Short Communication

Protozoan pathogens *Blastocystis* and *Giardia* spp. in roof-harvested rainwater: the need to investigate the role of the common brushtail possum (*Trichosurus vulpecula*) and other potential sources of zoonotic transmission

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ABSTRACT

Globally, protozoan pathogens are an increasingly important cause of reported disease outbreaks, with the majority of documented outbreaks between 2004 and 2010 reported in Australia. While the microbiological contamination of roof-harvested rainwater (RHRW) has been well studied, limited information is available regarding contamination with protozoan pathogens. In this study, rainwater ($n = 134$) and possum fecal samples ($n = 20$) were screened for the presence of several protozoan pathogens, including *Blastocystis* spp., *Cryptosporidium* spp., *Giardia* spp., *Dientamoeba fragilis*, and *Entamoeba histolytica* using the multiplex real-time polymerase chain reaction. While *Cryptosporidium* spp. was only detected in two possum fecal samples (10%) and *Giardia* spp. was only detected in three RHRW samples (2.23%, $n = 134$), *Blastocystis* spp. was detected in both possum feces (25%) and RHRW (5.22%) samples. *Dientamoeba fragilis* and *Entamoeba histolytica* were not detected in any samples. These findings highlight protozoan pathogens as a potentially important area of focus for rainwater quality assessment. Furthermore, while possums are suggested as a potential source of *Blastocystis* spp. in RHRW, sources of this pathogen in RHRW warrant further investigation.

Key words | *Blastocystis* spp., *Cryptosporidium* spp., *Giardia* spp., possum, public health, rainwater

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INTRODUCTION

Rainwater tanks have a significant history of usage in Australia, particularly in the rural and remote settings of the country (Commonwealth of Australia 2010). Currently, rainwater tank usage has grown significantly in urban areas, especially as a means to assist the existing centralized water supply (König & Sperfeld 2006). Tanks within a dual-household reticulation system are strongly encouraged and, in some cases, subsidized by the government of Australia (Commonwealth of Australia 2010; Gato-Trinidad & Gan 2014). The benefits of household rainwater tank usage include water conservation, especially within the scope of the impacts of climate change (Commonwealth of Australia 2010). An average of 42.5% reduction in water consumption per household was

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measured in Greater Melbourne (Gato-Trinidad & Gan 2014), for example. Another benefit from employing rainwater tanks includes a reduction in water runoff, which can damage the ecology of local creeks and other water bodies (Ahmed *et al.* 2011). Usage of collected rainwater can vary widely; it can be used as a source of drinking water, gardening water, or even as a measure of protection from bushfires (Hamilton *et al.* 2016). In 2007, 19% of households used rainwater tanks with approximately 10% using rainwater tanks as a source of drinking water. The largest number of rainwater tanks is recorded in South Australia, the driest state, where 45.4% of households use rainwater tanks (Commonwealth of Australia 2010).

The microbiological quality of rainwater in terms of potential contamination can be lower than that from a centralized supply, depending on various factors including catchment and tank maintenance, meteorological conditions, and wildlife present (Hamilton *et al.* 2017). Roof-harvested rainwater (RHRW) is often reported to be contaminated with fecal indicator bacteria, namely *Escherichia coli* and *Enterococcus* spp. (Dobrowsky *et al.* 2013; Hamilton *et al.* 2017; Leong *et al.* 2017) and bacterial pathogens and/or opportunistic pathogens such as *Campylobacter jejuni*, pathogenic *E. coli*, *Salmonella* spp., *Aeromonas hydrophila*, *Legionella pneumophila*, and *Pseudomonas aeruginosa* (Uba & Aghogho 2000; Albrechtsen 2002; Rodrigo *et al.* 2009; Ahmed *et al.* 2014; Hamilton *et al.* 2016, 2017). There is also anecdotal evidence that the prevalence of giardiasis and cryptosporidiosis are relatively higher in patients who drink rainwater in Southeast Queensland, Australia (Pers. Comm. General Practitioner). One report also indicated that giardiasis is a problem in aboriginal communities in New South Wales and may be associated with drinking RHRW (Nean & Pearce 2004; Noradilah *et al.* 2017). In a cross-sectional study of intestinal parasitic infections in primary schoolchildren and water sources (three reservoirs and two RHRW tanks with no treatment applied prior to consumption) in an area of Thailand with high *Blastocystis* prevalence, a match was found between a rainwater sample and an isolate from a *Blastocystis*-infected schoolchild using polymerase chain reaction (PCR) and restriction fragment length polymorphism analysis of the SSU ribosomal RNA gene (Leelayoova *et al.* 2008). Consumption of untreated rainwater in rural Australia was previously linked to a case of *Blastocystis* in three Australian patients (Roberts *et al.* 2014) and in a patient due to consumption of non-potable water (Angelici *et al.* 2018). However, information regarding *Blastocystis* and other protozoan pathogen occurrence in rainwater is scarce. Protozoan pathogens, such as *Blastocystis* spp., *Giardia* spp., and *Dientamoeba fragilis*, cause considerable disease burden in urban Sydney, yet exposures are not well characterized (Fletcher *et al.* 2014).

Traditionally, most tanks collect runoff water from the roof of a house. As a result, any contaminants found on the roof (i.e., dead insects, decomposed tree leaves, or animal fecal droppings) can enter the tank and contaminate water. A recent study determined an association between possum and bird feces and microbial quality of tank water in Southeast Queensland, Australia (Ahmed *et al.* 2016). The Common Brushtail Possum (*Trichosurus vulpecula*) is an Australian marsupial with a wide distribution and is especially prevalent in urban areas where it lives on roofs and in trees. Possums have been shown to harbor *Giardia* spp. and *Cryptosporidium* spp. that can infect and cause diarrheal illness in humans (Ahmed *et al.* 2012a), as well as *Blastocystis* spp. (Parkar *et al.* 2007). Nonetheless, the occurrence and diversity of protozoan pathogens in possum feces remain poorly understood. Therefore, possum feces were chosen for testing in the current study.

In this study, possum fecal samples were screened for the presence of the emerging protozoan pathogens *Blastocystis* spp., *Dientamoeba fragilis*, and *Entamoeba histolytica* along with *Cryptosporidium* and *Giardia* spp. In addition, RHRW samples were also tested for the presence of the above-mentioned protozoa pathogens. The intention of this study was to explore possum hosts as potential sources of protozoan pathogens in Australian rainwater tanks. While this study was not designed to assess a causal relationship between possums and human infection with protozoan pathogens, it can generate information useful for characterizing exposures to protozoa in RHRW. The results of this study may guide future management on animal feces as sources of contamination in RHRW and potentially aid in the management of RHRW quality and minimize public health risks from zoonotic infections.

MATERIALS AND METHODS

Possum fecal DNA samples

Possum fecal DNA samples used in this study ($n = 20$) were obtained from a previous study (Ahmed *et al.* 2016). Fecal samples were collected from Brisbane and a peri-urban Ecovillage located in the Gold Coast area of Southeast Queensland, Australia. Samples were transported on ice to the laboratory for storage at 4 °C and processed for DNA extraction within 24 h. DNA was extracted from 100 to 220 mg of fresh feces samples using the QIAamp stool DNA kit (Qiagen). The freshness of possum fecal samples was confirmed by the presence of a high moisture sheen. DNA samples were stored at −80 °C until use as detailed in Ahmed *et al.* (2016).

Rainwater DNA samples

The RHRW samples ($n = 134$) were collected from urban Brisbane and a peri-urban Ecovillage located in the Gold Coast area of Southeast Queensland, Australia as part of a previous study from March to November 2015 in which the protocol is described in detail elsewhere (Hamilton *et al.* 2016). The samples included 134 unique tanks constructed from a variety of materials with a variety of roofing materials. Sampling occurred under ambient conditions and covered both wet season and dry season months (Hamilton *et al.* 2016). Sample collection and DNA extraction procedures have been described elsewhere (Hamilton *et al.* 2016). Briefly, 10 L of samples were collected directly from RHRW tanks into sterile polyethylene containers and processed within 24 h. A hemoflow ultrafiltration procedure was used to concentrate the samples prior to filtering through a 0.45 μm nitrocellulose filter and storage at −80C until DNA extraction. DNA was extracted from the filters using a MO BIO PowerMax soil kit prior to conducting qPCR assays. Average recovery efficiency of pathogens analyzed in the study was demonstrated to be 84%, and PCR inhibition was tested using a Sketa22 assay (Hamilton *et al.* 2016). Inhibition was relieved with a 10-fold dilution.

Screening for protozoan pathogens

All DNA samples were screened for the presence of five protozoan pathogens (i.e., *Blastocystis* spp., *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia* spp.) using the EasyScreen™ Enteric Parasite Detection Kit (Stark *et al.* 2014; Dirani *et al.* 2019) in a multiplex real-time PCR format. The assay is performed on a Bio-Rad CFX96 thermal cycler using two PCR wells, with the first tube detecting (i) *D. fragilis* in the green channel, (ii) an extraction control in the yellow channel, and (iii) *Cryptosporidium* spp. in the red channel. The second tube detects (i) *Blastocystis* spp. in the green channel, (ii) an internal test positive control in the yellow channel, (iii) *E. histolytica* in the orange channel, and (iv) *Giardia* spp. in the red channel.

RESULTS AND DISCUSSION

For the screening of protozoan pathogens, we used our archived DNA samples (Ahmed *et al.* 2016; Hamilton *et al.* 2016). Five of 20 possum fecal DNA samples (25%) were positive for *Blastocystis* spp. Parkar *et al.* (2007) tested five Brushtail possum fecal samples from Perth, WA and reported that 40% samples were positive for *Blastocystis* spp. Two (10%) possum fecal DNA samples were also positive for *Cryptosporidium* spp. The presence of *Cryptosporidium* spp. in possum fecal samples has been reported in Australia (13% of 40 possum fecal samples) and New Zealand (12.8% of 30 possum fecal samples) (Chilvers *et al.* 1998; Ahmed *et al.* 2012a). The remaining protozoan pathogens (*D. fragilis*, *E. histolytica*, and *Giardia* spp.) could not be detected in any possum fecal DNA sample. This could be due to the fact that only a small number of samples were tested in this study. Out of 134 rainwater tank samples, 11 samples in total were positive for at least one enteric protozoa. Seven (5.22%) rainwater tank samples were positive for *Blastocystis* spp., and three (2.23%) for *Giardia* spp. (Table 1). The C_T values for *Blastocystis* spp. ranged from 26.7 to 38.5, suggesting the occurrence of variable concentrations of *Blastocystis* spp. in tank water DNA samples. The C_T values for *Giardia* spp. >35 suggested a low occurrence of *Giardia* spp. in tank water DNA samples.

Table 1 | Prevalence of protozoa pathogens in possum fecal and rainwater samples

Protozoa pathogens	Occurrence (%) in possum fecal samples ($n = 20$)	Occurrence (%) in tank water samples ($n = 134$)
Blastocystis spp.	5 (25)	7 (5.22)
Cryptosporidium spp.	2 (10)	ND
Dientamoeba fragilis	ND	ND
Entamoeba histolytica	ND	ND
Giardia spp.	ND	3 (2.23)

ND, not detected.

Limited measurements of *Blastocystis* spp. have been previously made in rainwater tank samples (Nean & Pearce 2004; Leelayoova *et al.* 2008; Noradilah *et al.* 2017), and the current study presents the first quantitative results for this protozoan in rainwater. The current results echo previous findings that *Blastocystis* spp. is present in RHRW and support anecdotal evidence of RHRW as a human exposure source.

The importance of these findings lies in the epidemiological properties of this protist genera: while there is a lack of consensus regarding the pathogenic potential of *Blastocystis* spp., its presence is a marker for fecal contamination. In this study, a direct causal link could not be established between possums and zoonotic enteric protozoa in the water tank samples. Establishing a link can be difficult because possums, other animals or birds, or even severe weather conditions can be factors in transporting protozoan cysts (Graczyk *et al.* 2008; Daniels *et al.* 2016). The small number of positive detections in both matrices precluded a robust correlation analysis; however, the findings suggest that possums can be a potential source. It cannot be ruled out from the current study that other wildlife species could be a source of protozoans in environments to which humans are exposed, including RHRW. A screening of indigenous Australian wildlife species for protozoan pathogens and molecular matching between animal and water samples is recommended for further study to better characterize these pathogen sources. Furthermore, the extent to which the conclusions of this study can be generalized to other geographic regions is not known but is also recommended for future investigation.

A recent study established an association between the degradation of microbial tank water quality with possum feces by analyzing possum-associated microbial source tracking markers in Australia (Ahmed *et al.* 2016). Additionally, previously, Ahmed *et al.* (2012a) established a link between rainwater quality degradation with possum and bird feces by analyzing clinically significant *E. coli* in rainwater tanks using a biochemical fingerprinting method. Clinically significant *E. coli* strains from a small number of tank water samples were identical to a number of *E. coli* found in bird and possum feces (Ahmed *et al.* 2012b). These studies demonstrate that fecal source tracking methods can be applied to rainwater tanks for attributing contamination sources. However, birds and other insects also cannot be ruled out as potential fecal sources of protozoa and require further investigation regarding the occurrence of *Blastocystis* spp. in fecal samples.

The numbers of rainwater tanks as a source of water for urban and rural households around the world are increasing. More people in rural and urban areas are installing rainwater tanks for potable and non-potable water supply. For example, 26% of Australian households used a rainwater tank as a source of water in 2010 compared with 19% in 2007 and 17% in 2004 (Commonwealth of Australia 2010). Among the 134 tank water samples tested in this study, 57 (49% of 117 survey respondents; not all of the 134 tank owners responded to the survey) are used as a potable source. Therefore, the presence of *Blastocystis* spp. and *Giardia* spp. in tank water samples may pose a potential health risk to tank owners. It has to be noted that the PCR method used in this study does not provide information regarding microorganism viability. However, protozoa like *Blastocystis* spp. may persist in tank water for a longer period of time. The degree of persistence of these organisms is not known. In addition, the zoonotic link between possums and humans is not well understood. However, *Blastocystis* sequences from a possum and human in Australia were also shown to be 100% similar to each other (Parkar *et al.* 2007). This study highlights the need for further investigation into the genetic diversity of *Blastocystis* found in possum feces and tank water samples, which could aid in supporting a potential zoonotic link for possums as reservoirs of *Blastocystis* spp. To minimize potential public health risks, it is also recommended that roof access points be blocked off if possible, such as a one-way door

flap to allow possums to leave the roof space but not to return.

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