkholderia* (MMB media)'. Gentamicin and colistin were chosen because of their previous use in *B. pseudomallei* selective media [7,15] and intrinsic resistance of *B. pseudomallei* to those antimicrobials [16,17]. Minimum inhibitory concentration (MIC) of gentamicin and colistin of ten local *B. pseudomallei* isolates was determined by agar dilution method. MIC values of both gentamicin and colistin of all ten local isolates were > 1024 µg/mL. This indicates that using these antimicrobials at a lower concentration will allow the growth of *B. pseudomallei*, but will suppress growth of other microbial flora in the sample. Initially, we tried two combinations of gentamicin and colistin concentrations. Although, the higher concentrations of gentamicin and colistin (10 mg/L + 500 mg/L) had greater ability to suppress soil flora, they caused diminished growth rate of *B. pseudomallei*. So, finally the lower concentration of gentamicin and colisitin (5 mg/L + 50 mg/L) was selected. Vancomycin 2.5 mg/L and amphotericin B 1 mg/L were added in MMB media to inhibit Gram positive organism and fungus present in soil. The MMB media with the combination of four antimicrobials was highly selective against a variety of Gram positive and Gram negative bacterial species. There was no growth of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 23853 and *S. aureus* ATCC 25923 on MMB media. MMB media was also enriched with glycerol at a concentration of 40 ml/L to prevent the moisture loss during prolonged incubation and for production of characteristic colony of *B. pseudomallei* as used by Ashdown [7]. Our new MMB was evaluated for its capability to support the growth and easy recognition of *B. pseudomallei* in comparison to selective Ashdown and MacConkey media. Equally good growth of *B. pseudomallei* was present in MMB, MacConkey and Ashdown media. In MMB media, *B. pseudomallei* produced characteristic pink and centrally depressed colonies.

The assessment of newly modified MMB media for the isolation of *B. pseudomallei* in spiked positive soil samples showed that the MMB media has sensitivity similar to Ashdown media. During evaluation of the media by culturing sterile soil spiked with *B. pseudomallei*, it was seen that, there was no significant difference in colony counts between MMB media and Ashdown media. There was 100% inhibition of common Gram negative soil bacteria namely *K. pneumoniae*, *E. coli* and *P. aeruginosa* in MMB media while in Ashdown media, growth of *P. aeruginosa* was observed when sterile soil seeded with 1×10^6 to 1×10^3 CFU of *P. aeruginosa* /gm was cultured. There were no growth of any of the bacteria at a concentration of 1×10^2 and 1×10^1 CFU/gm of sterile soil in MacConkey, Ashdown media and MMB media. This could be due to either very low number of bacteria inoculated to grow or due to presence of some unknown substances in soil which might have inhibited the growth of bacteria in culture [18]. When *B. pseudomallei* was spiked into natural unsterile soil, the inhibition of other bacterial flora of soil was found significantly high in MMB media

in comparison to Ashdown and MacConkey agar media. There was apparent decrease in colony count of gentamicin and colistin resistant soil bacteria on MMB media. Growth of other soil bacteria was 30-26 CFU/plate on MMB media compared to 40 CFU/plate and 66-70 CFU/plate on Ashdown and MacConkey media respectively. So, it was easy to identify *B. pseudomallei* colonies in MMB media by inhibiting other soil bacteria.

The present study has some limitations. The newly devised MMB media could not be evaluated at the field level for the detection of *B. pseudomallei* from soil and other environmental samples from different locations of the country. Also, the efficacy of MMB media needs to be assessed for better isolation of *B. pseudomallei* with clinical samples from unsterile sites.

The newly devised MMB medium can be prepared in small laboratories located in melioidosis endemic areas for isolation of *B. pseudomallei* from environmental and clinical samples from unsterile sites. Also in resource limited settings, this inexpensive and easy to prepare selective media can serve as a tool for large scale epidemiological surveys for detection of *B. pseudomallei*.

Conflict of interest*:*

The authors declare no conflict of interest.

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