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# Real-Time PCR Detection of Pathogenic Microorganisms in Roof-Harvested Rainwater in Southeast Queensland, Australia▽

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**In this study, the microbiological quality of roof-harvested rainwater was assessed by monitoring the concentrations of *Escherichia coli*, enterococci, *Clostridium perfringens*, and *Bacteroides* spp. in rainwater obtained from tanks in Southeast Queensland, Australia. Samples were also tested using real-time PCR (with SYBR Green I dye) for the presence of potential pathogenic microorganisms. Of the 27 rainwater samples tested, 17 (63%), 21 (78%), 13 (48%), and 24 (89%) were positive for *E. coli*, enterococci, *C. perfringens*, and *Bacteroides* spp., respectively. Of the 27 samples, 11 (41%), 7 (26%), 4 (15%), 3 (11%), and 1 (4%) were PCR positive for the *Campylobacter coli ceuE* gene, the *Legionella pneumophila mip* gene, the *Aeromonas hydrophila lip* gene, the *Salmonella invA* gene, and the *Campylobacter jejuni mapA* gene. Of the 21 samples tested, 4 (19%) were positive for the *Giardia lamblia* β-giardin gene. The binary logistic regression model indicated a positive correlation ($P < 0.02$) between the presence/absence of enterococci and *A. hydrophila*. In contrast, the presence/absence of the remaining potential pathogens did not correlate with traditional fecal indicators. The poor correlation between fecal indicators and potential pathogens suggested that fecal indicators may not be adequate to assess the microbiological quality of rainwater and consequent health risk.**

Water scarcity is a major problem due to frequent droughts and increased urbanization. To meet the growing demand for water, alternative sources are imperative. Among the alternatives, roof-harvested rainwater has been considered a potential source of water, and its use is common in many countries (36). In Australia, the use of rainwater tanks is quite common, particularly in rural and remote areas, where reticulated mains and town water are not feasible options. For example, in Southern Australia, 82% of the rural population uses rainwater as their main water source (19). Recent water restrictions implemented in several capital cities including Brisbane, Queensland, highlight the importance of water conservation, including the use of rainwater tanks as an alternative source. To encourage the use of rainwater, the Queensland state government has announced the "Home Water Wise Rebate Scheme," which provides rebates to Southeast Queensland residents who install rainwater tanks with a minimum capacity of 3,000 liters, from July 2006 (30). The tanks are employed for purposes for which nonpotable water may be used, such as garden watering.

There is a general community sense that rainwater is safe to drink, and this is partially supported by limited epidemiological evidence. However, little is known regarding the microbiological quality of the roof-harvested rainwater and the potential health risks. Pathogenic microorganisms such as *Aeromonas* spp., *Campylobacter* spp., *Legionella* spp. *Salmonella* spp., *Giardia* spp., and *Cryptosporidium* spp. are present in the feces of birds, mammals, and reptiles that have access to the roof. Therefore, following rain events, fecal matter could potentially be transported to the tank via roof runoff. A previous study has reported that roof-harvested rainwater quality is generally acceptable for drinking and household use (13). In contrast, the presence of potential pathogenic microorganisms in rainwater samples has been reported (24, 36, 37). Therefore, questions have arisen regarding the quality of roof-harvested rainwater.

Direct monitoring of pathogens in water is an attractive option, as it would provide invaluable information regarding public health risk. However, there are hundreds of different types of pathogens that can be found in water due to fecal pollution. Therefore, it is not economically, technologically, and practically feasible to routinely monitor the microbiological quality of water for all possible pathogens. Alternatively, traditional fecal indicators, such as fecal coliforms, *Escherichia coli*, enterococci, and *Clostridium perfringens*, have long been used as surrogates for the presence of pathogenic microorganisms. Epidemiological studies have established human health standards based on exposure to fecal indicator bacteria for recreational bodies of water (29). Most studies assess the quality of roof-harvested rainwater based on the concentration of traditional fecal indicators (namely, fecal coliforms and *E. coli*) (15, 34, 44). However, the major limitations of using fecal bacteria as indicators are a poor correlation between the bacterial concentration and the presence of pathogenic microorganisms in environmental water (20) and the indicator bacteria's ability to replicate outside the host, especially in tropical environments (11). Little is known regarding the correlation between the presence of traditional fecal indicators and pathogens in roof-harvested rainwater. The recent advances in molecular techniques such as PCR technology enable rapid, specific, and sensitive detection and the identification of potential pathogenic microorganisms in environmental waters that are

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difficult and/or laborious to culture using traditional microbiological methods.

The aims of this study were (i) to assess the microbiological quality of roof-harvested rainwater in Southeast Queensland, Australia; (ii) to investigate the prevalence of pathogenic microorganisms (*Aeromonas hydrophila*, *Campylobacter coli*, *Campylobacter jejuni*, *Legionella pneumophila*, *Salmonella* spp., and *Giardia lamblia*); and (iii) to establish a correlation between fecal indicators and potential pathogens. To achieve these aims, rainwater samples were tested for the presence of *E. coli*, enterococci, and *C. perfringens* spores, using traditional culture methods. Samples were also tested for the presence of the above-mentioned pathogenic microorganisms, using real-time PCR detection assays.

## MATERIALS AND METHODS

**Rainwater sampling.** One-off samples were collected from 27 residential houses representing 18 suburbs in Brisbane, within 1 to 4 days after a rain event. During a rain event, fecal droppings deposited by birds, lizards, rats, and possums are generally washed off the roof. The size of the tanks sampled ranged from 500 to 10,000 liters, and the end uses were (i) outdoor use (63%), including gardening and car washing; and (ii) indoor use (37%), including drinking and kitchen use. The residents were interviewed to identify the presence of possible sources of fecal pollution. Birds, bats, and possums were identified as potential sources of fecal pollution. Water samples were collected in sterilized 10-liter containers from the outlet taps located close to the base of the tanks. Before the tank was sampled, the tap was allowed to run for 30 to 60 s to flush out water from the tap. Samples were transported to the laboratory on ice and processed within 8 to 10 h.

**Isolation, identification, and enumeration of fecal indicators.** The membrane filtration method was used to process the water samples for bacterial enumeration. Sample serial dilutions were made and filtered through 0.45-μm-pore-sized (47-mm-diameter) nitrocellulose membranes (Advantec, Tokyo, Japan) and placed on modified mTEC agar (Difco, Detroit, MI), membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) agar (Difco), and oleandomycin-polymyxin-sulfadiazine perfringens (OPSP) agar with supplement (Oxoid, London, United Kingdom) for the isolation of *E. coli*, enterococci, and spore-forming *C. perfringens*, respectively. For the isolation of *C. perfringens* spores, water samples were heated at 60°C for 30 min before filtration. The OPSP agar plates were overlaid with 15 ml of molten OPSP agar before incubation. 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 (39), and mEI agar plates were incubated at 41°C for 48 h (38). OPSP agar plates (for *C. perfringens*) were incubated anaerobically at 44°C for 24 h. The confirmatory test for *C. perfringens* was performed according to the method described previously (43). For bacterial enumeration, all water samples were tested in triplicate.

**DNA extraction from rainwater samples.** For PCR analysis of *Bacteroides* spp., 100 ml of each water sample was filtered through a 0.45-μm-pore-size membrane (Advantec). The filter was lifted and suspended in sterile 2-ml tubes containing 700 μl of GITC buffer (5 M guanidine isothiocyanate, 100 mM EDTA [pH 8.0]), 0.5% Sarkosyl), and stored overnight at −80°C. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA).

For PCR analysis of the *A. hydrophila lip* gene, the *C. jejuni mapA* gene, the *C. coli ceuE* gene, the *L. pneumophila mip* gene, and the *Salmonella invA* and *spvC* genes, 1 liter of water sample from each tank was filtered through a 0.45-μm-pore-size membrane (Advantec). In case of membrane clogging during filtration, multiple membranes were used. The membranes were immediately transferred into 15-ml screw cap tubes containing 10 ml of sterile STE buffer (0.1 M NaCl, 10 mM Tris, and 1 mM EDTA [pH 7.6]). The tubes were vortexed vigorously for 8 to 10 min to detach the bacteria from the membranes and then centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 2 ml of sterile distilled water. DNA was extracted using a DNeasy blood and tissue kit (Qiagen) and stored at −80°C until use.

For PCR analysis of the *G. lamblia* β-giardin gene, a 2.5-liter sample of rainwater from each tank was filtered through a 3-μm-pore-size membrane (47-mm diameter; Advantec). After filtration, the membrane was transferred into a 2-ml sterile microcentrifuge tube. DNA was extracted directly onto the filter, using a DNeasy blood and tissue kit (Qiagen). In brief, 360 μl of buffer ATL was added to each sample and subjected to three cycles of freezing (−80°C) and thawing (56°C) in a water bath. After samples underwent freezing-thawing, 40 μl of proteinase K was added to each tube. The tubes were then incubated overnight at 56°C. After incubation, the DNA was extracted according to the manufacturer's instructions.

**DNA and plasmid extraction of PCR-positive controls.** Strains were purchased from the American Type Culture Collection (ATCC), as follows: *Bacteroides vulgatus* ATCC 8482, *A. hydrophila* ATCC 7966, *C. coli* ATCC 43478, *L. pneumophila* ATCC 33152, and *Salmonella enterica* serovar Typhimurium ATCC 14028. *C. jejuni* NCTC 11168 was kindly donated by Shreema Merchant, Queensland University of Technology, Australia. DNA was extracted from the broth cultures using a DNeasy blood and tissue kit (Qiagen). *G. lamblia* β-giardin-positive control DNA was isolated from sewage. In brief, the PCR-amplified product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into a pGEM-T Easy vector system (Promega, Madison, WI), transferred into *E. coli* JM109-competent cells, and plated on LB agar plates containing ampicillin, isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, as recommended by the manufacturer. A recombinant plasmid with a single copy of the β-giardin gene insert was purified using a plasmid mini kit (Qiagen). DNA sequencing was carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia).

**Real-time PCR analysis.** Amplification was performed in 25-μl reaction mixtures using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The PCR mixture contained 12.5 μl of SuperMix, 300 nM of each primer, and 2 μl of template DNA. For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. Each DNA sample was tested in triplicate to obtain positive/negative results.

The real-time PCRs were performed using a Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Australia). The corresponding PCR cycles and primers for each target are shown in Table 1. To separate the specific product from nonspecific products (if any), DNA melting curve analysis was performed for each PCR experiment. During melting curve analysis, the temperature was increased from 57 to 95°C at approximately 2°C/min. Amplified products were also visualized by electrophoresis, through 2% E-gels (Invitrogen), and exposure to UV light for further confirmation. Samples were considered positive when the visible band was the same as that of the positive control strain and had the same melting temperature as the positive control. To minimize PCR contamination, DNA extraction, the PCR set up, and gel electrophoresis were performed in separate laboratories.

**Real-time PCR detection limits.** To determine the lower limits of the real-time PCR detection, genomic DNA (from *A. hydrophila*, *C. jejuni*, *C. coli*, *L. pneumophila*, and *Salmonella* serovar Typhimurium) and plasmid DNA (from *G. lamblia*) were quantified using a spectrophotometer. Tenfold serial dilutions were made and tested with the real-time PCR.

**Testing for PCR inhibitors.** Environmental samples contain numerous organic and inorganic substances with the potential to inhibit PCRs (42). An experiment was conducted to determine the potential presence of PCR inhibitory substances in rainwater samples collected from five different tanks. Each sample (i.e., 1 liter) was concentrated using a membrane filtration technique as described above. DNA was extracted using a DNeasy blood and tissue kit (Qiagen) and tested by real-time PCR. DNA was also extracted from 1 liter of ultrapure DNase- and RNase-free sterile distilled water (Invitrogen) in the same manner. All samples were spiked with a known concentration of *Salmonella* serovar Typhimurium (5.1 pg), *C. jejuni* (2.2 pg), and *L. pneumophila* (3.4 pg) DNA. The threshold cycle ($C_T$) values obtained for the DNA samples from spiked rainwater were compared to those of the DNA samples from distilled water. The $C_T$ value reflects the PCR cycle number at which the fluorescence generated crosses the threshold. It is inversely correlated to the logarithm of the initial copy number.

**DNA sequencing.** To verify the identity of the PCR products obtained from rainwater tanks, up to three PCR-amplified products from each target were purified using a QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer's instruction (Qiagen). DNA sequences were aligned and compared using Bioware Jellyfish software.

**Statistical analysis.** A Mann-Whitney nonparametric test was performed to determine the differences between $C_T$ values obtained for distilled water and those obtained for rainwater samples. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used to perform the Mann-Whitney nonparametric test. The nonparametric Spearman rank correlation was performed to obtain correlations among fecal indicator bacteria concentrations. A binary logistic regression analysis was also performed to obtain correlations between the presence/absence results of pathogen detection by PCR and the concentration of fecal indicators. Logistic regression is the technique most commonly used to model such a binary (i.e., presence/absence) response. The presence/absence of pathogens was treated as the dependent variable (i.e., a binary variable). When a target organism was present, it was assigned the value 1, and when a target

TABLE 1. Primers and cycling parameters for real-time PCR assays

| Target | Primer sequences (5′–3′)[a] | Cycling parameters | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| *Bacteroides* 16S rRNA gene | AAC GCT AGC TAC AGG CTT (F), CAA TCG GAG TTC TTC GTG (R) | 15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 59°C, and 60 s at 72°C | 700 | 4 |
| *A. hydrophila lip* gene | AAC CTG GTT CCG CTC AAG CCG TTG (F), TTG CTC GCC TCG GCC CAG CAG CT (R) | 15 min at 95°C, 35 cycles of 60 s at 94°C, 60 s at 62°C, and 90 s at 72°C | 760 | 6 |
| *C. jejuni mapA* gene | GCT AGA GGA ATA GTT GTG CTT GAC AA (F), TTA CTC ACA TAA GGT GAA TTT TGA TCG (R) | 10 min at 95°C, 50 cycles of 15 s at 95°C and 30 s at 59°C | 72 | 28 |
| *C. coli ceuE* gene | CAA GTA CTG CAA TAA AAA CTA GCA CTA CG (F), AGC TAT CAC CCT CAT CAC TCA TAC TAA TAG (R) | 10 min at 95°C, 40 cycles of 15 s at 95°C and 30 s at 59°C | 67 | 28 |
| *L. pneumophila mip* gene | GCA ATG TCA ACA GCAA (F), CAT AGC GTC TTG CATG (R) | 15 min at 95°C, 35 cycles of 30 s at 94°C, 60 s at 54°C, and 60 s at 72°C | 159 | 41 |
| *Salmonella invA* gene | ACA GTG CTC GTT TAC GAC CTG AAT (F), AGA CGA CTG GTA CTG ATC GAT AAT (R) | 5 min at 94°C, 45 cycles of 30 s at 94°C, 35 s at 59°C, and 120 s at 72°C | 244 | 7 |
| *Salmonella spvC* gene | ACT CCT TGC ACA ACC AAA TGC GGA (F), ACA GTG CTC GTT TAC GAC CTG AAT (R) | 5 min at 94°C, 45 cycles of 30 s at 94°C, 35 s at 59°C, and 120 s at 72°C | 571 | 7 |
| *G. lamblia* β-giardin gene | CCT CAA GAG CCT GAA CGA TCTC (F), AGC TGG TCG TAC ATC TTC TTC CTT (R) | 10 min at 95°C, 40 cycles of 15 s at 95°C, and 60 s at 59°C | 74 | 16 |

[a] F, forward primer; R, reverse primer.

organism was absent, it was assigned the value 0. Minitab Release version 11.12 (State College, Pa.) software was used for logistic regression analysis. In all cases, a difference was considered significant if the *P* value for the model chi square was $<0.05$.

## RESULTS

**Concentration of fecal indicators.** The concentrations of *E. coli*, enterococci, and *C. perfringens* were determined using conventional culture-based methods. Of the 27 rainwater samples tested, 17 (63%) were found to be positive for *E. coli*. The number of *E. coli* in positive samples ranged between 4 ± 3 CFU/100 ml and 800 ± 235 CFU/100 ml. Similarly, 21 (78%) samples and 13 (48%) samples were also positive for enterococci and *C. perfringens*, respectively. The number of enterococci and *C. perfringens* in these samples ranged between 5 ± 1 CFU/100 ml and 200 ± 33 CFU/100 ml and between 2 ± 1 CFU/100 ml and 31 ± 17 CFU/100 ml, respectively. The concentration of *E. coli* exceeded that specified by Australian drinking water guidelines for 7 out of 10 samples of rainwater used for drinking. Of the 27 samples tested, 11 (41%) were positive for all three indicators, 17 (63%) were positive for at least two indicators, and 23 (85%) were positive for at least one indicator tested in this study. A nonparametric Spearman rank correlation was performed to determine whether any correlation exists among concentrations of fecal indicator bacteria. Significant correlations were found between *E. coli* and enterococci ($P < 0.04$) and between enterococci and *C. perfringens* ($P < 0.04$). However, the concentration of *E. coli* did not significantly correlate with the concentration of *C. perfringens* ($P > 0.18$). In all, 24 (89%) water samples were positive for *Bacteroides* spp., indicating recent fecal pollution. The presence of *Bacteroides* spp. was generally in agreement with the presence of *E. coli* or enterococci. However, two samples (i.e., T8 and T11) were positive for the *Bacteroides* spp., but none of the indicators could be detected.

**Lower detection limits of the real-time PCR assays.** These assays were performed using purified genomic DNA isolated from pure cultures of *A. hydrophila* ATCC 7966, *C. coli* ATCC 43478, *C. jejuni* NCTC 11168, *L. pneumophila* ATCC 33152, and *Salmonella* serovar Typhimurium 14028 and plasmid DNA containing the *G. lamblia* β-giardin gene. To determine the reproducibility of the assays, several replicates ($n = 6$) were tested. The results of these assays are summarized in Table 2. The real-time PCR detection limits were as low as 5 gene

TABLE 2. Lower detection limits of the real-time PCR assays using genomic and plasmid DNA for pathogenic microorganisms

| Pathogenic microorganism | Amt of genomic and plasmid DNA tested | No. of gene copies tested[c] | No. of replicates[b] | No. (%) of positive samples[c] |
|---|---|---|---|---|
| *A. hydrophila* | 2.51 pg, 25.1 fg | 500, 5 | 6 | 6 (100), 6 (100) |
| *C. coli* | 0.90 pg, 9.00 fg | 500, 5 | 6 | 6 (100), 6 (100) |
| *C. jejuni* | 0.87 pg, 17.5 fg | 500, 10 | 6 | 6 (100), 6 (100) |
| *L. pneumophila* | 1.85 pg, 18.5 fg | 500, 5 | 6 | 6 (100), 6 (100) |
| *Salmonella* serovar Typhimurium | 2.55 pg, 25.5 fg | 500, 5 | 6 | 6 (100), 6 (100) |
| *G. lamblia*[a] | | 700, 7 | 6 | 6 (100), 5 (83.3) |

[a] Plasmid DNA.
[b] Replicates containing different amounts of genomic and plasmid DNA.
[c] The first and second values in each pair correspond to experiments with high and low copy numbers, respectively.

TABLE 3. Effects of PCR inhibitors on the real-time PCR detection of spiked pathogenic microorganism in samples from rainwater tanks

| Samples | Mean $C_T$ value ± SD for real-time PCR detection of the following gene: | | |
|---|---|---|---|
| | *C. jejuni mapA* | *L. pneumophila mip* | *Salmonella* serovar Typhimurium *invA* |
| Distilled water | 33.9 ± 0.31 | 22.6 ± 0.49 | 22.7 ± 0.53 |
| Tank 1 | 35.6 ± 0.23 | 24.1 ± 0.43 | 23.1 ± 0.83 |
| Tank 2 | 36.1 ± 0.11 | 23.9 ± 0.13 | 24.3 ± 0.56 |
| Tank 3 | 33.4 ± 0.34 | 23.1 ± 0.19 | 23.6 ± 0.34 |
| Tank 4 | 38.3 ± 0.90 | 23.3 ± 0.65 | 23.9 ± 0.16 |
| Tank 5 | 33.1 ± 0.53 | 23.5 ± 0.39 | 22.9 ± 0.37 |

copies for the *A. hydrophila*, *C. coli*, *L. pneumophila*, and *Salmonella* serovar Typhimurium target genes. For *G. lamblia* and *C. jejuni*, the detection limits were 7 and 10 gene copies, respectively. Lower levels (<5 copies) were tested, but the results were not reproducible.

**PCR inhibitors.** To detect the presence of inhibitors, rainwater samples ($n = 5$) were spiked with $10^3$ gene copies each of *C. jejuni* DNA (equivalent to 2.2 pg), *L. pneumophila* DNA (equivalent to 3.4 pg), and *Salmonella* serovar Typhimurium DNA (equivalent to 5.1 pg). The real-time PCR $C_T$ values were compared to those obtained from the same concentration of DNA that was used to spike 1 liter of distilled water. For the spiked distilled water, the mean $C_T$ values for *C. jejuni*, *L. pneumophila*, and *Salmonella* serovar Typhimurium DNA are given in Table 3. For spiked rainwater samples, the mean $C_T$ values were 35.3 ± 2.0 (for *C. jejuni*), 23.6 ± 0.5 (for *L. pneumophila*), and 23.5 ± 0.7 (*Salmonella* serovar Typhimurium). The Mann-Whitney nonparametric test was performed to determine differences between the $C_T$ values obtained for distilled water and those obtained for rainwater samples. No significant differences were observed between the $C_T$ values for spiked distilled water and those for rainwater samples of *C. jejuni* ($P = 0.49$), *L. pneumophila* ($P = 0.13$), and *Salmonella* serovar Typhimurium ($P = 0.06$), indicating that the tested rainwater samples were free of inhibitors.

**Prevalence of potential pathogens and correlation with traditional fecal indicators.** *C. coli* organisms were the most prevalent among all the potential pathogens tested in this study. Of the 27 samples tested, 11 (41%) were positive for the *C. coli ceuE* gene (Table 4). The prevalence of other potential pathogens was as follows: 7 samples (26%) were positive for the *L. pneumophila mip* gene, 4 (15%) for the *A. hydrophila lip* gene, 3 (11%) for the *Salmonella* spp. *invA* gene, and 1 (4%) for the *C. jejuni mapA* gene. None of the samples was positive for the *Salmonella spvC* gene. Of the 21 samples tested, 4 (19%) were positive for the *G. lamblia* β-giardin gene. Of the 27 samples tested, 3 (11%) were PCR positive for three target pathogens, 9 (33%) were positive for two target pathogens, and 18 (67%)

TABLE 4. Concentration of fecal indicators and PCR presence/absence results for potential pathogens in roof-harvested rainwater samples

| Tank | Concn of fecal indicators (CFU/100 ml) | | | Pathogen presence (+)/absence (−) as detected by PCR | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *E. coli* | Enterococci | *C. perfringens* | *Bacteroides* spp.[b] | *A. hydrophila lip* gene | *C. jejuni mapA* gene | *C. coli ceuE* gene | *L. pneumophila mip* gene | *Salmonella invA* gene | *Salmonella spvC* gene | *G. lamblia* β-giardin gene[c] |
| T1[a] | 5 ± 1 | 9 ± 4 | 4 ± 2 | + | + | − | − | − | − | − | − |
| T2[a] | 800 ± 235 | 200 ± 33 | 11 ± 3 | + | − | + | − | − | − | − | − |
| T3[a] | 8 ± 3 | 5 ± 3 | 31 ± 17 | + | − | − | + | + | + | − | − |
| T4[a] | <1 | <1 | 16 ± 9 | + | − | − | − | − | − | − | − |
| T5[a] | 11 ± 6 | 180 ± 27 | 3 ± 1 | + | + | − | − | − | − | − | − |
| T6 | <1 | 43 ± 9 | <1 | + | − | − | + | − | − | − | NT |
| T7 | 5 ± 3 | 125 ± 34 | 9 ± 3 | + | − | − | − | + | + | − | + |
| T8 | <1 | <1 | <1 | + | − | − | + | + | − | − | NT |
| T9 | <1 | <1 | <1 | − | − | − | − | − | − | − | NT |
| T10 | 6 ± 4 | 5 ± 2 | <1 | + | − | − | − | − | − | − | NT |
| T11 | <1 | <1 | <1 | + | − | − | + | − | + | − | NT |
| T12 | 23 ± 7 | <1 | <1 | + | − | − | + | + | − | − | − |
| T13[a] | <1 | <1 | <1 | − | − | − | − | − | − | − | NT |
| T14 | 74 ± 17 | 5 ± 1 | <1 | + | − | − | + | + | − | − | + |
| T15 | 11 ± 5 | 5 ± 3 | <1 | + | − | − | − | + | − | − | − |
| T16[a] | 350 ± 109 | 85 ± 41 | <1 | + | + | − | + | − | − | − | − |
| T17 | 7 ± 6 | 41 ± 17 | 5 ± 4 | + | − | − | − | − | − | − | − |
| T18[a] | 6 ± 3 | 60 ± 9 | 4 ± 2 | + | − | − | − | − | − | − | + |
| T19 | 287 ± 57 | 180 ± 40 | 8 ± 6 | + | + | − | − | + | − | − | − |
| T20 | <1 | 39 ± 7 | 29 ± 6 | + | − | − | + | − | − | − | + |
| T21 | 4 ± 3 | 28 ± 4 | 2 ± 1 | + | − | − | − | − | − | − | − |
| T22[a] | 5 ± 1 | 10 ± 3 | <1 | + | − | − | − | − | − | − | − |
| T23[a] | <1 | 45 ± 7 | <1 | + | − | − | + | − | − | − | − |
| T24 | <1 | 8 ± 3 | <1 | − | − | − | + | − | − | − | − |
| T25 | 6 ± 2 | 19 ± 5 | 3 ± 1 | + | − | − | − | − | − | − | − |
| T26 | <1 | 35 ± 11 | <1 | + | − | − | + | − | − | − | − |
| T27 | 25 ± 6 | 28 ± 7 | 6 ± 3 | + | − | − | − | − | − | − | − |

[a] Tanks used for drinking water.
[b] Amounts of 100-ml water samples were processed.
[c] NT, not tested.

TABLE 5. Correlations between the concentrations of fecal indicator bacteria and the presence/absence of potential pathogens[a]

| Fecal indicator bacteria | *P* value for correlation to the indicated pathogen | | | | | |
|---|---|---|---|---|---|---|
| | *A. hydrophila lip* gene | *C. coli ceuE* gene | *C. jejuni mapA* gene | *L. pneumophila mip* gene | *Salmonella invA* gene | *G. lamblia* β-giardin gene |
| *E. coli* | 0.250 | 0.611 | 0.466 | 0.969 | 0.306 | 0.406 |
| Enterococci | 0.020[b] | 0.142 | 0.552 | 0.878 | 0.986 | 0.873 |
| *C. perfringens* | 0.759 | 0.752 | 0.909 | 0.469 | 0.107 | 0.316 |

[a] Data are results of binary logistic regression analysis (*P* values) performed to identify correlations between the concentrations of fecal indicator bacteria and the presence/absence of potential pathogens in roof-harvested rainwater samples.
[b] The correlation is significant.

were positive for at least one target pathogen. In contrast, none of these potential pathogens were detected in nine (33%) rainwater samples.

Binary logistic regressions were used to identify whether any correlation existed between the concentrations of fecal indicators (*E. coli*, enterococci, and *C. perfringens*) and the presence/absence results for *Bacteroides* spp. Significant correlations were found between *E. coli* ($P < 0.05$) and enterococci ($P < 0.01$) with *Bacteroides* spp. However, the concentration of *C. perfringens* and the presence/absence of *Bacteroides* spp. did not correlate significantly ($P > 0.09$). Binary logistic regressions were also performed to identify the correlations between the concentrations of fecal indicator bacteria and the presence/absence of potential target pathogens (Table 5). A positive correlation was found between enterococci and *A. hydrophila*. In contrast, the presence/absence of the remaining potential pathogens did not correlate with any of the indicator bacteria concentrations.

## DISCUSSION

In this study, roof-harvested rainwater samples were tested for the concentration of traditional fecal indicators, using conventional culture methods, as well as the presence of potential pathogenic microorganisms, using real-time PCR detection. Previous studies that have reported acceptable levels of microbiological quality of roof-harvested rainwater used conventional culture methods to detect traditional fecal indicators (8, 13, 15). However, there are several limitations to those culture methods, including the underestimation of the bacterial concentration due to the presence of injured or stressed cells (10). Furthermore, certain microorganisms in environmental waters could be viable but not culturable (27). In addition, some of the conventional test methods are time-consuming, labor-intensive, and not practical for routine monitoring of pathogenic microorganisms. However, the application of PCR-based methods has generated interest in the direct monitoring of pathogens in environmental waters. The advantages of PCR-based methods are that they are rapid and can detect organisms that are difficult to grow using conventional culture techniques. In addition, PCR has the potential to improve detection limits. For these reasons, PCR-based methods have been used to detect a wide range of pathogenic microorganisms in surface waters (31, 32).

In view of this, we applied a real-time PCR assay using SYBR Green I dye to detect potential zoonotic pathogens in samples from roof-harvested rainwater. The major advantage of the SYBR Green I system is that the detection method could easily be transferred to a quantitative real-time PCR method, which circumvents the need for redesigning new primers, and optimizing real-time PCR conditions. In addition, the SYBR Green I dye method is more tolerant of polymorphic targets than the probe-based methods, and as a result, it can be beneficial for testing environmental samples in which the targets may show slight genetic variations (1). However, a notable limitation of the SYBR Green system is that it may bind to nonspecific double-stranded DNA, and this results in the formation of primer dimer and other nonspecific products. In our study, nonspecific products were not observed with melting curve analysis for most of the targets, except for *A. hydrophila*. To reduce the level of primer dimer and other nonspecific products, we optimized the PCR condition for annealing temperature and primer concentration. Before we applied real-time PCR detection, the sensitivity of the assays was rigorously evaluated by amplifying known concentrations of DNA from target pathogens. The lower limits of detection ranged from 5 to 10 copies (Table 2), indicating that the detection sensitivity values of our real-time PCR assay were comparable to the values reported in the literature (3, 31). The cross-reactivity of each primer set for each target has also been assed by testing a number of other microorganisms, including *B. vulgatus* ATCC 8482, *A. hydrophila* ATCC 7966, *C. coli* ATCC 43478, *L. pneumophila* ATCC 33152, and *Salmonella* serovar Typhimurium ATCC 14028. *C. jejuni* NCTC 11168, *C. perfringens* ATCC 13124, *E. coli* ATCC 9637, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Pseudomonas aeruginosa* ATCC 27853, *Cryptosporidium* ATCC PRA-67D, *Citrobacter freundii* ATCC 8090, *Shigella sonnei* ATCC 29930, and *Klebsiella pneumoniae* ATCC 31314. The primers did not amplify any PCR products other than those products that were expected, suggesting a high specificity for the tested primers. We also tested for the effects of inhibitory substances on our PCR detection limit by spiking rainwater samples with known concentrations of target genomic DNA. The initial results indicated the absence of inhibitory substances in rainwater samples. For further confirmation, all ($n = 27$) rainwater samples were spiked with a lower level (10 gene copies) of positive DNA of targets and were retested using PCR. No PCR inhibitors were found in 24 (89%) out of 27 samples. However, three samples (i.e., T19, T21, and T22) contained PCR inhibitory substances. A 10-fold serial dilution of DNA was necessary to remove inhibitors from these samples. Based on our data, we recommend that PCR inhibitory checks need to be performed prior to PCR analysis.

Of the 27 samples tested, 17 (63%) exceeded the Australian

drinking water guidelines for 0 *E. coli*/100 ml of water (25), and 12 (44.4%) samples exceeded the Australia and New Zealand Environment and Conservation Council (ANZECC) recreational water quality guidelines for fresh and marine waters of 35 enterococci/100 ml for primary contact (2). In all, 24 (89%) samples were positive for *Bacteroides* spp., which are considered to be a good alternative indicator of fecal pollution due to a number of advantages including short survival rates outside the hosts, their exclusivity to the gut of warm-blooded animals, and the fact that they constitute a relatively larger portion (i.e., 1,000-fold) of fecal bacteria than traditional indicators (35). The presence of *Bacteroides* spp. in most of the samples confirms recent fecal pollution. Fecal pollution was expected in samples collected after rain events as roof runoff would have introduced fecal matter to the tanks, which could be present on the roof or in gutters, originating from birds, possums, lizards, or other animals that have access to the roof. Interestingly, three samples (i.e., T8, T11, and T13) were positive for the *Bacteroides* spp.; however, none of the traditional fecal indicators was detected, supporting the possibility that *Bacteroides* spp. could be a more sensitive indicator for the detection of fecal pollution in rainwater samples. To our knowledge, this is the first study that has used *Bacteroides* spp. as an alternative indicator to detect fecal pollution in roof-harvested rainwater.

Of the 27 samples tested, 12 were positive for the *C. coli ceuE* and *C. jejuni mapA* genes. The *C. coli* and *C. jejuni* in rainwater samples could potentially be from bird feces (21, 40). However, other potential sources such as possums or lizards cannot be ruled out. Interestingly though, *Campylobacter* spp. could not be isolated from the possum feces in New Zealand (12). The presence of *Campylobacter* spp. in roof-harvested rainwater samples has also been reported in New Zealand (33) and "campylobacteriosis" was found to be epidemiologically associated with the consumption of roof-harvested rainwater (14). Another study reported the outbreak of *Campylobacter* enteritis among resort staff in North Queensland, Australia, and the source was hypothesized to be rainwater consumption (25). We also detected the *A. hydrophila lip* gene, the *L. pneumophila mip* gene, and the *Salmonella invA* gene in rainwater samples from a number of tanks (Table 4). *A. hydrophila* has been reported as one of the most common *Aeromonas* spp. associated with human intestinal disease (22). The *Salmonella invA* genes enable the bacteria to invade cells and are reported to be present in all *Salmonella* serovars (i.e., 2,000 serovars). On the other hand, seven *Salmonella* serovars are known to contain the virulence plasmid that carries the *spvC* gene which plays a significant role in human diseases (7). However, none of the samples were positive for the *Salmonella spvC* gene. *Aeromonas* spp., *Legionella* spp., and *Salmonella* spp. have previously been detected in roof-collected rainwater cisterns and/or from tanks, using culture-based methods in the United States and New Zealand and in the tropics (5, 36). The high prevalence of *Giardia* cysts (45%) and *Cryptosporidium* oocysts (23%) in rainwater cisterns in the U.S. Virgin Islands has been reported (9). The *Giardia lamblia* β-giardin gene was detected in four samples tested in this study. To our knowledge, this is the first study in Australia that reports the presence of *C. coli*, *C. jejuni*, and *G. lamblia* in water samples from domestic rainwater tanks. In this study, we were able to detect all the potential target pathogens. However, their prevalence appears to be higher than that in a previously published report (36), except for *Giardia* (9). This could be due to the fact that we have used real-time PCR detection, which is known to be more sensitive than culture-based detection (18).

We validated our PCR positive results by analyzing DNA melting curves. The melting of the PCR amplicons at the correct temperature indicated true and positive amplification. The presence of positive amplicons was further confirmed by visualization on agarose gels. Finally, we sequenced up to three amplicons for each target and verified they were ≥97% identical to the published sequences (data not shown). It is acknowledged that our PCR results are expressed as the presence/absence for each potential pathogen and do not provide information regarding the pathogenicity of the targets and the degree of fecal pollution. A major limitation of PCR is its inability to distinguish between viable and nonviable pathogenic microorganisms. Therefore, in this study, it cannot be ruled out that PCR may have detected DNA from nonviable pathogenic microorganisms. However, the high prevalence of potential pathogens along with the presence of one or more indicators in samples collected after rainfall events indicate a poor level of microbial quality of rainwater and could represent a potential health risk to end users, especially those who use the water for drinking and kitchen use. It is possible that residents probably have acquired immunity to a range of pathogens and will not inform the public health department unless the illnesses are severe. Nonetheless, such poor quality could represent a risk to new consumers such as visitors or persons who have not consumed rainwater before (36).

*E. coli* bacteria were detected in 17 (63%) samples, and the concentration varied from sample to sample. From our data, it is evident that the assessment of the microbial quality of rainwater using fecal coliforms or *E. coli* may not be adequate. For example, *E. coli* could not be detected in rainwater samples from seven tanks (i.e., T4, T6, T8, T11, T20, T23, and T24) that were positive for at least one of the other indicators tested (Table 4). Previous studies that used only fecal coliforms or *E. coli* to assess the microbial quality of roof-harvested rainwater may have underestimated the degree of fecal pollution (13, 15, 44). However, our study also showed a poor correlation between the concentration of fecal indicators and the PCR presence/absence results. It has been reported previously that *E. coli* and enterococci do not correlate well with pathogenic *Salmonella* spp. (23), *Campylobacter* spp. (20), and *Cryptosporidium* and *Giardia* spp. (17, 20, 23) in surface waters and in reclaimed wastewater. The use of indicator bacteria alone to assess the quality of rainwater has been questioned in a previous study (24).

In conclusion, roof-harvested rainwater collected after a rain event in Southeast Queensland, Australia, appears to be of poor microbiological quality. The presence of one or more pathogenic microorganisms along with fecal indicators could represent a significant health risk to users. The results obtained also indicated a poor correlation between fecal indicators and potential pathogens tested. Therefore, testing fecal indicators may not be adequate to assess the microbiological quality of rainwater and consequent health risk. The real-time PCR detection of pathogens is rapid, and results can be obtained in less than 6 h, compared with the period of days required for using conventional culture-based methods. However, a limita-

tion of the current study is that the data interpretation is based on PCR presence/absence results of potential pathogens and does not provide information regarding the pathogenicity of the targets and the degree of fecal pollution. We are developing real-time PCR assays for the quantification of *C. jejuni*, *L. pneumophila*, and *Salmonella* spp. in roof-harvested rainwater. Our future research will focus on quantitative microbial risk assessment of roof-harvested rainwater.

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