

ATTACHMENT OF *BURKHOLDERIA PSEUDOMALLEI* TO PHARYNGEAL EPITHELIAL CELLS: A HIGHLY PATHOGENIC BACTERIA WITH LOW ATTACHMENT ABILITY

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Abstract. Respiratory infections are initiated by the attachment of bacteria to pharyngeal epithelial cells. We studied the attachment of *Burkholderia pseudomallei* to pharyngeal epithelial cells. After one, two, three, and four washes, there were 22.6 ± 8.9 , 15.7 ± 7.0 , 6.8 ± 3.1 , and 4.6 ± 1.1 (mean \pm SD) attached bacteria/cell, respectively. If the bacterial concentration was maintained at 1×10^8 colony-forming units (cfu)/ml and three washes were done, at concentrations of 2.5×10^4 , 5×10^4 , and 1×10^5 cells/ml there were 9.9 ± 3.6 , 3.3 ± 0.8 , and 2.5 ± 1.1 attached bacteria/cell, respectively. If the cell concentration was kept at 2.5×10^4 cells/ml and three washes were done, at bacterial concentrations of 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , and 1×10^9 cfu/ml, there were 0.3 ± 0.3 , 0.6 ± 0.6 , 1.0 ± 0.2 , 5.1 ± 2.3 , and 9.6 ± 1.9 attached bacteria/cell, respectively. There were 4.8 ± 1.9 , 5.5 ± 2.5 , 5.6 ± 1.9 , and 6.4 ± 2.6 attached bacteria/cell at 0, 30, 120, and 240 min of incubation, respectively. Pharyngeal cells from 10 persons (seven men and three women, mean \pm SD age = 30.7 ± 8.1 years, 12 experiments with a single isolate) showed that there were 7.8 ± 4.3 attached bacteria/cell. It was found that the efficiency of attachment of this bacteria was very low (7.0 ± 3.3 bacteria/cell). Electron microscopy revealed that there were no fimbriae but a thin capsular polysaccharide layer on the surface of *B. pseudomallei*. Attachment to pharyngeal epithelial cells appeared to be mediated by this structure.

Burkholderia pseudomallei, a gram-negative bacilli, is a natural saprophyte that can be isolated from soil, stagnant streams, ponds, and rice paddies in areas endemic for melioidosis. This bacteria is usually transmitted by cutaneous and respiratory routes; however cutaneous transmission is significantly more prevalent than the respiratory route.^{1–3} The most common form of this disease is a pulmonary infections that ranges from acute bronchitis to overwhelming necrotizing pneumonia. *Burkholderia pseudomallei* is one of the life-threatening causes of pneumonia in Southeast Asia and northern Australia. The initial step in the pathogenesis of respiratory infection is the attachment of the bacteria to the pharyngeal epithelial cells. Until now no studies have been done on the adherence of this bacteria to respiratory cells. A recent study has shown that *B. pseudomallei* was present in the pharynx of approximately half of the patients with pulmonary melioidosis, but absent in the controls.⁴ This indicates that colonization of the pharynx by *B. pseudomallei* might be associated with the pathogenesis of this infection. Therefore, this study was conducted to describe the basic aspects of attachment of *B. pseudomallei* to pharyngeal epithelial cells. This will lay the groundwork for exploring the pathogenic mechanisms of melioidosis.

MATERIALS AND METHODS

Bacteria. All eight strains of *B. pseudomallei* used were obtained from Chiang Mai University (Chiang Mai, Thailand) and their identification was confirmed with the API test system (Biomérieux S. A., Marcy l’Etoile, France).⁵ Strain SP 186 was the predominant strain used in this study. Bacteria were stocked in Mueller Hinton broth (Difco Laboratories, Detroit, MI) containing 5% horse blood and kept at -40°C until used. Bacteria were cultured on brain-heart infusion agar (BBL, Becton Dickinson Microbiology System, Cockeysville, MD) overnight at 37°C .

Virulence test in mice. To determine adverse effects on

the bacteria in laboratory conditions, virulence was validated by challenging mice with *B. pseudomallei*. Five-week-old, pathogen-free, female ICR mice (Shizuoka Agricultural Cooperation Association for Laboratory Animals, Shizuoka, Japan) were used. Animals were housed in clean conditions and were given sterile food and water. Mice were anesthetized by injecting them with 0.15 ml (7.5 mg) of pentobarbital sodium (Dainabot Company Ltd., Osaka, Japan) intraperitoneally. Groups of five mice were challenged intraperitoneally and intrabronchially with bacterial suspensions in sterile phosphate-buffered saline (PBS) containing 1×10^5 , 1×10^7 , and 1×10^9 colony-forming units (cfu)/ml in an inoculum volume of 0.2 ml. Control mice were challenged intrabronchially and intraperitoneally with 0.2 ml of sterile PBS. After 24 hr, mortality and morbidity were determined and mice were killed by cervical dislocation. After dissection, the heart and lungs were removed, suspended in an appropriate volume of sterile saline, homogenized, diluted in a 10-fold series in sterile saline, inoculated onto brain-heart infusion agar plates, and incubated overnight at 37°C .

Attachment assay. Pharyngeal cells were obtained by scrapping the human pharynx with a swab. Cells were suspended in phosphate buffer, pH 7.2, and washed three times by centrifugation at $80 \times g$ for 10 min (per wash) at room temperature. The attachment assay was done as described elsewhere with modifications.⁶ Unless otherwise stated, cell and bacterial concentrations of 2.5×10^4 cells/ml and 1×10^8 cfu/ml, respectively, were mixed in equal volumes, incubated in a shaking water bath at 37°C for 30 min, and washed three times by centrifugation at $80 \times g$ for 10 min (per wash) at room temperature to remove the nonattached bacteria. Smears were made on glass slides using a cytospin procedure (Shandon Southern Products Ltd., Astmoor, United Kingdom) and Gram staining was done. Fifty cells per slide were observed with the oil-immersion lens of a microscope to count the attached bacteria.

Electron microscopy. To reveal the surface structures on

TABLE 1

Mean number of colonies (cfu/g) isolated from lung and heart of mouse after different challenge doses of *Burkholderia pseudomallei*

Challenge dose* (cfu/ml)	Intraperitoneal route		Intrabronchial route	
	Lung	Heart	Lung	Heart
1×10^5	No growth	No growth	2×10^7	No growth
1×10^7	No growth	No growth	3×10^9	1.1×10^4
1×10^9	3.1×10^7	3×10^6	1.5×10^9	0.6×10^6

* The inoculum volume was 0.2 ml. cfu = colony-forming units.

B. pseudomallei and the involvement of these structures on attachment, bacteria were analyzed by electron microscopy after the attachment assay. Specimens were prepared for transmission electron microscopy and scanning electron microscopy according to a method described elsewhere.⁶

Statistical analysis. The Student's *t*-test was used for statistical analysis. Data were considered statistically significant when *P* values were less than 0.05.

RESULTS

After 24 hr, all mice challenged with 1×10^9 cfu/ml and two mice challenged with 1×10^5 cfu/ml (intrabronchially) died. Three of the mice challenged with 1×10^5 cfu/ml (intrabronchially) and all mice challenged with 1×10^7 cfu/ml were sick. All mice challenged intrabronchially and all challenged intraperitoneally with 1×10^9 cfu/ml had pneumonia. Hemorrhaging in the lungs were also found in this group. No peritoneal fluid was found in the challenged mice. No abnormalities were found in mice in the control group. The bacterial concentrations in the lung and heart cultures of control and challenged mice are shown in Table 1.

Burkholderia pseudomallei could attach to the surface as well as to the periphery of pharyngeal epithelial cells. In general, cells were found with attached bacteria; in a few instances, no bacteria or many bacteria were found attached. Morphologically, all cells were similar. After one, two, three, and four washes, there were 22.6 ± 8.9 , 15.7 ± 7.0 , 6.8 ± 3.1 , and 4.6 ± 1.1 (mean \pm SD) attached bacteria/cell, respectively (all differences were statistically significant). The background of the smears was clear of bacteria only after three and four washes. If the bacterial concentration was maintained at 1×10^8 cfu/ml and three washes were performed, at cell concentrations of 2.5×10^4 , 5×10^4 , and 1×10^5 cells/ml, there were 9.9 ± 3.6 , 3.3 ± 0.8 , and 2.5 ± 1.1 attached bacteria/cell, respectively. There were significant differences ($P < 0.05$ and $P < 0.005$) in attachment at cell concentrations of 5×10^4 and 1×10^5 cells/ml compared with 2.5×10^4 cells/ml. When the cell concentration was maintained at 2.5×10^4 cells/ml and three washes were done, at bacterial concentrations of 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 , and 1×10^9 cfu/ml, there were 0.3 ± 0.3 , 0.6 ± 0.6 , 1.0 ± 0.2 , 5.1 ± 2.3 , and 9.6 ± 1.9 attached bacteria/cell, respectively. Significantly ($P < 0.05$) greater attachment was observed at a bacterial concentration of 1×10^9 cfu/ml.

To determine the effects of incubation on the attachment ability, bacteria and cells at concentrations of 1×10^8 cfu/ml and 2.5×10^4 cells/ml, respectively, were incubated to-

TABLE 2

Attachment of seven strains of *Burkholderia pseudomallei* to pharyngeal epithelial cells*

Strain no.*	No. of attached bacteria per cell (mean \pm SD)
SP 335	3.7 ± 1.1
SP 140	2.7 ± 0.2
SP 235	4.2 ± 1.7
SP 237	2.3 ± 0.3
SP R3	2.8 ± 1.5
H 99	1.8 ± 1.0
U 232	1.9 ± 0.3

* SP, H, and U indicate strains isolated from sputum, blood and urine, respectively, from patients with melioidosis.

gether for different times and washed three times. There were 4.8 ± 1.9 , 5.5 ± 2.5 , 5.6 ± 1.9 , and 6.4 ± 2.6 attached bacteria/cell at 0, 30, 120, and 240 min of incubation, respectively. There was a significant difference ($P < 0.05$) only between the attachment at 0 and 240 min of incubation.

To elucidate the attachment ability of other isolates of *B. pseudomallei*, the attachment assay was done with seven strains isolated from different sources. All isolates showed similar, low-level attachment to pharyngeal epithelial cells (Table 2). Pharyngeal cells from 10 persons (seven males and three females, mean \pm SD age = 30.7 ± 8.1 years, 12 experiments with a single isolate) showed that there were 7.8 ± 4.3 attached bacteria/cell. In each attachment experiment, identification of the gram-negative bacilli that actually bind to the epithelial cells was confirmed by examining a negative control containing only epithelial cells. The negative control was treated in a similar way. The number of attached gram-negative bacilli ranged from 0 to 0.5/cell, with a mean of 0.07 attached bacteria/cell.

Other than 2–4 flagella/bacterium, no fimbriae were observed in any isolates of *B. pseudomallei*. There was a thin, granular, electron-dense, ruthenium red-positive material around the *B. pseudomallei* cell that appeared to mediate attachment (Figure 1). Scanning electron microscopy showed that *B. pseudomallei* used the microvillae (elevations on the surface of pharyngeal epithelial cells) of the cells for attachment (Figure 2).

DISCUSSION

To our knowledge, this is the first study on the attachment of *B. pseudomallei* to respiratory epithelial cells. Exploiting the attachment mechanism may facilitate new ways of treatment and prevention of *B. pseudomallei* infection. The attachment of *B. pseudomallei* to certain cells and different sites on the cells may be due to the distribution of receptors on the cell surface and access these receptors to the bacteria. Clearing of the background bacteria in smears and the initial weak attachment of the bacteria were presumably responsible for the gradual decrease in attached bacteria after each washing. It is known that the first step of attachment is relatively weak and reversible by washing.⁷ Therefore, we did not determine the effects of additional washing on attachment. When the bacterial concentration was kept constant and the cell concentration was increased, attachment of *B. pseudomallei* did not increase. This indicates that at a given

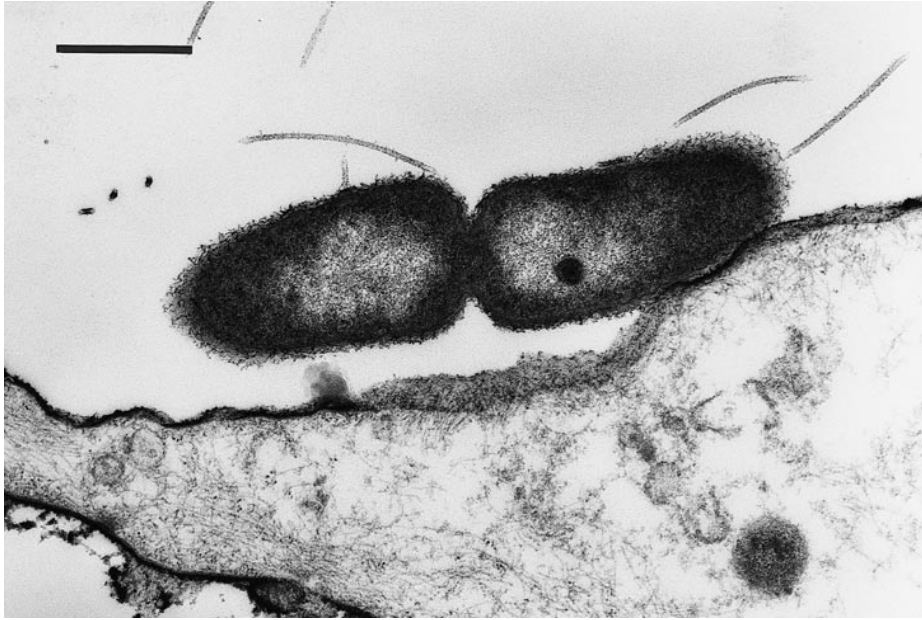


FIGURE 1. Transmission electron micrograph of *Burkholderia pseudomallei* attached to a pharyngeal epithelial cell stained with ruthenium red. There is a thin, granular, ruthenium red-positive layer on the surface of the bacteria and the cell, indicating a polysaccharide layer (bar = 500 nm).

concentration of bacteria, all are not able to attach. When the bacterial concentration was increased, there was an increase in attachment, although not statistically significant at all steps. Although the greatest attachment was observed at a concentration of 1×10^9 cfu/ml, several studies have suggested that this concentration is not present in clinical diseases in humans, in the environment, or *in vitro* culture.⁸⁻¹⁰ Therefore, in attachment experiments, one should use a concentration of 1×10^8 cfu/ml, which is easily achievable after

overnight broth culture, and showed a relatively higher number of bacteria attached to pharyngeal cells.

Our mouse model demonstrated that intrabronchial route was efficient in causing pneumonia, even at a concentration of 1×10^5 cfu/ml. However 1×10^9 cfu/ml were necessary to cause pneumonia in intraperitoneally challenged mice. A higher inoculum dose caused hemorrhagic pneumonia as well as bacteremia. It appears that exceeding a certain level of bacterial load in the lungs during pneumonia leads to

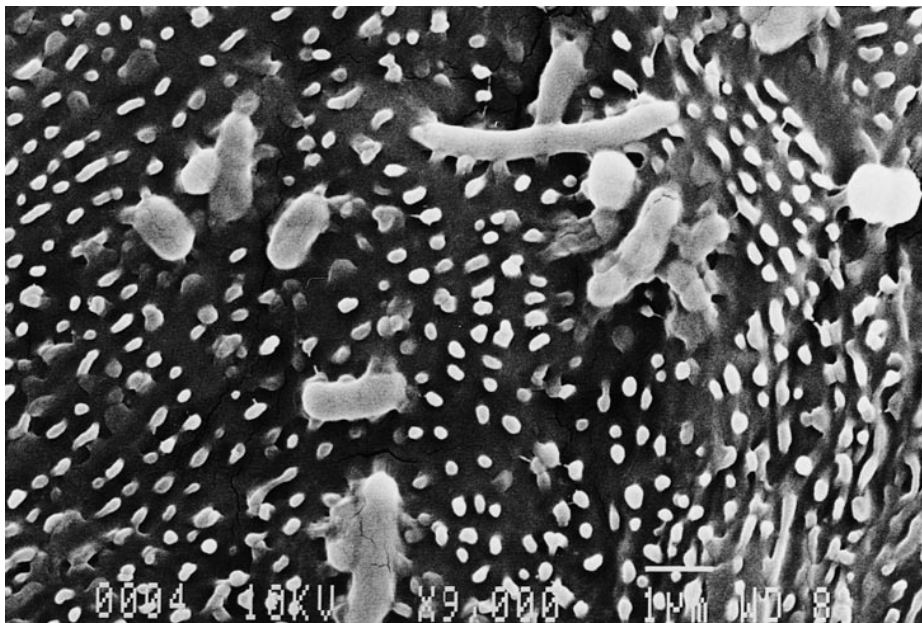


FIGURE 2. Scanning electron micrograph of *Burkholderia pseudomallei* attached to the microvillae (elevations on the surface of pharyngeal epithelial cells) (bar = 1 μ m).

bacteremia. Therefore, we may conclude from the mouse study that prolonged incubation did not have an adverse effect on the virulence of *B. pseudomallei*.

Melioidosis mainly affects malnourished and immunodeficient hosts.^{1,11} However, pulmonary melioidosis was common in U.S. soldiers (helicopter crewmen) in Vietnam. It was suggested that rotor blades of the helicopters dispersed greater amounts of *B. pseudomallei* in the air from the ground, and inhalation of this bacteria caused pulmonary melioidosis in these soldiers.¹² While there was no report of melioidosis among Japanese troops returned from Southeast Asia during and after World War II,¹³ they did not use helicopters. The low attachment ability of *B. pseudomallei* may explain why a greater bacterial load is necessary to cause infections in U.S. soldiers and why it affects mainly malnourished and immunodeficient hosts. In general, infectivity parallels adherence ability except for *Salmonella*, which, although poorly adherent *in vitro*, is moderately infective.¹⁴ A similar low level of adherence ability was also found in *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.¹⁵

As revealed by electron microscopic observation of other bacteria,⁶ *B. pseudomallei* was also found attached to the microplicae of pharyngeal epithelial cells. This may facilitate the delivery of bacterial toxin products efficiently. Electron microscopy also provided evidence that polysaccharide layer may be important in facilitating the attachment of *B. pseudomallei* to pharyngeal epithelial cells. Other studies also revealed this polysaccharide layer of *B. pseudomallei*.^{16,17} Attachment in bacteria without fimbriae is mediated through the capsular polysaccharide.^{18,19} In general, negatively charged bacterial polysaccharide appears to inhibit attachment to mammalian cells. Acapsular mutants of *Hemophilus influenzae* and Group B streptococcus showed increased adherence and entry into respiratory epithelial cells when compared with encapsulated wild type strains.^{20,21} Much still needs to be learned about the bacterial and host factors that determine the successful establishment of *B. pseudomallei* in the respiratory tract.

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