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Fecal Indicators and Zoonotic Pathogens in Household Drinking Water Taps Fed from Rainwater Tanks in Southeast Queensland, Australia

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Running title: Fecal indicators and pathogens in household taps

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ABSTRACT

In this study, the microbiological quality of household tap water samples fed from rainwater tanks was assessed by monitoring the numbers of *Escherichia coli* and enterococci from 24 households in Southeast Queensland (SEQ), Australia. Quantitative PCR (qPCR) was also used for the quantitative detection of zoonotic pathogens in water samples from rainwater tanks and connected household taps. The numbers of zoonotic pathogens were also estimated in fecal samples from possum and various species of birds using qPCR as possums and birds are considered to be the potential sources of fecal contamination in roof-harvested rainwater (RHRW). Among the 24 households, 63% rainwater tank and 58% connected household tap water (CHTW) samples contained *E. coli* and exceeded Australian Drinking Water Guidelines of < 1CFU *E. coli* per 100 mL water. Similarly, 92% rainwater tanks and 83% CHTW samples were also contained enterococci. In all, 21%, 4%, and 13% rainwater tank samples contained *Campylobacter* spp., *Salmonella* spp., and *Giardia lamblia*, respectively. Similarly, 21% rainwater tank and 13% CHTW samples contained *Campylobacter* spp. and *G. lamblia*, respectively. The number of *E. coli* ($P = 0.78$), enterococci ($P = 0.64$), *Campylobacter* spp. ($P = 0.44$), and *G. lamblia* ($P = 0.50$), in rainwater tanks did not differ significantly from numbers observed in the CHTW samples. Among the 40 possum fecal samples tested, the *Campylobacter* spp., *Cryptosporidium parvum*, and *G. lamblia* were detected in 60%, 13%, and 30% samples, respectively. Among the 38 bird fecal samples tested, the *Campylobacter* spp., *Salmonella* spp., *C. parvum*, and *G. lamblia* were detected in 24%, 11%, 5%, and 13% samples, respectively. Household tap waters fed from rainwater tanks tested in the study appear to be highly variable. Regular cleaning of roof and gutter along with pruning of overhanging tree branches might also prove effective in reducing animal fecal contamination to the rainwater tanks.

Keywords: Roof-harvested rainwater, fecal indicators; Zoonotic pathogens, qPCR, Health risks

Introduction

Roof-harvested rainwater (RHRW) has been used as potable and non-potable water sources in many countries (15, 16, 34). There is a general community feeling that RHRW is safe to drink, and this is particularly supported by limited epidemiological evidence (19). In contrast, the presence of potential pathogenic microorganisms such as *Aeromonas* spp., *Campylobacter* spp., *Salmonella* spp., *Giardia* spp., and *Cryptosporidium* spp. in RHRW samples has been reported (3, 5, 13, 30, 32). The most significant issue in relation to RHRW for potable and non-potable uses is the potential health risks associated with the exposure to these pathogenic microorganisms. Case control studies established links between gastroenteritis and consumption of untreated RHRW (9, 28).

Wild animals such as birds, mammals and reptiles are the most likely sources of fecal contamination in RHRW as these animals have access to the roof surface. Consequently, fecal matter from these animals and other organic debris originating from overhanging trees could be transported to the rainwater tanks via roof runoff following rain events.

The microbiological quality of RHRW is generally assessed by monitoring fecal indicator bacteria such as fecal coliforms, *Escherichia coli*, and enterococci which are commonly found in the gut of warm-blooded animals including humans (29, 31, 33). In addition, a number of studies on the microbial quality of RHRW reported the presence of zoonotic bacterial and protozoa pathogens in individual or communal rainwater tanks (3, 8, 13, 25, 30, 32). Most of these studies assessed the quality of the RHRW on the basis of the presence or absence of the specific pathogens, with little information available regarding their numbers or potential sources in RHRW.

Around 10% of Australian people currently use RHRW as a major source of their drinking water, and an approximate additional 5% use RHRW as potable replacement for showering, toilet flushing, and clothes laundering (1), however, it is usually not recommended to use RHRW for drinking where town water is available. For example, Queensland regulations do not prohibit the plumbing of rainwater tanks to supply drinking water. If a person, however, chooses to use rainwater for drinking or any other purpose, then that person is responsible for ensuring the quality of the water is fit for its intended use. Many householders who drink RHRW use under sink filtration (USF) system in order to reduce the exposure to pathogenic microorganisms, suspended solids and harmful chemicals.

Little is known regarding the prevalence of zoonotic pathogenic microorganism in wild animals such as birds and mammals which are most likely contaminating RHRW. Mammals can get access to the roof via over

hanging trees, electricity cable or climbing the roof via walls or other structures attached to the house. Birds can get access to the roof via overhanging trees or mounted structures on the roof such as TV aerials and solar panels. Knowing the source of pathogenic microorganisms is important in order to mitigate management strategies and to reduce public health risks from exposure to pathogenic microorganisms.

The aims of this study were (i) to investigate the prevalence and numbers of fecal indicators (*E. coli* and enterococci) zoonotic bacterial (*Campylobacter* spp., and *Salmonella* spp.) and protozoa (*Cryptosporidium parvum* and *Giardia lamblia*) pathogens in water samples from rainwater tanks and connected household taps; and (ii) to investigate the prevalence of above mentioned pathogens in fecal samples from possums and various species of wild birds. Conventional culture based methods were used to enumerate *E. coli* and enterococci and quantitative PCR (qPCR) were used to obtain the numbers of zoonotic pathogens in RHRW, CHTW and animal fecal samples.

MATERIALS AND METHODS

Study area and sanitary survey. The study area "Currumbin Ecovillage" is located on the southern end of the Gold Coast, SEQ, Australia. The Ecovillage is known for its sustainable residential developments and is often viewed as a blueprint for future urban developments. Twenty four households participated in this study. All households use captured RHRW for drinking and other non-potable uses such as car washing, cloth laundering, showering, gardening etc. A sanitary inspection was undertaken to identify factors (i.e., the presence of overhanging trees, TV aerials and wild life fecal contamination on the roof) that may contribute to the fecal contamination of the rainwater tanks. Information on the filtration methods of RHRW prior to drinking was also obtained from the householders (Table 1).

Roof-harvested rainwater and connected household tap water sampling. Two water samples were collected from each household (i.e., one from rainwater tank and one from the connected household tap) giving a total of 48 samples from 24 households. Samples were collected within one to four days after a rain event (> 100 mm) in 20 L sterile containers. The external taps were located 15-20 cm from the bottom of the rainwater tanks and connected household taps were located over the kitchen sinks. Before sampling, the external taps and connected household cold water taps were wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water. Samples were transported to the laboratory and processed within 2-4 h.

Enumeration of fecal indicators. The membrane filtration method was used to process water samples for bacterial enumeration (35, 36). Sample serial dilutions were made (where necessary) and filtered through

0.45 µm pore sized (47 mm diameter) nitrocellulose membranes (Millipore, Tokyo, Japan). The membranes were placed on modified mTEC agar (Difco, Detroit, MI) and membrane-Enterococcus indoxyl-β-D-glucoside (mEI) agar (Difco) for the isolation of *E. coli* and enterococci, respectively. Modified mTEC agar plates were incubated at 35°C for 2 h to recover stressed cells, followed by incubation at 44°C for 22 h (36), and mEI agar plates were incubated at 41ºC for 48 h (35).

Concentration of water samples. Approximately, 19 L water sample from each rainwater tank and household tap was concentrated by hollow-fiber ultrafiltration system (HFUS), using Hemoflow HF80S dialysis filters (Fresenius Medical Care, Lexington, MA, USA) as previously described by Hill et al., (20). Briefly, each water sample was pumped with a peristaltic pump (Masterflex: Cole-Parment Instrument Co, USA) in a closed loop with sterile high-performance, platinum-cured L/S 36 silicone tubing (Masterflex; Cole-Parmer Instrument Co.). Tubing was sterilised by soaking in 10% bleach, washed with deionised (DI) water and autoclaved at 121ºC for 15 mins. At the end of the concentration process pressurised air was passed through the filter cartridge from the top to recover as much water as possible. A new filter cartridge was used for each sample. The samples were concentrated to approximately 100 mL. Each 100 mL sample was further centrifuged at 3,000 *g* for 30 mins at 4ºC. The supernatant was discarded, and the pellet was resuspended in 5 mL of sterile distilled water.

DNA extraction. For qPCR analysis of bacterial pathogens, DNA was extracted from the pellet obtained from 1.5 mL of concentrated samples (i.e., 48 samples) using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) and stored at -80ºC until use. For qPCR analysis of protozoa pathogens, DNA was also extracted from the pellet obtained from 1.5 mL of concentrated samples using the same kit with some modification. In brief, 180 µl of buffer ATL was added to each pellet and subjected to three cycles of freezing (-80ºC) followed by thawing (56ºC) in a water bath. After samples underwent freeze thaw cycles, proteinase K (i.e., 20 µl) was added to each tube. The tubes were incubated overnight at 56ºC. After incubation, the DNA was extracted according to the manufacturer's instructions.

Animal fecal sampling and DNA extraction. Brush tail possum fecal samples (*n*=40) were obtained from the possum removal service in Brisbane (http://www.possumman.com.au). Bird fecal samples (*n*=38) were collected from botanical garden, bird sanctuary and a veterinary hospital. The bird species include plover, wood duckling, noisy minet, pacific black duckling, blue faced honey eater, magpie, crow, ibis, seagull, topknot pigeon, crested tern, juvenile black swan, pacific baza, fantail cuckoo, rainbow lorikeet, and tawny frogmouth. Up to three samples were collected from each species of bird. All samples were transported to the laboratory, stored at

4ºC and processed within 24 h. DNA was extracted from fresh feces (i.e., 80-220 mg) from each individual animal by using a QIAmp Stool DNA kit (Qiagen).

Positive controls and qPCR assays. Strains and prepared DNA from typed cultures were purchased from the American Type Culture Collection (ATCC), as follows: *S.* Typhimurium ATCC 14028, *Campylobacter jejuni* (33560D), *G. lamblia* (30888D), and *C. parvum* (PRA-67D). qPCR assays were performed using previously published primers and probes (Table 2). Standards for qPCR of the *Campylobacter* spp. 16S rRNA, *Salmonella invA*, *C. parvum* COWP and *G. lamblia* β-giardin genes were prepared from the genomic DNA. The concentration of genomic DNA was determined by measuring the A_{260} using a Beckman Coulter DU 730 spectrophotometer. The genomic copies were calculated, and a 10-fold dilution ranging from 10^6 to 10^0 copies per µl of DNA extract was prepared from the genomic DNA, and stored at –20°C.

Salmonella qPCR amplification was performed in 20-µl reaction mixtures using Sso Fast™ EvaGreen® Supermix (Bio-Rad Laboratories, Calif). The PCR mixture contained 10 µl of Supermix, 300 nM each primer, 4.25 µl of DNase- and RNase-free deionized water, and 5 µl of template DNA (12). *Campylobacter* spp., *C. parvum* and *G. lamblia* qPCR amplification was performed in 25-µl reaction mixtures using iQ Supermix (Bio-Rad Laboratories). The PCR mixture contained 12.5 µl of Supermix, 500 nM each primer, 400-600 nM corresponding probe and 5 µl of template DNA. (17, 24, 26). For each PCR experiment, negative control (i.e., sterile water) was included. The PCR was performed using the Bio-Rad iQ5 (Bio-Rad Laboratories).

qPCR reproducibility and limit of detection. The reproducibility of the qPCR was assessed by determining intra-assay repeatability and interassay reproducibility. The coefficient of variation (CV) was calculated using six dilutions (5×10^6 to 5×10^0 gene copies) of the *C. jejuni*. *S.* Typhimurium, *C. parvum* and *G. lamblia* genomic DNA. Each dilution was quantified in replicates. The CV for evaluation of intra-assay repeatability was calculated based on the C_T value by testing the six dilutions three times in the same experiment. The CV for interassay reproducibility was calculated based on the C_T values of six dilutions on three different days. To determine the qPCR limit of detection, known gene copies (5×10^3 to 5×10^0 gene copies) were measured from pure genomic DNA isolated from corresponding control strains and tested by qPCR. The lowest number of gene copies that was detected consistently in replicate assays was considered the qPCR limit of detection.

Testing for PCR inhibitors. An experiment was conducted to determine the potential presence of PCR inhibitory substances in DNA extracted from RHRW (n=3) and CHTW (n=3) samples from three different households (i.e., H1, H10, and H15). DNA isolated from possums (n = 3) and birds (n = 3) were also checked for

the potential PCR inhibitors. Ten-fold serial dilutions were made and all DNA samples (i.e., undiluted and diluted) were spiked with 10^3 gene copies of human adenoviruses type 41. The cycle threshold (C_T) values obtained for the DNA samples from spiked RHRW, connected household taps and animal fecal samples were compared to those of the DNA samples from distilled water (3).

Quality control. To prevent carryover contamination for water and fecal samples, method blank runs were performed to ensure that the disinfection procedure was effective in preventing carryover contamination between sampling events. In addition, to prevent carryover contamination during DNA extraction, reagent blanks were included for each batch of samples. No carryover contamination was observed. During the setup of the PCR assays, the PCR conditions for annealing temperature were optimized by performing gradient analysis (i.e., temperature ranged from 53 to 63ºC) for each target. During melting curve analysis, the temperature was increased from 57 to 95ºC at approximately 2ºC per min. Samples were considered positive when had the same melting temperature (±0.2ºC) as the positive control. To minimize PCR contamination, DNA extraction and PCR setup were performed in separate laboratories.

Statistical analysis. Analysis of variance (ANOVA) was performed to determine the differences between C_T values obtained for DNA isolated from distilled water, and those obtained for RHRW, CHTW and fecal samples. Prior to the statistical analysis, all indicators and pathogen numbers were Log_{10} transformed. Wilcoxon signed-rank test was applied to test the significance of difference in fecal indicators and pathogen numbers between RHRW and CHTW samples. The Pearson's multiple correlation was used to test the relationship between *E. coli* and enterococci numbers in RHRW and CHTW samples. In all cases, a difference was considered significant if the P value was < 0.05. All statistical analyses were performed with SAS version 9.2 software (SAS Institute, Cary, NJ).

RESULTS

Survey results. The size of the selected rainwater tanks ranged from 7,200 to 30,000 L and aged between one to five years (Table 1). Among the 24 households surveyed, six (25%) had overhanging trees ($n = 4$) or TV aerials ($n = 2$) mounted on the roof. Seven (29%) tanks had visible sign of fecal droppings on the roof. twenty of the tanks (88%) had first flush diverter installed. Among the 24 households, ten (42%) filtered the water before drinking. Of the ten households, nine (90%) had USF (i.e., cartridge type filter, 0.5 µm pore size) and one had both USF and UV installed (Table 1).

qPCR standards, reproducibility and limit of detection. Ten-fold dilutions of quantified *C. jejuni S.* Typhimurium, *C. parvum* and *G. lamblia* were analysed in order to determine the reaction efficiencies. The standard curves had a linear range of quantification from 10^6 to 10^1 genomic copies per µl of DNA extracts. The amplification efficiencies were > 95% and the correlation coefficient (r^2) was > 0.98 for all four assays. The mean intra-assay and interassay CV values and standard deviations, respectively, were 3.9% ± 1.0% and 2.3% ± 1.6% (for *Campylobacter* assay), 1.9% ± 0.8% and 1.9% ± 1.3% (for *Salmonella* assay), 3.9% ± 1.9% and 2.9% ± 1.6% (for *C. parvum* assay), and 3.2% ± 1.2% and 4.5% ± 2.1% (for the *G. lamblia* assay), indicating high reproducibility. The qPCR limit of detection was five gene copies for all target pathogens.

PCR inhibitors. For the spiked distilled water, the mean C_T value for the human-specific adenovirus DNA was 25.6 ± 0.4 (Table 3). For RHRW samples (n = 3), CHTW samples (n = 3), possum (n = 3) and bird (n = 3) fecal samples, the mean C_T value was 28 ± 0.3, 27 ± 1.0, 27 ± 1.1, and 26 ± 0.4, respectively when undiluted DNA was spiked. Values obtained for 10-fold and 100-fold dilutions are shown in Table 3. One-way analysis of variance (ANOVA) was performed to determine the differences between the C_T values obtained for distilled water and those obtained for RHRW samples, CHTW samples, possum and bird fecal samples. No significant differences were observed between the C_T values for spiked distilled water, undiluted DNA, and serially diluted DNA, thus indicating that the tested samples were free of PCR inhibitors.

Numbers of fecal indicators in roof-harvested rainwater and connected household tap water samples. Among the 24 households, *E. coli* were cultured from 15 (62%) RHRW and 14 (58%) CHTW samples. Similarly, 22 (92%) RHRW and 20 (83%) CHTW samples contained cultured enterococci (Table 4). The numbers of *E. coli* in these samples ranged from 1×10^0 to 2.3×10^2 per 100 mL (for RHRW) and 1×10^0 to 3.0×10^2 CFU per 100 mL (for CHTW) of water, respectively. For enterococci, these numbers were 2×10^0 to 1.1×10^2 CFU per 100 mL (for RHRW) and 1×10^0 to 1.1×10^2 (for CHTW) CFU per 100 mL, respectively. Enterococci were more frequently detected in both RHRW (22 of 24 samples contained enterococci) and CHTW (20 out of 24) than *E. coli* (15 out of 24; RHRW and 14 out of 24; CHTW). Among the 24 samples tested from RHRW tanks, 96% samples contained at least one fecal indicator and 58% samples contained both indicators. Similarly, among the 24 samples tested from the connected household taps, 92% samples contained at least one fecal indicator and 50% were positive for both indicators.

Numbers of zoonotic pathogens in roof-harvested rainwater and connected household tap water samples. Among the 24 households, five (21%), one (4%), and three (13%) RHRW samples contained *Campylobacter* spp. 16S rRNA, *Salmonella invA*, and *G. lamblia* β-giardin genes, respectively (Table 4).

Similarly, five (21%) and three (13%) of the CHTW samples contained *Campylobacter* spp. 16S rRNA, and *G. lamblia* β-giardin genes, respectively. *Salmonella invA* gene could not be detected in CHTW samples. For the estimation of pathogen numbers, genomic copies (determined by qPCR) of each pathogen was converted to bacterial cells or protozoa cysts (4, 17, 22).

After conversion of genomic copies to number of cells, the number of *Campylobacter* spp. in RHRW and household tap water samples ranged from 5×10^0 to 1×10^2 (in RHRW) and 1×10^1 to 1.9×10^1 (in CHTW) cells per L of water. Similarly the estimated number of *Salmonella* spp. was 7.3×10^3 (in RHRW) cells per L of water. The numbers of *G. lamblia* cysts ranged from 1.2×10^2 to 5.8×10^2 (in RHRW) and 1.1×10^2 to 1.4×10^2 (in CHTW) per L of water.

Correlation between zoonotic pathogens and indicators in roof-harvested rainwater and connected household tap water samples. The number of fecal indicators and pathogens were pooled for all RHRW and CHTW samples to determine whether the numbers correlated between RHRW and CHTW samples. The number of *E. coli* ($P = 0.78$), enterococci ($P = 0.64$), *Campylobacter* spp. ($P = 0.44$), and *G. lamblia* ($P = 0.50$), in RHRW did not significantly differ from those numbers in CHTW samples as determined by Wilcoxon's signed-rank test. The numbers of *E. coli* and enterococci were analysed to determine whether the numbers within the RHRW and CHTW correlated with each other. Significant correlations were observed between *E. coli* and enterococci in water samples from RHRW ($r_p = 0.33$; $P = 0.005$) and CHTW ($r_p = 0.28$; $P = 0.01$) as determined by Pearson's multiple correlation.

Numbers of zoonotic pathogens in animal fecal samples. Among the 40 possum fecal samples tested, the *Campylobacter* spp. 16S rRNA, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 60%, 13% and 30% samples, respectively (Table 5). After conversion of genomic copies to number of cells, the number of *Campylobacter* spp. in possum fecal samples ranged from 2×10^5 to 2×10^7. *G. lamblia* was detected in 12 samples, however, only seven were quantifiable. The numbers of *G. lamblia* in possum fecal samples ranged from 2.1×10^1 to 1.6×10^3 cysts per gm of feces. *C. parvum* COWP gene was not quantifiable and *Salmonella invA* gene could not be detected in DNA from possum fecal samples.

Among the 38 bird fecal samples tested, the *Campylobacter* spp. 16S rRNA, *Salmonella invA*, *C. parvum* COWP, and *G. lamblia* β-giardin genes were detected in 24%, 11%, 5%, and 13% samples, respectively. The numbers of *Campylobacter* spp. *Salmonella* spp. and *G. lamblia* organisms in bird fecal samples ranged from 6.6×10^4 to 6.6×10^6, 6.3×10^2 to 1.8×10^3, and 1.3×10^0 to 1.0×10^2 cysts per gm of feces, respectively. *C. parvum* COWP gene was not quantifiable.

DISCUSSION

In this study, 62% of the RHRW and 58% of the CHTW samples fed from the RHRW tanks exceeded Australian Drinking Water Guidelines (2) of < 1CFU *E. coli* per 100 mL water. The pooled numbers of *E. coli* and enterococci in the CHTW samples did not differ significantly from the numbers found in the RHRW samples. It has to be noted that 58% of households in this study did not use any filtration methods, therefore, the presence of fecal indicators in the CHTW samples was not unexpected. Ten (42%) households had USF installed, however, these systems do not appear to be effective in removing fecal indicators. For example, households H3, H8, H11, H12, H15, H18 and H35 had USF, however, the numbers of fecal indicators in CHTW samples did not differ significantly from rainwater samples.

Five (21%) of the 24 RHRW tanks tested in this study contained *Campylobacter* spp. H6, H10, H12 and H15 were four of the five households where there were overhanging trees (H10 and H12) or evidence of wild life fecal droppings (H6 and H15) on the roofs. Two households (H12 and H15) had USF installed, however, *Campylobacter* spp. was detected in the CHTW samples suggesting the poor efficacy of USF systems. For *Campylobacter* spp., most human infections (i.e., 95%) are caused by *C. jejuni* and *C. coli* (10) and therefore, All *Campylobacter* spp. PCR positive samples were further tested for the presence of *C. jejuni* and *C. coli*. Three RHRW tank and two CHTW samples contained *C. coli*. None of the RHRW tank and CHTW samples, however, contained *C. jejuni* (data not shown). *G. lamblia* was detected in three (13%) of the RHRW tanks tested in this study. H1 and H7 were two of the three households where there was evidence of wild life fecal droppings. All three CHTW samples contained *G. lamblia*. It has to be noted that these households did not apply any filtration methods for rainwater purification prior to drinking. The high numbers of *G. lamblia* in both RHRW and CHTW samples from households H1, H7 and H14 may pose serious health risks to the consumers because of the low infectious dose of *Giardia.*

To obtain an insight on the magnitude of health risks, genomic copies of *G. lamblia* were converted to cysts numbers. The *G. lamblia* β-giardin gene is expressed as a single-copy gene within the nucleus of each trophozoite (21). Cysts of *Giardia* contain two trophozoites that have undergone multiple steps of nuclear division, resulting in 16 copies of total genetic information within each cyst (7) resulting 16 copies of the β-giardin gene per *Giardia* cyst (17). The number of *G. lamblia* appeared to be one order of magnitude higher in rainwater samples in this study compared to our previous study (4). It has to be noted that in this current study, 20 L of water samples were tested and whereas in the previous study, a smaller volume (i.e., 2-2.5 L) of water samples was tested. Concentration of large volume of water samples may have increased the detection sensitivity

(23). *C. parvum* could not be detected in any of the samples tested, however, the presence of *Cryptosporidium* spp. in RHRW samples has been reported in US Virgin Islands and Denmark (5, 13). *Salmonella* spp. was detected only one rainwater tank and none of the CHTW samples were positive for *Salmonella* spp.

Wild animals such as birds, mammals and reptiles are the most likely sources of fecal contamination in RHRW as these animals have access to the roof surface. In all, 60% possum and 24% bird fecal samples contained *Campylobacter* spp. All bird fecal samples contained *C. jejuni*. None of the possum fecal samples contained *C. jejuni* (data not shown). Possum and bird fecal samples also contained *G. lamblia* and the numbers of cysts ranged from 2.1×10^1 to 1.6×10^3 (for possums) and 1.3×10^0 to 1.2×10^2 (for birds) per gm of feces. Previous research studies also reported the presence of *G. lamblia* in possum and bird feces in North Island, New Zealand (11, 27). In this study, five possum and two bird fecal samples were also positive for *C. parvum*. The prevalence of *C. parvum* in possum and bird fecal samples was lower than *G. lamblia*. Chilvers et al., (11) reported similar findings and suggested that this could be because the duration of *Cryptosporidium* infection is much shorter than *Giardia* infection. It has to be noted that *Giardia* cysts were also detected in fecal samples from cats, rats and mice and therefore, these animals may also contribute *Giardia* to rainwater tanks (11). Other animals such as lizards, frogs and flying foxes that have access to the roof cannot be ruled out as possible sources of bacterial and protozoa pathogens in rainwater tanks.

Around 10% of the population in Australia currently use RHRW as a major source of their drinking water (1). To date, several disease outbreaks and clinical cases associated with rainwater consumption have been reported (6, 9, 28). In contrast, an epidemiological study of young children in South Australia reported that the consumption of RHRW did not increase the risk of gastroenteritis as opposed to mains water (19). The results of the current study indicate that certain householders were potentially exposed to potential pathogenic bacteria and protozoa, however, no increase in reported cases of illnesses is evident. This could be due to the fact that there is a naturally high incidence of gastroenteritis in the community which may mask the actual disease (18). Before the disease can be reported in the Notifiable Diseases Surveillance System, it must first be identified, and not every individual will seek medical attention if the illness is mild and lasts only for a few days. Another factor is the possibility of individuals acquiring immunity to certain pathogens due to frequent exposure. It is acknowledged that the qPCR methods used in the study do not provide information on what fraction of PCR detected cells or cysts were viable and infective. This is one of the major limitations of PCR based methods. A number of possum ($n = 5$) and bird ($n = 3$) fecal samples were tested for the presence of *Campylobacter* spp.

using both conventional and PCR based methods. Four possums and all three bird fecal samples were positive for *Campylobacter* spp. with both methods (data not shown).

The fecal contamination of RHRW appears to be limited to improperly designed systems as well as systems that are not well maintained. It has been suggested that all RHRW systems should be appropriately maintained, including ensuring the cleanliness of the systems before rainfall events, especially roofs and gutters, which should be cleaned frequently, while the receiving tanks should be cleaned at least two times per year to improve the quality of water (14). The roof should be kept clear of overhanging trees which may provide access to wild animals. Indeed, the high numbers of bacterial and protozoa pathogens in possum and bird fecal samples indicates the need for good maintenance of roof and gutter and elimination of overhanging tree branches to minimize fecal contamination of RHRW. It is evident that further information relating to the occurrence of pathogens throughout the year and the viability of pathogens in rainwater tanks is needed. In addition, more information is required on the survival of bacterial and protozoa pathogens in rainwater tanks. In a previous study after estimating health risks associated with the rainwater use, it was suggested to disinfect rainwater before using it as potable water, especially for drinking (4). The householders were asked to provide information on the types of filters installed in their USF systems and as well as information on the maintenance regimes. Certain householders did not follow the manufacturer's instruction, and therefore, the presence of fecal indicators and pathogenic microorganisms in CHTW samples was not unexpected. The quality of the RHRW and CHTW can be improved by implementing effective point of use treatment procedures such as filtration followed by disinfection with UV treatment, ozone disinfection or ultra membrane filtration.

In conclusion, household tap waters fed from rainwater tanks in Currumbin Ecovillage appear to be highly variable and of poor microbiological quality. The presence of one or more fecal indicators along with the potential bacterial and protozoa pathogens suggest that RHRW may not be suitable for drinking. Although 42% householders filtered RHRW prior to drinking, however, the poor microbiological quality suggests the inefficacy of filtration methods that are being used. In view of this, it is recommended that RHRW should be disinfected using effective treatment procedures prior drinking. The high prevalence of bacterial and protozoa pathogens in possum and bird fecal samples indicates these animal species may be the sources of fecal contamination in rainwater tanks. Therefore, maintenance of good roof and gutter hygiene and elimination of overhanging tree branches and other structures where possible to prevent the flocking of possums and birds should be considered.

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TABLE 1. Survey results of rainwater tanks tested in this study

Household ID	Size of the tank (litres)	Age of the tanks(yr)	Presence of overhanging trees[a]	Presence of TV aerials[a]	Evidence of wild life fecal droppings on the roof[a]	First flush diverters installed[a]	Under sink filtration[a]
H1	20,000	2	N	N	Y	Y	N
H2	20,000	5	N	N	N	N	N
H3	22,500	2	N	N	N	Y	Y
H4	22,500	1	N	N	N	Y	N
H5	22,500	2	N	N	N	Y	N
H6	20,000	1	N	Y	Y	Y	N
H7	20,000	2	N	N	Y	Y	N
H8	30,000	1	N	N	N	Y	Y
H9	20,000	1	N	N	Y	Y	Y
H10	22,500	3	Y	N	N	Y	N
H11	20,000	2	N	N	N	Y	Y
H12	22,000	1	Y	N	N	Y	Y
H13	10,000	2	Y	N	N	Y	Y[b]
H14	20,000	2	N	N	N	Y	N
H15	15,000	3	N	N	Y	N	Y
H16	15,000	1	N	N	N	Y	N
H17	10,000	3	N	N	Y	Y	N
H18	7,200	2	N	N	Y	Y	Y
H19	20,000	3	N	N	N	Y	N
H20	20,000	2	N	N	N	Y	N
H23	22,000	2	Y	N	N	Y	Y
H25	20,000	3	N	N	N	N	N
H29	20,000	1	N	Y	N	Y	N
H35	18,000	2	N	N	N	Y	Y

[a] Y, yes: N, no

[b] UV installed in addition to under sink filtration

TABLE 2. Primers, probes and cycling parameters for qPCR assays used in this study

Target	Primer sequence (5'-3')	Cycling parameters	Amplicon size	Reference
Campylobacter spp. 16S rRNA	F : CACGTG CTA CAA TGG CAT AT R: GGC TTC ATG CTC TCG AGT T P: FAM-CAG AGAA CAA TCC GAA CTG GGA CA-BHQ1	10 min at 95°C, 45 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C	108	(24)
Salmonella invA gene	F: ACA GTG CTC GTT TAC GAC CTGAAT R: AGA CGA CTG GTA CTGATC GAT AAT	5 min at 94°C, 30 cycles of 30 s at 94°C, 35 s at 59°C, and 120 s at 72°C	244	(12)
Giardia lamblia β-giardin gene	F: CAT AAC GAC GCCATCGCGGCTCTCAGGAA R: TTT GTG AGC GCT TCT GTC GTG GCA GCG CTAA P : FAM-AGC TCA ACG AGA AGG TCG CAG AGG GCTT-TAMRA	3 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C	218	(26)
Cryptosporidium oocyst wall protein (COWP) gene.	F: CAAATTGATACCGTTTGTCCTTCTG R: GGCATGTCGATTCTAATTCAGCT P: HEX-TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA T-BHQ1	10 min at 95°C, 45 cycles of 15 s at 95°C, 1 min at 60°C.	150	(17)

F: Forward primer; R: Reverse primer; P: Probe

TABLE 3. Evaluation of PCR inhibition on the PCR detection of spiked sewage-associated adenoviruses in RHRW, CHTW and animal fecal samples as opposed to spiked distilled water samples

Samples	Threshold cycle (C_T) value (mean ± SD) for PCR		
	Undiluted DNA	10-fold dilution	100-fold dilution
Distilled water	25.6 ± 0.4		
RHRW			
H1	27.1 ± 0.3	26.4 ± 0.3	26.9 ± 0.3
H10	27.2 ± 0.6	26.3 ± 0.4	26.6 ± 0.5
H15	26.7 ± 0.2	26.5 ± 0.5	26.7 ± 0.4
CHTW			
H1	26.8 ± 0.1	26.8 ± 0.2	27.9 ± 0.3
H10	28.4 ± 0.3	26.3 ± 0.6	26.0 ± 0.4
H15	26.4 ± 0.4	25.9 ± 0.4	25.8 ± 0.6
Possum feces			
P3	26.0 ± 0.3	25.8 ± 0.6	25.8 ± 0.7
P14	26.0 ± 0.6	25.4 ± 0.8	25.9 ± 0.3
P26	28.0 ± 0.1	25.9 ± 0.5	25.9 ± 0.6
Bird feces			
B1	26.0 ± 0.4	26.0 ± 0.4	25.9 ± 0.9
B5	26.4 ± 0.5	25.6 ± 0.7	25.4 ± 0.4
B11	25.6 ± 0.2	25.2 ± 0.8	25.2 ± 0.6

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536 TABLE 4. Numbers of fecal indicators and zoonotic pathogens in roof-harvested rainwater and connected household tap water samples
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Household ID	Numbers (mean) of fecal indicators per 100 mL of water				Numbers (mean) of bacterial cells and protozoa cysts per L of water					
	E. coli		Enterococci		*Campylobacter* spp.		*Salmonella* spp.		*G. lamblia*	
	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW	RHRW	CHTW
H1	1.5×10^1	2×10^1	2.1×10^1	1.3×10^1	ND	ND	ND	ND	1.2×10^2	1.4×10^2
H2	3×10^0	4×10^0	1.2×10^1	1.3×10^1	ND	ND	ND	ND	ND	ND
H3	1×10^0	4×10^0	9.1×10^1	1×10^2	ND	ND	ND	ND	ND	ND
H4	2×10^0	ND	3×10^0	6×10^0	ND	ND	7.3×10^3	ND	ND	ND
H5	2×10^0	3×10^0	3×10^0	1×10^1	ND	ND	ND	ND	ND	ND
H6	2.3×10^2	6.7×10^1	2.7×10^1	ND	1.1×10^2	+ [a]	ND	ND	ND	ND
H7	1×10^0	2×10^0	3.7×10^1	8.2×10^1	ND	ND	ND	ND	1.6×10^2	1.4×10^2
H8	8.9×10^1	6×10^0	4.0×10^1	6.1×10^1	ND	ND	ND	ND	ND	ND
H9	ND	ND	4×10^0	1×10^0	ND	ND	ND	ND	ND	ND
H10	2×10^0	ND	1.7×10^1	2.5×10^1	4.7×10^1	ND	ND	ND	ND	ND
H11	5×10^0	9×10^0	2.8×10^1	3.6×10^1	ND	1.4×10^1	ND	ND	ND	ND
H12	1.2×10^1	6×10^0	ND	ND	+ [a]	1.1×10^1	ND	ND	ND	ND
H13	ND	ND	3×10^0	ND	ND	ND	ND	ND	ND	ND
H14	5×10^0	2×10^0	5.4×10^1	6.1×10^1	5×10^0	1.2×10^1	ND	ND	5.8×10^2	1.1×10^2
H15	1.2×10^1	3×10^2	7.5×10^1	1.1×10^2	3×10^1	1.9×10^1	ND	ND	ND	ND
H16	ND	ND	2.3×10^1	1.8×10^1	ND	ND	ND	ND	ND	ND
H17	ND	3×10^0	2×10^0	2×10^0	ND	ND	ND	ND	ND	ND
H18	ND	ND	4.9×10^1	4.1×10^1	ND	ND	ND	ND	ND	ND
H19	1×10^0	1×10^0	1.5×10^1	4×10^1	ND	ND	ND	ND	ND	ND
H20	ND	ND	ND	2.4×10^1	ND	ND	ND	ND	ND	ND
H23	ND	1.5×10^1	1.1×10^2	2.5×10^1	ND	ND	ND	ND	ND	ND
H25	ND	ND	5×10^0	3×10^0	ND	ND	ND	ND	ND	ND
H29	1×10^0	ND	2×10^0	ND	ND	ND	ND	ND	ND	ND
H35	ND	ND	4×10^0	3×10^0	ND	ND	ND	ND	ND	ND

538 ND: Not detected; [a]: Not quantifiable

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TABLE 5. Numbers of zoonotic pathogens in possum and bird fecal samples

Samples	No. of samples tested	No. of PCR positive samples (%) (range of bacterial cells and protozoa cysts per gm of feces)			
		Campylobacter spp.	*Salmonella* spp.	*C. parvum*	*G. lamblia*
Possums	40	24 (60) (2×10^5 to 2×10^7)	ND	5 (13) [a]	12 (30) (2.1×10^1 to 1.6×10^3)
Birds	38	9 (24) (6.6×10^4 to 6.6×10^6)	4 (11) (6.3×10^2 to 1.8×10^3)	2 (5) [a]	5 (13) (1.3×10^0 to 1.2×10^2)

ND: Not detected; [a]: Not quantifiable

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