Efficacy of Post Exposure Administration of Doxycycline in a Murine Model of Inhalational Melioidosis

H. Carl Gelhaus¹, Michael S. Anderson¹, David A. Fisher¹, Michael T. Flavin², Ze-Qi Xu²*, & Daniel C. Sanford¹

¹Battelle, Battelle Memorial Institute, Columbus, OH, USA, ²Advanced Life Sciences, Woodridge, IL, USA.

Burkholderia pseudomallei is the causative agent of melioidosis. Treatment of melioidosis is suboptimal and developing improved melioidosis therapies requires animal models. In this report, we exposed male BALB/c mice to various amounts of aerosolized B. pseudomallei 1026b to determine lethality. After establishing a median lethal dose (LD₅₀) of 2,772 colony forming units (cfu)/animal, we tested the ability of doxycycline administered 6 hours after exposure to a uniformly lethal dose of ~20 LD₅₀ to prevent death and eliminate bacteria from the lung and spleens. Tissue bacterial burdens were examined by PCR analysis. We found that 100% of mice treated with doxycycline survived and B. pseudomallei DNA was not amplified from the lungs or spleens of most surviving mice. We conclude the BALB/c mouse is a useful model of melioidosis. Furthermore, the data generated in this mouse model indicate that doxycycline is likely to be effective in post-exposure prophylaxis of melioidosis.

Burkholderia pseudomallei is a Gram-negative, motile, non-spore-forming, aerobic bacteria found in contaminated water, soil, and on market produce. It is the causative agent of melioidosis, an important infectious disease with highly endemic regions in southeast Asia and northern Australia and expanding known global distribution. The primary reservoir of B. pseudomallei is the soil and water. Transmission from the environment to humans most commonly occurs via open wounds and skin abrasions as well as via inhalation during severe weather and can also occur via ingestion although this occurs more in animals than humans. Melioidosis can manifest a broad range of symptoms, with both acute and chronic manifestations. Acute disease can be severe, including fever, weight loss, pneumonia, and death. In addition to acute disease, asymptomatic chronic infections can exist for up to 62 years. Although there is no documented history of the use of B. pseudomallei as a biological weapon, Ken Alibek claimed the Soviet Union weaponized B. pseudomallei. Because of heightened concerns about the use of B. pseudomallei as a biological weapon, ongoing research is aimed at developing novel and safe anti-bacterial therapeutics.

B. pseudomallei is naturally resistant to a diversity of antibiotics, including penicillin, ampicillin, first and second generation cephalosporins, gentamicin, tobramycin, streptomycin, and polymyxin. Treatment involves an intensive intravenous infusion phase using either cefazidime, meropenem, or imipenem for 10 to 14 days followed by a lengthy eradication phase of oral trimethoprim-sulfamethoxazole for up to 6 months. Given the multidrug resistance of B. pseudomallei and lengthy treatment for melioidosis, there is a need to develop improved therapies. This requires well-characterized animal models of melioidosis with defined endpoints relevant to human disease for preclinical testing of new therapeutics and for screening of potential vaccine candidates. Additionally, given the low incidence of naturally acquired melioidosis in most parts of the world, the testing of new vaccine candidates and therapeutics may require demonstration of efficacy for licensure via the FDA Animal Rule (21 CFR 601.90–95). Therefore, the overall goal of our research is aimed at developing novel and safe anti-bacterial therapeutics.

Numerous mouse models of B. pseudomallei infections have been developed, although most of these models used intranasal, intraperitoneal, or intravenous routes of infection, with LD₅₀ ranging from < 5 to 1.5 × 10⁴ cfu/animal. Differences in LD₅₀ values are attributable to a number of factors include mouse strain, B. pseudomallei strain, and route of infection. Given the likelihood a biological weapon will utilize aerosol dissemination, the route of B. pseudomallei infection impacts disease manifestation, and an aerosol model has benefits not found in parenteral challenges. There have been recent efforts to develop mouse models of aerosolized
We expand the nose-only exposure of male BALB/c mice to aerosolized *B. pseudomallei*, including the efficacy of post-exposure antibiotic administration. In particular, we examined doxycycline as it was used in older treatment protocols, we have our experience with administering doxycycline treatments in mice, and the vast majority of *B. pseudomallei* strains are susceptible to this antibiotic. We report on the mouse model of inhalational melioidosis and the efficacy of post-exposure doxycycline administration in preventing the development of melioidosis.

### Results

#### Lethality of the aerosol exposure

In order to have the greatest confidence in the LD50 estimate, we desired the maximum number of mice to be exposed to a dose near to the actual LD50. To best target the aerosol dose, mice were divided into three phases, with the targeted doses for phases II and III being determined by the cumulative lethality results from the previous phase(s). The targeted and actual aerosol doses are shown in Table 1. The average mass median aerodynamic diameter (MMAD) of the aerosol particles generated for phases I, II, and III was 2.34 μm, 2.03 μm, and 1.80 μm, respectively. The MMAD of the aerosol particles generated for this study are consistent with lower respiratory tract deposition. There were two animals (one in group I-E and one in group II-D) that were found dead immediately after removal from the aerosol exposure system and were excluded from all analyses. Analysis of these data found a statistically significant dose-response relationship (p = 0.0001) with higher dosages of the agent resulting in a higher probability of death. Figure 1 depicts the dose lethality curve. The LD50 was estimated at 2,772 cfu/animal with an upper 95 percent confidence bound of 3,888 cfu/animal. The LD90 was estimated to be 8,728 cfu/animal with an upper 95 percent confidence bound of 15,159 cfu/animal.

#### Survival of mice with or without doxycycline

Having established the LD50, we next wanted to examine the efficacy of doxycycline in preventing death following exposure to aerosolized *B. pseudomallei* 1026b. Mice inhaled an estimated 1.17 × 10^2 to 1.30 × 10^3 cfu aerosolized *B. pseudomallei* 1026b. One group of mice was treated with doxycycline twice daily for 14 days beginning 6 hours after aerosol exposure. The control group received sterile water for injection on the same schedule. Figure 2 plots the Kaplan-Meier curves for the time-to-death data. All doxycycline treated mice survived 28 days after exposure to aerosolized *B. pseudomallei* 1026, while all water treated mice died 3–4 days after exposure. Fisher’s exact comparing the survival rate between the two groups was performed and the differences in survival were significant (p < 0.0001).

#### Impact of doxycycline on *B. pseudomallei* in tissues

As *B. pseudomallei* infections can manifest as either a lethal acute infection or a sub-lethal chronic infection, we wanted to assess the number of bacteria present in the lungs and spleens of animals at the time of

![Figure 1](https://www.nature.com/scientificreports/srep01146/figures/figure1.png)

**Figure 1** | Estimated logistic regression curve and 95 percent confidence interval with points showing the proportion of animals that died. The probability of death of each group of male BALB/c mice is plotted against the inhaled dosage of *B. pseudomallei* 1026b in cfu. The percentage of animals alive 14 days after exposure to aerosolized *B. pseudomallei* 1026b was plotted against the inhaled dosage of *B. pseudomallei* 1026b in cfu. The percentage of animals alive 14 days after exposure to aerosolized *B. pseudomallei* 1026b was plotted against the inhaled dosage of *B. pseudomallei* 1026b in cfu. The percentage of animals alive 14 days after exposure to aerosolized *B. pseudomallei* 1026b.
BALB/c mice were exposed to aerosolized *B. pseudomallei* in each group surviving on each day following exposure to doxycycline was administered twice daily for 14 days. The percentage and the control group was treated with WFI (solid line). The 40 mg/kg exposure, one group of mice was treated with doxycycline (dashed line) and the control group was treated with WFI (solid line). The 40 mg/kg doxycycline was administered twice daily for 14 days. The percentage of mice in each group surviving on each day following exposure to *B. pseudomallei* 1026b is shown.

**Discussion**

Similar to previous studies, we show that *B. pseudomallei* causes a lethal disease in BALB/c mice and that doxycycline can be used as a treatment. Doxycycline treatment resulted in no detectable bacteria in 19 of 20 lungs and 16 of 20 spleens. In previous reports, whole body exposure to aerosolized *B. pseudomallei* 1026b resulted in an LD<sub>50</sub> 1 × 10<sup>8</sup> cfu/mouse, well below 2.8 × 10<sup>9</sup> we found for nose-only aerosol in male BALB/c mice. While this difference may be attributable to the differences in the *B. pseudomallei* aerosolized, differences in route of exposure (whole body vs. nose only), and sex differences also existed. It is important to note that we examined male BALB/c mice rather than females used in six previous reports<sup>19,20,22,29–31</sup>. It is worth noting that 70–75% of melioidosis cases are male, indicating a sex difference in melioidosis susceptibility<sup>5,32,33</sup>. However, it has been suggested that sex differences observed in clinical melioidosis may be due to increased exposure due to outdoor occupations of men<sup>33</sup>.

Doxycycline was effective at preventing death from exposure to aerosolized *B. pseudomallei*, similar to what was shown by Sivalingam et al., who demonstrated that 100% of mice survived when doxycycline was administered at the time of challenge and 80% of mice survived when doxycycline was administered 10 hours after challenge<sup>25</sup>. The sterility achieved in the lungs in 19 of 20 mice was similar to what was previously shown with no bacteria being detected by nine days post-challenge. In conclusion, the BALB/c mouse is a useful model of melioidosis and post-exposure prophylaxis by doxycycline is likely to be effective, based on the mouse model.

**Methods**

**Aerosolization of B. pseudomallei.** *B. pseudomallei* 1026b was obtained from BEI Resources (cat #NR-4074) and a working cell bank of single use glycerol stock vials are cryopreserved at −70°C at Battelle. The *B. pseudomallei* was propagated from frozen culture in Luria broth plus 4% glycerol. Mid to late-log phase cultures were used to generate aerosols. The aerosol exposure system used for the mouse aerosol challenge testing consisted of a system capable of exposing multiple animals (up to 30) with the addition of impinger samplers, an aerosol particle size analyzer, temperature and humidity monitoring, mass flow meters (MFM) and mass flow controllers (MFC) to monitor the aerosol flows. To generate *B. pseudomallei* 1026b containing aerosols, forced air entered Battelle’s custom system through high efficiency particulate air (HEPA) filters and was divided into a continuous air stream (continuous air) and an air stream that either flowed into the Collison nebulizer (during aerosol generation) or by-passes it (between aerosol generations). Mass flow controllers (MFC) regulated the flow for each of the air streams. The *B. pseudomallei* aerosol, created by the nebulizer, was allowed to mix with continuous air before being delivered to the exposure chamber. A nose-only aerosol exposure system (CH Technologies Tower) was utilized to deliver the desired *B. pseudomallei* aerosol. From the exposure chamber the aerosol was sampled for concentration dose determination of *B. pseudomallei* using an impinger (model 7541, Ace Glass, Inc.). The liquid in the nebulizer and impinger was diluted and enumerated by the spread plate and/or filtering technique to quantify viable *B. pseudomallei* bacteria counts per mL. The *B. pseudomallei* concentration (based on enumerations) of the nebulizer and impinger samples were used to determine the actual exposure dose for each challenge group. Using Guyton’s formula and the concentration of *B. pseudomallei* collected from air
sampled from the exposure system, the inhaled dose was determined from the enumeration results.

**Animals.** A total of 160 male BALB/c mice approximately 6–8 weeks of age were obtained from Charles River. Mice were obtained at different times for either the LD₅₀ determination or for the efficacy of doxycycline determination. Mice were observed twice daily throughout the experiments for signs of morbidity and mortality. Animal use procedures were approved by Battelle’s Institutional Animal Care and Use Committee (IACUC) and the Department of Defense Animal Care and Use Review Office (ACURO).

**LD₅₀ determination.** A total of 120 male BALB/c mice were used to estimate the LD₅₀ in three phases. In phase I, eight groups of six mice were exposed to escalating targeted doses of aerosolized *B. pseudomallei* 1026b ranging from 5 to 5,000 cfu/mouse. Phase II consisted of six groups of six mice were exposed to escalating targeted doses of aerosolized *B. pseudomallei* 1026b ranging from 7,000 to 100,000 cfu/mouse. Phase III consisted of six groups of six mice were exposed to escalating targeted doses of aerosolized *B. pseudomallei* 1026b ranging from 2,000 to 8,000 cfu/mouse. LD₅₀ estimates were made following each phase to plan targeted doses for the subsequent phase.

**Antibiotic efficacy testing.** A total of 40 male BALB/c mice were used to test the efficacy of doxycycline in post-exposure prophylaxis. Mice were divided into doxycycline and sterile water for injection (WFI) groups of 20 mice each. Five mice from each group were exposed to aerosolized *B. pseudomallei* 1026b on four different runs to a target of 5.6 × 10⁴ cfu (~20 LD₅₀). Following the aerosol challenge, antibiotic therapy began approximately 6 (±1) hr post median challenge time. Mice infected with a doxycycline treatment group received doxycycline at a dose of 40 mg/kg twice a day (BID) for 14 days [every 12 (±1 hr) hours]. This dosing regimen was selected to ensure that the area under the concentration time curve for a 24 hour period (AUC₀–₂₄) was similar in mice compared to the human label dose. This approach is favored as the antimicrobial pattern of doxycycline predicts that AUC₀–2₄ will determine efficacy. Doxycycline dose indicates 100 mg every 12 hours for the average person. However, published human data was only available for a 200 mg dose, with an AUC₀–₂₄ of 30 µg·h/mL. Based on trapezoidal calculation using data from Newton 2005, this data was used as the basis of a 100 mg twice daily regimen and calculated the AUC₀–₂₄ was approximately 30 µg·h/mL. In mice, the AUC₀–₂₄ has been reported to be 13.7 µg·h/mL with a dose of 25 mg/kg. In order to achieve an AUC₀–₂₄ near 30 µg·h/mL, BID dosing of 40 mg/kg was used. Doxycycline (Vibramycin® calcium syrup oral suspension, Pfizer Labs, NY, NY) was obtained from a local pharmacy. Control mice received WFI for 14 days [every 24 (±1 hr) hr] until death at a volume of 5 mL/kg QD. Dosing occurred via orogastric intubation. Mice were observed for clinical signs of disease and survival for 28 days following aerosol exposure.

**Tissue bacterial burden.** The bacterial burden in the lungs and spleen was determined by quantitative PCR from all mice found dead or euthanized from the antibiotic efficacy testing. At the time a mouse was found dead or following euthanasia, a specimen of lung and spleen at gross necropsy was collected for bacterial burden analysis. The lung and spleen specimens were weighed, manually homogenized using a tissue grinder and total nucleic acid was isolated using the NuclSens easyMAG (bioMerieux, Durham, NC). Briefly, 100 µl of sample was added to the easyMAG lysing buffer and the instrument was then dispensed easyMAG Lysis Buffer (bioMerieux, Durham, NC) and incubated the samples for 10 minutes. Afterwards, easyMAG Magnetic Silica (bioMerieux, Durham, NC) was added to the samples. The instrument then incubated the samples for 10 minutes, collected the silica into pellets, and washed them subsequently with easyMAG Extraction Buffer 1 (bioMerieux, Durham, NC), easyMAG Extraction Buffer 2 (bioMerieux, Durham, NC), and easyMAG Extraction Buffer 3 (bioMerieux, Durham, NC). The purified nucleic acid was then eluted from the silica with 100 µl of easyMAG Extraction Buffer 3 (bioMerieux, Durham, NC) and assayed. Based on published sequence data, oligonucleotides were designed to amplify a small fragment within the coding region of the rpoB target because it contained a sequence that demonstrated a quantity within the coding region of the rpoB target because it contained a sequence that demonstrated a quantity.
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