ROLE OF *BURKHOLDERIA PSEUDOMALLEI* IN THE INVASION, REPLICATION AND INDUCTION OF APOPTOSIS IN HUMAN EPITHELIAL CELL LINES

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Abstract. *Burkholderia pseudomallei* is a bacterial pathogen causing melioidosis. In the present study, we demonstrated the effects of *B. pseudomallei* capsular polysaccharide on the invasion, intracellular replication, induction of cytotoxicity and apoptosis of human epithelial HeLa and A549 cells. The *B. pseudomallei* capsule mutant had a significantly greater ability to invade both cell lines ($p<0.05$). The *B. pseudomallei* capsule mutant had a greater ability to induce apoptosis in A549 cells than wild type *B. pseudomallei*. These results indicate the capsular polysaccharide of *B. pseudomallei* plays an important role in inhibiting invasion by the bacteria but has no effect on intracellular multiplication, induction of cytotoxicity or apoptosis in epithelial cells.

Key words: *Burkholderia pseudomallei,* capsular polysaccharide, invasion, replication, cytotoxicity , apoptosis

INTRODUCTION

Burkholderia pseudomallei, a gram-negative bacillus, is the causative agent of melioidosis, a severe disease endemic in Southeast Asia and northern Australia (White, 2003) and is considered to be a potential biological weapon (Deshazer, 2007). The mortality rate in acute cases exceeds 40%, with 10% to 15% of the survivors experiencing a relapse despite prolonged antibiotic treatment (White, 2003). Infection usually follows skin inoculation, inhalation or drinking contaminated water with the causative bacterium, which is present in soil, stagnant streams, ponds, and rice paddies in the endemic region. While 20-36% of melioidosis cases have no evident predisposing risk factor, the vast majority of fatal cases have an identified risk factor, the most important of which are diabetes, alcoholism and chronic renal disease. Half of all cases present with pneumonia, but there is great clinical diversity, from localized skin ulcers or abscesses without systemic illness to fulminant septic shock with multiple abscesses in the lungs, liver, spleen and kidneys, (Currie, 2003). The clinical features vary from acute fulminant septicemia to chronic debilitating localized infection (White, 2003). Abscess formation can be found in any organ, with the lungs being the most commonly affected organ in those with chronic melioidosis (Dhiensiri *et al*, 1988; Currie *et al*, 2000). The patients often present with cough and fever as a result of primary lung abscesses or secondary septicemia.

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B. pseudomallei is known as a facultative intracellular pathogen. The bacteria may be found in a patient long after exposure; as long as 26 years later (Mays and Ricketts, 1975). This evidence illustrated the prolonged latency with recrudescence, difficult diagnosis, and resistance to therapy of chronic melioidosis. A prolonged infection may become an acute, fulminating fatal infection (Koponen *et al*, 1991) or the bacteria may enter a dormant state where it can avoid immune surveillance, most probably in an intracellular location (Adler *et al*, 2009). Several reports have demonstrated the bacteria can multiply in both phagocytic and nonphagocytic cells (Harley *et al*, 1998; Ahmed *et al*, 1999). After internalization, the bacterium escapes from a membrane-bound phagosome into the cytoplasm (Jones *et al*, 1996). *B. pseudomallei* is able to induce cell-to-cell fusion leading to multinucleated giant cell (MNGC) formation (Kespichayawattana *et al*, 2000). It has been reported the bacteria can escape macrophage killing by interfering with the expression of inducible nitric oxide synthase (iNOS) (Utaisincharoen *et al*, 2001). A number of components produced by the bacteria have been purposed as virulence factors, including quorum sensing, type III secretory system, lipopolysaccharide, surface polysaccharides, flagella and a number of factors crucial for the intracellular survival of *B. pseudomallei* (Wiersinga and van der Poll, 2009).

Capsule production has been associated with virulence in numerous pathogenic bacteria, particularly those causing serious invasive infections of humans (Boulnois and Roberts, 1990). Although the importance of this capsule in virulence has been previously established (Reckseidler *et al*, 2001; Reckseidler-Zenteno *et al*, 2005; Sarkar-Tyson *et al*, 2007), the specific contribution of the capsule to the pathogenesis of *B. pseudomallei* has not been defined. *B. pseudomallei* can produce four distinct capsular polysaccharides. These are types I, II, III and IV O-antigenic polysaccharide (O-PS) (Sarkar-Tyson *et al*, 2007). The role of the capsular polysaccharide involved in the virulence of this pathogen was revealed by using the subtractive hybridization technique. A sequence of the glycosyltransferase gene of wild type *B. pseudomallei* 1026b was interrupted; therefore, the mutant was unable to produce extracellular polysaccharide capsule -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1- which was demonstrated to be an essential virulence determinant of *B. pseudomallei* in an animal model (Reckseidler *et al*, 2001). A mutation on the capsular biosynthetic operon also altered the virulence of *B. pseudomallei* in a mouse model (Atkins *et al*, 2002).

Many bacteria have been demonstrated to induce apoptosis in host cells, including phagocytic and epithelial cells (Grassme *et al*, 2001). There is growing evidence apoptosis plays an important role in modulating the pathogenesis of a variety of infectious diseases, which involves a variety of host-pathogen interactions (Gao and Kwaik, 2000). A number of studies demonstrated that *B. pseudomallei* can kill host cells by apoptosis using the caspase-1 pathway and RpoS induction (Sun *et al*, 2005; Lengwehasatit *et al*, 2008).

Even though the importance of the capsule in virulence has been demonstrated previously, the role of the *B. pseudomallei* capsule in internalization, multiplication, cell cytotoxicity and apoptosis in human epithelial cell lines has not been established. The aim of this study, was to investigate the effects of a capsular polysaccharide of *B. pseudomallei* on internalization, intracellular multiplication, cytotoxicity and apoptosis induction of

infection in the lung epithelial, A549 and human cervix carcinoma, HeLa cell lines.

MATERIALS AND METHODS

Cell line and experimental conditions

The human lung adenocarcinoma, A549 (ATCC, Rockville, MD) and the human cervix carcinoma, HeLa cell lines were used in this study. The A549 cells were cultured and maintained in Ham's F12 medium (Hyclone, Logan, UT) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, Gland Island, NY) and 2 mM L-glutamine (Sigma Chemical, St Louis, MO). The HeLa cell lines were cultured and maintained in modified Eagle medium (MEM, Gibco BRL, Grand Island, NY) supplemented with 10% FBS. Cells were cultured at 37ºC in a humidified incubator under an atmosphere of 95% air/5% CO_2.

Bacterial isolation and growth conditions

B. pseudomallei 1026b, a clinically isolated strain and SR1015, a capsule mutant strain (kindly provided by Professor DE Woods, University of Calgary Health Sciences Center, Calgary, Alberta, Canada) were used for the study. The mutant was created from its wild type parental strain 1026b as described previously (Reckseidler *et al*, 2001).

The *B. pseudomallei* 1026b was grown in Luria-Bertani (LB) medium while *B. pseudomallei* SR1015 was grown in the same medium supplement with 50 mg/ml tetracycline. *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Escherichia coli* HB101 were used as prototypes of intracellular and non-invasive extracellular bacteria, and cultured in Trypticase soy (TS) medium. To obtain mid-log phase cultures, 1% of the overnight culture in appropriate medium was inoculated into fresh medium and incubated with agitation at 200 rpm at 37ºC. The desired concentration of bacteria was measured at an optical density of 550 nm and the colony-forming unit (cfu) were calculated from the precalibrated standard curve. The growth rate constant and generation time for both *B. pseudomallei* 1026b and the capsule mutant in LB were also calculated.

Internalization of *B. pseudomallei* by HeLa and A549 cell lines

Infection of the cells with *B. pseudomallei* wild type and capsule mutant strains, *S.* Typhi and *E. coli* were performed essentially as described previously (Jones *et al*, 1996). The cells ($3x10^5$ cells) were cultured in a 24-well plate overnight before co-cultured with the mid-log phase bacteria at a multiplicity of infection (MOI) of 2:1 and 10:1 for HeLa and A549, respectively. Consequently, the plates were briefly and gently shaken on a rotating platform at 120 rpm to distribute the inocula throughout the tissue culture media. The plates were further incubated for 2 hours thereafter to remove extracellular bacteria and then the cells were washed three times with prewarmed tissue culture medium. Residual bacteria were completely eliminated by incubation in the culture medium containing 250 μg/ml kanamycin for 2 hours. To determine intracellular bacteria, the infected cells were washed with prewarmed tissue culture medium and subsequently lysed with 0.1% Triton X-100. The released bacteria were serially plated on TS agar. The number of intracellular bacteria expressed as colony forming units was determined by bacterial colony counting. The internalization assays were performed in triplicate and repeated in three independent experiments.

Intracellular survival and multiplication of *B. pseudomallei* in culture cell lines

The HeLa and A549 cells were infected and treated as described above for the internalization experiment. After the antibiotic protection assay, the infected cell lines were washed with fresh culture medium and subsequently incubated with complete culture medium containing 20 μg/ml kanamycin for 6, 12 and 24 hours for HeLa cells and 6, 12, 16 and 18 hours for A549 cells. The numbers of intracellular bacteria were monitored as colony forming units. Growth rate constants and generation times for both *B. pseudomallei* 1026 and the capsule mutant in HeLa and A549 cells were calculated.

Detection of cytotoxicity of infected cell lines

To determine cytotoxicity in the cell lines infected with bacteria, the media from the overnight cultures in the HeLa and A549 cell lines seeded at $4x10^4$ cells/well in a 96-well plate, was removed. The mid-log phase of bacteria was added to give MOI of approximately 25:1, 50:1 and 100:1, then further incubated for 2 hours at 37ºC in 5%CO_2 as described in the internalization assay. The released cytolytic lactate dehydrogenase (LDH) was determined using the CytoTox96 kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief, a 100-μl aliquot of centrifuged supernatant obtained from each well was added to an enzymatic assay plate (ELISA plate). The assays were performed in triplicate. The LDH release (% cytotoxicity) was calculated using the following equation: (OD_{490} experimental release-OD_{490} spontaneous release)/(OD_{490} maximum release-OD_{490} spontaneous release)x100. The spontaneous release was the amount of LDH release from the cytoplasm of uninfected cells, whereas the maximum release was the amount released by total lysis of uninfected cells by Triton X-100.

Detection of apoptosis by DNA fragmentation and ELISA

Analysis of DNA fragmentation. The HeLa and A549 cells were seeded as $1x10^6$ cells/well in a 6-well plate and incubated for 18 to 20 hours. After co-culturing with bacteria at a MOI of approximately 50:1 for 2 hours, the cells were washed twice with prewarmed tissue culture medium followed by antibiotic protection for 2 hours. The cells were then washed with prewarmed in tissue culture medium and incubated in the culture medium, containing 20 μg of kanamycin per ml, to inhibit the growth of residual extracellular bacteria for 24 hours. After incubation, the infected cells were harvested and washed twice with PBS. The DNA of the infected cells was isolated and purified by the QIAGEN system (QIAamp® DNA Mini Kit; QIAGEN, Germany) according to the manufacturer's protocol. In brief, 300 μl lysis buffer [0.2% Triton X-100, 20 mM Tris (pH 7.4), 10 mM EDTA(pH 8.0)] was added, followed by centrifugation at 10,000*g* for 5 minutes. The pellet was mixed with 300 μl of protease solution and incubated in a water bath at 65ºC for 5 minutes. Nucleic acids were then precipitated by the addition of 300 μl of isopropanol and centrifuged for 7 minutes at 10,000*g*. The pellet was treated with 300 μl 70% ethanol and gently vortexed. After centrifugation, the pellets were air dried and dissolved in TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA] by incubating for 30 minutes at 65ºC in a water bath. The DNA solution was incubated at 37ºC for 1 hour in the presence of RNase (0.1 mg/ml) before it was subjected to electrophoresis in 1.8% agarose gel. The gel was then

stained with ethidium bromide, and the DNA ladders were viewed under an UV light. For a positive control in apoptosis, the uninfected culture cell lines were treated with 50 μmole of etoposide for 4 hours.

Apoptotic assay by immunodetection of oligonucleosomes. Mononucleosomes and oligonucleosomes released from the nucleus into the cytoplasm of apoptotic cells were detected with the use of a sandwich enzyme-linked immunosorbent assay (The Cell Death Detection-ELISA^{PLUS}) (Roche Applied Sciences). The assay was based on a quantitative sandwich enzyme-linked immunosorbent assay principle, with mouse monoclonal antibody directed against DNA histones. The HeLa and A549 cell lines seeded at 3 x 10^5 cells/well, were co-culutred with mid-log phase bacteria at a MOI of 50:1 in 24-wells and briefly and gently shaken on a rotating platform to distribute the inoculum throughout the tissue culture medium. The plate was then incubated for 2 hours for invasion incubation. One group of triplicate wells was used to quantify the bacterial invasion to provide a baseline for the number of intracellular organisms present at time zero. Twenty-four and 48 hours post-infection, the plates were centrifuged at 200*g* for 10 minutes and the supernatant carefully removed. Cells were lysed in incubation buffer for 30 minutes. The lysate was centrifuged at 200*g* for 10 minutes. The supernatant was transferred carefully into a streptavidin-coated microplate for analysis according to the manufacturer's protocol.

Statistical analysis

Unless otherwise indicated, all experiments in this study were conducted independently at least three times. All tests for significance were performed using the Student's *t*-test and Excel software 97 (Microsoft). Results were considered significant at a *p*-value of <0.05.

RESULTS

The effect of the capsule in *B. pseudomallei* internalization in HeLa and A549 cell lines

The capsule mutant strain of *B. pseudomallei* was able to enter the HeLa cell line at a MOI of 2:1 with a percentage of 1.026±0.379 while the wild type had a percentage of 0.587±0.219 (Table 1). When *B. pseudomallei* strains were cultured in the A549 cell lines at a MOI of 10:1, the capsule mutant had a percentage of internalization of 3.586 ± 1.221, and the wild type strain had a percentage of internalization of 1.265 ± 0.593. The results indicate the capsule mutant had significantly higher invasive potential in both cell lines than the wild type judging by the number of intracellular bacteria. In the HeLa cell line (MOI 2:1), the invasion of both *B. pseudomallei* wild type and capsule mutant strains were significantly lower than the *S. enterica* serovar Typhi (3.883±1.332) invasion. In the A549 cell line (MOI 10:1) the *B. pseudomallei* capsule mutant was able to invade the cells at a slightly higher rate than the *S. enterica* serovar Typhi (3.151 ± 0.463). Both wild type and capsule mutant invaded HeLa (MOI 2:1) and A549 cells (MOI 10:1) at a higher level than *E. coli* HB101 (0.0028±0.003 and 0.002 ± 0.0019, respectively) (Table 1).

Effect of capsule on intracellular replication of *B. pseudomallei* in HeLa and A549 cell lines

The results of the experiments to determine the effect of *B. pseudomallei* capsule on intracellular survival and replication in HeLa cell lines are shown in Table 2. Once internalized, both *B. pseudomallei* wild type

Table 1
Internalization of *B. pseudomallei*, *S.* Typhi and *E. coli* into cultured human epithelial cell lines, as measured by the kanamycin protection assay.

	HeLa (MOI = 2:1)			A549 (MOI = 10:1)		
	Inoculum (no. of cells) (cfu)	Mean no. of intracellular organisms (cfu/well)	% Internalization (cfu/well)	Inoculum (no. of cells) (cfu)	Mean no.of intracellular organisms (cfu/well)	% Internalization (cfu/well)
B. pseudomallei 1026b	9.25×10^5	$(5.42 \pm 0.38) \times 10^3$	0.587 ± 0.219	3.51×10^6	$(3.99 \pm 1.05) \times 10^4$	1.265 ± 0.593
B. pseudomallei SR1015	7.50×10^5	$(7.69 \pm 0.54) \times 10^3$	1.026 ± 0.379	4.72×10^6	$(1.69 \pm 0.97) \times 10^5$	3.586 ± 1.221
S. enterica serovar Typhi	9.50×10^5	$(3.68 \pm 0.74) \times 10^4$	3.883 ± 1.332	3.75×10^6	$(1.18 \pm 0.17) \times 10^4$	3.151 ± 0.4632
E. coli HB101	28.62×10^5	$(1.00 \pm 0.89) \times 10^2$	0.0028 ± 0.003	4.40×10^6	$(1.66 \pm 0.81) \times 10^2$	0.002 ± 0.0019

Table 2
Intracellular multiplication of *B. pseudomallei*, *S.* Typhi and *E. coli* in cultured human epithelial cell lines.

Time post-invasion (hours)	HeLa		A549	
	B. pseudomallei 1026b	*B. pseudomallei* SR1015	*B. pseudomallei* 1026b	*B. pseudomallei* SR1015
0	$(1.15 \pm 0.33) \times 10^3$	$(3.90 \pm 1.58) \times 10^3$	$(9.70 \pm 6.40) \times 10^3$	$(2.96 \pm 0.80) \times 10^4$
6	$(9.43 \pm 3.69) \times 10^3$	$(1.16 \pm 0.52) \times 10^4$	$(1.35 \pm 0.30) \times 10^5$	$(2.18 \pm 1.21) \times 10^5$
12	$(1.12 \pm 0.22) \times 10^5$	$(2.47 \pm 1.29) \times 10^5$	$(2.50 \pm 0.89) \times 10^6$	$(6.20 \pm 2.49) \times 10^6$
18	ND	ND	$(1.10 \pm 0.11) \times 10^7$	$(1.71 \pm 0.64) \times 10^7$
24	$(5.03 \pm 1.20) \times 10^5$	$(1.00 \pm 0.50) \times 10^6$	ND	ND

ND, Not determined

Table 3
Growth rate constant and generation time of *B. pseudomallei* in LB broth, HeLa and A549 cell lines.

Bacterial strains	LB broth		HeLa		A549	
	Growth rate constant (h^{-1})	Generation time (minutes)	Growth rate constant (h^{-1})	Generation time (minutes)	Growth rate constant (h^{-1})	Generation time (minutes)
B. pseudomallei 1026b	0.732	56.8	0.253	164.3	0.391	106.3
B. pseudomallei SR1015	0.683	60.8	0.231	180	0.353	117.7

and capsule mutant survived and multiplied. *B. pseudomallei* 1026b (the wild type) and *B. pseudomallei* SR1015 (capsule mutant) were found intracellularly at 4 hours in amounts of 1.15 (±0.33) x 10^3 organisms, or 0.38% of inoculum and 3.90 (±1.58) x 10^3 or 1.3% of inoculum, respectively. After an additional 24 hours of incubation, the number of intracellular organisms increased to 5.03 (±1.20) x 10^5 and 1.00 (±0.50) x 10^6, respectively. These results reveal a statistically significant 2-log-unit increase ($p<0.05$). Both bacterial strains replicated inside the human epithelial cell line after internalization until 24 hours after infection. The generation time and growth rate constant for the wild type and capsule mutant strains were 0.253 and 0.231 h^{-1} and 164.3 and 180 minutes, respectively (Table 3). The results suggest the rate of replication of the capsule mutant inside the HeLa cell line was not significantly different from the wild type ($p>0.05$).

Both bacterial strains replicated inside the human epithelial cell line after internalization until 18 hours after infection. After the initial 4-hour incubation period *B. pseudomallei* 1026b (the wild type) and *B. pseudomallei* SR1015 (capsule mutant) were found in A549 cells at 9.70 (±6.40) x 10^3 organisms or 3.2% of the inoculum and 2.96 (±0.80) x 10^4 organisms or 9.87% of the inoculum. After 18 hours of incubation, the number of intracellular *B. pseudomallei* 1026b (the wild type) and *B. pseudomallei* SR1015 (capsule mutant) increased to 1.10 (±0.11) x 10^7 and 1.71 (±0.64) x 10^7, representing a statistically significant increase of 3-logs and 2-logs, respectively. The generation time and growth rate constant in the wild type and capsule mutant strains were 0.391 and 0.353 h^{-1} and 106.3 and 117.7 minutes, respectively (Table 3). The results suggest the rate of replication of the

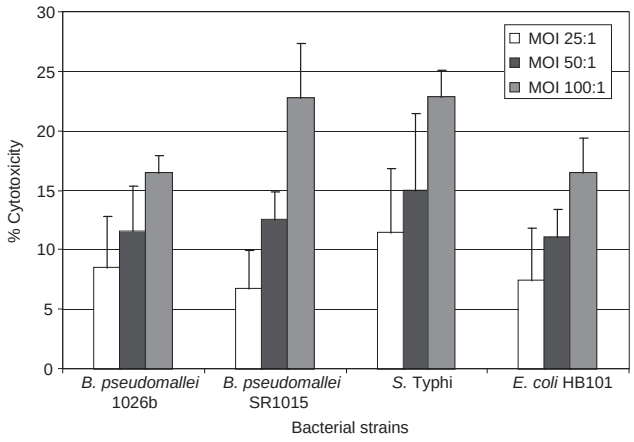


Fig 1–Cytotoxic effects of *B. pseudomallei* 1026b and SR1015 strains on HeLa cells. HeLa cells were infected with different MOIs of these bacterial strains for 2 hours. Cell cytotoxicity was determined using the LDH- release assay. Each value represents the means ± SD of three independent experiments.

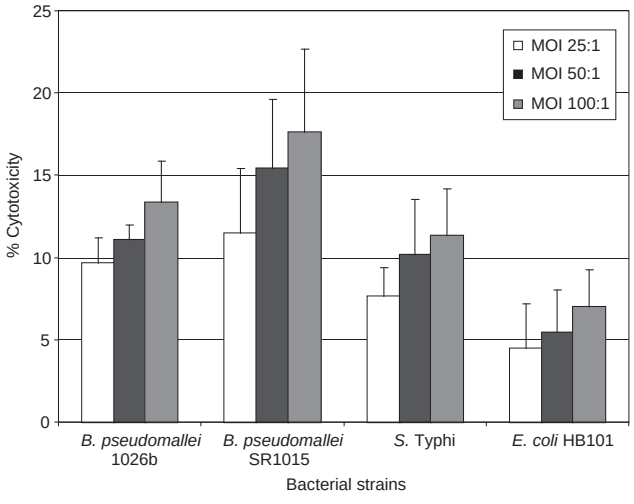


Fig 2–Cytotoxic effects of *B. pseudomallei* 1026b and SR1015 strains on A549 cells. A549 cells were infected with different MOIs of these bacterial strains for 2 hours. Cell cytotoxicity was determined using the LDH release assay. Each value represents the means ± SD of three independent experiments.

capsule mutant inside the A549 cell line was not significantly different from the wild type (p>0.05). At 24 hours of infection, intracellular bacteria could not be determined because the infected cells were damaged and detached from the surface of the tissue culture plates.

Determination of cytotoxicity of *B. pseudomallei* capsule on HeLa and A549 cell lines

The cytotoxicity in the HeLa cell lines infected with four bacterial strains was expressed in a dose-dependent manner (Fig 1). The capsule mutant (SR1015) induced LDH leakage in infected HeLa cells at a higher level than the wild type at a MOI of 50:1 and 100:1.

The cellular cytotoxicity in the A549 cell lines infected with four bacterial strains is shown in Fig 2. The cytotoxic effects of A549 infected with the four bacterial strains demonstrated a dose-dependent effect. *B. pseudomallei* SR1015 resulted in the highest level of LDH release at all three MOIs. The wild type *B. pseudomallei* also caused more LDH release than the invasive controls. The level of LDH released in *B. pseudomallei* infected A549 cells was higher than in the infected HeLa cells at a MOI of 25:1 but was lower at a MOI of 100:1.

Apoptosis

The capsule mutant and wild type *B. pseudomallei* did not induce DNA fragmentation in infected HeLa cells at a MOI of 50:1 at 24 and 48 hours after infection (data not shown). In contrast, the A549 infected with wild type and capsule mutant *B. pseudomallei* at a MOI of 50:1 at 24 and 48 hours clearly induced A549 cell

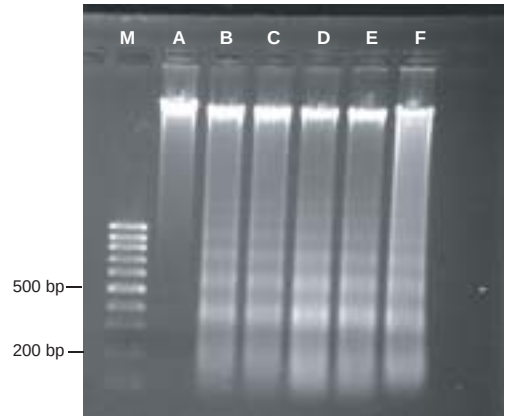
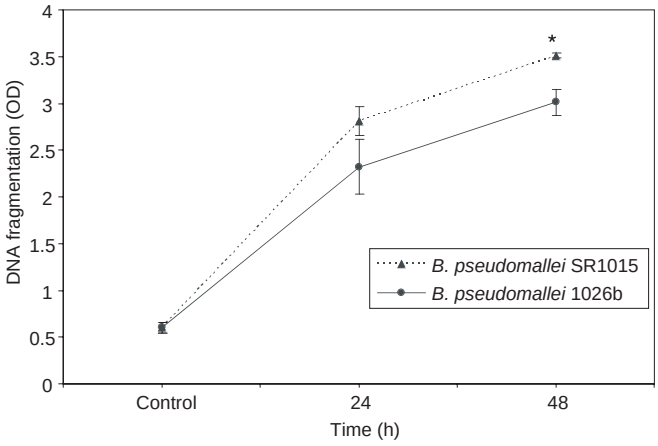


Fig 3–DNA fragmentation of A549 cells caused by *B. pseudomallei*. The uninfected A549 cell lines (A) and A549 cells infected with wild-type *B. pseudomallei* at an MOI 50:1 (B-C) or capsule mutant *B. pseudomallei* at an MOI 50:1 (D-E) after 24 hours of infection. The A549 cells treated with etoposide 85 μM for 24 hours (F) were used as a positive control for apoptosis. Lane M is a 100 bp standard marker. Electrophoresis was performed with 1.6% agarose gel, and DNA was stained with ethidium bromide.



* Significant difference ($p<0.05$) between capsule mutant and wild-type *B. pseudomallei*

Fig 4–*B. pseudomallei* induced DNA fragmentation on A549 cells was quantified using the Cell Death ELISA^{plus} kit and the histone-associated DNA fragments as indicators of apoptosis. A549 cells were infected with capsule mutant and wild-type *B. pseudomallei* at an MOI 50:1 for 24 and 48 hours. Data shown as mean ± SD of three independent experiments.

apoptosis, as shown by the ladder formation (Fig 3). Interestingly, the capsule mutant strain induced apoptosis in infected A549 at a significantly higher rate than the wild type ($p<0.05$) using the ELISA kit. Histone-associated DNA fragmentation occurred in a time-dependent manner (Fig 4). Observations showed both wild type and capsule mutant *B. pseudomallei* induced multinucleated giant cell formation in A549 at 24 hours after infection (data not shown).

DISCUSSION

The present study demonstrated *B. pseudomallei* SR1015 (capsule mutant) could more efficiently invade both HeLa and A549 cells than *B. pseudomallei* 1026b (wild type) ($p<0.05$). These results suggest the capsule may hamper internalization of *B. pseudomallei* into nonphagocytic cells. This observation was also been reported in *K. pneumoniae*, where the capsule impedes adhesion and invasion of *K. pneumoniae* into nonphagocytic cells and epithelial cell lines (Fumagalli *et al*, 1997; Oelschlaeger and Tall, 1997; Sahly *et al*, 2000). The null capsule mutant of *Hemophilus influenzae* and Group B streptococcus show increased adherence and entry into respiratory epithelial cells when compared with encapsulated wild type strains (St Geme and Falkow, 1991). In the case of *Streptococcus suis*,

capsular polysaccharide may interfere with adhesion molecules located on the streptococcal surface, since the non-encapsulated mutant strain showed significantly higher adherence and invasiveness than its parental strain (Benga *et al*, 2004). Regueiro *et al* (2006) demonstrated the *K. pneumoniae* capsule mutant was internalized by A549 cells more efficiently than wild-type *K. pneumoniae*. In *E. coli*, the K1 capsule-deletion mutant was also shown to have higher binding and internalization rates in the mutant than in the parent *E. coli* K1 strain in human brain microvascular endothelial cells (Kim *et al*, 2003).

The results of this study demonstrate intracellular multiplication in the non-phagocytic cells, HeLa and A549, by *B. pseudomallei* 1026b (wild type) and *B. pseudomallei* SR1015 (capsule mutant) were similar (Table 2). The growth rate of *B. pseudomallei* wild type in both HeLa and A549 cell lines, was slightly higher than the capsule mutant (Table 3). These observations were related to the growth rate of both bacterial strains in LB broth (Table 3).

The LDH assay is a general indicator of loss of cell membrane integrity and a well-established and quantitative assay of cell viability. The results from this study demonstrate *B. pseudomallei* SR1015 (capsule mutant) may induce host cell cytotoxicity at a slightly higher level than *B. pseudomallei* 1026b. This observation is correlated with ability of the mutant strains to invade host cells more easily than the wild type (Figs 1 and 2). Internalized bacteria that still have other virulence factors may cause damage to the host cells. Therefore, intracellular mutant strains can lead to more cellular cytotoxicity in host cells than the wild type. It has been reported high molecular weight polysaccharide, rhamnolipid toxin, phospholipase C and RpoS of *B. pseudomallei*, have cellular cytotoxic effects (Wongwanich *et al*, 1996; Haussler *et al*, 1998; Balaji *et al*, 2004; Korbsrisate *et al*, 2007). This phenomenon can also be demonstrated in *Yersinia pestis*, as the encapsulated *Y. pestis* loses the ability to cause cytotoxicity in the J774.A1 a murine macrophage cell line (Weeks *et al*, 2002).

The present study demonstrated that *B. pseudomallei* can invade HeLa and A549 epithelial cells within hours after infection. Apoptosis analysis of infected A549 cells with both wild type and mutant strains revealed DNA ladder formation by agarose gel and the Cell Death Detection ELISA^{plus}. The histone-associated DNA fragmentation was time-dependent. The DNA ladder formation in HeLa cells infected with *B. pseudomallei* 1206b and the capsule mutant could not be demonstrated by gel electrophoresis but could be observed with the ELISA kit, which is a more sensitive method. Kespichayawattana *et al* (2000) demonstrated that *B. pseudomallei* can readily induce apoptosis in HeLa cells, as shown by DNA ladder formation, but some strains of *B. pseudomallei* appeared to cause less extensive damage 24 hours after infection. Apoptosis of A549 cells infected with the capsule mutant was slightly more common than in cells infected with the wild type and is related to the number of internalized bacteria and cellular cytotoxicity. Apoptosis of infected HeLa and A549 cell may be a protective mechanism of the host to prevent systemic host infection by clearing bacteria infected epithelial cells (Kim *et al*, 1998). A major role of apoptosis during bacterial infection is to delete the population of infected epithelial cells from the tissue and to restore normal epithelial cell growth. From the perspective of the host, deleting those populations of epithelial cells by apoptosis can restore normal regulation of epithelial

cells growth and differentiation, while preserving the integrity of the epithelial cell barrier (Eckmann *et al*, 1997).

One of the unique characteristics of *B. pseudomallei* is its ability to induce cell-to-cell fusion, resulting in multinucleated giant cell (MNGC) formation which may facilitate bacteria spread from one cell to another (Harley *et al*, 1998; Kespichayawattana *et al*, 2000). The bacteria may induce MNGC formation in both phagocytic and nonphagocytic cells (Kespichayawattana *et al*, 2000). Utaisincharoen *et al* (2006) reported that RpoS interferes with MNGC formation in mouse macrophage cell lines (RAW 264.7). The *rpoS* mutant failed to initiate these changes. They demonstrated, the wild type *B. pseudomallei* failed to induce MNGC when the bacterial protein synthesis was inhibited after the bacteria had been internalized, suggesting that it is the bacterial factors expressed after being internalized that play an important role in the induction of MNGC formation. In this study, MNGC formation was observed in HeLa and A549 infected cells with either *B. pseudomallei* wild type and the mutant strain at 24 and 48 hours after infection. The results reveal the capsule does not affect MNGC formation in these epithelial cells.

It has been reported the addition of a capsule was not toxic to hamsters, since hamsters inoculated with 100 μg of purified capsule alone survived for the duration of the experiment without any ill effects (Reckseidler-Zenteno *et al*, 2005). It has also been reported that *B. pseudomallei* SR1015 is markedly less virulent than the parent strain in animal models (Reckseidler *et al*, 2001). The purified capsule was shown to increase the virulence of the capsule mutant in a Syrian hamster model with acute melioidosis (Reckseidler-Zenteno *et al*, 2005). The controversial differences in results between *in vivo* and *in vitro* studies imply different conditions, such as the immune system.

Pneumonia is a common manifestation of melioidosis (Cheng and Currie, 2005). More apoptosis was seen in human alveolar lung epithelial (A549) cells than in human cervical epithelial (HeLa) cells. It is possible the role of apoptosis may be associated with the pathogenesis of *B. pseudomallei* in the lung. Inhalation was initially thought to be the primary mode of acquisition in melioidosis (Cheng and Currie, 2005).

A correlation between cellular cytotoxicity and apoptosis was observed in our study. The capsule mutant *B. pseudomallei* showed more cytotoxicity in an epithelial cell model than wild type *B. pseudomallei*. These results suggest the *B. pseudomallei* capsule does not play a major role in induction of cytotoxicity and apoptosis, but other factors might be involved.

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