Evidence of Avian and Possum Fecal Contamination in Rainwater Tanks as Determined by Microbial Source Tracking Approaches

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ABSTRACT

Avian and possum fecal droppings may negatively impact roof-harvested rainwater (RHRW) water quality due to the presence of zoonotic pathogens. This study was aimed at evaluating the performance characteristics of a possum feces-associated (PSM) marker by screening 210 fecal and wastewater samples from possums ($n = 20$) and a range of nonpossum hosts ($n = 190$) in Southeast Queensland, Australia. The host sensitivity and specificity of the PSM marker were 0.90 and 0.95 (maximum value, 1.00), respectively. The mean concentrations of the GFD marker in possum fecal DNA samples (8.8×10^7 gene copies per g of feces) were two orders of magnitude higher than those in the nonpossum fecal DNA samples (5.0×10^5 gene copies per g of feces). The host sensitivity, specificity, and concentrations of the avian feces-associated GFD marker were reported in our recent study (W. Ahmed, V. J. Harwood, K. Nguyen, S. Young, K. Hamilton, and S. Toze, *Water Res* 88:613–622, 2016, <http://dx.doi.org/10.1016/j.watres.2015.10.050>). The utility of the GFD and PSM markers was evaluated by testing a large number of tank water samples ($n = 134$) from the Brisbane and Currumbin areas. GFD and PSM markers were detected in 39 of 134 (29%) and 11 of 134 (8%) tank water samples, respectively. The GFD marker concentrations in PCR-positive samples ranged from 3.7×10^2 to 8.5×10^5 gene copies per liter, whereas the concentrations of the PSM marker ranged from 2.0×10^3 to 6.8×10^3 gene copies per liter of water. The results of this study suggest the presence of fecal contamination in tank water samples from avian and possum hosts. This study has established an association between the degradation of microbial tank water quality and avian and possum feces. Based on the results, we recommend disinfection of tank water, especially for tanks designated for potable use.

IMPORTANCE

The use of roof-harvested rainwater (RHRW) for domestic purposes is a globally accepted practice. The presence of pathogens in rainwater tanks has been reported by several studies, supporting the necessity for the management of potential health risks. The sources of fecal pollution in rainwater tanks are unknown. However, the application of microbial source tracking (MST) markers has the potential to identify the sources of fecal contamination in a rainwater tank. In this study, we provide evidence of avian and possum fecal contamination in tank water samples using molecular markers. This study established a potential link between the degradation of the microbial quality of tank water and avian and possum feces.

Growing water scarcity has led to the increased reliance on alternative and decentralized potable and nonpotable water resources in recent decades. Australia is the driest inhabited continent on earth and suffered from a severe “millennium” drought from 2001 to 2009 (1). As a result of the water scarcity in this region, the use of roof-harvested rainwater (RHRW) (stored in tanks) for domestic purposes is a widely accepted practice. This is beneficial for simultaneously conserving water and reducing storm water runoff. The presence of multiple microbial pathogens, including opportunistic pathogens in rainwater tanks, has been reported by several studies, supporting the necessity for the management of potential health risks (2–4).

Pathogens could be introduced to tanks via roof runoff containing fecal matter from birds, insects, bats, possums, and reptiles. The microbiological quality of RHRW stored in tanks is generally assessed by monitoring *Escherichia coli*, which is commonly found in the gut of warm-blooded animals (2, 5, 6). The presence of *E. coli* in tank water generally indicates fecal contamination and the potential for public health risks. Drinking water guidelines have been used to assess the microbial quality of the tank water. For most guidelines, this entails the nondetection of *E. coli* in 100 ml of water (7, 8). Even when tank water is not used for drinking, assessment of the microbial quality is usually undertaken by mon-

itoring *E. coli* (2, 9–11). One major limitation of *E. coli* for monitoring is that it fails to predict the presence of pathogens in water sources (12–14). In a previous study, the presence of *E. coli* did not correlate with the presence of potential pathogens, including opportunistic pathogens, such as *Aeromonas hydrophila*, *Campylobacter jejuni*, *Campylobacter coli*, *Legionella pneumophila*, *Salmonella* spp., and *Giardia lamblia*, in tank water samples (3, 9).

Another limitation of *E. coli* is that its presence does not provide information regarding its sources (15, 16). Identification of the source(s) of fecal contamination in tank water is critical for

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implementing appropriate remediation and protecting potential human health risks associated with designated water use. Water quality researchers are currently using microbial source tracking (MST) markers to detect fecal contamination in environmental waters (13, 17, 18). However, the application of MST markers to identify the sources of fecal contamination in rainwater tank samples is rare. Previously, an attempt was made to identify the likely sources of clinically significant *E. coli* in rainwater tanks by analyzing *E. coli* isolates from tank water and fecal samples from birds and possums. The biochemical phenotypes of *E. coli* strains carrying virulence genes from a small number of tank water samples were identical to a number of biochemical phenotypes of *E. coli* strains carrying the same virulence genes from bird and possum feces. These findings suggest that these animals may be the likely sources of *E. coli* strains identified in tank water samples (19).

A recent study reported the development of a quantitative PCR (qPCR) assay for the identification of avian feces-associated GFD marker of unclassified *Helicobacter* spp. in various host groups in the United States, Canada, and New Zealand (20). The distributions of the GFD marker in avian feces across the United States, Canada, and New Zealand suggest it might have broad applicability for MST studies in other parts of the world (20). A recent follow-up study in Australia and the United States also reported the high host specificity of the GFD marker and suggested that this marker could be used as a reliable marker to detect the presence of avian fecal contamination in environmental waters (21). Another study developed a possum PCR marker based on the *Bacteroidales* 16S rRNA sequences (22). The authors did not name the marker; here, we designate it the PSM marker. The host sensitivity (0.83) and specificity (0.96) values of the PSM marker in tested possum fecal ($n = 36$) and nonpossum fecal samples ($n = 233$) were high (22).

The primary aim of this study was to evaluate the host sensitivity and specificity of the PSM marker by analyzing fecal samples from a variety of host groups in Southeast Queensland, Australia. The host specificity and sensitivity values of the GFD marker were reported in a recent study for Southeast Queensland (21). Tank water samples (presumed to be affected by fecal contamination) were collected from Brisbane and the Currumbin Ecovillage. GFD and PSM markers were quantified using qPCR from tank water samples, and *E. coli* was enumerated using a culture-based method. The host sensitivity and specificity of the GFD and PSM markers along with their distribution in tank water samples were then used to validate the presence of avian and possum fecal contamination in tank water samples.

MATERIALS AND METHODS

Animal fecal and wastewater sampling. To determine the host sensitivity and specificity of the PSM marker, individual and composite fecal and wastewater samples were collected from various host groups in the Brisbane and Currumbin areas (Table 1). Additional information on the fecal and wastewater samples is given in Note S1 in the supplemental material. All samples were transported on ice to the laboratory, stored at 4°C, and processed within 24 h.

Concentration of cattle and pig wastewater samples. Cattle and pig wastewater samples were concentrated with Amicon Ultra-15 (30 K) centrifugal filter devices (Merck Millipore Ltd., Tokyo, Japan). In brief, 10 ml of wastewater sample was added to the Amicon device and centrifuged at $4,750 \times g$ for 10 min. Entire volumes (180 to 200 μ l) of concentrated samples were collected from the filter device sample reservoir using a

TABLE 1 Percentage of possum and nonpossum fecal DNA samples PCR positive for the possum marker

Host group	No. of samples	Sample source	No. (%) of PCR-positive samples
Bird	10	Wild, farm	0 (0)
Cat	13	Veterinary hospital	4 (31)
Cattle feces	4	Farms	0 (0)
Cattle wastewater	8	Abattoir	0 (0)
Deer	12	Sanctuary	2 (17)
Dog	13	Veterinary hospital and parks	0 (0)
Emu	14	Emu park	0 (0)
Goat	10	Veterinary hospital	0 (0)
Horse	19	Horse racecourse	0 (0)
Human feces	3	Human	0 (0)
Human wastewater	20	Wastewater treatment plants	0 (0)
Kangaroo	10	Sanctuary	0 (0)
Koala	12	Sanctuary	0 (0)
Pig wastewater	20	Abattoir	0 (0)
Possum	20	Wild	18 (90)
Sheep	14	Veterinary hospital	3 (21)
Waterfowl	8	Parks	1 (13)
Host sensitivity			0.90
Host specificity			0.95

pipette (23). The concentrated samples were stored at -20°C for a maximum of 24 h prior to DNA extraction.

Study areas and sanitary inspection. One hundred thirty-four rainwater tanks were sampled from various areas of Brisbane ($n = 84$) and the Currumbin Ecovillage ($n = 50$), both located in Southeast Queensland, Australia, during March to September 2015. Brisbane is the capital city of Queensland, Australia. The Brisbane metropolitan area has a population of 2.3 million people. The Ecovillage is a decentralized residential development that employs a range of strategies to conserve water and energy, including a cluster-scale sewage treatment/water reclamation plant, rainwater storage tanks, solar panels, and source-separated urine usage. One hundred percent and 20% of rainwater tanks are used for potable use in Brisbane and Currumbin, respectively, in addition to other nonpotable uses, such as cooking, showering, and gardening.

The size of the tanks ranged from $<3,000$ to $>40,000$ liter. The Brisbane tanks were most commonly made from polyethylene (68%), and the Currumbin tanks were primarily galvanized metal (83%). Each tank has an opening on the top. Of the 134 tanks, 65 (49%) had a first-flush diverter installed to bypass the first flush consisting of contaminants. Eighty-four (63%) tanks had never been cleaned (desludged) since installation. The remaining tanks were cleaned every 2 to 3 years. On each property, a visual sanitary inspection was undertaken by the residents for 10 to 15 min to identify factors (the presence of overhanging trees, television [TV] aerials, wildlife fecal droppings, and the presence/absence of first flush diverters) that can affect the quality of RHRW stored in tanks (see Form S4 in the supplemental material).

Tank water sampling. The tap/spigot connected directly to the rainwater tank was wiped with 70% ethanol, and the water was run for 15 s prior to filling a 10-liter sterile container. In the absence of a tap, samples were collected directly from openings in the top of the tank. The samples were transported to the laboratory, kept at 4°C, and processed within 6 to 72 h.

Enumeration of *Escherichia coli*. Colilert test kits (Idexx Laboratories, Westbrook, ME, USA) were used to determine the concentrations of *E. coli* in 100 ml of each tank water sample. The test kits were incubated at $37 \pm 0.5^{\circ}\text{C}$ for 18 to 24 h, as per the manufacturer's recommendation.

Concentration of rainwater samples. An approximately 10-liter water sample from each tank was concentrated by a hollow-fiber ultrafiltration system (HFUF) using Hemoflow FX 80 dialysis filters (Fresenius Medical Care, Bad Homburg, Germany), as previously described (24). Briefly, 1 g of sodium hexametaphosphate (NaPP; Sigma-Aldrich, St. Louis, MO, USA) was added to each 10-liter rainwater sample to achieve a concentration of 0.01% (wt/vol). Each water sample was pumped with a peristaltic pump (Adelab Scientific, South Australia, Australia) in a closed loop with sterile high-grade Norprene A60-F tubing (Adelab Scientific). The tubing was sterilized by soaking in 3% bleach, washing with deionized water, and autoclaving at 121°C for 15 min. The sample was concentrated to approximately 150 to 200 ml, depending on turbidity. At the end of the concentration process, pressurized air was passed through the filter cartridge from the top to recover as much water as possible. To improve recovery, after each sample was processed through the HFUF, 500 ml of elution solution (0.5% Tween 80 [Sigma-Aldrich], 0.01% NaPP, and 0.001 Antifoam A [Sigma-Aldrich]) was recirculated through the filter for 5 min and then concentrated in the same manner as the sample to 150 ml. This elution solution was added to the concentrated sample to achieve a final volume of approximately 300 to 400 ml and stored at −4°C. A new filter cartridge was used for each sample. The combined concentrate was filtered through a 0.45 µm-pore-size, 90-mm-diameter cellulose filter paper (Advantec, Tokyo, Japan) and stored (48 to 72 h) at −80°C until DNA extraction. In case of filter clogging, multiple filter papers were used for each sample.

DNA extraction. DNA was extracted from the concentrated cattle and pig wastewater samples using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). A QIAamp stool DNA kit (Qiagen) was used to extract DNA from 100 to 220 mg of fresh animal feces and 250 µl of raw human wastewater samples. A PowerSoil Max DNA kit (Mo Bio, Carlsbad, CA, USA) was used to extract DNA directly from the filter. The filter(s) was inserted into 50 ml of PowerBead tube containing 15 ml of PowerBead solution. DNA was extracted according to the manufacturer's instructions and stored at −80°C until use. The protocol was modified slightly with 2 ml of DNA elution buffer C6 instead of 5 ml (25). DNA concentrations were determined using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technology, Wilmington, DE, USA).

PCR inhibition. An experiment was conducted to determine the presence of PCR-inhibitory substances in tank water DNA samples using a Sketa22 real-time PCR assay (26). Of the 134 samples, 23 (17%) had the sign of PCR inhibition (2-quantification cycle [C_q] delay was considered to be having PCR inhibitors). These inhibited samples were 10-fold serially diluted and further tested with the Sketa22 real-time PCR assay. The results indicated the relief of PCR inhibition. Based on the results, neat DNA samples (PCR-uninhibited samples) and 10-fold diluted (PCR-inhibited) samples were tested with GFD and PSM qPCR assays.

Preparation of qPCR standards. Standards for the GFD qPCR assay were prepared from a gene fragment amplified from bird feces and cloned into the pGEM-T Easy vector system II (Promega, Madison, WI, USA). Plasmid DNA was isolated using the plasmid minikit (Qiagen, Valencia, CA, USA). A purified recombinant plasmid containing a 152-bp GFD target (TGC AAG TCG AGG GGT AAC AGG GCC TAG CAA TAG GCC GCT GAC GAC CGG CGC ACG GGT GAG TAA CAC GTA TCC AAC CTG CCG ATA ACT CGG GGA TAG CCT TTC GAA AGA AAG ATT AAT ACC CGA TAG CAT AAG GAT TCC GCA TGG TCT CCT TA) was purchased from Integrated DNA Technologies (Coralville, IA, USA). The purified recombinant plasmids were serially diluted to create standards ranging from 1×10^6 gene copies to 1 gene copy per µl of DNA extract. A 3-µl template from each serial dilution was used to prepare a standard curve for each qPCR assay. For each standard, the genomic copies were plotted against the cycle number at which the fluorescence signal increased above the C_q value. The amplification efficiency (E) was determined by analysis of the standards and was estimated from the slope of the standard curve to be $E = 10^{-1/\text{slope}}$.

qPCR assays. qPCR assays were performed using previously published primers, probes, and cycling parameters (see Table S1 in the supplemental material for more details). GFD and PSM qPCR amplifications were performed in a 20-µl reaction mixture using SsoFast EvaGreen supermix (Bio-Rad Laboratories, CA, USA). The qPCR mixtures contained 10 µl of supermix, 100 nM each primer (GFD assays), 250 nM each primer (PSM assay), and 3 µl of template DNA. To separate the specific product from nonspecific products, including primer dimers, a melting-curve analysis was performed for each qPCR run. During melting-curve analysis, the temperature was increased from 65 to 95°C at 0.5°C increments. Melting-curve analysis showed a distinct peak at temperature $84.0^\circ\text{C} \pm 0.2^\circ\text{C}$ (for the GFD assay) and $85.5^\circ\text{C} \pm 0.2^\circ\text{C}$ (for the PSM assay), indicating positive and correct amplifications. Standards (positive controls) and sterile water (negative controls) were included in each qPCR run. All qPCRs were performed in triplicate using a Bio-Rad CFX96 thermal cycler.

qPCR performance characteristics. qPCR standards were analyzed to determine the amplification efficiencies (E) and the correlation coefficient (r^2). The qPCR lower limit of quantification (LLOQ) was also determined from the standard series. The lowest concentration of gene copies from the standard series detected in all triplicate samples was considered the qPCR LLOQ.

Quality control. Method blank runs were performed to ensure that the disinfection procedure was effective in preventing carryover contamination between sampling events. In addition, to prevent DNA carryover contamination, reagent blanks were included for each batch of DNA samples. No carryover contamination was observed. To minimize qPCR contamination, the DNA extraction and qPCR setup were performed in separate laboratories.

Statistical analysis. The host sensitivity and specificity of the PSM marker were determined as follows: host sensitivity = $a/(a + b)$, and specificity = $c/(c + d)$, where a is true positive (possum fecal samples were positive for the PSM marker), b is false negative (nonpossum fecal samples were negative for the PSM marker), c is true negative (nonpossum fecal samples were negative for the PSM marker), and d is false positive (nonpossum fecal samples were positive for the PSM marker) (21). Samples were considered quantifiable when the PSM marker levels were above the qPCR LLOQ. Samples that fell below the LLOQ and generated PCR amplification were considered positive but not quantifiable. The concentrations of *E. coli* and GFD and PSM markers in tank water samples were not normally distributed (as determined by Kolmogorov-Smirnov and Shapiro-Wilk normality tests). Therefore, nonparametric Spearman rank correlation with a two-tailed P value was also used to establish the relationship between *E. coli* and markers (GFD and PSM) in tank water samples.

RESULTS

Sanitary inspection results. Roofs connected to the rainwater tanks from Brisbane had more overhanging trees present (17%) than at Currumbin (7%) (Fig. 1). The sanitary inspection also identified more debris on Brisbane roofs (63%) than at Currumbin (43%). Eighty-one percent of the Currumbin tanks had first-flush diverters installed, whereas only 29% of the Brisbane tanks had the first-flush devices. Brisbane roofs also had more TV aerials (76%) installed than at Currumbin (26%).

qPCR performance characteristics and LLOQ. qPCR standards were analyzed to determine the performance characteristics, such as slope, amplification efficiencies, and correlation coefficient values. The standards had a linear range of quantification from 1×10^6 gene copies to 1 gene copy per µl of DNA extract. The qPCR performance characteristics for individual assays were within the values prescribed by the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (see Table S2 in the supplemental material). The small-

FIG 1 Sanitary inspection results for rainwater tanks from Brisbane ($n = 84$) and Currumbin ($n = 50$) in Southeast Queensland, Australia.

est amount of diluted gene copies detected in triplicate samples was considered the qPCR LLOQ. The qPCR LLOQ was determined to be 3 gene copies for the GFD assay and 30 gene copies for the PSM assay.

Host specificity and sensitivity of the GFD and PSM markers.

The host sensitivity and specificity values of the GFD marker have been reported in a previous study (21) using the same set of fecal and wastewater samples analyzed for the PSM marker in this study. The host sensitivity of the GFD marker in avian feces ($n = 36$) was 0.58 (maximum value, 1). The host specificity of the GFD marker was also high (0.94) for nonavian fecal samples ($n = 190$). In this study, among the 20 possum fecal samples tested, 18 were PCR positive for the PSM marker (Table 1). Therefore, the host sensitivity of the PSM marker in DNA samples from possum feces was 0.90 (maximum value, 1). Among the 190 nonpossum fecal DNA samples tested, 180 samples were negative for the PSM marker, yielding a host specificity value of 0.95. Small numbers of cat ($n = 4$), deer ($n = 2$), sheep ($n = 3$), and waterfowl ($n = 1$) fecal DNA samples, however, were positive for the PSM marker. Several horse ($n = 5$), human ($n = 2$), koala ($n = 6$), and emu ($n = 8$) fecal DNA samples also showed PCR amplifications for the PSM marker; however, they had different melting peaks of $<84.5^{\circ}\text{C}$ or $>86.5^{\circ}\text{C}$ compared to the correct melting peak of 85.5°C for the PSM marker.

Concentrations of PSM marker in possum and nonpossum fecal samples. The concentrations of the PSM marker in possum fecal DNA samples (from Brisbane) were highly varied per gram of feces (Fig. 2). The mean concentrations in these samples ranged from 1.7×10^5 to 1.1×10^9 gene copies per g of feces. The mean concentration of the PSM marker in nonpossum host groups ranged from 1.1×10^4 to 6.4×10^4 (cat), 3.7×10^4 to 1.1×10^5 (deer), 1.5×10^4 to 2.5×10^5 (sheep), and 4.3×10^6 (waterfowl) gene copies per g of feces. The overall mean concentration of the PSM marker in possum was 8.8×10^7 gene copies per g of feces, which was two orders of magnitude higher than that of the nonpossum host groups (5.0×10^5 gene copies per g of feces). A t test for equal means indicated that the mean concentration of PSM marker in possum feces was significantly different ($P = 0.02$) than that of nonpossum feces.

FIG 2 Concentrations of possum (PSM) markers in possum and nonpossum fecal DNA samples in Southeast Queensland, Australia. The inner box lines represent the medians, while the outer box lines represent the 5th and 95th data percentiles, and the whiskers extend to the range.

Concentrations of *E. coli* and GFD and PSM markers in tank water samples.

Of the 84 tank water samples tested from Brisbane, 59 (70%) were positive for *E. coli*, whereas of the 50 tank water samples tested from the Currumbin Ecovillage, 34 (68%) were positive for *E. coli*. The concentrations of *E. coli* in positive samples are shown in Fig. 3. The concentrations of *E. coli* ranged from 1 to $>2,420$ most probable number (MPN) per 100 ml for Brisbane tank water samples and from 1 to 435 MPN per 100 ml of water for Currumbin tank water samples. The *E. coli* concentrations were significantly ($P = 0.03$) higher in Brisbane tank water samples than in Currumbin samples. A t test for equal means indicated that the mean concentration of *E. coli* in Brisbane tank water samples was significantly different ($P = 0.01$) than that at Currumbin.

Of the 84 tank water samples tested from Brisbane, 27 (32%) and 5 (6%) samples were PCR positive for the GFD and PSM markers, respectively. Similarly, of the 50 tank water samples tested from Currumbin, 12 (24%) and 6 (12%) samples were PCR positive for the GFD and PSM markers, respectively. Of the 84 tank water samples from Brisbane, 31 (37%) contained at least one

FIG 3 Box-and-whisker plots of the concentrations (MPN per 100 ml) of *Escherichia coli* in positive tank water samples from Brisbane and Currumbin. The inner box lines represent the medians, while the outer box lines represent the 5th and 95th data percentiles, and the whiskers extend to the range.

FIG 4 Box-and-whisker plots of the concentrations (gene copies per liter) of avian feces-associated GFD and possum feces-associated PSM markers in positive tank water samples from Brisbane and Currumbin. The inner box lines represent the medians, while the outer box lines represent the 5th and 95th data percentiles, and the whiskers extend to the range.

marker, and 1 (1%) tank contained both markers. Of the 50 tank water samples from Currumbin, 16 (32%) tanks contained at least one marker, and 2 (4%) tanks contained both markers. The GFD marker was more prevalent than the PSM marker in both Brisbane and Currumbin areas. The concentrations of GFD and PSM markers in positive tank water samples are shown in Fig. 4. The GFD marker concentrations ranged from 9.3×10^2 to 3.0×10^5 gene copies per liter of water (Brisbane) and 3.7×10^2 to 8.5×10^5 gene copies per liter of water (Currumbin). The PSM marker concentrations ranged from 2.7×10^3 to 6.8×10^3 gene copies per liter of water (Brisbane) and 2.0×10^3 to 6.1×10^3 gene copies per liter of water (Currumbin). The *t* test for equal means indicated that the mean concentration of the GFD marker in tank water samples from Brisbane was significantly different ($P = 0.007$) than in samples from Currumbin. However, the PSM marker concentration did not differ significantly in the Brisbane and Currumbin tank water samples. Pearson's correlation was used to test the relationship between *E. coli* concentrations with the GFD and PSM marker concentrations. The concentrations of the GFD marker negatively correlated with concentrations of *E. coli* ($r = -0.07$, $P = 0.04$). The concentrations of the PSM marker did not correlate with concentrations of *E. coli* ($r = 0.09$, $P = 0.25$).

Agreement and disagreement between the presence of markers and sanitary inspection results. In all, 47 of 134 tank water samples from Brisbane and Currumbin had either the GFD or PSM marker. The presence/absence of GFD and PSM markers and visual sanitary inspection results were compared pairwise for these tank water samples. From the pairwise comparison, the percentage of agreement (cooccurrence) (i.e., presence of a marker in the presence of a factor potentially affecting the quality tank water) and agreement (co-nonoccurrence) (i.e., the absence of a marker in the absence of a factor) were calculated. The percentage of total disagreement (i.e., the presence of a marker in the absence of a factor or absence of a marker in the presence of a factor) for each pairwise comparison was also calculated by subtracting the percentage of agreement (cooccurrence and co-nonoccurrence) from 100%. On average, 36% (cooccurrence) and 17% (co-nonoccurrence) agreements were found for the GFD marker with sanitary inspection results. These values for the PSM marker were 7%

FIG 5 Percentage agreement and disagreement between the presence of avian feces-associated GFD and possum feces-associated PSM markers and sanitary inspection results.

and 36% for cooccurrence and co-nonoccurrence, respectively (Fig. 5). The GFD marker and the presence of wildlife droppings and TV aerial had the highest percentage (47% each) of cooccurrence agreement, followed by 38% cooccurrence agreement for first-flush diverters. The PSM marker and the presence of TV aerial and wildlife droppings had 11% and 9% cooccurrence agreements, respectively.

DISCUSSION

The numbers of rainwater tanks used as a source of water for urban and rural households around the world are increasing. For example, 26% of Australian households used a rainwater tank as a source of water in 2010 compared with 19% in 2007 and 17% in 2004 (27). There has been a marked increase in the proportion of households with a rainwater tank in Queensland, Australia (17% in 2004 to 36% in 2010). In our previous studies, we have reported the presence of potential bacterial pathogens, including opportunistic pathogens and protozoa in rainwater tank samples from Southeast Queensland, Australia (3, 28). If the untreated tank water is used for drinking, there are potential disease risks for people consuming this water. Therefore, it is essential to obtain information on the sources of fecal contamination to design management strategies and minimize public health risks from exposure to these pathogens.

In this study, we investigated the potential sources of fecal contamination in a large number of tank water samples from urban (Brisbane) and periurban (Currumbin Ecovillage) settings in Southeast Queensland, Australia. During the visual sanitary inspection, debris was spotted on the roofs and gutters for certain tanks. Possums, bats, and different avian species were identified as potential sources of fecal contamination on the roofs by the residents. Since monitoring *E. coli* does not provide definitive information on the sources of fecal contamination, two newly developed MST markers targeting avian and possum hosts were chosen for this study (20, 22). The performance characteristics of the GFD marker were evaluated in a recent study (21). Although the GFD

marker exhibited high host specificity (0.94), the host sensitivity value (0.58%) was low. On the other hand, little is known regarding the host specificity and sensitivity of the PSM marker.

The host sensitivity (0.83) and specificity (0.96) of the PSM marker were reported to be high in the original study that developed this marker, in which 36 possum and 233 nonpossum fecal samples in New Zealand were screened (22). The authors recommended that host sensitivity and specificity of the PSM marker be tested prior to field application in a new location, since bacterial markers do not often exhibit absolute host specificity and sensitivity (13, 22, 29). In this study, the host sensitivity and specificity of the PSM marker were determined to be 0.90 and 0.95, respectively, which were well within the recommended guidelines by the U.S. EPA (16) and also similar to the values reported by Devane and colleagues (22).

The concentrations of the PSM marker in an individual possum fecal sample varied by 3 to 4 orders of magnitude. However, the mean concentration (8.8×10^7 gene copies per g of feces) obtained in this study was similar to the range (1.6×10^7 to 1.0×10^7 gene copies per g of feces) reported by Devane and colleagues (22). The variation of the PSM marker in individual possum fecal samples could be attributed to factors, such as diet, which may vary both regionally and seasonally (30, 31). This has implications, because a marker with varied and/or low concentrations in its host(s) can be difficult to detect in waters due to dilution (32). Further study would be required to shed light on the variability of this marker in a large number of possum fecal samples to identify factors that may be responsible for such variability.

In this study, approximately 70% of the tank water samples tested exceeded the Australian drinking water guideline of zero *E. coli* cells per 100 ml (33). The frequency of detection and concentrations of *E. coli* were significantly higher in Brisbane tank water samples than in Currumbin samples. This might be due to the fact that the Currumbin Ecovillage is a new subdivision, and most of the rainwater tanks installed there are relatively new compared to tanks in Brisbane. Since all Currumbin tanks are used for drinking, residents put more effort into maintaining the quality of water by installing first-flush diverters and using other cleanliness practices, such as trimming of overhanging trees and cleaning the gutters more frequently. Such practices were not observed for the Brisbane area, as only 20% tanks are used for potable use. These factors collectively may have contributed to the high frequency of detection and concentrations of *E. coli* in Brisbane tank water samples.

Overall, the concentrations of *E. coli* were highly varied, ranging from 1 to >2,420 MPN per 100 ml of water, suggesting the occurrence of fecal contamination. The results were in accordance with the fact that 29% and 8% of 134 tank water samples from Brisbane and Currumbin were PCR positive for the GFD and PSM markers, respectively. The GFD marker was more frequently detected in tank water samples than the PSM marker. This could be due to the fact that *Helicobacter*-associated GFD marker may have better survival ability in the tank environment than the *Bacteroides*-associated PSM marker, which is an obligate anaerobe. It is also possible that birds are more likely to be on the roofs than possums. The frequencies of GFD and PSM markers detection were higher for Brisbane tank water samples than for Currumbin samples. Again, this might be related to the poor maintenance practices of Brisbane tanks.

The GFD (21) and PSM markers (this study) were detected in

small numbers of cat, dog, deer, kangaroo, sheep, and waterfowl fecal DNA samples. Their presence in dog, deer, kangaroo, and sheep may not be problematic due to the fact that roof contamination with feces from these animals is unlikely. During the sanitary inspection, we did not observe any cats or waterfowls on the roof. However, tank water contamination from these sources cannot be ruled out. This phenomenon may not be a critical issue as long as the concentrations of the GFD and PSM markers remain low in nonavian and nonpossum host groups. The mean concentrations of the GFD and PSM markers in nonavian and nonpossum fecal DNA samples were 2 to 3 orders of magnitude lower than those in avian and possum fecal DNA samples. The concentrations of the markers in certain tank water samples were as high as 8.5×10^5 (GFD) and 6.8×10^3 (PSM) gene copies per liter of water. This has public health implications, as bird and possum feces are known to contain *Campylobacter* spp., *Cryptosporidium* spp., *Giardia* spp., and clinically significant *E. coli* strains (19, 28, 34, 35).

Overall, 70% and 90% of the tank water samples were PCR negative for the GFD and PSM markers, respectively. The presence of low levels of avian and possum fecal contamination in these tank water samples cannot also be ruled out, because the lower limit of detection (LLOD) of the qPCR assays ranged from 1 to 3 gene copies per μ l of DNA, which translates to approximately 67 to 200 gene copies per liter of water sample that would need to be present for qPCR detection. Forty-three percent of the tank water samples had >1 *E. coli* cell per 100 ml of water. However, these samples were negative for the GFD and PSM markers. The sources of fecal contamination in these tanks may have originated from bats, insects, frogs, and lizards. Since both the GFD and PSM markers did not show absolute sensitivity when tested against avian and possum hosts, it is also possible that avian and possum fecal contamination may be occurring in certain tanks, but the markers were absent in the feces of those animals contaminated tank water samples and therefore could not be detected with qPCR assays.

Concentrations of *E. coli* did not correlate with the concentrations of the GFD and PSM markers in tank water samples from both Brisbane and Currumbin. Therefore, this study is also in accordance with findings that *E. coli* monitoring is not likely to be a reliable surrogate for general fecal contamination and presence of pathogens, as was previously reported (3, 9). The lack of a relationship between *E. coli* and MST markers likely reflects differences in methodologies, in which *E. coli* analysis provides a viable concentration of *E. coli* and MST marker detection/quantification provides the information on the presence/absence of host-specific fecal contamination and its magnitude. In addition, MST markers come from a specific host group, whereas *E. coli* comes from all warm-blooded animals. Furthermore, the fate (inactivation) of the GFD and PSM markers could be different from that of *E. coli* (36). A similar lack of correlation has been reported for other MST marker concentrations with *E. coli* concentrations (13, 37).

An attempt was taken to determine what sanitary factors might have contributed to the presence of GFD and PSM markers in tank waters. The data indicated that wildlife droppings and the presence of TV aerials had the highest percentage of cooccurrence with the presence of both GFD and PSM markers. However, these data should be interpreted with care, because it is difficult to differentiate among factors that may have contributed to the presence of GFD and PSM markers in the tank water. For example, the roof connected to tank T62 (see Table S3 in the supplemental material) had overhanging trees, fecal droppings, no first-flush diverter, and

a TV aerial. The presence of the GFD in the tank water samples could be associated with one or more of these factors. Thirty-eight percent and 6% agreements were observed for the GFD and PSM markers with the presence of first-flush diverters, suggesting that this device may not be effective in removing microbial contaminants.

The presence of GFD and PSM markers suggests the occurrence of fecal contamination in tank water samples from avian and possum hosts. This study has established a potential link between the degradation of the microbial quality of tank water with avian and possum feces. There are several factors that may have contributed to the avian and possum fecal contamination in tank water samples. Therefore, maintenance of good roof and gutter hygiene and elimination of overhanging tree branches, blocking off possum access points by placing timber or mesh, or placing fake predators, like owls and hawks, on the roofs may be a clever way to trick birds into staying off the roof.

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