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# Association of the Melioidosis Agent *Burkholderia pseudomallei* with Water Parameters in Rural Water Supplies in Northern Australia

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Running Title: *Burkholderia pseudomallei* in Bore Water in Australia.

19 **Abstract:**

20

21 We analysed water parameters and the occurrence of the melioidosis agent, *Burkholderia*
22 *pseudomallei* in 47 water bores in Northern Australia. *B.pseudomallei* was associated with soft,
23 acidic bore water of low salinity but high iron levels. This finding aids in identifying water
24 supplies at risk of contamination with this pathogenic bacterium.

25

**Text**

Melioidosis is a severe, emerging disease caused by the Gram-negative bacterium *Burkholderia pseudomallei (B.ps),* a hydrophilic soil saprophyte endemic in Southeast Asia and northern Australia (3,17,18). Melioidosis is the commonest cause of fatal community-acquired bacteraemic pneumonia in northern Australia (4).

Melioidosis outbreaks have been attributed to contaminated unchlorinated water supplies in northern Australia causing fatalities among humans and animals (5,8)(own observation). Rural water bores – of which there are 5,000 around Darwin alone - are mostly unchlorinated due to concerns of bore owners about taste, by-products and maintenance of chlorination. We have analyzed the association of *B.ps* occurrence with environmental and physico-chemical water properties in unchlorinated bore water from rural Darwin in northern Australia. This study is the first report on the habitat of *B.ps* in water supplies.

**Water sampling.** Bore water was collected from 47 properties (188 samples) in an area of 22x13 km in the 2008 dry season. 25 bores (53%) were fed by carbonate rock aquifers and 22 by aquifers in fractured weathered rock (6,13). 26 were resampled in the wet season (103 samples). Per bore, 1L of water was collected after 1, 30 and 60 minutes of water pumping to represent water from the bore head, shaft and aquifer. Water was collected from tanks linked to the bore. Water filtration and *B.ps* culture was done as previously described (7). Briefly, *B.ps* was cultured in modified Ashdown's-Broth and Tryptone-Soy-Broth and sub-cultured onto Ashdown's agar. Colonies exhibiting *B.ps* morphology were confirmed by latex agglutination and PCR targeting TTS1 (14). Water samples were tested for pH, temperature, electro-conductivity (TPS), total

nitrates, total iron, phosphates (Hanna-Instruments) and total hardness (Aquaspex). Water samples were cultured for total coliform counts (3M).

**Occurrence of *B.ps*.** In the dry season, 12 of 47 bores (26%) tested positive for *B.ps*. In the wet season, these 12 were revisited together with 14 bores negative for *B.ps* matched for aquifer type and location. 11 of the 12 initially positive bores were again positive and 4/14 (29%) previously negative bores were newly positive in the wet season.

Multilocus variable-number tandem repeat analysis (MLVA-4) of *B.ps* isolates (10) revealed 33 different MLV-4 patterns with identical or closely related genotypes also found in *B.ps* from soil or clinical cases within a 50 km radius. A median of 1.5 different genotypes was found per bore visit (95%CI 1-2). Isolates with identical or closely related patterns in the dry and wet season were retrieved from seven of the bores indicating persistent colonization. No geographical clustering of positive bores nor MLV-4 types was obvious.

No significant variation in bore characteristics was evident between *B.ps* positive and negative bores. These included bore age, depth, aquifer type, presence of concrete casing/slab, likelihood of water pooling at the bore and origin of sample (bore head, shaft, aquifer, tank). Effective bore capping showed reduced *B.ps* prevalence (21% vs 44%; p=0.21) (Table 1).

We analyzed the association between the occurrence of *B.ps* and water parameters (Table/Figure 1). A significant association was found between the occurrence of *B.ps* and more acidic water. These results support data showing the preference of *B.ps* for more acidic soil (2,10,15).

Water hardness, i.e. calcium carbonate levels, salinity and pH showed a significant positive correlation with each other (Spearman's correlation, $P<0.001$), attributed to the buffering capacity of carbonates and salts. *B.ps* was significantly associated with low hardness, i.e. soft

69 water and low salinity. This correlates with *in vitro* research showing *B.ps* counts dropping
70 rapidly in salt concentrations above 2.5% (9) or seawater (16). Soft water can be corrosive and
71 compromise bore piping, potentially facilitating *B.ps* introduction into bores and creating a rough
72 inner bore surface promoting biofilms (1).

73 Coliform counts were significantly higher in *B.ps* positive bores suggesting the presence of
74 nutrients for microbial growth. There was also a significant association between increased
75 turbidity and *B.ps*. Higher turbidity likely reflects failure of the bore casing in the subterranean
76 environment or backflow of surface water into the bore. Contamination of the bore with soil may
77 well explain the origins of *B.ps* and other microbes in these bores, but this requires formal study.
78 More organic matter in these bores would be favourable to the saprophytic *B.ps* and
79 decomposition of organic matter would contribute to acidification of the water.

80 The occurrence of *B.ps* was strongly associated with high iron levels. This finding supports
81 previous research showing enhanced *B.ps* growth in iron-rich media (19) and red-coloured soil
82 (indicating oxidized iron) (11). Clinical conditions causing iron overload such as thalassemia are
83 associated with increased melioidosis rates (3) and *B.ps* is able to produce siderophores under
84 limited iron supply (19). It is of interest that acidic pH associated with *B.ps* positives bores
85 increases bio-availability of iron by reduction of precipitated $Fe^{3+}$ to soluble $Fe^{2+}$, especially
86 under more anaerobic conditions such as if water is pumped up from aquifers (12).

87 A comparison of dry and wet season data showed that water parameters in *B.ps* positive bores
88 were even more favourable for *B.ps* in the wet season. Heavy rainfall might explain reduced
89 salinity in the wet season, with higher iron levels due to water influx from shallow aquifers in an
90 iron-rich lateritic layer which are only active in the wet season.

Clustered multivariable analysis showed that the most significant predictors for presence of *B.ps* in water were high iron levels and the interaction of a low pH with low salinity (Table 1).

**Conclusion**. We have found a close association between *B.ps* presence in bore water and water parameters such as low pH, low salinity and high iron levels. This indicates that *B.ps* occurrence in bores is not only the result of an initial contamination event but also depends on water conditions favourable for *B.ps*. The strong association of *B.ps* with an abiotic fingerprint aids in identification of water bores at high risk of *B.ps* contamination. Future studies will examine whether changing levels of abiotic parameters such as through pH-correction filters create an environment unfavourable for *B.ps* growth and thus, could be used as a preventive measure against *B.ps* persistence in unchlorinated water supplies.

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166 **TABLE 1** Summary of Water Parameters

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| | Dry Season | | Wet Season | | Multivariable Analysis |
|---|---|---|---|---|---|
| **Variable** | **Median (95% CI)** | **Mann-Whitney** | **Median (95% CI)** | **Mann-Whitney** | **OR (95% CI) P value** |
| **** Total Iron** (mg/L) | | | | | |
| *B.ps* positive | 2 (1-3) | P<0.001 | 4 (2-5) | P=0.002 | 1.34 (1.12-2.62) |
| *B.ps* negative | 1 (1-1) | | 1 (1-1) | | P=0.002 |
| **** pH** | | | | | |
| *B.ps* positive | 6.8 (6.5-7.1) | P<0.001 | 6.3 (6.1-6.6) | P<0.001 | Interaction of pH and Salinity |
| *B.ps* negative | 7.3 (7.2-7.4) | | 7.4 (7.3-7.6) | | |
| *** Salinity** (mS/cm) | | | | | <0.01 (<0.01-0.36) |
| *B.ps* positive | 0.07 (0.02-0.25) | P=0.037 | 0.02 (0.01-0.05) | P<0.001 | P=0.027 |
| *B.ps* negative | 0.25 (0.17-0.27) | | 0.25 (0.09-0.28) | | |
| *** Hardness** (mg/L) | | | | | |
| *B.ps* positive | 100 (40-170) | P=0.066 | 25 (10-80) | P<0.001 | |
| *B.ps* negative | 180 (150-200) | | 170 (80-180) | | |
| **Phosphate** (mg/L) | | | | | |
| *B.ps* positive | 2 (1-3) | P=0.114 | 6 (4-6) | P=0.069 | |
| *B.ps* negative | 3 (2-3) | | 3 (3-6) | | |
| **** Turbidity** (NTU) | | | | | |
| *B.ps* positive | 14.0 (6.0-22.0) | P<0.001 | 15.0 (10.0-26.0) | P=0.001 | |
| *B.ps* negative | 3.1 (2.6-4.0) | | 6.4 (5.0-8.0) | | |
| *** Coliforms** (CFU/mL) | | | | | |
| *B.ps* positive | 100 (20-220) | P=0.033 | 120 (21-220) | P=0.004 | |
| *B.ps* negative | 12 (5-25) | | 9 (2-24) | | |
| **Effective Bore Capping** | *B.ps* positive | Fisher's Exact | *B.ps* positive | Fisher's Exact | |
| Yes (38 bores) | 21 % | P=0.205 | 29 % | P=0.438 | *0.38 (0.14-1.06)* |
| No (9 bores) | 44 % | | 44 % | | *P=0.067* |

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169

**TABLE 1**: Summary statistics of the occurrence of *B.ps* in water bores and bore parameters. Using Stata/IC 10.0 (USA), 95% CI are percentile bootstrap estimates, odds ratios for *B.ps* positive bores were calculated using multivariable logistic regression clustered by bore and allowing standard errors for intra-group correlation and including season. The model was specified correctly as tested by a linktest. * indicates statistically significant result with P<0.01 in either dry or wet season and ** a significant result in dry and wet season. Nitrate levels are not reported as most were below test detection levels of 10 mg/L.

177 **FIGURE LEGEND**

178

179 **FIGURE 1:** Box-and-whisker plots of water parameters in B.ps negative ("neg") and positive
180 ("pos") bores of the dry and wet season. The box spans the interquartile range of the data and the
181 median is marked with a vertical line. * indicates a significant difference between B.ps negative
182 and positive bores with $P < 0.01$ (Mann-Whitney U test). Unit of salinity (conductivity) is
183 mS/cm (mS/cm x 640 = ppm total salts).

184

**Box plots of water parameters of *B. pseudomallei* positive and negative bores**