The Effect of Free Chlorine on *Burkholderia pseudomallei* in Potable Water.

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Abstract

Chlorine is widely used in public water supplies to provide a disinfection barrier. The effect of chlorine disinfection on the water-borne pathogen *Burkholderia pseudomallei* was assessed using multiple techniques. After exposure to chlorine viable bacteria were undetectable by conventional plate count techniques, however persistence of *B. pseudomallei* was verified by flow cytometry and bacteria were recoverable following a simple one step broth procedure. The minimum residual chlorine concentration and contact time as prescribed by potable water providers in Australia was insufficient to reduce a *B. pseudomallei* population by more than 2 $\log_{10}$. Chlorine had a bacteriostatic effect only on *B. pseudomallei*; viable bacteria were recovered from water containing up to 1000 ppm free chlorine. This finding has practical implications for water treatment in regions where *B. pseudomallei* is endemic. Future work to assess the effect of alternative water disinfection processes either singly or in sequence is necessary.

Keywords: *Burkholderia pseudomallei*, chlorine, disinfection

1.0 Introduction

Burkholderia pseudomallei causes the potentially fatal infection melioidosis, with a high mortality rate (Beeker *et al*., 1999). *B. pseudomallei* is endemic to Southeast Asia (Leelarasamee, 1998; Perret *et al*., 1998) and Northern Australia (Brook *et al*., 1997) where it was first recognised during the early 20th century. Since the 1960s *B. pseudomallei* has come to prominence in other parts of the world through infection of Vietnam veterans and, more recently, as a potential biological weapon (Josephson, 2001). The principal means of contracting melioidosis is through recreational or occupational exposure to contaminated soil or surface water (Leelarasamee and Bovornkitti, 1989), particularly via direct wound inoculation. However, infection by inhalation of an aerosol or ingestion of contaminated material has been proposed (Currie *et al*., 2001).

It was shown recently that the potable water supply was the likely source of a small outbreak of melioidosis in a remote Australian community (Inglis *et al*., 1999). The outbreak occurred during failure of the chlorine supply to the community's water treatment plant (Inglis *et al*., 2000). Other melioidosis case clusters also show evidence that the water supply may have been the principal vehicle of infection (Ketterer *et al*., 1986; Currie *et al*., 2000). Chlorine is the most widely used method for disinfecting water supplies against possible bacteriological contamination (Miche and Balandreau, 2001), however, there may be an increased resistance of bacterial strains to chlorine inactivation (Mir *et al*., 1997). Furthermore, bacteria may be partially damaged by exposure to sublethal levels of chemical biocides. This may manifest as the inability to grow and form colonies (McFeters *et al*., 1986) and result in an underestimation of bacterial contamination.

Drinking water chlorination in Western Australia aims to achieve a 1 ppm residual chlorine concentration with a contact time of not less than 30 min. In the current study, we set out to test the efficacy of these criteria for chlorine disinfection of *B. pseudomallei* in water. This study also aimed to determine the chlorine

susceptibility of a range of clinical and environmental isolates of *B. pseudomallei* and to ascertain if chlorine tolerance was the result of prior exposure to chlorine.

2.0 Materials and Methods

2.1 Bacterial strains

The origin of *B. pseudomallei* strains used in these studies is listed in Table 1. With the exception of some of the environmental isolates from Western Australia, none of the strains had been in prior contact with chlorine. All strains had been in culture for greater than 12 months except for the environmental strains 65A1and 90B1, which were isolated 3 months prior to testing, and BCC122 which was isolated 1 month prior to testing. Type strains of *Pseudomonas aeruginosa* (ATCC 27583) and *Escherichia coli* (ATCC 25922) were used as controls.

All bacteria were stored in 15% glycerol in brain heart infusion broth at -70°C and incubated on blood agar (BA) for a maximum of 3 days, then transferred to 10 mL trypticase soy broth (TSB) for 18 h incubation at 37°C in the dark.

Prior to use in the experiments, overnight cultures in 10 mL polycarbonate centrifuge tubes were washed twice in cold sterile water after centrifugation at 300 *g* for 15 min at room temperature. They were incubated at room temperature for 2 h before exposure to chlorine, to bring the cultures to mid-lag phase.

2.2 Assessment of viability

Four complementary methods were employed to assess viability of bacterial cultures after chlorine exposure. A standard plate counting method was used to indicate the number of colony forming units able to grow within 48 h. Viability staining measured by flow cytometry was used to quantify the viable organisms in cultures. All cultures were put through a qualitative recovery procedure to determine whether viable bacteria remained after 72 h. The most probable number (MPN) method was used to determine surviving bacteria after chlorine exposure by utilising this recovery procedure.

2.2.1 Colony count method

Bacterial suspensions were plated in triplicate onto plate count agar (PCA) using a spiral plater (Don Whitely Scientific Ltd, Shipley, UK) after 10:1 dilution with water to reduce the effect of chlorine.

2.2.2 Flow cytometry

Bacterial suspensions of 10^6 organisms/mL in filter sterilised distilled water were stained for 10 min with 1 μL/mL each of SYTO®BC bacterial stain in DMSO (Molecular Probes, Oregon, USA) and propidium iodide (20 mM in DMSO) for quantification of viable cells by flow cytometry (Becton Dickinson FACSCalibur®). Samples were collected using two thresholds: side-scatter and fluorescence following SYTO staining. The amount of non-cellular material contributed by the stains and diluent was assessed with a series of reagent controls prior to sample collection and was always less than 0.2%. Viability was calculated as the percentage of live gated events from a minimum 20 000 events. An assumption is made that the error of flow gated events is linear for the measurement range. The percentages of live gated events were converted to viable organisms from plate counts of initial suspension concentrations. Data analysis was performed using Becton Dickinson CELLQuest® v3.1.

2.2.3 Qualitative recovery procedure

The cultures were diluted and centrifuged at 300 *g* for 10 min at room temperature after chlorine exposure. The bacterial pellet was re-suspended in 10 mL TSB, left at room temperature for 24 h, and then incubated at 37°C for 48 h. Recovery of *B. pseudomallei* was confirmed by plating onto *B. pseudomallei* selective agar (BPSA) and PCA.

2.2.4 Most probable number of surviving bacteria

To determine the MPN of surviving growth units the cultures were diluted and washed and the recovered bacteria were resuspended in TSB and filtered through a 10 µ filter to reduce any clumping effect before 10 µL was aliquoted into 1 mL of TSB. Ten and 100 fold dilutions were also done in the same manner, with 100 replicates for each dilution. All tubes were left at room temperature for 24 h, then incubated at 37°C for 48 h. The MPN was calculated (Hurley and Roscoe, 1983) using an average of the repeats and the 95% confidence interval (CI) calculated.

2.3 Susceptibility of bacteria to chlorine

Chlorine was added to cell suspensions as a solution of sodium hypochlorite. Concentrations of free chlorine (Cl_2, HOCl, OCl^-) were measured (± 0.02 mg/L) using a pocket colorimeter analysis system (HACH Test Kit, HACH Company, Colorado, USA). All water used for experiments was micro filtered at 18Ω. Suspensions of 10^6 organisms/mL did not create a measurable chlorine demand and, after 30 min exposure, the residual chlorine was similar to the original dose.

Overnight cultures of 45 strains of *B. pseudomallei* (Table 1) were washed and diluted to 10^6 cfu/mL. The number of viable cells was determined by flow cytometry. Bacterial suspensions were then exposed to 1 ppm chlorine (pH 6.25 - 7, 22 - 25°C) and further viable counts were taken at 20, 30 and 60 min. At 60 min plate counts (3 replicate plates) were also taken. After 48 h incubation there was no growth. The two reference strains (NCTC 13177 and 10276) and the persistently mucoid strain (BCC11) were exposed for 2 h and the proportion of viable cells determined at 30 min intervals by flow cytometry. NCTC 13177 was exposed to 0, 0.25, 0.5, 0.75 and 1 ppm chlorine for 30 min after a 1 h preparatory incubation (early lag phase), with viability readings at 5 min intervals. Early lag phase cultures of *P. aeruginosa* (ATCC

27583), *E. coli* (ATCC 25922) and *B. pseudomallei* (NCTC 13177) were exposed to 1 ppm chlorine for 30 min for a comparison of population response to chlorine.

Using 3 of the more tolerant strains (NCTC 13177, NCTC 10276 and BCC11) plate counts (5 replicate plates) were taken after 1, 5 and 10 min exposure to 1 ppm chlorine.

All experiments were repeated and were compared to control cultures with no chlorine exposure.

2.4 Survival of bacteria in chlorine

The MPN of reference strains NCTC 13177 and 10276 were determined after the cultures were subjected to 1 ppm chlorine for 30 min. This experiment was performed three times.

Six of the more tolerant strains of *B. pseudomallei* were subjected to 0, 10, 50, 100, 200, 300, 500 ppm chlorine, with the two reference strains and BCC11 also subjected to 1000 ppm chlorine. *P. aeruginosa* and *E. coli* were exposed to up to 10 ppm chlorine only. After 30 min exposure, the solution was diluted with sterile water (1:100) to end effective chlorine exposure. This was followed immediately with two centrifugation and washing steps and the recovery procedure. Recovery of the two reference strains and BCC11 from 1000 ppm was repeated. Strains subjected to 100 ppm were stored at -70°C for further experimentation.

2.5 Effect of pH on survival in chlorinated water

The reference strain NCTC 13177 and the persistently mucoid strain (BCC11) were prepared as overnight cultures, washed and resuspended in water that had been adjusted to pH 4, 5, 6, 7, or 8 by addition of HNO_3 or NaOH. The addition of chlorine was varied to achieve 1 ppm at each pH. The pH of these bacterial suspensions was checked periodically throughout the experiment and did not change by more than 0.4 units from the original value.

2.6 Previous exposure to chlorine

A comparison was made between viable cell counts in strains previously exposed to 100 ppm chlorine and their parent strains after subsequent exposure to 1 ppm chlorine. Flow cytometer readings were taken at 0 and 30 min. Viability of NCTC 13177 (previously survived 100 ppm chlorine) and its control were read at 10 min intervals in duplicate.

3.0 Results

3.1 Susceptibility to chlorine

The survival of 45 strains of *B. pseudomallei* following 30 min exposure to 1 ppm chlorine is shown in Figure 1a. After 60 min exposure there was a 100 fold reduction in viability. In mid-lag phase the viability of the isolates was rapidly reduced by 1 ppm chlorine during the first 10 to 20 min, after which the rate of decrease in viability slowed and levelled off (Figure 1b). The decline after 60 min was similar to the decline due to osmotic shock observed in control cultures suspended in water. In early lag phase survival was increased by more than 5% when there was a reduction in chlorine concentration (Figure 1c). *E. coli* and *P. aeruginosa* populations were reduced more rapidly by chlorine than *B. pseudomallei* with a 30% decline in viable organisms within the first 5 min of contact. Reduction in viability of *E. coli* was limited to the initial 5 min exposure (Figure 1d).

In contrast, when plate counts were used to measure viability, no viable *B. pseudomallei* were detected after 10 min exposure to 1 ppm chlorine (Figure 3). The persistently mucoid strain was non-culturable over all periods of chlorine exposure tested (Figure 2). However, three test strains were recovered after subsequent incubation of the remaining solution in TSB. Plate counting was not used further as a measure of survival in these experiments.

3.2 Survival of bacteria in chlorine

The more chlorine tolerant group of isolates stabilised after 10 min exposure to 1 ppm while the susceptible group had a more linear rate of decrease over contact time (Figure 3). The number of viable organisms/mL of the susceptible group was 0.5 $\log_{10}$ less than the tolerant group by 30 min.

Clinical isolates from the Northern Territory were more tolerant of chlorine ($p=0.001$) than the other groups. There was no significant difference between the rest of the groups ($p=0.69$) (Figure 4). Further analysis revealed that two pairs of

strains from the Northern Territory clinical group were indistinguishable, while the remaining 7 were classified as distinct by DNA macro restriction with *XbaI* (Inglis *et al*., 2002). There was no significant difference in chlorine tolerance between soil and water environmental isolates from Western Australia; a result that was anticipated since most of these isolates were from a single cluster and had a high homology by PFGE.

An end point for the killing curve for *B. pseudomallei* was not found. The two reference strains and BCC11 were recovered from 1000 ppm chlorine. Strains BCC51 and BCC52 were recovered from 500 ppm (the highest concentration tested for these strains) while BCC49 was not recovered at 500 ppm but survived at 300 ppm chlorine. All recovered populations had pellicles within 48 h of 37°C incubation. The MPN of NCTC 13177 and 10276 was 370 MPN/mL (LL 95% CI of 290 and UL of 470) and 300 MPN/mL (LL of 240 and UL of 370 MPN/mL) respectively.

There was a 30% recovery rate after exposure to 1 ppm chlorine for *P. aeruginosa*, while *E. coli* was non-recoverable after any chlorine exposure.

3.3 Effect of pH on survival in chlorinated water

Chlorine was a more effective disinfectant at higher pH. Survival was increased in an acidic environment by 10 fold in both strains (Figure 5). The survival of the persistently mucoid strain varied more than the reference strain over the pH range tested.

3.4 Cumulative effect of chlorine on *B. pseudomallei*

There was less than 1% difference in the survival of strains that had previous or no previous contact with chlorine, with only one exception; the persistently mucoid strain BCC11. When BCC11 had previous exposure to chlorine there was a small increase in survival at 30 min in 1 ppm chlorine compared to no previous exposure. In

contrast when examined at 10 min intervals, the survival of NCTC 13177 was 10 times lower in the isolates previously exposed to chlorine (Figure 6). When previously exposed the rate of decrease during the contact time was more linear than in the isolate not previously exposed to chlorine.

4.0 Discussion

The efficacy of chlorination for the control of *B. pseudomallei* in potable water has not been extensively studied. This study highlights the capacity of *B. pseudomallei* to survive in chlorine treated water. *B. pseudomallei* survived up to 1000 times the chlorine concentration used to disinfect drinking water supplies in Australia. It is also notable that a conventional bacteriological plate count method used to determine the presence of this species significantly underestimated viable *B. pseudomallei* in water.

Chlorine treatment produced a 2 to 3 log reduction in viability but had a bacteriostatic effect only on the remainder of the bacterial population. Some of these cells were subsequently able to regrow under suitable incubation conditions. Using a plate count method Thomas (1991) previously showed that 0.5 to 1 ppm chlorine readily killed one strain of *B. pseudomallei* within a contact time of 5 min. Our bacterial plate count results are consistent with those of Thomas (1991). However, a significant portion of the total population of *B. pseudomallei* is not enumerated using conventional techniques, possibly resulting in inaccurate water quality determinations. Flow cytometry is recognised as a useful tool in aquatic and environmental microbiology (Vives-Rego *et al*., 2000). Here it has provided useful supplementary data on the chlorine disinfection process showing the decline in population over time. Coupled with MPN calculations, flow cytometer determinations allow a tentative quantification of the remaining viable cells.

P. aeruginosa responded to chlorine in a manner similar to *B. pseudomallei* with a continued decline in viability over time, but *B. pseudomallei* is a more chlorine tolerant organism. Chlorine reduced the viability of *E. coli* cultures by more than 30% in the first 5 min, and rendered the remaining cells non-recoverable. These results show that other bacterial species cannot be used as indicator species for disinfection processes for control of *B. pseudomallei*.

The majority of *B. pseudomallei* cells died within 10 - 20 min of chlorine contact. It is likely that the surviving cells either adapted to chlorine by 30 min or were

intrinsically resistant. McFeters and Camper (1978) showed that *E. coli* damaged by chlorine recovered after a 2 h repair period in a rich broth. The difference between the plate and flow cytometry viability counts suggests that damaged *B. pseudomallei* had entered a non-culturable state in the chlorinated water (Havelaar *et al*., 1993; Leclerc and Moreau, 2002). The recovery of culturable bacteria from high concentrations of chlorine may be due to the regrowth of a few culturable cells or resuscitation of viable but non culturable cells (Dukan *et al*., 1997).

Waters with lower pH produced a greater tolerance to chlorine in the *B. pseudomallei* strains tested. This result was unexpected because at low pH the chlorine solution produces more HOCl, the most effective bactericide of the chlorine species, and should therefore be a more effective disinfectant. *B. pseudomallei* had been found to grow as well at pH 4.5 as at pH 7 (Kanai *et al*., 1994). It is therefore suggested that either *B. pseudomallei* rapidly employs survival strategies more readily or excludes chlorine under low pH conditions.

Tolerance to chlorine did not increase with subsequent exposure to chlorine, in contrast to the finding of Ridgway and Olson (1982) who showed that other bacteria from chlorinated systems were more resistant than those from non-chlorinated systems. In the current study, the strains previously exposed to chlorine appear to have exhausted their capacity to cope with chlorine exposure. This suggests that isolates previously exposed to chlorine accumulate sublethal damage from their prior exposure. An explanation for this was suggested by Dukan and Touati (1996) who found when *E. coli* pretreated with low concentrations of HOCl are challenged with higher HOCl concentrations, part of their ability to scavenge HOCl is exhausted. They also found that the kinetics of HOCl consumption was slower in pretreated cells.

Whether the higher tolerance to chlorine of the clinical isolates from Northern Territory can be linked to greater virulence is not known. There are conflicting reports in the literature on virulence and injured bacteria. For example, McFeters

et al. (1986) reported that injured waterborne enteropathogenic bacteria can remain virulent, while chlorine induced a temporary loss of virulence among sublethally injured enterotoxigenic *E. coli* (Walsh and Bissonnette, 1983, 1987).

4.1 Future directions

This study has clearly shown that chlorination of drinking water supplies needs careful attention in areas endemic for *B. pseudomallei*. With the knowledge gained from this study, identification of high risk water supplies and the establishment of preventative strategies can be implemented. However, there are other aspects that must be considered. The resistance of *B. pseudomallei* to chlorine may be further enhanced by an ability to form biofilms (Mah and O'Toole, 2001) or to survive within free living protozoa (King *et al*., 1988; Inglis *et al.,* 2000). Additionally, whether *B. pseudomallei* employs survival strategies more readily under conditions of favourable pH and temperature is not known. When maintained in a low nutrient environment, such as drinking water, strains are more resistant to disinfection by chemical agents than strains that have been maintained on a rich medium (Kuchta *et al*., 1985; Taylor *et al*., 2000) by persisting in non-growth or slow-growth states with low metabolic activity (Wai *et al*., 1999).

4.2 Conclusion

Our results suggest that while chlorination may be a satisfactory method for controlling coliforms and preventing growth of *B. pseudomallei* in the potable water supply, this method does not eradicate all viable *B. pseudomallei* in water. Shock doses of chlorine may provide an adequate countermeasure against *B. pseudomallei* in the event of contamination of the water supply - but alternative treatments are needed to eradicate *B. pseudomallei.* It remains to be seen whether chlorine damaged *B. pseudomallei* is more virulent than untreated populations.

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Table 1. Source of strains of *Burkholderia pseudomallei* used to determine the effect of exposure to chlorine in water.

Place of isolation	Source of strain	Strain identification	
		NCTC	BCC
Western Australia	Clinical	13177	1, 2, 3, 4, 14, 16, 30, 33, 74, 87
	Environmental		21, 24, 26, 27, 28, 31, 44, 45, 46
Northern Territory	Clinical		11*, 18, 49, 50, 51, 52, 53, 54, 69, 70*, 122
	Environmental		65A1, 90B1
Queensland	Clinical		75, 78, 79, 80, 81, 82, 83, 84, 85, 86
Overseas	Clinical	10276	H1

* persistently mucoid colony form
NCTC =National Collection Type Culture
BCC =Burkholderia culture collection number – held in PathCentre, Perth, Western Australia.

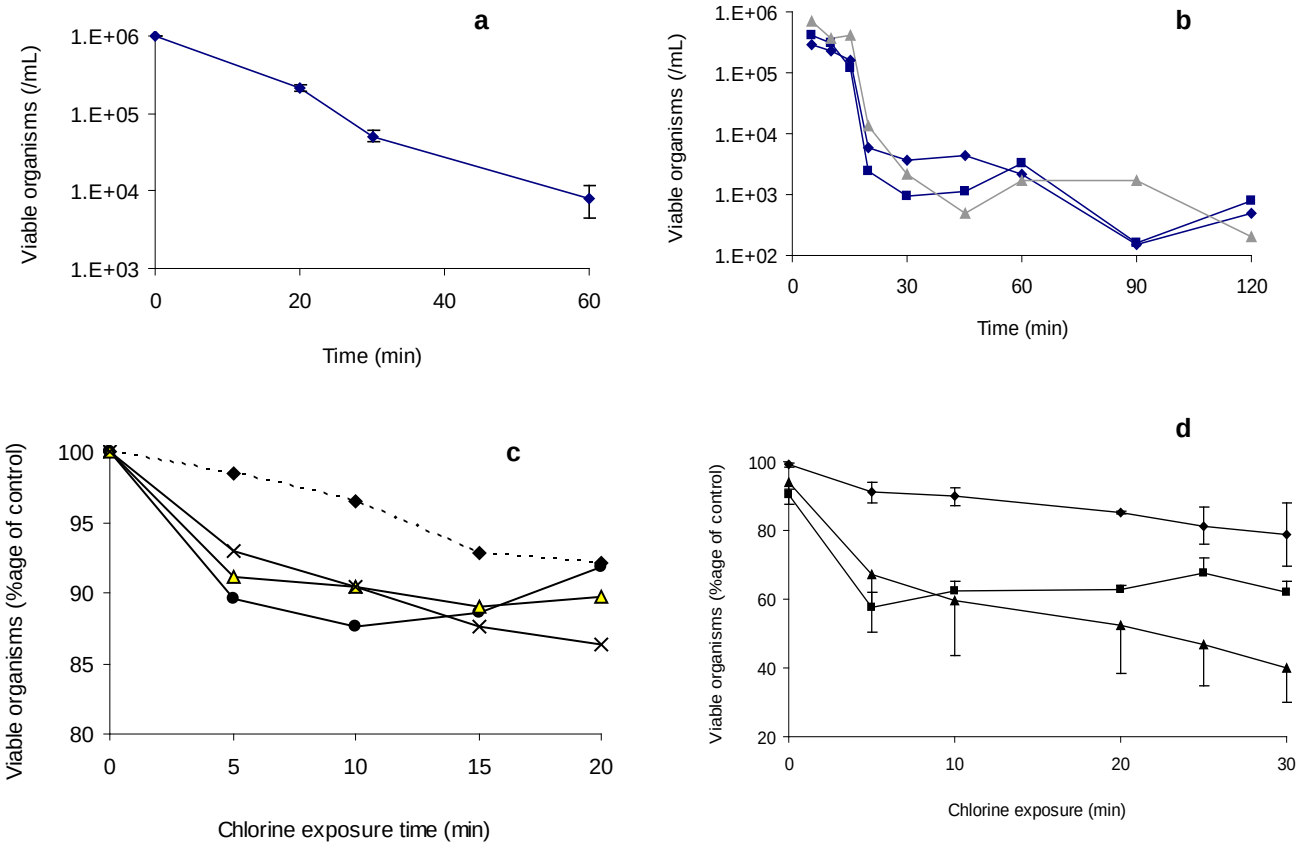

Figure 1 a) Survival of 45 strains of *Burkholderia pseudomallei* in 1 ppm chlorine. b) Longer term survival of 3 strains of *B. pseudomallei* (NCTC 13177 ◆ , NCTC 10276 ■ and BCC11 ▲) in 1 ppm chlorine in water. c) Survival of *B. pseudomallei* (NCTC 13177) at varying chlorine concentrations (0.25 ◆ , 0.50 ● , 0.75 ▲ , and 1 ppm **X**) as compared to control (0 ppm chlorine). d) Survival of *B. pseudomallei* (NCTC 13177 ◆), *Pseudomonas aeruginosa* (ATCC 27853 ▲) and *Escherichia coli* (ATCC 25922 ■) in 1 ppm chlorine in water. Viability determined by flow cytometer. Error bars = 95% confidence interval.

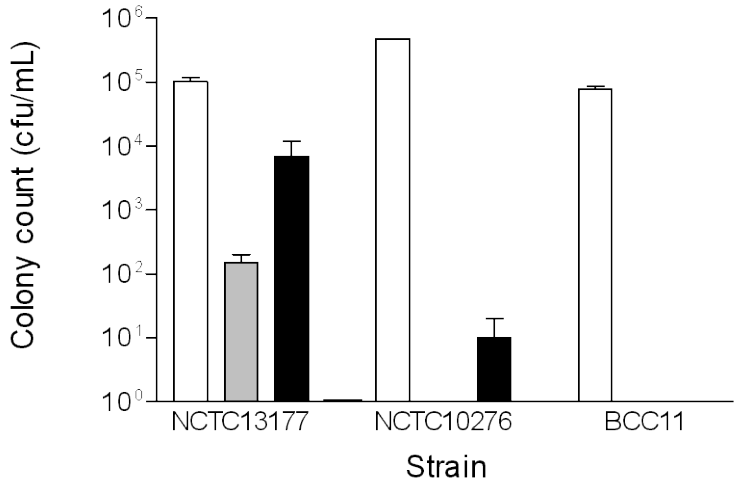

Figure 2 Recovery of *Burkholderia pseudomallei* strains after exposure to 1 ppm chlorine in water for 0 (□), 1 (▒) 5 (■) and 10 min (▨). Viability was determined by plate counting only. Error bars = standard error the mean.

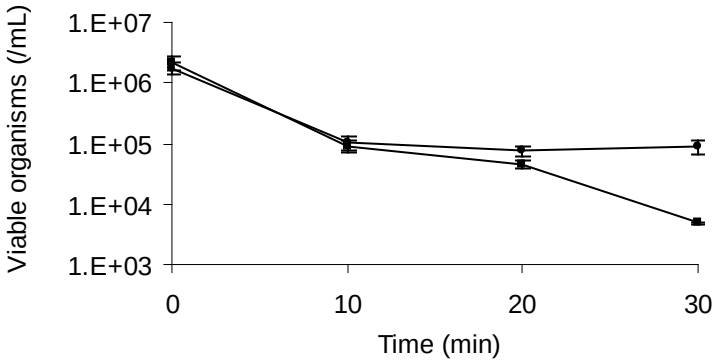

Figure 3 Survival of strains tolerant ● (n=6) and susceptible ■ (n=3) to 1 ppm chlorine in water. Viability determined by flow cytometer. Error bars = standard error of the mean.

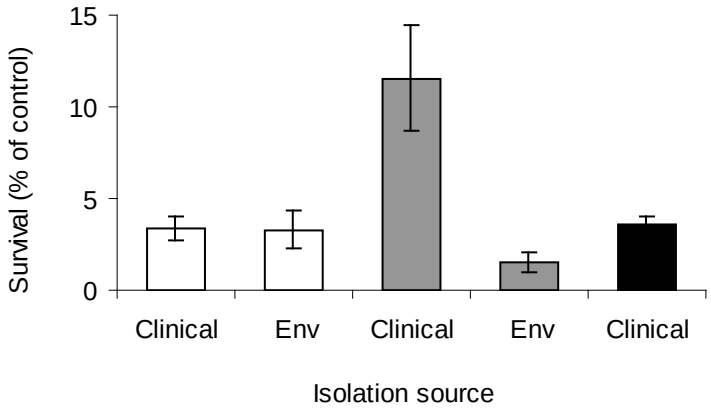

Figure 4 Survival of *Burkholderia pseudomallei* strains grouped by state (Western Australia □ , Northern Territory ▩ and Queensland ■) and isolation source (clinical or environmental) after 30 min exposure to 1 ppm chlorine in water expressed as percentage of control cultures. Viability determined by flow cytometry. Error bars = standard error of the mean.

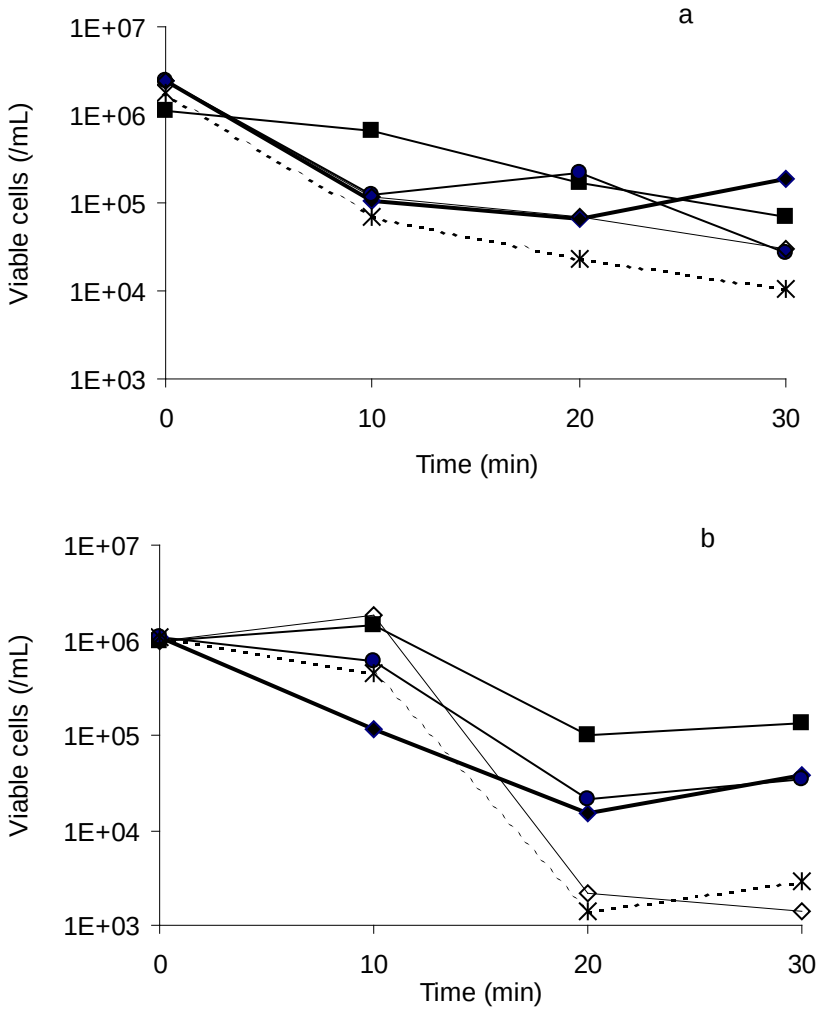

Figure 5 Survival of *Burkholderia pseudomallei* (a = NCTC 13177, b = BCC11) in 1 ppm chlorine in water adjusted to pH 4 ◆, 5 ■, 6 ●, 7 ◇, 8 ✳ Viability was determined by flow cytometry.

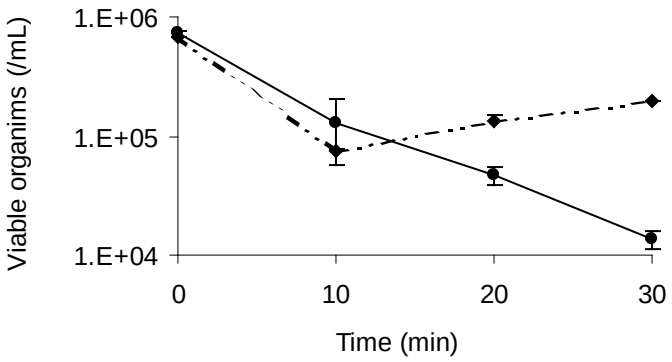

Figure 6 Survival of *Burkholderia pseudomallei* NCTC 13177 with no prior (◆) and prior (●) exposure to 100 ppm chlorine after subsequent exposure to 1 ppm chlorine. Error bars = standard error of the mean.