QUANTITATIVE DETECTION OF PATHOGENS IN ROOF HARVESTED RAINWATER IN QUEENSLAND INDIACTES POTENTIAL HEALTH RISKS

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ABSTRACT

The microbiological quality of roof-harvested rainwater was assessed by monitoring faecal indicators and pathogens using PCR/qPCR based methods. In the 84 rainwater samples tested, 57 (65%) were found to be positive for *E. coli* and 72 (82%) were found to be positive for enterococci. A number of samples were also positive for *Aeromonas hydrophila*, *Campylobacter coli*, *Campylobacter jejuni*, *Legionella pneumophila*, *Salmonella* spp., and *Giardia lamblia*. The presence/absence of potential pathogens did not correlate with traditional faecal indicators. The presence of one or more pathogenic microorganisms along with fecal indicators could represent a significant health risk to users.

INTRODUCTION

Roof-harvested rainwater has received significant attention as a potential alternative source of potable water supply in water-scarce regions. To encourage the use of roof harvested rainwater, governmental bodies of many countries such as Australia, Denmark, Germany, India and New Zealand are providing rebates to residents who use rainwater for domestic purposes. The use of rainwater is quite common in Australia, particularly in rural, and remote areas, where reticulated mains or town water are not available. Recent water scarcity in several capital cities prompted the use of rainwater as an alternative source. For instance, the Queensland State Government has announced the 'Home Water Wise Rebate Scheme' that provides rebates to Southeast Queensland residents who use rainwater for domestics purposes (Queensland Government 2007).

There is a general community sense that roof-harvested rainwater is safe to drink, and this is partially supported by limited epidemiological evidence (Heyworth et al. 2006). Studies have reported that roof-harvested rainwater quality is generally acceptable for potable use (Dillaha and Zolan 1985). In contrast, the presence of potential pathogens in rainwater samples has been reported (Lye 2002; Simmons et al. 2001).

Direct routine monitoring of microbiological quality of water for all possible pathogens is not economically, technologically and practically feasible. Traditional faecal indicators, such as faecal coliforms, *Escherichia coli* and enterococci have long been used as surrogates to determine the presence of pathogens. Most studies commonly assess the quality of roof-harvested rainwater based on the concentration these indicators (Dillaha and Zolan 1985 Sazakil et al. 2007). However, the major limitation in using faecal bacteria as indicators is the poor correlation between the bacterial concentration and the presence of pathogenic microorganisms in environmental water (Hörman et al. 2004).

The recent advances in molecular techniques such as Polymerase Chain Reaction (PCR) technology enable rapid specific and sensitive detection of pathogens. It also enables the quantification of potential pathogenic microorganisms in environmental waters that are otherwise difficult and/or laborious to culture using traditional microbiological methods.

The aims of the research study were: (I) to investigate the prevalence of faecal indicators (*E. coli* and enterococci) and pathogenic microorganisms (*Aeromonas hydrophila*, *Campylobacter coli*, *Campylobacter jejuni*, enterohaemorrhagic *E. coli*, *Legionella pneumophila*, *Salmonella* spp., *Giardia lamblia* and *Cryptosporidium parvum* (II) to establish whether or

not a correlation between faecal indicators and potential pathogens exists and (III) to quantify selected pathogens to provide data for Quantitative Microbial Risk Assessment (QMRA). To achieve these aims, rainwater samples were tested using traditional culture based and PCR based methods.

EXPERIMENTAL DESIGN

Sources of samples: In all, 84 samples were collected from 66 residential houses in Brisbane and Gold Coast regions in South East Queensland. Water samples were collected in sterilized 10 L containers from the outlet taps located close to the base of the tanks. Before sampling, the tap was 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.

Enumeration of *Escherichia coli* and enterococci: The membrane filtration method was used to process the water samples for *E. coli* and enterococci enumeration. Sample serial dilutions were made, and filtered through 0.45-µm pore size nitrocellulose membranes (Advantec, Tokyo, Japan), and placed on modified mTEC agar (Difco, Detroit, MI, USA) and 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 and mEI agar plates were incubated at 41°C for 48 h.

DNA extraction from rainwater samples: For PCR detection and quantification of pathogenic microorganisms 1-2.5 L water sample from each tank was filtered through 0.45-*µ*m pore size membrane (Advantec). DNA was extracted directly on the membrane using DNeasy blood and tissue kit (Qiagen, Valencia, CA), and stored at -80°C until use.

Specificity of the PCR primers. Previously published primers were used for this study. The primer sequence and cycling parameters have been described elsewhere (Ahmed et al. 2008). The specificities of primers were determined against known microbial genomes and sequence by Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/BLAST/) to ensure no homology was observed with known gene sequences of other pathogenic microorganisms commonly found in waters. The cross reactivity of each primer set was also evaluated by testing DNA isolated from other non-target species of microorganisms commonly found in environmental waters.

Preparation of standard curves. For quantitative PCR (qPCR) assays, the standards were prepared from the genomic/plasmid DNA of selected pathogens (*Campylobacter jejuni*, *Legionella. pneumophila Salmonella enterica* serovar. Typhimurium and *Giardia lamblia*). The concentration of genomic/plasmid DNA was determined by measuring the absorbance at A_{260} using a spectrophotometer. The gene copies were calculated, and a tenfold dilution was prepared from the genomic and plasmid DNA, ranging from 10^6 to 10^0 copies/*µ*l of DNA extract using CAS-1200^{TM} precision liquid handling system (Corbett Life Sciences, Brisbane, Australia) and stored at -20°C until use. For each standard, the concentration was plotted against the cycle number at which the fluorescence signal increased above the threshold value (C_T value).

PCR detection and quantification: 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 SuperMix, 300 nM of each primer, 6.75-µl of DNase and RNase free deionised water and 4-µl of template DNA. For each PCR experiment, corresponding positive DNA and negative controls (i.e., sterile water) were included. The PCR/qPCR reactions were performed using the Rotor-Gene 6000 real-time cycler (Corbett Life Sciences). To separate the specific product from non-specific products (if any), DNA melting curve analysis was performed for each PCR experiment. During melting curve analysis, the temperature was increased from 62-95°C at approximately 2°C/min. Amplified products were also visualized by electrophoresis through 2% E-gel® (Invitrogen), and exposure to UV light for further confirmation. Samples were considered to be 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.

PCR limit of detection (LOD): To determine the PCR lower limits of the detection (LOD), known gene copies of the pathogens (5 X 10^3 to 5 X 10^0 gene copies) were tested with the PCR. The lowest concentration of gene copies that were detected consistently in replicate assays was considered as PCR LOD.

PCR reproducibility: The reproducibility of the qPCR was assessed by determining intra-assay repeatability and inter-assay reproducibility. The Coefficient of Variation (CV) was calculated using six dilutions (10^6 to 10^1 gene copies) of the genomic/plasmid DNA of selected pathogens. 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 6 dilutions 6 times in the same experiment. The CV for inter-assay reproducibility was calculated based on the C_T value of 6 dilutions on 6 different days.

PCR inhibitors: An experiment was conducted to determine the potential presence of PCR inhibitory substances in rainwater samples collected from 3 different tanks. Each sample (i.e. 1 L) was concentrated using the membrane filtration technique as described above. DNA was extracted using DNeasy blood and tissue kit (Qiagen), and tested with the PCR. DNA was also extracted from 1 L of ultra pure DNAse and RNase free sterile distilled water (Invitrogen) in the same manner. All samples were spiked with 10^3 gene copies of *S.* Typhimurium DNA. The C_T values obtained for the DNA samples from spiked rainwater were compared to 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.

Detection efficiency of the qPCR assay in rainwater samples: The detection efficiency was determined by spiking deionised water (*n*=3) and rainwater samples (*n*=3) with known concentration of *S.* Typhimurium cells. Initially, samples were collected from several rainwater tanks and were tested for the presence of *Salmonella* spp. using PCR. Water samples which were PCR negative for *Salmonella* Spp. were selected for this experiment. The samples were autoclaved to destroy background microbial flora. The *S.* Typhimurium strain was grown overnight in LB broth and cell concentrations were determined using membrane filtration method. Ten-fold serial dilutions were made and spiked into 250 ml of deionised and rainwater samples. The samples were filtered through membranes, and DNA extraction was performed according to the method described above. Samples were tested in triplicate for each concentration, and the recovery efficiency (%) was calculated using the following equation: Recovery (%) = (No. of cells after filtration/No. of cells before filtration) X 100.

RESULTS AND DISCUSSION

Concentration of *E. coli* and enterococci: Of the 84 rainwater samples tested, 57 (65%) were found to be positive for *E. coli.* The concentrations of *E. coli* in these positive samples were: 18 (20%) samples ranged between 1 and 10 CFU/100 ml, 16 (18%) ranged between 11 and 100 CFU/100 ml, 17 (19%) ranged between 101 to 1000 CFU/100 ml, and 6 (7%) had >1001 CFU/100 ml. In the 84 samples tested, 57 (65%) exceeded the Australian drinking water guidelines for 0 *E. coli*/100 ml of water (ANZECC 2000).

In the 84 rainwater samples tested, 72 (82%) were found to be positive for enterococci and the concentrations were: 16 (18%) samples ranged between 1 to 10 CFU/100 ml, 27 (31%) ranged between 11 to 100 CFU/100 ml, 20 (23%) ranged between 101 to 1000 CFU/100 ml, and 9 (10%) had >1001 CFU/100 ml. In the 84 samples tested and 56 (64%) 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 (ANZECC 2000). In the 84 samples tested 54 (61%) were positive for both indicators, and 75 (89%) were positive for at least one indicator.

Cross-reactivity of PCR primers: The cross-reactivity of each primer set for each target has also been assessed by testing a number of other microorganisms commonly found in environmental waters such as *Bacteroides vulgatus*, *Citrobacter freundii*, *Clostridium perfringens*, *Enterococci faecalis*, *Enterococci faecium*, and *Shigella sonnei* etc. The primers we used in this study did not amplify any PCR products other than those

products that were expected, suggesting a high specificity for the tested primers.

Prevalence of pathogenic microorganisms using PCR: There are several limitations of traditional culture based methods such as the underestimation of the bacterial concentration due to the presence of injured or stressed cells (Delgado-Viscogliosi et al. 2005). Furthermore, certain microorganisms in environmental waters could be viable but not culturable (Oliver 2000). The application of PCR-based methods has generated interest in the direct monitoring of pathogens in 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 view of this, we applied PCR assays 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 qPCR method, which circumvents the need for redesigning new primers, and optimises PCR conditions. However, a notable limitation of the SYBR Green system is that it may bind to non-specific double-stranded DNA, and this results in the formation of primer dimer and other non-specific products. In this research study, non-specific products were not observed with melting curve analysis of the targets.

C. coli organisms were the most prevalent among all the bacterial pathogens tested in this study (Table1). However, only one sample was positive for *C. jejuni*. The *C. coli* and *C. jejuni* in rainwater samples could potentially be from bird faeces (Kapperud and Rosef 1983). However, other potential sources such as possums or lizards cannot be ruled out. The presence of *Campylobacter* spp. in roof-harvested rainwater samples has also been reported in New Zealand (Savill et al. 2001) and "campylobacteriosis" was found to be epidemiologically associated with the consumption of roof-harvested rainwater (Eberhart-Phillips et al. 1987). 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 1). *A. hydrophila* has been reported as one of the most common *Aeromonas* spp. associated with human intestinal disease (Kühn et al. 1997). 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). *Aeromonas* spp., *Legionella* spp., and *Salmonella* spp. have previously been detected in in the United States and New Zealand and in the tropics in roof-collected rainwater cisterns and/or from tanks, using culture-based methods (Broadhead et al. 1998; Simmons et al. 2001). None of the samples tested were positive for *E. coli* O157 LPS, VT1, VT2, and *Salmonella spvC* gene. In the 84 samples tested, 15 (18%) were positive for the *G. lamblia* β-giardin gene. *Giardia* could be derived from bird faecal matters deposited on the roof. To our knowledge, this is the first study in Australia that reports the presence of *G. lamblia* in water samples from domestic rainwater tanks. None of the samples were positive for *C. parvum* COWP gene.

PCR limit of detection: The PCR detection limits were as low as 5 gene copies for bacterial pathogens For *G. lamblia* β-giradin gene and C. parvum COWP gene the detection limit was 7 gene copies. Lower levels (i.e., 1 copy) were tested for each target, but the results were not reproducible for all replicates. Nonetheless, the detection sensitivity values in the PCR assays were comparable to the values reported in the research literature (Sails et al. 2002).

TABLE 1. PCR positive results for potential pathogens

Target pathogens	PCR positive results/Number of samples tested	% of samples positive
A. hydrophila lip gene	7/84	8.3
C. coli ceuE gene	10/27	37
C. jejuni mapA gene	1/84	1.1
E. coli O157 LPS gene	0/84	0
E. coli VT1 gene	0/84	0
E. coli VT2 gene	0/84	0
L. pneumophila mip gene	8/84	9.5
Salmonella invA gene	17/84	20
Salmonella spvC gene	0/27	0
G. lamblia β-giradin gene	15/84	18
C. parvum COWP gene	0/84	0

PCR reproducibility: The reproducibility of the PCR analysis was determined by assessing intra-

assay and inter-assay Coefficient of Variation (CV) of the standards. These values were less than 4% and 5% for *C. jejuni mapA* gene, *L. pneumophila mip* gene, and *Salmonella invA* gene, respectively, thus indicating high reproducibility. For *G. lamblia* β-giradin gene, these values were less than 5% (i.e., intra-assay) and 7% (inter-assay).

PCR inhibitors: To detect the presence of inhibitors, rainwater samples (n = 5) were spiked with 10^3 gene copies of *Salmonella* serovar Typhimurium DNA. The qPCR C_T values were compared to those obtained from the same concentration of DNA that was used to spike 1 L of distilled water. For the spiked distilled water, the mean C_T values for *Salmonella* serovar Typhimurium DNA are given in Table 5. For rainwater samples, the mean C_T value was 23.5 ± 0.4 when undiluted DNA was spiked. For 10- fold, 100-fold and 1000-fold dilutions of DNA, these values were 23.5 ± 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 surface water samples. No significant differences were observed between the C_T values for spiked distilled water, undiluted DNA, and serially diluted (i.e., 10-fold, 100-fold, and 1000-fold) thus indicating that the tested rainwater samples were free of PCR inhibitory substances. However, three samples out of 84 contained PCR inhibitory substances. A 10-fold serial dilution of DNA was necessary to remove inhibitors from these samples. Based on data derived, it is recommend that PCR inhibitory checks need to be performed prior to PCR analysis.

Detection efficiency: The detection efficiency was determined by spiking autoclaved distilled water and surface water with known concentration of S. Typhimurium cells. The estimated detection efficiency in autoclave distilled water samples ranged between 93% and 68% with the greatest variability occurring at lower cell counts. The mean detection efficiency was 80% ± 10% (Table 3). The estimated detection efficiency in autoclaved rainwater samples ranged between 91% and 58% with the greatest variability occurring at lower cell counts. The mean detection efficiency was 75% ± 12%.

Concentrations of selected pathogens: Quantitative PCR assays were performed on selected pathogens considering their prevalence and infectious dose. Though *C. jejuni mapA* gene was detected in one sample, the concentration was below qPCR detection limit. *L. pneumophila*, *Salmonella*, and *Giardia lamblia* were detected in several samples (Table 2). *L. pneumophila mip* and *Salmonella invA* are single copy gene and were converted to cells (i.e., 1 gene copy = 1 cell). *G. lamblia* β-giradin gene copies numbers were converted to cysts (16 gene copies = 1 cyst).

TABLE 2 – Quantitative PCR results of the potential pathogens

Target pathogens	PCR positive results	Range of gene copies/100 ml
C. jejuni mapA gene	1/1[a]	-
L. pneumophila mip gene	8/2[a]	$1.2 \times 10^1 – 3.4 \times 10^1$
Salmonella invA gene	17/8[a]	$3.0 \times 10^1 – 1.4 \times 10^2$
G. lamblia β-girdin gene	15/4[a]	$1.8 \times 10^1 – 1.0 \times 10^2$

[a] Number of samples below qPCR detection limit

Correlation between faecal indicators and pathogens: 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 3). The presence/absence of the potential pathogens did not correlate with any of the indicator bacteria concentrations. The poor correlation between faecal indicators and pathogens especially viral and protozoans has been reported previously (Hörman et al. 2004).

Quantitative Microbial Risk Assessment (QMRA):

The implications of these findings in terms of public health risk is currently being undertaken. The exposure of users to *C. jejuni*, *L. pneumophila*, *Salmonella* spp. and *G. lamblia* in roof-harvested rainwater will be quantified and Quantitative Microbial Risk Assessment (QMRA) will be applied

using Monte Carlo analysis to determine the likely numbers of infections resulting from these exposures. These outcomes in terms of the impact of using roof-harvested rainwater on the disease burden of South East Queensland region of Australia will be interpreted.

TABLE 3 - The relationships between faecal indicators and the presence and absence of pathogens in samples from rainwater tanks.

Indicators vs. pathogenic microorganisms	Nagelkerke's *R* square [a]	*P*-value [b]	Odd ratio
E. coli vs. *A. hydrophila*	.055	0.460	1.00
E. coli vs. *C. jejuni*	.008	0.775	1.00
E. coli vs. *L. pneumophila*	.006	0.640	1.00
E. coli vs. *Salmonella*	.048	0.198	1.00
E. coli vs. *G. lamblia*	.019	0.484	1.00
Ent vs. *A. hydrophila*	.006	0.700	1.00
Ent vs. *C. jejuni*	.001	0.943	1.00
Ent vs. *L. pneumophila*	.007	0.555	1.00
Ent vs. *Salmonella*	.016	0.388	1.00
Ent vs. *G. lamblia*	.001	0.928	1.00

[a] Nagelkerke's *R* square, which can range from 0.0 to 1.0, denotes the effect size (the strength of the relationship); stronger associations have values closer to 1.0.

[b] *P*-value for the model chi 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.

CONCLUSIONS

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. Currently we are performing QMRA analysis in order to asses the public health risks associated with the use of roof-harvested rainwater.

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