Original Article

DOI: 10.7860/JCDR/2022/57824.17135

Successful Isolation of *Burkholderia pseudomallei* from Soil by Extended Incubation of Ashdown's Agar: A Cross-sectional Study

Microbiology Section

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ABSTRACT

Introduction: Melioidosis is an infectious disease of humans and animals caused by an environmental saprophyte *Burkholderia pseudomallei*. Although the organism is associated with soil and water, environmental isolation is rarely successful which could be due to the existence of viable but non culturable forms.

Aim: To isolate *B. pseudomallei* from the soil to detect the environmental presence of this organism in and around Puducherry, India.

Materials and Methods: A descriptive cross-sectional study was carried out from July 2018 to January 2021 at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. A total of 473 soil samples were collected from areas surrounding the residence and workplaces of seven culture-proven melioidosis cases, from Puducherry and three districts of Tamil Nadu (Cuddalore, Nagapattinam and Villupuram) during the dry and wet seasons. Soil samples were enriched in Ashdown's broth and cultured on Ashdown's agar. The plates were incubated at 37°C and examined daily for seven days with a further extended period of incubation till the tenth day for samples that did not show growth. Suspected isolates were subjected to Vitek 2 system for biochemical identification. Confirmation of the isolates was carried out by antigen detection and Polymerase Chain Reaction (PCR).

Results: From 473 soil samples processed, bacteria with colony morphology similar to *B. pseudomallei* were isolated in 56 (11.83%) samples. Only one isolate, which was detected on the tenth day of incubation was confirmed as *B. pseudomallei* using antigen detection and PCR. This sample was collected during the wet season (December 2020) from Endur, in the Villupuram district of Tamil Nadu, India.

Conclusion: The study findings highlight the importance of extended incubation of culture plates at 37°C for up to ten days to improve the chances of isolation from the soil.

Keywords: Environment, Gram negative, Melioidosis

INTRODUCTION

Melioidosis is an emerging infection of humans and animals caused by *Burkholderia pseudomallei*, a Gram negative, motile, non spore-forming environmental bacterium [1,2]. Although originally prevalent in Southeast Asia and Northern Australia, melioidosis cases are now being progressively identified in almost all other continents [3]. Based on epidemiological modelling, the global incidence of melioidosis is ~165,000 and in India ~52,500 cases [4]. This highly pathogenic bacterium is commonly found as a saprophyte in the soil and hence acquiring infection is likely among those exposed to such an environment. A higher incidence of melioidosis is reported during monsoon when the subterranean organisms migrate to the surface [1]. The presence of non cultivable forms as well as the existence of a non virulent, closely related species, *B. thailandensis* makes isolation of *B. pseudomallei* from the soil a challenging task [5,6]. Both conventional culture-based methods and molecular tests using PCR have been employed for this purpose [7]. Ashdown's agar is the selective medium used for the isolation of *B. pseudomallei*. The presence of crystal violet and gentamicin inhibits the growth of unwanted bacteria. Glycerol helps to enhance the growth of *B. pseudomallei*. Both neutral red and crystal violet give *B. pseudomallei* colonies a characteristic purple colour [7]. Additionally, *B. pseudomallei* can be detected using specific PCR assay targeting a Tat domain protein [7].

Although environmental *B. pseudomallei* has been isolated from other parts of the world, there are not many studies reported from India. There are only three reports of successful isolation, one from Cuddalore (Tamil Nadu), the second from the Malabar coastal region of Kerala and third from Mangalore (Karnataka). Among these Tamil Nadu and Karnataka are highly endemic areas for the disease [8-10].

Prior exposure to *B. pseudomallei* in the study population was documented by a seroprevalence of 19.8% among the high-risk population [11]. So far, no attempts have been made to study the environmental distribution of *B. pseudomallei*. This information is critical in identifying high-risk zones for acquiring melioidosis. Thus, the aim of the study was to isolate *B. pseudomallei* from soil surrounding the residence and workplaces of patients diagnosed with melioidosis by culture.

MATERIALS AND METHODS

A descriptive cross-sectional study was carried out from July 2018 to January 2021 at JIPMER, Puducherry, India. This study was approved by the Institutional Ethics Committee for Human Studies, JIPMER, Puducherry, India, (JIP/IEC/2018/0230) and written informed consent was obtained from the occupants to collect the soil samples.

Selection of Study Sites and Method of Sampling

Seven locations in and around Puducherry were sampled between 2018 and 2021 during the dry and wet seasons [Table/Fig-1] [12]. From each site, soil samples were collected from the residence of the patients and/or their workplace [Table/Fig-2] following a standard technique depending on the total area of house/field [13]. Based on the site, ten or more sampling points were selected maintaining a distance of 2.5 m and 5 m between the sampling points, from residence and/or workplace, respectively [Table/Fig-2]. With the use of protective gear (masks, gloves and rubber boots) soil samples were collected from a depth of 30 cm from the surface, using a metal gardening shovel. Approximately 20-40 g of soil from each sampling point was collected and transferred to

District, State	Date	Season	Location (Latitude/Longitude)	Max-Min (°C)	Average relative humidity (%)	Average rainfall (mm)*
Cuddalore, TN†	16-07-2018	Dry	Subramaniyapuram (11.65110N, 79.70340E)	38-27	58	65
Nagapattinam, TN†	18-09-2018	Dry	Sirkazhi (11.23910N, 79.73610E)	34-25	81	110.2
Cuddalore, TN†	09-10-2018	Wet	Subramaniyapuram (11.65110N, 79.70340E)	31-24	86.5	298.9
Cuddalore, TN†	09-10-2018	Wet	Kurinjipadi (11.56420N,79.5960E)	31-24	86.5	298.9
Villupuram, TN†	15-12-2020	Wet	Endur (12.20890N, 79.78900E)	28-22	84.3	131.5
Villupuram, TN†	15-12-2020	Wet	Marakkanam (12.18990N, 79.92490E)	28-22	84.3	131.5
Puducherry, PY‡	13-01-2021	Wet	Thattanchavady (11.94510 N, 79.80520 E)	28-24	89.5	23.5
Puducherry, PY‡	15-01-2021	Wet	Kuruvinatham (11.79540 N, 79.73300 E)	28-24	88.3	

[Table/Fig-1]: Details of climatic conditions existing at the time of soil sampling.
Source: https://www.timeanddate.com/weather/india/puducherry/climate [12]; †: Tamil Nadu; ‡: Puducherry

Month and year	Location	Number of soil samples from home	Number of soil samples from workplace	Total
1. July 2018	Subramaniyapuram	10	21 (field)	31
2. September 2018	Sirkazhi	19	0	19
3. October 2018	Subramaniyapuram	10	49 (field)	59
4. October 2018	Kurinjipadi	10	38 (field)	48
5. December 2020	Marakkanam	100	0	100
6. December 2020	Endur	100	0	100
7. January 2021	Thattanchavady	28	0	28
8. January 2021	Kuruvinatham	25	63 (field)	88
Total		302	171	473

[Table/Fig-2]: Details of soil sampling with year, location and number of samples collected.
Samples numbered 1 and 3 were from the same location collected during the dry (March-September) and wet (October-January) seasons

sterile containers, labelled and transported on the same day to the laboratory without exposure to direct sunlight. The metal shovel was cleaned in between the sampling with clean water and 70% alcohol to remove all the visible debris.

Processing of Soil Samples and Isolation of *B. pseudomallei*

The soil samples were processed in the laboratory as per the following protocol. To 10 g of soil, 10 mL of Ashdown's broth containing colistin 50 mg/L was added, vortexed for 30 seconds and incubated at 37°C for 48 hours after which 10 µL of the upper layer of the medium was plated onto Ashdown's agar to achieve isolated colonies. The plates were incubated at 37°C and examined daily for seven days with a further extended period of incubation till the tenth day for samples that did not show growth. Characteristic purple, dry wrinkled colonies of *B. pseudomallei* were purified further by subculture on 5% sheep blood agar and MacConkey agar.

B. pseudomallei was provisionally identified based on the following results: Motile Gram negative bacilli with bipolar staining [Table/Fig-3], oxidase positive, oxidative utilisation of lactose, arginine dihydrolase positive and lysine decarboxylase negative and resistant to gentamicin (10 µg) and colistin (10 µg) [14]. Vitek 2 system (bioM-rieux, Marcy-l'Étoile, France) was also used for biochemical identification.

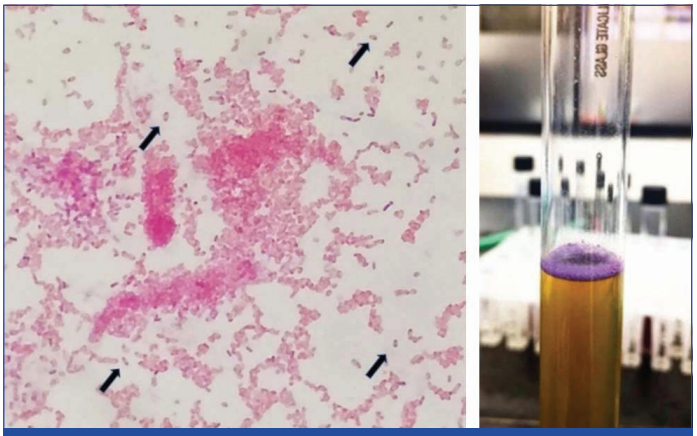
Isolates which were provisionally identified as *B. pseudomallei*/*B. cepacia* by Vitek 2 system were tested using the InBiOS AMD Active Melioidosis Detect (InBiOS, Seattle, WA) kit for capsular polysaccharide antigen [15]. Further confirmation of the isolates was carried out by PCR targeting a Type III secretion system gene cluster (TTS1) using previously published primers and protocol [16].

STATISTICAL ANALYSIS

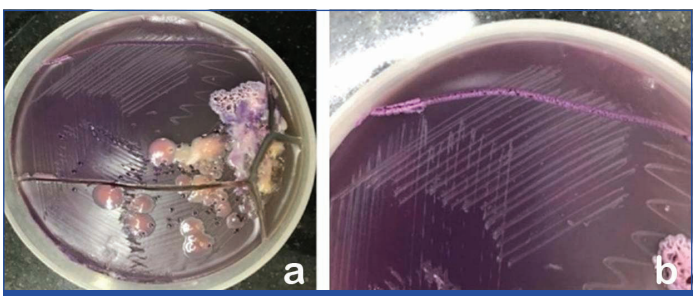
Descriptive analysis was performed by entering the data in Microsoft excel sheet and expressing the results as numbers and percentage.

RESULTS

Soil samples were collected from the residence/workplace of seven confirmed melioidosis patients. A total of 473 soil samples were processed during the dry and wet seasons from different locations [Table/Fig-1]. Many of the samples produced surface pellicle when incubated in Ashdown's broth with colistin [Table/Fig-4]. Fifty-six (11.8%) soil samples yielded isolates with colony morphology similar to *B. pseudomallei* on Ashdown's agar. Of these, 1 (0.2%) was detected on the tenth day of incubation. These 56 isolates were tested using Vitek 2 system. Only one isolate was identified as *B. pseudomallei* while seven (1.5%) others were identified as *B. cepacia*. All these 8 (1.7%) isolates were subjected to antigen detection and PCR. The single isolate identified by Vitek 2 system as *B. pseudomallei* gave a positive result with these two tests. This was the isolate that grew on the tenth day of incubation, along the cracks which had developed in the medium [Table/Fig-5]. This single positive isolate was obtained from Endur, in the Villupuram district of Tamil Nadu.



[Table/Fig-3]: *B. pseudomallei* exhibiting bipolar staining (Gram stain,100x).
[Table/Fig-4]: Shows the surface pellicle formation by *B. pseudomallei* after 48 hours of incubation in Ashdown's broth with colistin 50 mg/L. (Images from left to right)



[Table/Fig-5]: a) Ashdown's agar with growth of *B. pseudomallei* along with the mixed microbial flora. b) Magnified view showing *B. pseudomallei* growth along the cracks in the medium.

DISCUSSION

B. pseudomallei is an environmental saprophyte of soil and surface water and is known to cause a community-acquired infectious disease, melioidosis [17]. Environmental sampling is important to develop a risk map for melioidosis, to take preventive measures and raise awareness among clinicians and laboratory personnel in these endemic areas [17]. Diabetics and farmers are at increased risk of acquiring this disease with a male preponderance documented in other studies [1,18]. Even though melioidosis affects both animals and humans, evidence of zoonotic transmission or human to human spread is lacking [5].

The only source of infection is exposure to *B. pseudomallei* contaminated soil or water [5]. In endemic areas, *B. pseudomallei* is found in irrigated sports grounds, golf courses and irrigated rice fields [19]. More number of melioidosis cases are reported from Southern India which can be attributed to similar climatic conditions and occupation when compared with other endemic areas [20]. However, isolation rates of *B. pseudomallei* from soil in Southern India remain very low. One of the reasons for this could be poor sampling methods.

Detection of *B. pseudomallei* in soil depends on the selection of sampling sites. It is recommended to identify melioidosis cases and target their residence and workplace as performed in our study [17]. Use of Geographic Information System (GIS) can improve the rate of isolation for large environmental surveys [17]. Insufficient numbers of sampling sites, away from *B. pseudomallei* hotspots may give a false negative result [17]. Researchers found *B. pseudomallei* by collecting 100 samples from the site yielding positive result. Isolation rates also depend on distance between the sampling sites as adjacent sampling points tend to give similar results [17]. Therefore, depending on the total area of the collection site, a distance of 2.5 m and 5 m between each sampling site from the residence and fields, respectively was maintained. Soil sampling at a depth of 30 cm is recommended and was followed in this study, as rate of isolation is higher at this point compared to deeper layers [17].

Use of selective broth in soil culture helps in recovery of *B. pseudomallei*. Threonine-basal Salt Solution TBSS-50 (with colistin 50 mg/liter) and Ashdown's broth are commonly used enrichment broths [7,19,21,22]. TBSS-50 contains L- threonine as sole source of carbon and nitrogen. Wuthiekanun V et al., found more luxuriant growth in TBSS-50 when compared to Ashdown's broth [23]. Brook MD et al., reported improved recovery rates of *B. pseudomallei* with Ashdown's broth during dry season, as the stressed cells without water grew remarkably well, whereas TBSS-50 was found to be more useful during the wet months suppressing competitive organisms with selective agents [24]. In the Indian studies on environmental isolation, Ashdown's broth was successfully used [8,10]. This was replicated in the present study.

The presence of dry, rough and wrinkled surface pellicle at the broth-air interface of Ashdown's broth was found. Pellicle formation in liquid cultures of *B. pseudomallei* could be due to positive aerotaxis. Possibly during monsoon when dry topsoil gets moistened with rain, *B. pseudomallei* moves upwards due to aerotaxis, towards the soil surface to find air-water interfaces between soil particles [5]. This occurs due to rising water tables during or immediately after monsoons. The chances of isolation of *B. pseudomallei* from surface water are more during the rainy season due to this reason [25]. In our study too we were able to isolate *B. pseudomallei* from the soil when collected during the rainy season when the soil was wet and muddy.

Even though a higher incubation temperature of 40°C is preferred as it inhibits the growth of other soil microflora, 37°C can also be used as an alternate based on availability [17]. The media were incubated at 37°C for ten days. Due to dehydration, cracks developed within the medium and luxuriant growth of *B. pseudomallei* was observed along the cracks for one sample alone [Table/Fig-5]. Therefore, extending the incubation to 10 days may be recommended to improve the isolation rate. Further studies are required to prove that this is not a chance finding.

There are reports of misidentification of *B. pseudomallei* as *B. cepacia* by Vitek 2 system [26]. Moreover, all the eight isolates were further tested with antigen detection and PCR to confirm the identity and only the isolate identified by Vitek 2 system as *B. pseudomallei* was positive by the above two tests.

In endemic areas, *B. thailandensis* is frequently recovered from the soil along with *B. pseudomallei*. Even though they share similar colony morphology, there are differences in their polysaccharide-related genes [6]. We did not find *B. thailandensis* in our study. Other bacterial flora such as *Klebsiella pneumoniae*, *Ralstonia pickettii* and *Yersinia intermedia* were identified in several soil samples despite using enrichment broth and selective media with antibiotics [Table/Fig-5].

During times of stress in the dry season, *B. pseudomallei* may persist in the environment in a Viable But Non Culturable (VBNC) state. *B. pseudomallei* is known to survive in distilled water for several years, in unfavourable conditions without any nutrients, it can also tolerate a wide pH spectrum, temperature range and ultraviolet radiation [5]. In present study, failure to isolate the *B. pseudomallei* during the dry season could also be due to this viable but non culturable state of bacteria. However, the evidence for seasonal variation in isolation was inconclusive as three studies reported a higher isolation rate in the wet season while two studies reported isolation in dry seasons in a systematic review by Limmathurotsakul D et al., [17].

Similar to this study, isolation of *B. pseudomallei* from soil was investigated by other researchers in India. Prakash A et al., attempted isolation of this pathogen from Cuddalore district, roughly 30 km from Subramaniapuram and 40 km from Kurunjipadi, the two sites in this study. While Prakash A et al., isolated *B. pseudomallei* from eight soil samples just after the rainy season in 2010, researchers collected soil samples in both dry and wet seasons (July and October 2018) [Table/Fig-1] [8]. Their sampling sites were immediately next to a river (Vellar river) while they collected inland. This may have been responsible for the failure to isolate *B. pseudomallei* in this study. Interestingly, the only site where they were able to isolate was next to a lake (Endur lake). In another study by Chandrasekar S and Dias M, in the district of Mangalore, *B. pseudomallei* was isolated at a site next to a river (Gurupura river) [10]. Similar findings were noted in a study by Peddayelachagiri BV et al., with successful recovery of *B. pseudomallei* from river banks of Kerala Backwaters [9]. This is an interesting finding as isolation was common next to freshwater bodies and their catchment areas. Thus, sampling nearby water bodies may greatly improve the isolation of *B. pseudomallei* and investigators may consider this fact while planning further studies.

Moist and humid conditions during the monsoon are known to increase the pathogen virulence and environmental *B. pseudomallei* load [1]. It has been suggested that *B. pseudomallei* can be recovered from soil culture when collected within the first 90 cm from the surface where it is more likely to prevail and cause health hazard [5]. One of our sites of soil collection had a hand pump for water supply along with dense trees that provided continuous moisture, shade and ambient temperature for the pathogen to remain viable in surface layers of soil for a longer period. Therefore, we could recover *B. pseudomallei* from the same site. Inaccurate choice of sampling site could also be a reason for failure to isolate *B. pseudomallei* from culture. In our study, soil samples collected

from other areas of the same residence as well as the residence of other melioidosis cases yielded negative results, even though they were collected from the deeper layers of dry soil.

Regular watering and use of fertilisers rich in phosphates, nitrates and urea are found to increase the load of *B. pseudomallei* in soil. Phosphates are utilised by *B. pseudomallei* during oxidative stress response, motility and biofilm formation. *B. pseudomallei* reduces nitrates to nitrites that is used for anaerobic respiration. Urea hydrolysed to ammonia is used in biosynthetic pathways. Nitrifying soil bacteria oxidise urea to nitrates. Higher occurrence of *B. pseudomallei* in soil was found near animal sheds, due to high urea concentration [19]. *B. pseudomallei* prefers an acidic range [2,19]. A study from the U.S, reported the presence of clay resulted in anaerobic conditions unfavourable for the bacteria whereas the sand particles increased the oxygen content of the soil thereby favouring the growth and survival of *B. pseudomallei* [27]. Even though we collected soil with an increased fraction of sand from one of the sites, we were unable to isolate the pathogen.

Based on simplicity, specificity and cost, culture is considered a standard method for detection of *B. pseudomallei* from the soil although it is labour intensive [17]. Limitations of culture to detect *B. pseudomallei*, in its viable but non culturable state, during stress and dry season is well known [7]. Molecular techniques have a greater potential for screening the environmental samples compared to culture. The PCR assays could be used for epidemiological investigations, but do not indicate the viability of the pathogen [7]. In endemic areas, rate of isolation of *B. pseudomallei* from soil was found to be ten times more sensitive by conventional PCR than by culture [24]. Lau SK et al., found that in 1420 samples, use of conventional PCR improved detection in 6.8% of the samples as opposed to 0.6% positivity using culture alone [7]. Another study by Chen YS et al., reports culture negativity in 46.1% of soil samples, that were positive by Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) [22]. Kaestli M et al., report the most sensitive method of detection of *B. pseudomallei* with the use of TTS1 real-time PCR [28]. Studies on genetic relatedness between the environmental and the corresponding clinical isolate are ongoing and would help to establish soil as the source of infection.

Limitation(s)

Prior chemical analysis of the soil which would have indicated the likely sites of positive isolation was not carried out. Use of real-time PCR may have improved the rate of detection of *B. pseudomallei*.

CONCLUSION(S)

This study unequivocally demonstrates the presence of *B. pseudomallei* in the soil of Endur village located 51 km from Villupuram District of Tamil Nadu, India. Additional environmental studies will determine the distribution of *B. pseudomallei* from this area and the surrounding villages aiding to map the risk of melioidosis in this geographical region. Based on the present study the authors recommend an extended incubation time of ten days to improve the isolation rate of this important environmental pathogen.

Acknowledgement

The authors sincerely thank JIPMER for the intramural research grant provided to conduct this study.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: May 19, 2022
- Manual Googling: Jul 25, 2022
- iThenticate Software: Aug 02, 2022 (5%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **May 17, 2022**
Date of Peer Review: **Jul 19, 2022**
Date of Acceptance: **Aug 05, 2022**
Date of Publishing: **Nov 01, 2022**