Virulence of clinical and environmental isolates of *Burkholderia oklahomensis* and *Burkholderia thailandensis* in hamsters and mice

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**Keywords**

*Burkholderia oklahomensis*; *Burkholderia thailandensis*; *Burkholderia pseudomallei*.

**Abstract**

*Burkholderia pseudomallei* is the etiologic agent of the tropical disease melioidosis and is considered to be a potential biological weapon. Two *B. pseudomallei*-like species, *Burkholderia oklahomensis* and *Burkholderia thailandensis*, have been described in the literature. Infections with both of these microorganisms have occurred in the United States, but little is known about the relative virulence of these isolates in animal models of infection. In this study, *B. oklahomensis* and *B. thailandensis* CDC2721121 were determined to be avirulent in hamsters and mice at all challenge doses examined. The virulence of *B. thailandensis* CDC3015869, on the other hand, was more similar to the virulence of isolates of *B. thailandensis* from Southeast Asia.

**Introduction**

*Burkholderia pseudomallei* is the cause of the disease melioidosis in Southeast Asia and northern Australia (White, 2003; Cheng & Currie, 2005). The Gram-negative pathogen is present in water and soil in tropical and subtropical regions and spreads to humans through direct contact with these contaminated sources. Clinical manifestations in humans vary widely and range from chronic visceral and soft tissue abscesses to acute septicemia. Many animal species are susceptible to melioidosis, including sheep, goats, horses, swine, cattle, dogs, and cats (Sprague & Neubauer, 2004).

*Burkholderia pseudomallei* is highly virulent in Syrian hamsters and BALB/c mice, exhibiting a 50% lethal dose (LD$_{50}$) of $<10$ and $\sim 100$ bacteria, respectively (Smith et al., 1997; Brett et al., 1998; Leakey et al., 1998; Ulett et al., 2001).

Today, melioidosis is regarded as an emerging infectious disease and a potential bioterrorism threat (Yabuuchi & Arakawa, 1993; Rotz et al., 2002; Aldhous, 2005).

Two *B. pseudomallei*-like species have been described in the literature: *Burkholderia thailandensis* (Brett et al., 1998) and *Burkholderia oklahomensis* (Glass et al., 2006b). *Burkholderia thailandensis* was first isolated from soil and water in Thailand and was mistakenly identified as *B. pseudomallei* because of its similar environmental, biochemical, and morphological characteristics (Wuthiekanun et al., 1996). However, notable genetic and phenotypic differences between *B. thailandensis* and *B. pseudomallei* have been identified and these organisms can now be differentiated relatively easily (Trakulsomboon et al., 1997; Chaiyaroj et al., 1999; Dharakul et al., 1999; Thepthai et al., 2001; Liu et al., 2002; Sonthayanon et al., 2002; Wuthiekanun et al., 2002; Inglis et al., 2003). One of the most obvious differences between these species is their virulence capacity in humans and animals. Only two human infections with *B. thailandensis* have been recorded in Southeast Asia, suggesting that it is much less virulent than *B. pseudomallei* (Dharakul et al., 1999; Lertpatanasuwan et al., 1999). The LD$_{50}$ of *B. thailandensis* in hamsters is $\sim 10^6$ bacteria (at 48 h postinoculation), which is $>10^3$-fold higher than *B. pseudomallei* in this animal model of infection (Brett et al., 1997). Similarly, the LD$_{50}$ in BALB/c mice is $\sim 10^6$ bacteria, which is $>10^8$-fold higher than *B. pseudomallei* (Smith et al., 1997; Ulett et al., 2001). It should be noted, however, that the *B. pseudomallei* LD$_{50}$ in this animal model of infection can vary considerably between strains (Ulett et al., 2001).
Two infections with *B. thailandensis* have occurred in the United States over the past decade in Texas and Louisiana, but the organism has not been isolated from environmental sources in this country to date (Glass et al., 2006a). The United States isolates were closely related to *B. thailandensis* isolates from Southeast Asia, but they formed a distinct subcluster using multilocus sequence typing (MLST) (Glass et al., 2006a). Nothing is currently known about the virulence of these isolates in animal models of infection.

*Burkholderia oklahomensis* was originally isolated in Oklahoma, US, in 1973 from a farmer involved in a tractor accident (McCormick et al., 1977; Glass et al., 2006b). The strain isolated from the purulent discharge of a pelvic wound was described as *B. pseudomallei*-like because of cultural and biochemical similarities, but could be differentiated from *B. pseudomallei* using serology and fatty acid composition. In addition, a guinea-pig inoculated with the Oklahoma isolate did not die or exhibit any of the pathological signs indicating infection with *B. pseudomallei*. Two additional *B. oklahomensis* strains were isolated from the soil near the site of the accident, suggesting that soil was the source of infection. A second clinical isolate of *B. oklahomensis* was obtained from a man involved in an automobile accident in Georgia, USA, in 1977 (Nussbaum et al., 1980; Glass et al., 2006b), but no additional infections with this organism have been reported for 30 years.

In this study, the relative virulence of the United States isolates of *B. thailandensis* and *B. oklahomensis* in mice and hamsters is described and compared with two *B. thailandensis* isolates from Thailand. The results demonstrate that *B. oklahomensis* is avirulent in commonly used animal models of *Burkholderia* infection and that *B. thailandensis* isolates from the United States differ in their virulence capacity in these models of infection.

### Materials and methods

#### Bacterial strains and growth conditions

The *B. thailandensis* and *B. oklahomensis* strains used in this study were grown at 37°C on Luria–Bertani (LB) agar or in LB broth, and are described in Table 1.

#### Animal studies

Six- to 8-week-old female Syrian hamsters, five per group, were infected intraperitoneally with 10⁶, 10⁷, or 10⁸ CFU of *B. thailandensis* and *B. oklahomensis* and deaths were recorded daily for 14 days. On day 15, the surviving animals from each group were sacrificed. Six- to 7-week-old female BALB/c mice, 10 per group, were infected intraperitoneally with 10⁷ CFU of *B. thailandensis* or *B. oklahomensis* and deaths were recorded daily for 14 days. On day 15, the surviving animals from each group were sacrificed. GRAPHPAD PRISM 5 (San Diego, CA) was used to calculate per cent survival at each time point for each group of infected BALB/c mice. Kaplan–Meier survival plots were generated, and survival between groups was compared with the log-rank (Mantel–Cox) test.

All animals used in this research project were cared for and used humanely according to the following policies: the United States Public Health Service Policy on Humane Care and Use of Animals (1996); the Guide for the Care and Use of Laboratory Animals (1996); and the United States Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (1985). All NCI-Frederick animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>B. thailandensis</td>
<td>Type strain; genomic sequence completed (<a href="http://www.tigr.org/">http://www.tigr.org/</a>)</td>
<td>Brett et al. (1998), Yu et al. (2006)</td>
</tr>
<tr>
<td>Phuket 4W-1</td>
<td>Isolated from water in Phuket, Thailand in 1965</td>
<td>Finkelstein et al. (2000)</td>
</tr>
<tr>
<td>CDC3015869</td>
<td>Blood isolate from a 2-year-old male involved in an automobile accident in Texas, US in 2003</td>
<td>Glass et al. (2006a)</td>
</tr>
<tr>
<td>CDC2712121</td>
<td>Pleural wound isolate from a 76-year-old Louisiana, US man in 1997</td>
<td>Glass et al. (2006a)</td>
</tr>
<tr>
<td>B. oklahomensis</td>
<td>Pelvic wound isolate from a 27-year-old male injured in a farming accident in 1973 in Oklahoma, US, type strain</td>
<td>McCormick et al. (1977)</td>
</tr>
<tr>
<td>C7532</td>
<td>Soil isolate from the 1973 Oklahoma, US farming accident site</td>
<td>McCormick et al. (1977)</td>
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Results and discussion

*Burkholderia thailandensis* CDC2721121 is avirulent in Syrian hamsters

The 48 h LD$_{50}$ for *B. thailandensis* E264 in Syrian hamsters is $c. 10^6$ bacteria (Brett *et al.*, 1997). In comparison, *B. pseudomallei* has an LD$_{50}$ of $< 10$ CFU in this animal model of infection. In this study, the virulence of two Southeast Asian isolates of *B. thailandensis* (E264 and Phuket 4W-1) were compared with two USA isolates of *B. thailandensis* (CDC3015869 and CDC2721121) in hamsters. Groups of five hamsters were inoculated with $10^5$, $10^6$, or $10^7$ CFU and observed for a period of 14 days postinfection. Figure 1a–c shows that all hamsters infected with E264, Phuket 4W-1, and CDC3015869 died within the first week of challenge, regardless of the challenge dose. In a previous study, it was observed that hamsters challenged with a high dose ($> 10^6$ CFU) of *B. thailandensis* E264 did not have lesions on their spleens or livers at the time of death and *B. thailandensis* could be isolated from the bloodstream of these animals at a concentration of $> 10^4$ CFU mL$^{-1}$ (Brett *et al.*, 1997). Therefore, no attempts were made in this study to examine gross histopathology or isolate *B. thailandensis* from the bloodstream of challenged hamsters.

The results presented in Fig. 1 demonstrate that CDC3015869 displays a virulence capacity in hamsters that is very similar to that of *B. thailandensis* strains from Southeast Asia. In contrast, *B. thailandensis* CDC2721121 did not kill any hamsters at $10^5$ CFU (Fig. 1a), $10^6$ CFU (Fig. 1b), or $10^7$ CFU (Fig. 1c). This suggests that there is a distinct difference in the genetic makeup of this strain relative to other strains, including CDC3015869. Interestingly, there were no differences detected between CDC2721121 and CDC3015869 by MLST or 16S rRNA gene sequence analysis (Glass *et al.*, 2006b).

CDC2721121 was isolated from a 76-year-old male with a pleural wound, but little additional clinical information is available for this patient because no case report was published (Glass *et al.*, 2006b). Thus, it is difficult to make a statement about how the weak virulence in hamsters compares with the virulence manifested in this human case. *Burkholderia thailandensis* is considered by most to be nonpathogenic or weakly pathogenic. However, it may be possible to identify genes that contribute to the ability of this species to kill hamsters at high challenge doses using the closely related US strains described here. The genetic factor(s) responsible for the diminished virulence capacity of *B. thailandensis* CDC2721121 could potentially be identified in future studies by subtractive hybridization and/or comparative whole genome sequencing.

Finkelstein *et al.* (2000) conducted a study from 1964 to 1967 to define the geographic distribution of *B. pseudomallei*.
in Thailand. The researchers exploited the exquisite sensitivity of the hamster for \emph{B. pseudomallei} by inoculating soil and water sources intraperitoneally into each of five hamsters. The animals were observed for 1 week postinfection and \emph{B. pseudomallei} strains were subsequently isolated by spreading heart blood from sick or dead animals onto selective media. \emph{Burkholderia thailandensis} Phuket 4W-1 was isolated from a water source in Phuket in 1965 using this technique. Phuket 4W-1 was originally designated as a strain of \emph{B. pseudomallei}, but recent studies at the Centers for Disease Control and Prevention demonstrated that it assimilated l-arabinose and was 99.9% similar to \emph{B. thailandensis} by 16S rRNA gene sequencing (Mindy B. Glass, pers. commun.). The hamster experiments presented here also support this conclusion because hamsters infected with $10^5$ CFU of \emph{B. pseudomallei} survive for $\leq 48$ h, whereas hamsters infected with \emph{B. thailandensis} Phuket 4W-1 did not begin to die until day 3 with this challenge dose (Fig. 1a). Furthermore, the results obtained using Phuket 4 W-1 at higher challenge doses (Fig. 1b and c) also support this conclusion. It appears that the original water source in Phuket contained high enough levels of \emph{B. thailandensis} ($>10^4$ CFU mL$^{-1}$) to kill hamsters within the 1 week of observation. Alternatively, it is possible that \emph{B. pseudomallei} and \emph{B. thailandensis} were both present in the water source and that the colony chosen as Phuket 4W-1 from the selective agar plate happened to be \emph{B. thailandensis}.

\textbf{Burkholderia oklahomensis} strains are avirulent in Syrian hamsters

The virulence of \emph{B. oklahomensis} has not been assessed in an animal model of infection since a single guinea-pig was inoculated intraperitoneally with $10^5$–$10^6$ CFU of \emph{B. oklahomensis} C6786 over three decades ago (McCormick et al., 1977). The guinea-pig did not die or display any obvious symptoms of infection. In this study, groups of five hamsters were inoculated with $10^3$, $10^6$, and $10^7$ CFU of \emph{B. oklahomensis} C6786, C7532, C7533, and E0147 (Table 1) and observed for 2 weeks postinfection (Fig. 1a–c). No deaths occurred due to \emph{B. oklahomensis} at any challenge dose examined during the 14 days of observation, suggesting that \emph{B. oklahomensis} is not pathogenic for hamsters. \emph{Burkholderia oklahomensis} C6786 and E0147 were isolated from patients involved in relatively severe accidents involving the deposition of soil into wounds and this may have provided a niche not normally accessible to this docile soil saprophyte. A comparative genomics approach with \emph{B. pseudomallei} or \emph{B. thailandensis} might reveal why this species is avirulent in guinea-pigs and hamsters. The Naval Medical Research Center recently submitted the \emph{B. oklahomensis} C6786 and \emph{B. oklahomensis} E0147 whole genome shotgun (WGS) projects to GenBank with the project accession numbers NZ_ABBG00000000 and NZ_ABBF00000000, respectively. BLASTN searches (http://www.ncbi.nlm.nih.gov/blast/) of the nonredundant nucleotide database using \emph{B. pseudomallei} gene clusters encoding the capsular polysaccharide (Reckseidler et al., 2001), a type III secretion system (Stevens et al., 2002), and a type VI secretion system (Shalom et al., 2007) did not identify similar gene clusters in \emph{B. oklahomensis} C6786 or \emph{B. oklahomensis} E0147. These are confirmed (capsule and type III secretion) or likely (type VI secretion) virulence determinants in \emph{B. pseudomallei} and it is possible that \emph{B. oklahomensis} is avirulent because it does not harbor comparable gene clusters.

\textbf{Relative virulence of \emph{B. thailandensis} and \emph{B. oklahomensis} strains in BALB/c mice}

Previous studies demonstrated that the LD$_{50}$ for \emph{B. thailandensis} in BALB/c mice challenged via the intraperitoneal route of infection is $\sim 10^5$ CFU (Smith et al., 1997). By comparison, a dose of $10^6$ CFU \emph{B. thailandensis} E264 was lethal for BALB/c mice when delivered intranasally (Wiersinga et al., 2007). In this study, groups of 10 mice were challenged via the intraperitoneal route of infection with $10^5$ CFU of \emph{B. thailandensis} and observed for 14 days (Fig. 2). Infection with \emph{B. thailandensis} E264 and Phuket 4W-1, two Southeast Asian environmental isolates, resulted in the death of 90% and 80% of the BALB/c mice, respectively. By comparison, only 20% of the mice died when infected with CDC3015869 and no mice died when infected with CDC2721121 (Fig. 2). Kaplan–Meier survival plots were generated and survival between groups was compared with the log-rank (Mantel–Cox) test. \emph{Burkholderia thailandensis} Southeast Asian isolates E264 and Phuket 4W-1 were

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Survival of BALB/c mice challenged with \emph{Burkholderia thailandensis} and \emph{Burkholderia oklahomensis}. Mice were exposed to an intraperitoneal challenge of $10^7$ CFU and followed for 14 days postchallenge.}
\end{figure}
 significantly more virulent than *B. thailandensis* US isolates CDC3015869 and CDC2721121 (*P* < 0.001).

Taken together, the mouse and hamster experiments (Figs 1 and 2) strongly suggest that CDC2721121 has a low pathogenic potential. Furthermore, the results suggest that US isolates of *B. thailandensis* are somewhat less pathogenic than Southeast Asian isolates. A recent study demonstrated subtle MLST differences between Southeast Asian and US isolates of *B. thailandensis* (Glass et al., 2006a), suggesting that they form distinct subclusters within this species. Whole genome comparisons between Southeast Asian and US isolates may clarify their phylogenetic relationship and help identify genes that confer a greater virulence capacity on the former. *Burkholderia thailandensis* E264, the type strain of this species, is the only strain that has been sequenced to date; whole genome comparisons will have to wait until a US isolate(s) has been sequenced.

Nothing is currently known about the virulence of *B. oklahomensis* in mice and so groups of 10 mice were challenged via the intraperitoneally route of infection with $10^7$ CFU of *B. oklahomensis* C6786, C7532, C7533, and E0147 and observed for 14 days (Fig. 2). Similar to what was found in hamsters and a guinea-pig, no *B. oklahomensis*-infected mice died or exhibited symptoms of infection after this relatively high challenge dose. The results clearly demonstrate that this species is not pathogenic when injected intraperitoneally into laboratory animals commonly used to study virulence in the *Burkholderia* genus. Furthermore, it seems likely that the reason why there have been no *B. oklahomensis* infections reported for more than three decades is due to the fact that this organism is weakly pathogenic for humans also.

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**References**


