Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in southeast Queensland, Australia

W. Ahmed, A. Goonetilleke, and T. Gardner

Abstract: The study aimed to evaluate the suitability of *Escherichia coli*, enterococci, and *Clostridium perfringens* for assessing the microbiological quality of roof-harvested rainwater and assessing whether the concentrations of these faecal indicators can be used to predict the presence or absence of specific zoonotic bacterial or protozoan pathogens. From a total of 100 samples tested, 58%, 83%, and 46% of samples were found to be positive for, respectively, *E. coli*, enterococci, and *Clostridium perfringens* spores, as determined by traditional culture-based methods. Additionally, in the samples tested, 7%, 19%, 1%, 8%, 17%, and 15% were PCR positive for *Aeromonas hydrophila lip*, *Campylobacter coli ceuE*, *Campylobacter jejuni mapA*, *Legionella pneumophila mip*, *Salmonella invA*, and *Giardia lamblia* β-giardin genes, respectively. However, none of the samples was positive for *E. coli* O157 lipopolysaccharide, verocytotoxin 1, and verocytotoxin 2 and *Cryptosporidium parvum* oocyst wall protein genes. The presence or absence of these potential pathogens did not correlate with any of the faecal indicator bacterial concentrations as determined by a binary logistic regression model. The roof-harvested rainwater samples tested in this study appeared to be of poor microbiological quality, and no significant correlation was found between the concentration of faecal indicators and pathogenic microorganisms. The use of faecal indicator bacteria raises questions regarding their reliability in assessing the microbiological quality of water and particularly their poor correlation with pathogenic microorganisms. The presence of one or more zoonotic pathogens suggests that the microbiological analysis of water should be performed and that appropriate treatment measures should be undertaken, especially in tanks where the water is used for drinking.

Key words: faecal indicators, enteric pathogens, roof-harvested rainwater, PCR, public health risk.

Résumé : Cette étude visait à évaluer la pertinence de *Escherichia coli* des entérocoques et de *Clostridium perfringens* pour estimer la qualité microbiologique de l'eau de pluie récolté des toitures, et pour estimer si les concentrations de ces indicateurs fécaux pourraient être utilisés pour prédire la présence ou l'absence de bactéries zoonotiques spécifiques ou de protozoaires pathogènes. Sur un total de 100 échantillons, 58 %, 83 % et 46 % se sont révélés respectivement positifs à *E. coli*, aux entérocoques et aux spores de *Clostridium perfringens*, tel que déterminé par des méthodes traditionnelles de cultures. De plus, 7 %, 19 %, 1 %, 8 %, 17 % et 15 % de ces échantillons étaient positifs en PCR aux gènes *lipC* de *Aeromonas hydrophila*, *ceuE* de *E. coli, mapA* de *Campylobacter jejuni*, *mip* de *Legionella pneumophila*, *invA* de *Salmonella* et β-giardine de *Giardia lambalia*. Cependant, aucun des échantillons n'était positif au LPS O157, aux gènes VT1 et VT2 d'*E. coli*, ni au gène COWP de *Cryptosporidium parvum*. La présence ou l'absence de ces pathogènes potentiels n'était pas corrélée avec la concentration des indicateurs fécaux bactériens, selon un modèle de régression binaire. Les échantillons d'eau de pluie récoltée de toitures testés dans cette étude semblent de piètre qualité microbiologique et aucune corrélation significative n'a été trouvée entre la concentration des indicateurs fécaux et les microorganismes pathogènes. L'utilisation de ces indicateurs fécaux bactériens soulève des questions relativement à leur fiabilité dans l'estimation de la qualité microbiologique de l'eau, et particulièrement sur le faible degré de corrélation avec les microorganismes pathogènes. La présence d'un ou de plusieurs pathogènes zoonotiques suggère qu'une analyse microbiologique de l'eau doit être réalisée, et que des mesures de traitement appropriées soient entreprises, spécialement dans les réservoirs où l'eau est puisée pour la consommation.

Mots-clés : indicateurs fécaux, pathogènes entériques, eau de pluie récoltée des toitures, PCR, risque pour la santé humaine.

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Introduction

The demand on potable water supply is increasing in line with economic growth and increases in industrial output and commerce, as well as population growth. This is further exacerbated by the adverse impacts of climate change on water supply. Consequently, water authorities are keen to explore alternative water sources to meet the ever-increasing demand. Among the alternatives, roof-harvested rainwater (RHRW) has been considered a potential source for potable and nonpotable uses in many countries (Uba and Aghogho 2000; Evans et al. 2007; Despins et al. 2009). In Australia, the use of rainwater tanks is becoming increasingly common in most major cities in addition to rural and remote areas. To cope with severe drought conditions, subsidies and other regulatory measures have been introduced in recent years to encourage rainwater tank installation in several capital cities, including Brisbane, Queensland, highlighting their importance as an alternative source.

The most significant issue in relation to RHRW reuse is the potential public health risks associated with microbiological pollutants (Simmons et al. 2001; Ahmed et al. 2008). Various microorganisms, including pathogens, could be present in the faeces of birds, insects, small mammals, and reptiles. Consequently, faecal matter and other organic debris could be introduced to the tank via roof runoff following rain events. The microbiological quality of RHRW is generally assessed by monitoring faecal indicator bacteria such as faecal coliforms, *Escherichia coli*, and enterococci (Dillaha and Zolan 1985; Appan 1997; Ghanayem 2001; Plazinska 2001; Vasudevan et al. 2001).

There is a general community perception that rainwater is safe to drink without having to undergo prior treatment. This is partially supported by limited epidemiological studies (Heyworth et al. 2006). Additionally, a previous research study has reported that RHRW quality is generally acceptable for drinking and household use (Dillaha and Zolan 1985) and poses no increased risk of gastrointestinal illnesses when compared with mains water (Heyworth et al. 2006). In contrast, a number of studies have reported the presence of specific pathogens, including opportunistic pathogens in RHRW (Crabtree et al. 1996; Uba and Aghogho 2000; Simmons et al. 2001; Lye 2002; Birks et al. 2004; Ahmed et al. 2008). Therefore, questions have arisen regarding the microbiological quality of rainwater and consequent public health risks. This in turn highlights the most important limitation of faecal indicator bacteria (i.e., faecal coliforms, *E. coli*) arising from their poor correlation with pathogenic microorganisms in environmental waters (Hörman et al. 2004; McQuaig et al. 2006; Ahmed et al. 2009). This limitation is also common in sewage (Harwood et al. 2005). This is not surprising, considering that faecal indicator bacteria exhibit differential survival rates compared to pathogens, especially viruses and protozoans. Furthermore, faecal indicators may replicate in external environments (Desmarais et al. 2002; Anderson et al. 2005; Byappanahalli et al. 2006). Currently, there is a paucity of knowledge in relation to the occurrence and concentrations of pathogens in RHRW and their relationships with traditional faecal indicator bacteria.

Direct monitoring of pathogens in water sources could be an attractive option, as it would provide invaluable information regarding public health risks. However, isolation and identification of specific pathogens using traditional culture-based methods could be cumbersome. In recent times, PCR-based methods have been widely used for the detection and (or) quantification of various pathogens in environmental waters (Sails et al. 2002; Guy et al. 2003; Hörman et al. 2004; Ahmed et al. 2009). An important feature of the PCR-based methods is that they can be used to detect and quantify pathogens that are difficult to isolate using traditional culture-based and microscopic methods. It has to be noted that, to date, only a limited number of studies have applied PCR to detect specific pathogens in RHRW (Ahmed et al. 2008).

A primary aim of this study was to investigate the prevalence of faecal indicator bacteria, such as *E. coli,* enterococci, and *Clostridium perfringens*, and a wide range of bacterial and protozoan pathogens in RHRW samples. Samples were collected from the Brisbane, Gold Coast, and Sunshine Coast regions in Queensland and tested for the presence of faecal indicators using traditional culture-based methods and specific pathogens using PCR detection. Secondly, the study also aimed to assess whether the concentrations of faecal indicator bacteria were suitable for predicting the presence or absence of specific pathogens.

Materials and methods

Sources of samples

Initially, 27 RHRW samples were collected from 27 residential houses in Brisbane in southeast Queensland, Australia; these results have been published elsewhere (Ahmed et al. 2008). For this study, a total of 73 new RHRW samples were collected from 55 residential houses located in the Brisbane, Gold Coast, and Sunshine Coast regions, giving a total number of 100 RHRW samples from 82 residential houses. The size of the water tanks sampled ranged from 500 to 15 000 L, and they were made of polyethylene. The end uses were (1) outdoor use (65%), including gardening and car washing, and (2) indoor use (35%), including drinking and kitchen use. Samples were collected within 1–4 days after a rain event (ranging from 35 to 130 mm). Samples were collected in sterilized 10 L containers from the outlet taps located close to the base of the tanks. Before the rainwater was sampled, the tap was sterilized with 96% ethanol and allowed to run for 30–60 s to flush out water from the tap. Samples were transported to the laboratory on ice and processed within 8–10 h.

Isolation and enumeration of faecal indicators

The membrane filtration method was used to process the water samples (100 mL each) for faecal indicator bacterial enumeration. Sample serial dilutions were made and filtered through nitrocellulose membranes (47 mm diameter) with 0.45 μm pores (Advantec, Tokyo, Japan) and placed on modified mTEC agar (Difco, Detroit, Mich.), membrane-*Enterococcus* indoxyl-β-D-glucoside (mEI) agar (Difco), and oleandomycin–polymyxin–sulfadiazine perfringens (OPSP) agar with supplement (Oxoid, London, UK) for the isolation

of *E. coli*, enterococci, and spore-forming *Clostridium perfringens*, respectively. For the isolation of *Clostridium 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 (US Environmental Protection Agency 2002), and mEI agar plates were incubated at 41 °C for 48 h (US Environmental Protection Agency 1997). OPSP agar plates (for *Clostridium perfringens*) were incubated anaerobically at 44 °C for 24 h. The confirmatory test for *Clostridium perfringens* was performed according to the method described previously (Wohlsen et al. 2006). For bacterial enumeration, all water samples were tested in triplicate.

DNA extraction from water samples

For PCR analysis of potential bacterial pathogens, 1 L sample of water from each tank was filtered through a nitrocellulose membrane with 0.45 μm pores (Advantec). In case of membrane clogging during filtration, multiple membranes were used. The membranes were immediately transferred into a 15 mL screw-cap tube containing 10 mL of sterile STE buffer (0.1 mol/L NaCl, 10 mmol/L Tris, and 1 mmol/L EDTA (pH 7.6)). The tubes were vortexed vigorously for 8–10 min to detach the bacteria from the membranes followed by centrifugation at 8000*g* 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 the DNeasy blood and tissue kit (Qiagen, Valencia, Calif.) and stored at –80 °C until use.

For PCR analysis of protozoan *Giardia lamblia* β-giardin and *Cryptosporidium parvum* oocyst wall protein (COWP) genes, a 3–5 L sample of water from each tank was filtered through a membrane (47 mm diameter) with 3 μm pores (Advantec). After filtration, the membrane was transferred into a Petri dish. DNA was extracted directly on the filter using the DNeasy blood and tissue kit (Qiagen). In brief, 360 μL of buffer ATL was added to each filter paper. The filter paper was scraped very well and discarded. Each sample was transferred into a 1.5 mL microcentrifuge tube and subjected to 3 cycles of freezing–thawing. After 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.

PCR-positive controls

The following strains were purchased from the American Type Culture Collection (ATCC): *Aeromonas hydrophila* ATCC 7966, *Campylobacter coli* ATCC 43478, *Legionella pneumophila* ATCC 33152, and *Salmonella enterica* serovar Typhimurium ATCC 14028. *Escherichia coli* NCTC 12079 strain was kindly donated by Mr. Jack Tucker from the University of the Sunshine Coast, Queensland, Australia. DNA prepared from ATCC genuine cultures was purchased for *Campylobacter jejuni* (33560D), *G. lamblia* (30888D), and *Cryptosporidium parvum* (PRA-67D).

Specificity of the PCR primers

PCR detection of pathogenic bacteria and protozoans was done using previously published primers. The primer sequences for corresponding target genes are shown in Table 1. Primer specificity was determined by searching for similar sequences in microbial genomes using the Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/). This ensured that no homology was observed with known gene sequences of other pathogenic microorganisms commonly found in environmental waters. The cross-reactivity of each primer set was also evaluated by testing DNA isolated from other nontarget species commonly found in environmental waters. These included (1) *A. hydrophila*, (2) *Bacteroides vulgatus*, (3) *Campylobacter coli*, (4) *Campylobacter jejuni*, (5) *Citrobacter freundii*, (6) *Clostridium perfringens*, (7) *Enterococcus faecalis*, (8) *E. coli*, (9) *Klebsiella pneumoniae*, (10) *L. pneumophila*, (11) *Pseudomonas aeruginosa*, (12) *Salmonella* Typhimurium, (13) *Shigella sonnei*, (14) *Cryptosporidium parvum*, and (15) *G. lamblia*.

PCR detection of potential pathogenic microorganisms

Amplification was performed in 25 μL reaction mixtures using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, Calif.). The PCR mixture contained 12.5 μL SuperMix, 300 nmol/L of each primer, and 2 μL of template DNA. For each PCR experiment, corresponding positive (i.e., target DNA) and negative controls (sterile water) were included. The PCR was performed using the Rotor-Gene 6000 real-time cycler (Corbett Research, Mortlake, Victoria, Australia). Cycling parameters for the *A. hydrophila lip* gene were 2 min at 50 °C, 15 min at 95 °C for initial denaturation, and 35 cycles of 94 °C for 1 min, 62 °C for 1 min for annealing, and 72 °C for 1.5 min, followed by a final extension step of 72 °C for 5 min; for *Campylobacter coli ceuE* and *Campylobacter jejuni mapA* genes, 2 min at 50 °C, 10 min at 95 °C for initial denaturation, and 40 cycles of 95 °C for 15 s, 59 °C for 30 s for annealing; for *L. pneumophila mip* gene, 2 min at 50 °C, 15 min at 95 °C for initial denaturation, and 35 cycles of 94 °C for 30 s, 54 °C for 1 min for annealing, and 72 °C for 1 min, followed by a final extension step of 72 °C for 5 min; for the *Salmonella invA* and *spvC* genes, 2 min at 50 °C, 5 min at 94 °C for initial denaturation, and 45 cycles of 94 °C for 30 s, 59 °C for 35 s for annealing, and 72 °C for 2 min, followed by a final extension step of 72 °C for 10 min; for *E. coli* O157 lipopolysaccharide (LPS), verocytotoxin 1 (VT1), and verocytotoxin 2 (VT2) genes, 2 min at 50 °C, 10 min at 95 °C for initial denaturation, and 40 cycles of 95 °C for 30 s, 59 °C for 30 s for annealing, and 72 °C for 30 s, followed by a final extension step of 72 °C for 5 min; for *G. lamblia* β-giardin and *Cryptosporidium parvum* COWP genes, 2 min at 50 °C, 10 min at 95 °C for initial denaturation, 40 cycles of 94 °C for 15 s, and 59 °C for 1 min for annealing.

Quality control

To prevent false-positive results for RHRW samples, a method blank was included for each batch (n = 10) of water samples. In brief, a 1 L sample of distilled water was filtered through a membrane with 0.45 μm pores (Advantec). The filter paper was washed with sterile STE buffer followed by centrifugation as described previously. The

Table 1. Target genes and primers used for pathogen detection.

Target	Primer sequence (5′→3′)	Amplicon size (bp)	Reference
Aeromonas hydrophila lip gene	F: AACCTGGTTCCGCTCAAGCCGTT R: TTGCTCGCCTCGGCCCAGCAGCT	760	Cascón et al. 1996
Campylobacter coli ceuE gene	F: CAAGTACTGCAATAAAAACTAGCACTACG R: AGCTATCACCCTCATCACTCATACTAATAG	67	Price et al. 2006
Campylobacter jejuni mapA gene	F: GCTAGAGGAATAGTTGTGCTTGACAA R: TTACTCACATAAGGTGAATTTTGATCG	72	Price et al. 2006
Escherichia coli O157 LPS gene	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259	Pass et al. 2000
Escherichia coli verocytotoxin gene 1 (VT1)	F: ACGTTACAGCGTGTTGCTGGGATC R: TTGCCACAGACTGCGTCAGTTAGG	121	Pass et al. 2000
Escherichia coli verocytotoxin gene 2 (VT2)	F: TGTGGCTGGGTTCGTTAATACGGC R: TTGCCACAGACTGCGTCAGTTAGG	102	Pass et al. 2000
Legionella pneumophila mip gene	F: GCAATGTCAACAGCAA R: CATAGCGTCTTGCATG	159	Wilson et al. 2003
Salmonella invA gene	F: ACAGTGCTCGTTTACGACCTGAAT R: AGACGACTGGTACTGATCGATAAT	244	Chiu and Ou 1996
Salmonella spvC gene	F: ACTCCTTGCACAACCAAATGCGGA R: ACAGTGCTCGTTTACGACCTGAAT	571	Chiu and Ou 1996
Cryptosporidium oocyst wall protein (COWP) gene	F: CAAATTGATACCGTTTGTCCTTCTG R: GGCATGTCGATTCTAATTCAGCT	150	Guy et al. 2003
Giardia lamblia β-giardin gene	F: CCTCAAGAGCCTGAACGATCTC R: AGCTGGTCGTACATCTTCTTCCTT	74	Guy et al. 2003

supernatant was discarded, and the pellet was resuspended in sterile distilled water. DNA was extracted using the DNeasy blood and tissue kit (Qiagen). To prevent false-positive results during DNA extraction, a reagent blank was included for each batch (n = 10) of samples. During setup of the PCR assays, the PCR conditions for annealing temperature were optimized by performing gradient analysis (i.e., temperature ranged from 53 to 63 °C) for each target. The primer concentrations (100–500 nmol/L) were also optimized to reduce the level of primer dimer for each target. To separate the specific product from nonspecific products, 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 a 2% E-gel (Invitrogen) and exposure to ultraviolet light for further confirmation (if required). Samples were considered positive when the visible band was the same as that of the positive control strain and had the same melting temperature (±0.2 °C) as the positive control. To minimize PCR contamination, DNA extraction, PCR setup, and gel electrophoresis were performed in separate laboratories.

PCR limit of detection

To determine the PCR lower limits of detection (LOD), known gene copies (i.e., 10^3–10^0) of each target gene were tested by PCR. The lowest concentration of gene copies detected consistently in replicate assays was considered PCR LOD.

PCR inhibitors

An experiment was conducted to determine the potential presence of PCR inhibitory substances in rainwater samples collected from 3 different tanks from the Brisbane region. Each sample (i.e., 1 L) was concentrated using the membrane filtration technique described previously. DNA was extracted using the DNeasy blood and tissue kit (Qiagen), serially diluted, and tested with PCR. DNA was also extracted from ultrapure DNase- and RNase-free sterile distilled water (Invitrogen) in the same manner for comparison with the tank water. All samples (undiluted, diluted, and distilled water DNA) were spiked with 10^3 gene copies of human-specific HF183 *Bacteroides* markers (Bernhard and Field 2000). The cycle threshold (C_T) values obtained for the DNA samples from spiked tank water were compared to those of the DNA samples from distilled water.

DNA sequencing

To verify the identity of the PCR products obtained from water samples, up to 3 PCR-amplified products from each target were purified using the QIAquick PCR purification kit (Qiagen), as recommended by the manufacturer (Qiagen), and cloned in duplicate into the pGEM-T Easy Vector system (Promega, Madison, Wis.), as recommended by the manufacturer. Plasmids were extracted using the QIAprep Spin-Miniprep kit (Qiagen). Bidirectional sequences were obtained using T7 and SP6 long sequencing primer targeting sites on either side of the insert. DNA sequencing was carried out at the Australian Genome Research Facility (St. Lucia, Queensland, Australia). The sequences were ana-

lysed using Bioware Jellyfish Software and verified against the published sequence.

Statistical analysis

The Spearman rank correlation was used to test the relationship between faecal indicator concentrations in RHRW samples. A binary logistic regression analysis was also performed to obtain correlations between the presence–absence of pathogen detection by PCR and the concentrations of faecal 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 a value of 1, and when a target organism was absent, it was assigned a value of 0. For this analysis, the concentration of faecal indicator bacteria found in 100 mL water samples was converted to 1 L (to be compared with bacterial pathogens) and 3 L (to be compared with protozoan pathogens). Minitab Release version 11.12 (State College, Pa.) software was used for the Spearman rank correlation and logistic regression analysis. In all cases, a difference was considered significant if the P value for the model χ^2 was <0.05.

Results

Specificity of PCR primers

The specificity of each primer set for each target was assessed by testing a panel of other microorganisms that could be found in RHRW. The primers used in this study did not amplify any PCR products other than those that were expected.

PCR inhibitors

For the spiked distilled water, the mean C_T value for the HF183 DNA was 23.8 ± 0.4. For rainwater samples, the mean C_T value was 23.6 ± 0.4 when undiluted DNA was spiked. For 10-fold, 100-fold, and 1000-fold dilutions of DNA, these values were 23.4 ± 0.3, 23.4 ± 0.1, and 23.3 ± 0.2, respectively. 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 rainwater 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 rainwater samples were free of PCR inhibitors.

PCR limit of detection

The LOD assays were performed by analysing purified genomic DNA from bacterial and protozoan strains containing corresponding target genes. To determine the reproducibility of the assay, several replicates (n = 10) were tested. The PCR detection limits were as low as 5 gene copies for *A. hydrophila lip*, *Campylobacter coli ceuE*, *Salmonella invA*, *Salmonella spvC*, and *L. pneumophila mip* genes. For *Campylobacter jejuni mapA* and *E. coli* O157 LPS, VT1, and VT2 genes, the detection limit was 10 gene copies. For *G. lamblia* β-giardin and *Cryptosporidium parvum* COWP genes, the detection limits were 7 and 10 gene copies, respectively. Lower levels (i.e., 1 copy) were tested for these targets, but the results were not reproducible for all replicates.

Prevalence of faecal indicator bacteria

The concentration of *E. coli* in water samples from RHRW ranged from <1 colony-forming unit (CFU)/100 mL to 3060 ± 456 CFU/100 mL of water. For enterococci and *Clostridium perfringens* spores, these figures were <1 CFU/100 mL to 3400 ± 700 CFU/100 mL and <1 CFU/100 mL to 200 ± 30 CFU/100 mL, respectively. Of the 100 samples tested, 42% samples had <1 CFU/100 mL of *E. coli* (Table 2). Similarly, 17% and 54% of samples had <1 CFU/100 mL of enterococci and *Clostridium perfringens* spores, respectively. Enterococci were more frequently detected (83 of 100 samples were positive) in water samples than *E. coli* (58 of 100 samples) and *Clostridium perfringens* spores (46 of 100 samples). Of the 100 samples tested, 89% were positive for at least 1 faecal indicator, 62% were positive for at least 2 indicators, and 36% were positive for all 3 indicators tested in this study. The concentrations of faecal indicators were pooled for all tanks and analysed to determine whether the concentrations correlated with each other. Significant correlations were observed between *E. coli* and enterococci (Spearman's r_s = 0.57; P = 0.0001) and enterococci and *Clostridium perfringens* spores (r_s = 0.22; P = 0.0258). However, the concentrations of *E. coli* did not correlate with those of *Clostridium perfringens* spores (r_s = 0.10; P = 0.3056).

Prevalence of pathogenic microorganisms

Of the 100 samples tested, 7% of samples were positive for *A. hydrophila lip* gene (Table 3). *Campylobacter jejuni mapA* gene was detected in only 1 sample. However, *Campylobacter coli* was more prevalent, and 19% of the samples were positive for *Campylobacter coli ceuE* gene. *Legionella pneumophila mip* and *Salmonella invA* genes were detected, respectively, in 8% and 17% of the samples. *Salmonella spvC* and *E. coli* O157 LPS, VT1, and VT2 genes were not detected in any samples tested in this study. Additionally, 15% of the samples were positive for *G. lamblia* β-giardin gene. However, none of the samples were positive for *Cryptosporidium parvum* COWP gene. Most of the pathogens were detected in samples collected from the Brisbane region, followed by the Gold Coast region. None of the samples from the Sunshine Coast region were positive for any pathogens tested. Of the 100 samples tested, 1% were positive for at least the 4 target genes, 8% were positive for at least 3 target genes, 18% were positive for at least 3 target genes, and 40% were positive for at least 1 target gene. However, none of the potential pathogens were detected in 60% of RHRW samples.

Correlation between faecal indicator and pathogenic microorganisms

Discrepancies were observed in terms of the occurrence of faecal indicators and zoonotic pathogens. For example, 12% of samples had <1 *E. coli* but were positive for 1 or more target pathogens. Similarly, 6% and 19% of samples had <1 enterococcus and <1 *Clostridium perfringens* spore, respectively, but were positive for 1 or more target pathogens. Binary logistic regression was used to test the hypothesis that faecal indicator concentrations can predict the presence or absence of pathogens in samples collected from RHRW tanks. PCR results of bacterial and protozoan patho-

Table 2. Percentage of roof-harvested rainwater samples positive for faecal indicators.

	% of samples		
CFU/100 mL	*Escherichia coli*	Enterococci	*Clostridium perfringens*
<1	42	17	54
1–10	18	17	21
11–100	17	36	22
101–500	14	14	3
501–1000	4	7	0
>1000	5	9	0

gens (only those that gave positive and negative signals) were converted to binary data. When a pathogen was present, it was assigned a value of 1, and when a pathogen was absent, it was assigned a value of 0. The presence or absence of pathogens did not correlate with any of the indicator bacterial concentrations (Table 4).

Discussion

Most of the past research studies have assessed microbiological quality of RHRW by monitoring traditional faecal indicators, namely faecal coliforms and *E. coli* (Dillaha and Zolan 1985; Yaziz et al.1989; Evans et al. 2006). Only a limited numbers of research studies to date have investigated the presence of specific pathogens in RHRW. These studies have invariably found limitations relating to the suitability of traditional faecal indicators (Simmons et al. 2001; Lye 2002; Ahmed et al. 2008).

Detection of specific pathogens using traditional culture-based methods is laborious and lacks sensitivity (Toze 1999). Because of these limitations, we used PCR assays for the rapid detection of specific pathogens. Before application, the specificity of each primer and the PCR detection limit for each assay was rigorously evaluated. One major problem associated with PCR detection of pathogens in water is the presence of PCR inhibitors. Environmental waters generally contain organic and inorganic substances with the potential to inhibit PCR (Wilson 1997). The influence of such inhibitory substances on PCR detection was evaluated by spiking rainwater DNA samples with known concentrations of human-specific *Bacteroides* HF183 marker (Bernhard and Field 2000). This marker was chosen for spiking because it is unlikely that the source of faecal contamination in rainwater tanks would be of human rather than animal origin. Only 5% of samples contained PCR inhibitory substances, and a 10-fold serial dilution of DNA was required to remove the inhibitory effects.

In the 100 samples tested, 58% samples had >1 CFU *E. coli*/100 mL of water, exceeding Australian Drinking Water Guidelines. The concentrations of *E. coli* and enterococci were highly variable in the water samples, and some rainwater tanks (i.e., 5%) had >1000 CFU *E. coli* and enterococci per 100 mL of water, suggesting high levels of faecal pollution. It has to be noted that samples were collected within 1–4 days after rainfall, when faecal and other organic matter deposited on the roof enters tanks via roof runoff. This was done to obtain information regarding the magnitude of faecal pollution in the worst-case scenario. Enterococci were more prevalent than *E. coli*, and of the 100 samples tested, more were positive for enterococci (83%) than for *E. coli* (58%).

A number of samples (25 of 100 samples) were positive for culturable enterococci, but negative for culturable *E. coli*, possibly because enterococci persist in the water longer than *E. coli* (McFeters et al. 1974). The study results highlight the importance of testing multiple indicators for rainwater quality monitoring. The absence of a single indicator (i.e., *E. coli* alone) does not necessarily rule out the presence of faecal pollution and microorganisms of public health significance. In the 100 samples tested, 54% had <1 CFU *Clostridium perfringens* spores per 100 mL of water. In all, 46 of 54 (85%) samples were positive for *E. coli*, enterococci, or both. These data clearly indicate that *Clostridium perfringens* spores may not provide reliable information regarding faecal pollution in RHRW tanks. However, it may provide additional information regarding the magnitude of faecal pollution in RHRW samples.

In the 100 samples tested, 19%, 17%, 15%, 8%, 7%, and 1% were PCR positive for *Campylobacter coli*, *Salmonella* spp., *G. lamblia*, *L. pneumophila*, *A. hydrophila*, and *Campylobacter jejuni*, respectively. Samples from the Brisbane and Gold Coast regions were positive for 1 or multiple pathogens. However, none of the samples tested from the Sunshine Coast region were positive. Of the 16 samples tested, 5 were negative for all 3 indicators. The remaining 11 samples were positive for at least 1 indicator. The concentrations of faecal indicators and occurrence of pathogens in samples from the Sunshine Coast were relatively low compared to those in the Brisbane and Gold Coast regions. The samples from the Sunshine Coast region were collected from a new subdivision, where none of the residential houses had any overhanging trees or antennas on the rooftop, eliminating the high likelihood of bird faecal pollution. However, with time this situation is likely to change.

In all, 40% of the samples were positive for at least 1 target pathogen, and of these, 18 tanks were used for drinking. The presence of *Aeromonas* spp., *Campylobacter* spp., *Legionella* spp., and *Giardia* spp. in samples from RHRW has been reported in the United States, New Zealand, and in the tropics (Broadhead et al. 1998; Savill et al. 2001; Simmons et al. 2001). In this study, *Salmonella spvC*, *E. coli* O157 LPS, VT1, and VT2, and *Cryptosporidium parvum* COWP genes were not detected. To our knowledge, enterohaemorrhagic *E. coli* has not been previously isolated from RHRW samples. However, *Cryptosporidium* spp. has been found in rainwater cisterns in the US Virgin Islands (Crabtree et al. 1996). It has to be noted that a larger volume of water samples (i.e., 100 L) were screened for the detection of *Cryptosporidium* spp. in the United States. However, in this study, water samples of up to 5 L were screened for the PCR assay. Therefore, the assay used in this study could have underestimated the concentrations of *Cryptosporidium* spp. In this study, different volumes of water samples were tested for faecal indicators enumeration and the occurrence of bacterial and protozoan pathogens. It has to be noted that this is a common practice for microbiological water quality monitoring, because the concentration of pathogens may vary depending on the magnitude of faecal pollution and their persistence in the water. In addition, different concentration

Table 3. PCR positive results for pathogens in roof-harvested rainwater samples.

	No. of PCR-positive samples / No. of samples tested			
Pathogen	Brisbane	Gold Coast	Sunshine Coast	Total
Pathogenic bacteria				
A. hydrophila lip gene	7/66	0/18	0/16	7/100
Campylobacter jejuni mapA gene	1/66	0/18	0/16	1/100
Campylobacter jejuni ceuE gene	16/66	3/18	0/16	19/100
E. coli O157 gene	0/66	0/18	0/16	0/100
E. coli verocytotoxin gene 1 (VT1)	0/66	0/18	0/16	0/100
E. coli verocytotoxin gene 2 (VT2)	0/66	0/18	0/16	0/100
L. pneumophila mip gene	8/66	0/18	0/16	8/100
Salmonella invA gene	15/66	2/18	0/16	17/100
Salmonella spvC gene	0/66	0/18	0/16	0/100
Pathogenic protozoans				
Cryptosporidium oocyst wall protein (COWP) gene	0/66	0/18	0/16	0/100
G. lamblia β-giardin gene	13/66	2/18	0/16	15/100

Table 4. Binary logistic regression of the relationships between faecal indicators and the presence or absence of pathogens in water samples collected from rainwater tanks.

Indicator vs. pathogenic microorganism	Concordance (%)	*P* value[a]	Odds ratio
E. coli vs. *A. hydrophila*	6.50	0.972	1.00
E. coli vs. *Campylobacter jejuni*	94.9	0.376	1.00
E. coli vs. *Campylobacter coli*	22.0	0.597	1.00
E. coli vs. *L. pneumophila*	22.7	0.544	1.00
E. coli vs. *Salmonella* spp.	32.0	0.096	1.00
E. coli vs. *G. lamblia*	34.9	0.131	1.00
Enterococci vs. *A. hydrophila*	59.4	0.092	1.00
Enterococci vs. *Campylobacter jejuni*	12.1	0.887	1.00
Enterococci vs. *Campylobacter coli*	44.4	0.240	1.00
Enterococci vs. *L. pneumophila*	11.5	0.974	1.00
Enterococci vs. *Salmonella* spp.	44.1	0.172	1.00
Enterococci vs. *G. lamblia*	32.1	0.490	1.00
Clostridium perfringens vs. *A. hydrophila*	51.3	0.580	1.00
Clostridium perfringens vs. *Campylobacter jejuni*	5.10	0.948	1.00
Clostridium perfringens vs. *Campylobacter coli*	36.9	0.415	1.01
Clostridium perfringens vs. *L. pneumophila*	34.8	0.463	1.00
Clostridium perfringens vs. *Salmonella* spp.	51.3	0.580	1.00
Clostridium perfringens vs. *G. lamblia*	34.0	0.807	1.00

[a]*P* value for the model χ^2 square was <0.05, and the confidence interval for the odds ratio did not include 1.0. Greater odds ratios indicate a higher probability of change in the dependent variable with a change in the independent variable.

of faecal indicators and pathogens are shed in the faeces of warm-blooded animals. Therefore, a large volume of water samples need to be analysed to detect pathogens. One major limitation of PCR-based methods is that they do not provide information regarding the viability and infectivity of target pathogens. Nonetheless, the presence of these zoonotic pathogens is a cause for concern, especially in tanks where the water is used for drinking.

A binary logistic regression was performed to identify the correlations between faecal indicator bacterial concentrations and the PCR presence–absence of *A. hydrophila*, *Campylobacter jejuni*, *Campylobacter coli*, *L. pneumophila*, *Salmonella* spp., and *G. lamblia*. None of the faecal indicator concentrations correlated with the presence–absence of pathogens. Some samples had no measurable concentrations of faecal indicators, although they were positive for 1 or more target pathogens. For example, 10 samples had <1 CFU *E. coli*/100 mL. However, all these samples were positive for 1 or more target pathogens. Similarly, 6 samples (for enterococci) and 14 samples (*for C. perfringens* spores) had <1 CFU *E. coli* and *C. perfringens* spores per 100 mL, respectively, but were positive for 1 or more target pathogens. These results suggest that pathogens could be present in tank water samples in the absence of faecal indicator bacteria, raising serious questions regarding the reliability of employing faecal indicators to assess the microbiological quality of water. In this study, a one-off sample was collected from most of the tanks immediately after rain events. Therefore, limited data are available regarding the persistence of these pathogens. Such information is val-

uable for health risk assessment. We are currently undertaking a longitudinal study to investigate the prevalence and concentrations of these pathogens using quantitative PCR methods. In addition, we are also using a suite of methods (quantitative PCR and culture based) to obtain information on how many quantified pathogens are indeed viable. This information will be used to quantify microbial risk associated with potable and nonpotable uses of RHRW.

In conclusion, the RHRW samples tested in this study appeared to be of poor microbiological quality. A specific number of water samples tested in this study contained high levels of *E. coli*, enterococci, and *Clostridium perfringens* spores. A significant number of samples were also positive for zoonotic bacterial and protozoan pathogens. The use of faecal indicator bacteria raises questions regarding their reliability in assessing the microbiological quality of water, particularly because of their poor correlation with pathogenic microorganisms. The presence of 1 or more zoonotic pathogens suggests that the microbiological analysis of water should be performed and appropriate treatment measures be undertaken, such as under-sink filtration units, ultraviolet disinfection units, or simply boiling the water, especially when tanks contain water used for drinking.

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