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Public health implications of *Acanthamoeba* and multiple potential opportunistic pathogens in roof-harvested rainwater tanks

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ABSTRACT

A study of six potential opportunistic pathogens (*Acanthamoeba* spp., *Legionella* spp., *Legionella longbeachae*, *Pseudomonas aeruginosa*, *Mycobacterium avium* and *Mycobacterium intracellulare*) and an accidental human pathogen (*Legionella pneumophila*) in 134 roof-harvested rainwater (RHRW) tank samples was conducted using quantitative PCR (qPCR). All five opportunistic pathogens and accidental pathogen *L. pneumophila* were detected in rainwater tanks except *Legionella longbeachae*. Concentrations ranged up to 3.1×10^6 gene copies per L rainwater for *Legionella* spp., 9.6×10^5 gene copies per L for *P. aeruginosa*, 6.8×10^5 gene copies per L for *M. intracellulare*, 6.6×10^5 gene copies per L for *Acanthamoeba* spp., 1.1×10^5 gene copies per L for *M. avium*, and 9.8×10^3 gene copies per L for *L. pneumophila*. Among the organisms tested, *Legionella* spp. (99% tanks) were the most prevalent followed by *M. intracellulare* (78%). A survey of tank-owners provided data on rainwater end-uses. Fecal indicator bacteria (FIB) *Escherichia coli* and *Enterococcus* spp. were enumerated using culture-based methods, and assessed for correlations with opportunistic pathogens and *L. pneumophila* tested in this study. Opportunistic pathogens did not correlate well with FIB except *E. coli* vs. *Legionella* spp. (tau=0.151, P=0.009) and *E. coli* vs. *M. intracellulare* (tau=0.14, P=0.015). However, *M. avium* weakly correlated with both *L. pneumophila* (Kendall's tau=0.017, P=0.006) and *M. intracellulare* (tau=0.088, P=0.027), and *Legionella* spp. also weakly correlated with *M. intracellulare* (tau=0.128, P=0.028). The presence of these potential opportunistic pathogens in tank water may present health risks from both the potable and non-potable uses documented from the current survey data.

1. Introduction

Increasing water scarcity has led to a greater reliance on alternative and decentralized potable and non-potable water resources in recent decades (Hanjra et al., 2012). Australia is the driest inhabited continent on Earth and suffered from a severe "millennium" drought from 2001 to 2009 (van Dijk et al., 2013). As a result of water scarcity in this region, the use of roof-harvested rainwater (RHRW) for domestic purposes is a widely accepted practice. This is beneficial for simultaneously conserving water and reducing stormwater runoff.

Pathogens could be introduced to tanks via roof runoff containing fecal matter from birds, insects, bats, possums and reptiles. The microbiological quality of RHRW stored in tanks is generally assessed by monitoring *Escherichia coli* (*E. coli*) and *Enterococcus* spp., which are commonly found in the gut of warm-blooded animals (Albrechtsen, 2002; Lee et al., 2010). The presence of *E. coli* in tank water generally indicates fecal contamination and the presence of potential pathogens. Drinking water guidelines have been used to assess the microbial quality of the tank water. For most guidelines, this entails the non-detection of *E. coli* in 100 mL of water (NHMRC-NRMMC, 2004; WHO, 2004). Fecal indicator bacteria should be able to predict human health outcomes. From a public health perspective, the relationship between fecal indicator bacteria and pathogens is critical.

Although case-control studies have established associations between untreated rainwater consumption and gastroenteritis (Brodribb et al., 1995; Merritt et al., 1999), epidemiological studies have not supported a strong linkage (Heyworth et al., 2006; Rodrigo et al., 2011). The presence of multiple non-gastroenteritis-associated microbial pathogens (opportunistic in nature) in rainwater tanks have been reported (Tuffley and Holbeche, 1980; Dobrowsky et al., 2014; Chidamba and Korsten, 2015), supporting the need to assess potential health risks. Only a few studies

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(Ahmed et al., 2014; Kobayashi et al., 2014) have quantified opportunistic pathogens such as *Aeromonas hydrophila*, *Legionella* spp., and *Staphylococcus aureus* by analyzing small numbers of tank water samples.

Opportunistic pathogens infrequently cause illnesses in healthy individuals, and primarily affect those with weakened immune systems, children, and/or the elderly. These pathogens include *Legionella* spp., *Mycobacterium avium* complex [(MAC), a group of related bacteria that includes both *Mycobacterium avium* and *Mycobacterium intracellulare*], *Pseudomonas aeruginosa*, and *Acanthamoeba* spp. *Acanthamoeba* spp. are both pathogens and hosts for other opportunistic bacteria, potentially enhancing their growth and virulence (Thomas et al., 2010; Thomas and Ashbolt, 2011; Falkinham et al., 2015a).

Legionella spp. are ubiquitous in water sources and include *L. pneumophila*, an accidental human pathogen (Mekkour et al., 2013), the most common causative agent of the severe pneumonia-like illness Legionnaires' Disease, as well as the less severe form, Pontiac fever (Diederen, 2008). Legionellosis is the only disease associated with this group of pathogens that is a nationally notifiable disease in Australia (Australian Government Department of Health, 2016). The rate of Legionellosis in Australia was 13 people per million people in 2012, which was higher than rates reported for Europe (9.2 people per million), the United States (10.8 people per million), Canada (4 people per million), Japan (2–7 people per million for 2005 – 2009), and Singapore (6.5 people per million) but lower than New Zealand (14 people per million) (Phin et al., 2014). However, these estimates are not likely to be directly comparable due to varied approaches for case definition, clinical diagnostics, and reporting practices (Phin et al., 2014). Eighty cases of Legionellosis were reported in Queensland, Australia in 2015 (rate of 1.7 per 100,000) (Australian Government Department of Health, 2016). *L. longbeachae* has also been isolated from soils and composts linked to outbreaks, and is more common in Australia than other countries (Steele et al., 1990; Pravinkumar et al., 2010). However, the openings on the top of tanks by which water enters are covered with only mosquito mesh, leaving space for dust, soil, fecal matter, on-site compost piles, or other debris to enter the tank. This is hypothesized as a route for potentially introducing *L. longbeachae. P. aeruginosa* is associated with bacteremia in immunocompromised patients, pneumonia in cystic fibrosis patients, and community-acquired ear, eye, and skin infections. However, systematic information regarding the *P. aeruginosa* disease burden is not available in Australia (Falkinham et al., 2015). MAC causes soft tissue infections and cervical lymphadenitis in immune-competent patients and disseminated infections in immune-compromised patients (Falkinham, 1996). Additional known risk factors for MAC are environmental exposures to aerosols containing the bacteria (hot tubs, soil dusts), and host characteristics; in particular, taller, slender post-menopausal women are affected more often by NTM infections than their demographically matched controls (Kartalija et al., 2013). MAC are the most common source of bacterial infection in AIDS patients (Kunimoto et al., 2003; O'Brien et al., 2000), and most frequently identified isolate in non-tuberculosis *Mycobacterium* spp. (NTM) cases in the Northern Territory, Australia during 1989 – 1997 (O'Brien et al., 2000). However, *M. intracellulare* was identified as the most common pathogen in NTM isolates in Queensland in 2005 (Thomson et al., 2013). In the Northern Territory, the yearly incidence of pulmonary MAC disease not associated with human immunodeficiency virus infection was 21 cases per million people in 1997, while in Queensland, the incidence of MAC cases has increased over time from 6.3 per million people in 1985 to 32 per million people in 2005 (O'Brien et al., 2000; Thomson et al., 2013).

Given the increasing importance of these opportunistic pathogens in municipal drinking water systems, and as a portion of the Australian and global waterborne disease burdens (Falkinham et al., 2001; Falkinham et al., 2015b; Schoen et al., 2011; Rusin et al., 1997), it is warranted to further explore their occurrence in RHRW tanks. To the author's knowledge, only two other studies have measured *Mycobacterium* spp. (*M. intracellulare*, *M. avium*, *M. gordonae*, and *M. terrae-M. triviale-M. nonchromogenicum* complex) in RHRW and none to date have quantified these opportunistic pathogens in tank water (Albrechtsen, 2002; Tuffley and Holbeche, 1980).

Culture-based methods have historically been preferred in microbial water quality monitoring efforts. However, their application can be challenging and time-intensive due to the strict growth requirements of certain pathogens, and failure to detect viable but non-culturable (VBNC) pathogens (Bonetta et al., 2010). For example, samples containing *Legionella* spp. can take up to 96 h to grow on BCYE agar, and require visual examination of colonies, and further biochemical testing for accurate identification (CDC, 2005). In contrast, qPCR is a more rapid and sensitive method for quantifying pathogens that are difficult to grow using culture-based methods. In this study, qPCR methods were chosen for the quantification of seven opportunistic pathogens and culture-based methods were utilized for the enumeration of fecal indicator bacteria (FIB) *Escherichia coli* and *Enterococcus* spp. Although FIB have historically been used as regulatory water quality monitoring standards for RHRW, little is known regarding their correlations with opportunistic pathogens in tank water. The aims of this study were therefore to (1) quantify seven potential opportunistic pathogens of public health significance in tank water; (2) assess their correlations with FIB, and (3) highlight the implications of the presence of these opportunistic pathogens in tank water. The quantitative data presented in this study would aid in the quantitative microbial risk assessment (QMRA) of RHRW for various domestic uses.

2. Materials and methods

2.1. Study areas and survey

134 rainwater tanks were sampled from various areas of Brisbane ($n=84$) and the Currumbin Ecovillage ($n=50$), both located in Southeast Queensland (SEQ), Australia during March to September 2015. The Ecovillage is a decentralized residential development that employs a range of strategies to conserve water and energy, including a cluster-scale sewage treatment/water reclamation plant, rainwater storage tanks, solar panels, and source-separated urine usage (Hood et al., 2009). Rainwater tank owners were sent an online survey regarding end uses (potable and non-potable), and treatment practices. 121 (90%) tank owners provided responses to the survey. On site, a sanitary inspection was undertaken during sampling to identify factors (the presence of overhanging trees, TV aerials, and wildlife fecal contamination on the roof), and to verify survey results.

2.2. Tank water sampling

The tap/spigot connected directly to the rainwater tank ($n=129$) was sterilized with 70% ethanol, and the water was run for 15 s before filling a 10 L sterile container. In the absence of a tap, the sample was collected directly from the opening in the top of the tank ($n=5$). Samples were transported to the laboratory, kept at 4 °C, and processed within 6–12 h.

2.3. Enumeration of fecal indicator bacteria (FIB)

Colilert® and Enterolert® (IDEXX Laboratories, Westbrook, Maine, USA) Test kits were used to determine the concentrations