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CORRESPONDENCE

Production of oxalate in the culture supernate of *Burkholderia pseudomallei*

Burkholderia pseudomallei, a gram-negative, facultatively aerobic bacterium found in soil and water, is responsible for melioidosis in man and animals. This disease is prevalent in the tropics, particularly in northern Australia and south-east Asia, including Singapore [1–2]. The clinical spectrum of melioidosis is broad, ranging from subclinical to a fulminant, rapidly fatal septicaemia. Little is known of the pathogenic mechanisms involved in this infection, although secreted products such as the thermolabile lethal toxin associated with dermonecrotoxicity and proteolytic activity, exotoxin capable of inhibiting macromolecular synthesis in macrophages, proteases, haemolysin, phosphatases, lecithinase and lipase, as well as cell-associated factors such as lipopolysaccharide, flagella, capsule and pili are potentially significant in the pathogenesis of this disease [3]. While looking for new exotoxins of *B. pseudomallei*, instead of proteins, we found unusually large amounts of crystalline material after precipitating the culture supernate with high concentrations of ammonium sulphate, acetone, ethanol and isopropyl alcohol. In this communication, we report that *B. pseudomallei* is able to produce copious amounts of oxalate in broth culture. To our knowledge this is the first preliminary report of oxalate being produced by a human bacterial pathogen.

Three local strains and one reference strain (ATCC 15682) of *B. pseudomallei* from human, animal, soil and water were cultured. Strain KHW was from a national serviceman who died of melioidosis. He had acquired the infection during training in *B. pseudomallei*-infested terrain from which strain DB was isolated from drain water [4]. Isolate Soil GS was from the soil of a house in which a German Shepherd dog had died of pulmonary melioidosis. The *B. pseudomallei* strains were propagated on Tryptic Soy Agar (Difco) plates. After overnight culture, colonies were transferred into 10 ml of Todd Hewitt Broth (THB) and grown at 37°C for 6 days without shaking. The broth cultures were centrifuged at 10 000 *g* for 20 min at 4°C and the supernates were transferred to clean tubes. The culture supernates were then utilised for two studies, the first was for chemical characterisation and the second was for the estimation of oxalate. *B. pseudomallei* strain KHW was used for the first study. Ethanol was added to the supernate to achieve a 70% concentration (*c.* 2.33 volumes). The tube was held at 4°C for 20 min and the precipitate was recovered by centrifugation at 10 000 *g* for 20 min at 4°C. After dissolving the precipitate in water, ethanol precipitation was repeated. The white precipitate was lyophilised and stored at 4°C. Spectroscopic analyses were carried out. ^{1}H NMR spectroscopy (Bruker AMX 500 spectrometer) of the precipitate showed a characteristic active acidic proton broad signal at 4.8 ppm that was also supported by its IR absorption [ν_{max} (cm^{-1}) 3428.8 (*br* OH) and 1640 (C=O)], indicating that an acid was present in the sample. The ^{13}C NMR spectrum, recorded by a Bruker AMX 500 (500 MHz) spectrometer, showed the presence of one carboxyl signal [δ_C 175.80] in the ^{13}C chemical shift spectrum indicating that only one type of carboxyl carbon existed in the molecule. As it was only possible for formic acid and oxalic acid or their salts to display only one ^{13}C signal, and as no formaldehyde proton signal was detected at 8.48 ppm in the ^{1}H NMR spectrum, the precipitate contained oxalic acid or its salt. By comparing our results with published NMR and IR spectral data [5–7] and the solubility of the precipitate in water, it is most likely that the culture supernate contained mainly a mixture of sodium oxalate and oxalic acid. Elemental analysis by a Perkin-Elmer 2400 CHN elemental analyser showed the presence of 0.75% of hydrogen element, indicating that some molecules were monosodium oxalate or oxalic acid (Table 1).

Oxalate concentration in the culture supernates was determined with a colorimetric kit (Sigma; cat. no. 591-D) based on the oxidation of oxalate by oxalate oxidase, followed by measurement of H_2O_2 produced during the reaction by a peroxidase-catalysed reaction. Briefly, 1 ml of the culture supernate was added to an equal volume of the sample diluent. The mixture was added to an appropriately labelled sample purifier tube and mixed intermittently for 5 min. After centrifugation at 1500 *g* for 5 min, 50 μl of the supernate were mixed with 1 ml of oxalate reagent A and 0.1 ml of oxalate

Table 1. Elemental analysis of ethanol-precipitated Todd Hewitt broth culture supernate of *B. pseudomallei* strain KHW

Elements	Expected value (W%)	Determined value (W%)	Theoretical molecules	Determined molecules
C	12	18.58	2	1.55
Na	23	34.48	2	1.50
H	...	0.75	...	...

reagent B by gentle inversion and incubated at room temperature for 5 min. The A_{590} of blank (water) standard, test and control (culture medium alone) samples was read with a spectrophotometer (UV-2401PC, Shimadzu) and the concentration of oxalate was calculated. All four strains of *B. pseudomallei* produced large amounts (4.46–6.92 g/L), of oxalate in the culture supernate, compared with a negligible 0.02 g/L estimated from the THB control (Table 2).

Based on the above data, it was evident that *B. pseudomallei* isolates, from various sources, were capable of producing oxalate in the culture supernate. It was also co-incidental that *B. glumae*, a plant pathogen that causes grain rot and seedling rot of rice, produced oxalate in culture [8]. Oxalic acid and its salts are found in some plants (rhubarb leaves, 'dumbcane', spinach), fungi and mammals [9]. Oxalate is a product of metabolism in many moulds, and some species of *Penicillium* and *Aspergillus* are able to convert sugar into calcium oxalate [10]. It can be produced by metabolic sources such as ethylene glycol, glyoxylic acid, glycolic acid and glyoxal. Sodium oxalate is highly toxic through all routes of exposure [10]. The symptoms of acute poisoning may include severe gastrointestinal pain, cardiovascular collapse, central nervous depression, neuromuscular symptoms, kidney damage and death. Based on mol. wts, the relative toxicity of oxalate and oxalic acid appear to be about equal. What then are the probable role(s) of oxalates in melioidosis? The primary target organ for oxalate toxicity seems to be the kidney, followed by the nervous system. Renal damage results from precipitation of insoluble oxalic crystals in the renal tubules. In man, oxalate is a normal constituent of human urine, being excreted as a useless end-product of intermediary metabolism or from dietary sources [8]. Its insoluble salt of calcium is the main component of kidney stones [11]. Therefore, it is tempting to postulate that the production of kidney stones or urolithiasis in some melioidosis patients may be attributable to chronic low-level exposure to oxalate-producing *B. pseudomallei.* This does not necessarily preclude the role of oxalate in other manifestations of the disease.

We are currently studying whether oxalate is a possible virulence factor in melioidosis and also to determine how oxalate is produced in *B. pseudomallei*, whether from glyoxylate or oxaloacetate as immediate precursors or by some other metabolic pathway.

Table 2. Concentration of oxalate in the supernate of 6-day-old static Todd Hewitt broth cultures of *B. pseudomallei* at 37°C

Strain	Source	mM	g/L
KHW	Human	51.67	6.92
ATCC 15682	Monkey	46.60	6.25
Soil GS	Soil	33.27	4.46
DB	Water	40.31	5.40
Todd-Hewitt Broth	–	0.14	0.02

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