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Occurrence of Intestinal and Extraintestinal Virulence Genes in *Escherichia coli* Isolates from Rainwater Tanks in Southeast Queensland, Australia▽†

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In this study, 200 *Escherichia coli* isolates from 22 rainwater tank samples in Southeast Queensland, Australia, were tested for the presence of 20 virulence genes (VGs) associated with intestinal and extraintestinal pathotypes. In addition, *E. coli* isolates were also classified into phylogenetic groups based on the detection of the *chuA*, *yjaA*, and TSPE4.C2 genes. Of the 22 rainwater tanks, 8 (36%) and 5 (23%) were positive for the *eaeA* (belonging to enteropathogenic *E. coli* [EPEC] and Shiga-toxigenic *E. coli* [STEC]) and ST1 (belonging to enterotoxigenic *E. coli* [ETEC]) genes, respectively. VGs (*cdtB*, *cvaC*, *ibeA*, *kpsMT* allele III, PAI, *papAH*, and *traT*) belonging to extraintestinal pathogenic *E. coli* (ExPEC) were detected in 15 (68%) of the 22 rainwater tanks. Of the 22 samples, 17 (77%) and 11 (50%) contained *E. coli* belonging to phylogenetic groups A and B1, respectively. Similarly, 10 (45%) and 16 (72%) contained *E. coli* belonging to phylogenetic groups B2 and D, respectively. Of the 96 of the 200 strains from 22 tanks that were VG positive, 40 (42%) were carrying a single VG, 36 (37.5%) were carrying two VGs, 17 (18%) were carrying three VGs, and 3 (3%) had four or more VGs. This study reports the presence of multiple VGs in *E. coli* strains belonging to the STEC, EPEC, ETEC, and ExPEC pathotypes in rainwater tanks. The public health risks associated with potentially clinically significant *E. coli* in rainwater tanks should be assessed, as the water is used for drinking and other, nonpotable purposes. It is recommended that rainwater be disinfected using effective treatment procedures such as filtration, UV disinfection, or simply boiling prior to drinking.

Roof-harvested rainwater (RHRW) has been considered a potential source for potable and nonpotable uses in many countries, such as Australia, Canada, Denmark, Germany, India, South Korea, New Zealand, Thailand, and the United States (16, 18, 53). The most significant issue in relation to RHRW use is the potential public health risk associated with the presence (1, 3, 51) of pathogenic microorganisms. Various microorganisms, including human pathogens, could be present in the feces of birds, insects, small mammals, and reptiles. Consequently, fecal matter and other organic debris could be introduced into the tank via roof runoff following rain events. The microbiological quality of RHRW is generally assessed by monitoring fecal indicator bacteria, such as fecal coliforms, *Escherichia coli*, and enterococci, which are commonly found in the guts of warm-blooded animals, including humans. *E. coli* has traditionally been used as an indicator of fecal contamination in RHRW in many countries (1, 44, 49, 52).

RHRW can be contaminated with either traditional fecal indicator microorganisms or pathogens (4). One of the significant limitations of using fecal indicators to assess the microbial quality of water is their often poor correlation with the presence of pathogenic bacteria, protozoa, and viruses (1, 27). There has been little information, however, documenting whether any correlation exists between fecal indicators and pathogens in rainwater tanks. Two research studies reported that there was a poor correlation between fecal indicators and pathogenic microorganisms (2, 48). In these studies, pathogenic bacteria and protozoa were detected in the absence of *E. coli*. This is not surprising, considering that fecal indicator bacteria exhibit survival rates different from those of pathogenic microorganisms. Furthermore, fecal indicators may replicate in external environments and can become naturalized to beach sand, soil, and algae (6, 10, 13, 15, 29).

E. coli is often characterized as a commensal or harmless bacterium (26). However, certain strains of *E. coli* can be pathogenic and responsible for both intestinal and extraintestinal infections (35, 39). It has been reported that feces of some warm-blooded animals may contain high numbers of *E. coli* bacteria carrying virulence genes (VGs) (30). These VGs allow pathogenic *E. coli* to cause a wide array of infections, such as diarrhea, urinary tract infections (UTIs), neonatal meningitis, soft-tissue infections, and bacteremia (5, 14, 34, 35, 39, 41).

Pathogenic *E. coli* strains that are capable of causing diseases in humans and animals can be categorized as intestinal pathogenic *E. coli* (InPEC) or extraintestinal pathogenic *E. coli* (ExPEC) (47). *E. coli* pathotypes that are responsible for intestinal infections are known as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga-toxigenic *E. coli* (STEC), enteroinvasive *E. coli*, enteroaggregative *E. coli*,

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TABLE 1. Characteristics and end uses of rainwater tanks tested in this study

Tank	Location	Size (liters)	Age (yr)	Material	Presence of overhanging trees[c]	Evidence of wildlife droppings on roof[c]	Desludging	End use(s)
T1	Periurban	20,000[a]	2	Galvanized steel	N	Y	Never	Potable, nonpotable
T2	Periurban	20,000	5	Galvanized steel	N	N	4 yr ago	Potable
T5	Periurban	22,500[a]	2	Galvanized steel	N	N	Never	Potable, nonpotable
T6	Periurban	22,500[a]	1	Galvanized steel	N	N	Never	Potable, nonpotable
T8	Periurban	30,000[a]	1	Colorbond	N	N	Never	Potable, nonpotable[b]
T9	Urban	20,000[a]	1	Galvanized steel	N	Y	Never	Potable[b]
T10	Periurban	22,500[a]	3	Polyethylene	Y	N	Never	Potable, nonpotable
T12	Periurban	22,500[a]	1	Galvanized steel	N	N	Never	Potable, nonpotable[b]
T13	Periurban	10,000[a]	2	Galvanized steel	Y	N	Never	Potable, nonpotable[b]
T14	Periurban	20,000[a]	2	Polyethylene	N	N	Never	Potable, nonpotable
T15	Periurban	15,000	3	Colorbond	N	Y	1 yr ago	Potable, nonpotable[b]
T16	Periurban	15,000[a]	1	Galvanized steel	N	N	Never	Potable, nonpotable
T18	Periurban	7,200[a]	2	Stainless steel	N	Y	3 mo ago	Potable, nonpotable[b]
T20	Periurban	20,000[a]	2	Concrete	N	N	Never	Potable, nonpotable
T21	Periurban	22,500[a]	2	Galvanized steel	N	Y	3 mo ago	Potable, nonpotable[b]
T22	Periurban	22,500[a]	2	Stainless steel	N	N	Never	Potable, nonpotable
T23	Periurban	22,000[a]	2	Stainless steel	Y	Y	Never	Potable, nonpotable[b]
T31	Urban	5,000	3	Polyethylene	N	N	1 mo ago	Nonpotable
T32	Urban	20,000	4	Polyethylene	Y	Y	Never	Potable[b]
T33	Urban	10,000	3	Polyethylene	Y	Y	1 yr ago	Nonpotable
T34	Urban	5,000	3	Polyethylene	Y	Y	1 yr ago	Nonpotable
T37	Urban	5,000	3	Polyethylene	Y	Y	Never	Nonpotable
T42	Periurban	15,000	1.5	Polyethylene	N	Y	Never	Nonpotable
T43	Periurban	5,000	7	Polyethylene	N	Y	Never	Potable, nonpotable[b]
T44	Periurban	20,000	10	Polyethylene	Y	Y	Never	Potable, nonpotable[b]
T45	Periurban	1,000	25	Galvanized steel	Y	Y	1 yr ago	Potable
T46	Periurban	5,000	12	Galvanized steel	Y	Y	5 yr ago	Potable[b]
T47	Periurban	10,000[a]	0.5	Polyethylene	Y	Y	Never	Potable, nonpotable
T48	Periurban	12,000	4	Polyethylene	Y	Y	Never	Nonpotable
T49	Periurban	10,000	20	Galvanized steel	N	Y	Never	Potable, nonpotable[b]

[a] First-flush diverter installed.
[b] Under-sink filtration and UV installed.
[c] Y, yes; N, no.

and diffusely adherent *E. coli* (39). These pathotypes contain various combinations of VGs for the attachment and elaboration of hemolysins and enterotoxins (7). ExPEC strains have the special ability to cause extraintestinal infections such as UTIs, neonatal meningitis and sepsis, and wound infections which can lead to serious complications and death (14, 41, 47). ExPEC strains possess VG combinations that are distinct from those of strains that cause intestinal infections.

Furthermore, *E. coli* strains belong to four main phylogenetic groups (A, B1, B2, and D) (12). InPEC and commensal strains belong to phylogenetic groups A and B1 (12). ExPEC strains derive predominantly from phylogenetic group B2 and to a lesser extent from group D (12, 32). Detailed information about InPEC and ExPEC can be found in review articles (32, 35, 39).

Despite increasing evidence that *E. coli* strains from several animal hosts contain VGs and the fact that some have been shown to cause intestinal and extraintestinal diseases in humans (39), none of the studies have determined whether *E. coli* bacteria found in rainwater tanks carry VGs and are potentially able to cause intestinal or extraintestinal infections in humans. The primary aim of this research study was to investigate the presence of 20 VGs associated with the InPEC and ExPEC pathotypes in a collection of *E. coli* isolates from rainwater tank samples collected in Southeast Queensland (SEQ), Australia. This was done to highlight the human health risk associated with potentially pathogenic *E. coli* found in rainwater tanks.

MATERIALS AND METHODS

Survey of rainwater tanks and sampling. In all, 30 rainwater tanks representing seven suburbs in Brisbane and the Gold Coast region in SEQ, Australia, were selected for this study (Table 1). These tanks were located in periurban and urban areas and were selected on the basis of end uses. Among the 30 tanks, the water in 24 was used for both potable and nonpotable purposes and that in the remaining 6 was used for nonpotable purposes. A sanitary survey was undertaken to identify physical characteristics of the RHRW systems such as tank size, tank age, tank material, and factors that may contribute to fecal contamination of the tanks, such as the presence of trees overhanging the roof. The roofs were also surveyed for the presence of possible wildlife fecal contamination. A single water sample was collected from each rainwater tank within 3 to 7 days after a rain event (i.e., >80 mm). Water samples were collected in 2 sterilized 10-liter containers from the outlet tap located close to the base of the tank. Before the tank was sampled, the tap was wiped with 70% ethanol and allowed to run for 30 to 60 s to flush water from the tap. Samples were transported to the laboratory and processed within 2 to 4 h.

Enumeration and isolation of *E. coli*. The membrane filtration method was used to process water samples for bacterial enumeration (54). Serial sample dilutions were made with phosphate-buffered saline, filtered through 0.45-μm-pore-size (47-mm-diameter) nitrocellulose membranes (Millipore, Tokyo, Japan), and placed on modified mTEC agar (Difco, Detroit, MI) for the isolation of *E. coli*. The plates were incubated at 35°C for 2 h to recover stressed cells; this was followed by incubation at 44°C for 22 h (54). For bacterial enumeration, all water samples were tested in triplicate.

DNA extraction and confirmatory test for *E. coli* isolates. Up to 10 *E. coli* isolates were selected from replicate mTEC agar plates, giving a total of 200

TABLE 2. *E. coli* pathotypes and associated VGs tested in this study

Pathotype	VG(s)					
	Adhesins	Toxins[b]	Invasins	Siderophores	Capsule synthesis	Other
STEC	*eaeA*[a]	stx_1, stx_2, *hlyA*[a]				
ETEC		LT1, ST1				
EPEC	*eaeA*[a]	*cdtB*[a], *hlyA*[a]				
ExPEC	*bmaE*, *papG* allele II, *papG* allele III, *papAH*, *papEF*, *focG*	*cdtB*[a], *cvaC*	*ibeA*	*iutA*	*kpsMT* allele III, *kpsMT* allele K1	PAI, *traT*

[a] Gene found in more than one *E. coli* pathotype.
[b] Animal fecal samples were tested for these genes.

E. coli isolates from 22 rainwater tanks. *E. coli* was not isolated from the remaining 8 of 30 tanks. Single, well-isolated colonies were picked from agar plates and inoculated into 1.5-ml screw-cap tubes containing 2 ml nutrient broth (Oxoid, Basingstoke, United Kingdom). The tubes were kept in an incubator shaker at 100 rpm overnight. DNA was extracted from 1 ml of pure culture using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Strains were confirmed as *E. coli* by PCR amplification of the *uidA* gene as described elsewhere (20).

Animal fecal sampling and DNA extraction. Altogether, 40 fecal samples were collected from fresh fecal droppings of brushtail possums ($n = 20$) and various species of birds ($n = 20$) from Brisbane and the Gold Coast region in SEQ. The bird species include plover, wood duckling, blue-faced honey eater, magpie, crow, ibis, seagull, topknot pigeon, crested tern, Pacific baza, fantail cuckoo, rainbow lorikeet, and tawny frogmouth. Up to two samples were collected from each species of bird. All samples were transported to the laboratory, stored at 4°C, and processed within 24 h. DNA was extracted from fresh feces (i.e., 160 to 220 mg) from each individual animal by using the QIAamp Stool DNA kit (Qiagen) and stored at −20°C for further analysis.

Phylogenetic group classification and detection of VGs. Confirmed *E. coli* isolates from rainwater tank samples were tested for phylogenetic groups using multiplex PCR with the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 according to the method described by Clermont et al. (12). All isolates were further tested for the presence of 20 *E. coli* VGs associated with intestinal and extraintestinal diseases. The VGs screened in this study are shown in Table 2. DNA extracted from animal fecal samples was also tested for the presence of seven *E. coli* toxin VGs associated with intestinal and extraintestinal diseases (Table 2). PCR detection of the *uidA* gene (20), phylogenetic group classification (12), and detection of VGs (31, 42, 46) were undertaken using previously published primers. For the sequences of the primers used for the VGs, see Table S2 in the supplemental material.

PCR detection. PCR amplification of VGs associated with InPEC was performed in 25-μl reaction mixtures using SYBR green iQ Supermix (Bio-Rad Laboratories, Richmond, CA). The PCR mixture contained 12.5 μl SuperMix, 300 nM each primer, and 2 μl of template DNA. For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. The PCR was performed using the Bio-Rad iQ5 (Bio-Rad Laboratories). Multiplex PCR amplification of VGs associated with ExPEC was performed as described elsewhere (11). The PCR mixture contained 2.5 μl 10× reaction buffer (Qiagen), 1.5 mM $MgCl_2$, 400 μM deoxynucleoside triphosphates, 600 nM each primer, and 2 μl template DNA. For each PCR experiment, corresponding positive (i.e., target DNA) and negative (sterile water) controls were included. PCRs were conducted using an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). To separate the specific product from nonspecific products, DNA melting curve analysis was performed for VGs associated with InPEC. To detect the amplification product (where necessary), a 3-μl aliquot of the PCR product was visualized by electrophoresis through a 1.5% agarose gel (Progen, Toowong, Queensland, Australia) in 1× TAE buffer (50× TAE is 242 g Tris base and 57.1 ml glacial acetic acid made up to 1 liter with distilled water). Bands were identified by comparison with molecular size markers of 100 bp and a 1-kb ladder (GeneWorks) after staining with ethidium bromide and exposure to UV light. To minimize PCR contamination, DNA extraction, PCR setup, and gel electrophoresis were performed in separate laboratories.

RESULTS

Survey results. The sizes of the tanks ranged from 5,000 to 30,000 liters, and their ages ranged from 1 to 20 years (Table 1). Of the 30 tanks surveyed, 12 (40%) had trees overhanging the roof and 18 (60%) had visible signs of fecal droppings on the roof. Of the 30 tanks, 16 (53%) had a first-flush diverter installed, 13 (43%) treated the water before consumption, and 22 (73%) had never been desludged since installation (i.e., 1 to 20 years ago). Of the 30 tanks, 24 (80%) were used for both potable and nonpotable purposes and the remaining 6 (20%) were used only for nonpotable purposes.

Numbers of *E. coli* bacteria in RHRW. Of the 30 tested samples from rainwater tanks, 22 (73%) were found to be positive for *E. coli*. The number of *E. coli* bacteria in positive samples ranged from 2 ± 0 to 986 ± 61 CFU/100 ml of water. Eight (27%) tanks had <1 CFU of *E. coli*/100 ml of water (Table 3). All eight of these tanks either had first-flush diverters (which reduce the contamination level by passing the first 2 mm of rainfall) installed or were not characterized by either visible signs of fecal droppings or trees overhanging the roof. In 16 (53%) of 30 samples of water used for drinking, the number of *E. coli* CFU exceeded that specified by Australian drinking water guidelines, i.e., 0 CFU/100 ml.

Phylogenetic group classification. Samples from the 22 rainwater tanks that were positive for *E. coli* were further tested for phylogenetic groups. Of the 22 samples, 17 (77%) and 11 (50%) contained *E. coli* belonging to phylogenetic groups A and B1, respectively. Similarly, 10 (45%) and 16 (72%) contained *E. coli* belonging to phylogenetic groups B2 and D, respectively. Of the 200 tested isolates from these 22 tanks, 64 (32%), 32 (16%), 45 (22.5%), and 59 (29.5%) belonged to groups A, B1, B2, and D (Table 3).

Occurrence of InPEC and ExPEC VGs. Of the 20 VGs tested, 10 (50%) genes were detected in 17 (77%) of the 22 rainwater samples that were positive for *E. coli*. These included *eaeA*, ST1, *cdtB*, *cvaC*, *ibeA*, *kpsMT* allele III, *kpsMT* allele K1, PAI, *papAH*, and *traT*. The remaining VGs, *hlyA*, stx_1, stx_2, *bmaE*, *focG*, *iutA*, *papG* allele II, *papG* allele III, and *papEF*, were not detected in any of the 200 tested isolates from 22 rainwater tanks. *eaeA*, belonging to the EPEC and STEC pathotypes, and ST1, belonging to the ETEC pathotype, were detected in 8 (36%) and 5 (23%) of the 22 tanks (Table 4). VGs belonged to ExPEC were detected in 15 (68%) of the 22 tanks. Of the 200 isolates tested, 30 (15%) and 8 (4%) were positive for the *eaeA* and ST1 VGs. Of the ExPEC VGs, *kpsMT* allele III was the most prevalent (17.5%), followed by *papAH* (13.5%), *ibeA* (13%), and *traT* (12.5%).

Of the 30 *eaeA*-positive isolates, 18, 7, 3, and 2 belonged to phylogenetic groups B2, D, A, and B1, respectively. Similarly, of the eight ST1-positive isolates, three, two, two, and one

TABLE 3. Numbers of tested *E. coli* strains from rainwater tanks and their distribution among phylogenetic groups

Tank	Mean no. of *E. coli* CFU/100 ml ± SEM	No. of *E. coli* isolates tested	No. of *E. coli* isolates classified into phylogenetic groups/no. tested (%)			
			A	B1	B2	D
T1	15 ± 1	10			5/10 (50)	5/10 (50)
T2	3 ± 1	10	1/10 (10)		7/10 (70)	2/10 (20)
T5	2 ± 0	4			3/4 (75)	1/4 (25)
T6	226 ± 14	9	2/9 (22)	2/9 (22)	3/9 (33)	2/9 (22)
T8	89 ± 11	9	6/9 (67)	3/9 (33)		
T10	2 ± 1	5	1/5 (20)		1/5 (20)	3/5 (60)
T12	12 ± 2	11			6/11 (56)	5/11 (44)
T14	5 ± 2	8	8/8 (100)			
T15	12 ± 6	10	4/10 (40)	3/10 (30)	1/10 (10)	2/10 (20)
T31	273 ± 61	9	5/9 (56)	4/9 (44)		
T32	2 ± 1	7			7/7 (100)	
T33	6 ± 2	10	2/10 (20)	1/10 (10)		7/10 (70)
T34	56 ± 8	10	2/10 (20)			8/10 (80)
T37	190 ± 20	10	3/10 (30)	6/10 (60)		1/10 (10)
T42	253 ± 35	10	1/10 (10)		7/10 (70)	2/10 (20)
T43	20 ± 7	10	5/10 (50)	3/10 (30)		2/10 (20)
T44	6 ± 2	9			5/9 (56)	4/9 (44)
T45	630 ± 26	10	6/10 (60)	3/10 (30)		1/10 (10)
T46	11 ± 3	10	8/10 (80)	2/10 (20)		
T47	366 ± 57	9	5/9 (56)	4/9 (44)		
T48	986 ± 61	10	4/10 (40)	1/10 (10)		5/10 (50)
T49	11 ± 5	10	1/10 (10)			9/10 (90)
Total[a]		200	64/200 (32)	32/200 (16)	45/200 (22.5)	59/200 (29.5)

[a] $n = 22$.

belonged to groups D, B2, B1, and A, respectively. In all, 79 isolates were positive for one or more ExPEC VGs. Of these, 44 (56%) belonged to group D and 28 (35%) belonged to group B2. The remaining five isolates (6%) belonged to either group B1 or A. Of the 96 VG-positive isolates, 40 (42%) were carrying a single VG, 36 (37.5%) were carrying two VGs, 17 (18%) were carrying three VGs, and 3 (3%) had four or more VGs.

TABLE 4. Occurrence of VGs in *E. coli* isolated from rainwater tanks

Tank	No. of *E. coli* isolates harboring VGs/no. tested (%)	Distribution of VGs in *E. coli* isolated from tank water (%)									
		STEC, *eaeA*	ETEC, ST1	ExPEC							
				cdtB	*cvaC*	*ibeA*	*kpsMT* allele III	*kpsMT* allele K1	PAI	*papAH*	*traT*
T1	9/10 (90)			3/10 (30)		6/10 (60)	6/10 (60)			6/10 (6)	
T2	9/10 (90)	7/10 (70)				1/10 (10)	5/10 (50)			1/10 (10)	1/10 (10)
T5	4/4 (100)	4/4 (100)									
T6	2/9 (22)							2/9 (22)			
T8	0/9 (0)										
T10	4/5 (80)					1/5 (20)	1/5 (20)			4/5 (80)	
T12	5/11 (45)			3/11 (27)		1/11 (9)	1/11 (9)			3/11 (33)	1/11 (9)
T14	0/8 (0)										
T15	3/10 (30)	3/10 (30)									
T31	0/9 (0)										
T32	6/7 (86)					2/7 (29)	5/7 (71)		2/7 (29)	5/7 (71)	
T33	6/10 (60)			1/10 (10)			6/10 (60)				4/10 (40)
T34	8/10 (80)			8/10 (80)			7/10 (70)				
T37	0/10 (0)										
T42	9/10 (90)	6/10 (60)	1/10 (10)			4/10 (40)	1/10 (10)		1/10 (10)	2/10 (20)	8/10 (80)
T43	3/10 (30)	3/10 (30)									
T44	9/9 (100)	3/9 (33)		1/9 (11)	1/9 (11)	3/9 (33)	1/9 (11)			1/9 (11)	7/9 (77)
T45	1/10 (10)										1/10 (10)
T46	1/10 (10)		1/10 (10)								
T47	3/9 (33)	2/9 (22)	1/9 (11)	2/9 (22)							
T48	7/10 (70)		1/10 (10)	1/10 (10)		4/10 (40)	1/10 (10)			3/10 (30)	2/10 (20)
T49	7/10 (70)	2/10 (20)	4/10 (40)			4/10 (40)	1/10 (10)	3/10 (30)	1/10 (10)	2/10 (20)	1/10 (10)
Total[a]	96/200 (48)	30/200 (15)	8/200 (4)	19/200 (9.5)	1/200 (0.5)	26/100 (13)	35/200 (17.5)	5/200 (2.5)	4/200 (2)	27/200 (13.5)	25/200 (12.5)

[a] $n = 22$.

Occurrence of InPEC and ExPEC toxin VGs in animal fecal samples. Of the 20 possum fecal samples tested, 1 (5%) and 2 (10%) were positive for the stx_2 and *cdtB* genes, respectively. The stx_1, *hlyA*, LT1, ST1, and *cvaC* genes were not detected in any of the possum fecal samples. Of the 20 bird fecal samples, 3 (15%), 1 (5%), 1 (5%), and 3 (15%) were positive for the stx_2, stx_1 ST1, and *cdtB* toxin genes, respectively. The *hlyA*, LT1, and *cvaC* genes were not detected in any of the bird fecal samples tested.

DISCUSSION

E. coli has been historically used as an indicator of water quality, and guidelines have been developed to monitor the microbial quality of water for potable and recreational uses based on the numbers of *E. coli* CFU (40, 55). For most guidelines, this entails nondetectability (i.e., 0 CFU of *E. coli*/100 ml of water) (40, 55). Of the 30 rainwater samples tested, 22 (73%) exceeded the Australian drinking water guideline of 0 *E. coli* CFU/100 ml of water (40), indicating fecal contamination. It has to be noted that the occurrence of fecal pollution in most of the rainwater tanks was expected in samples collected after rainfall events. Roof runoff would have introduced into the tanks fecal matter present on the roof or in gutters and originating from birds, possums, lizards, or other animals that have access to the roof. Several other research studies have reported high detection frequencies and numbers of *E. coli* bacteria in RHRW (1, 44, 49, 52). However, to our knowledge, none of the studies in the research literature investigated the presence of InPEC and ExPEC VGs in water samples from rainwater tanks.

E. coli pathotypes such as STEC, EPEC, ETEC, and ExPEC and their associated VGs were selected in this study, as other studies have reported the presence of these pathotypes, originating from sewage and/or animal feces, in environmental water (23, 24, 25, 38, 46). Overall, 30 (15%) of 200 isolates from eight tanks were positive for the *eaeA* gene, which belongs to STEC or EPEC (25). However, none of the isolates was positive for the stx_1, stx_2, and *hlyA* genes. *E. coli* O157:H7 is known to harbor stx_1, stx_2, or both toxin genes along with the *eaeA* gene (9, 37, 43). All *eaeA*-positive strains were further tested for the presence of the *bfpA* (bundle-forming pilus) gene, which indicates whether the strains are EPEC or not (25). None of these strains were positive for the *bfpA* gene, and therefore these strains can be classified as atypical EPEC (aEPEC), as suggested by Kaper et al. (35). The role of aEPEC is not well understood in terms of the pathogenesis of InPEC. It has been suggested that these strains likely derive from STEC bacteria that have lost bacteriophages carrying the stx_1 or stx_2 gene or strains that have lost the *bfpA*-encoding EAF plasmid (8, 36).

Overall, eight (4%) isolates from five tanks were positive for the ST1 toxin gene associated with the ETEC pathotype. However, none of the isolates was positive for the LT1 toxin gene. ETEC strains carrying both the LT and ST genes or the ST gene along with colonization factors which allow the organisms to readily colonize the small intestine have been shown to cause relatively more severe disease than those carrying the LT gene alone (45). A number of ExPEC-associated VGs with the potential to cause UTIs, neonatal meningitis, and neonatal sepsis were also detected in this study. Among these genes, the *cdtB* toxin gene was detected in 19 (9.5%) isolates from seven tanks. *E. coli* carrying the *cdtB* gene is known to cause extraintestinal, as well as intestinal, infections (33). Furthermore, 26 (13%) isolates from nine tanks were positive for the *ibeA* gene, which is associated with neonatal meningitis and endothelial cell invasion and has an essential role in UTIs (21). ExPEC-associated VGs such as *kpsMT* allele III, *kpsMT* allele K1, PAI, *papAH*, and *traT*, which cause UTIs or septicemia, have also been detected in this study (31). However, none of the isolates tested contained complete sets of *pap* family genes. Overall, ExPEC-associated genes were more prevalent than STEC- or ETEC-associated genes. The high prevalence of ExPEC in environmental waters has been reported previously (23, 24, 38).

The presence of toxin genes in *E. coli* isolates from rainwater tanks can be alarming, as toxins are the most obvious virulence factors found in pathogenic *E. coli*. Although STEC-associated VGs stx_1 and stx_2 were not detected, these genes have been detected in *E. coli* isolated from wild birds (28, 50). The presence of the ST1 gene in *E. coli* isolates from pigeon and goose feces has been reported in the literature (19, 50). It is acknowledged that in this study, we did not investigate the presence of toxin genes in *E. coli* isolates from possum and bird fecal samples. However, we investigated the presence of toxin genes in total fecal DNA isolated from a small number of possum ($n = 20$) and bird ($n = 20$) fecal samples. A number of samples were positive for the stx_1, stx_2, ST1, and *cdtB* toxin genes. Ewers et al. (19) reported the presence of ExPEC-associated *pap*, *ibeA*, and *traT* along with other VGs in the feces of chickens and wild birds. Avian isolates carrying VGs are known as avian pathogenic *E. coli* (APEC). These VGs were detected in this study, suggesting the presence of APEC strains in RHRW tanks. The sanitary survey indicated the presence of fecal droppings on 60% of the roofs surveyed and trees overhanging 40% of the roofs surveyed. Indeed, in this study, a number of possum and bird fecal DNA samples were positive for *E. coli* toxin VGs.

All of the *E. coli* isolates collected from rainwater tank samples were also tested for phylogenetic classification, as proposed by Clermont et al. (12). In this study, most commensal *E. coli* strains (i.e., those not carrying any VGs) belonged to group A or B1. STEC- and EPEC-associated isolates harboring the *eaeA* gene belonged mainly to the B2 group. *E. coli* pathotypes such as STEC also belonged to the A or B1 group, whereas EPEC strains were distributed across all four groups (17, 33). Of the eight ST1-positive isolates, four belonged to group A or B1 and the remaining four belonged to group D. Most of the ExPEC isolates in this study (74 of 79) belonged to either group B2 or D. The remaining five isolates belonged to either group B1 or A, although these isolates were carrying ExPEC-associated VGs. It has to be noted that classification of *E. coli* into phylogenetic groups may vary and change over time due to factors such as dietary habits and a better level of hygiene (17).

PCR analysis of VGs in this study has revealed that *E. coli* isolates from rainwater tanks may carry one or more VGs. However, the mere possession of a single VG or a few VGs does not endow a strain with pathogenic status unless that strain has acquired the appropriate combination of VGs to cause disease (22). VG acquisitions, however, can reequip such

isolates with the potential to develop into pathogens (11). Of the 94 isolates carrying VGs, 58% were carrying two or more VGs. For example, two isolates from T49 were carrying both the ST1 and *eaeA* genes and therefore can be considered potentially clinically significant (45) For the *E. coli* isolates carrying one or more VGs, see Table S1 in the supplemental material.

In conclusion, this study reports the presence of InPEC and ExPEC VGs associated with *E. coli* strains in rainwater tank samples in SEQ, Australia. The presence of multiple VGs in *E. coli* strains may pose a health risk mainly to users who use RHRW for drinking. The significance of these strains in terms of health implications needs to be assessed by comparing strains from rainwater tanks and feces of householders drinking rainwater. In addition, better characterization of these strains from rainwater and stool samples from the householders is required by serotyping, genotyping, or testing for multiple-drug resistance. In view of this, it is recommended that rainwater be disinfected using effective treatment procedures such as filtration, UV disinfection, or simply boiling the water prior to drinking.

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REFERENCES

1. **Ahmed, W., F. Huygens, A. Goonetilleke, and T. Gardner.** 2008. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. Appl. Environ. Microbiol. **74:**5490–5496.
2. **Ahmed, W., A. Goonetilleke, and T. Gardner.** 2010a. Implications of faecal indicator bacteria for the microbiological assessment of roof-harvested rainwater quality in Southeast Queensland, Australia. Can. J. Microbiol. **56:**471–479.
3. **Ahmed, W., A. Vieritz, A. Goonetilleke, and T. Gardner.** 2010. Health risk from the use of roof-harvested rainwater in Southeast Queensland, Australia, as potable or nonpotable water, determined using quantitative microbial risk assessment. Appl. Environ. Microbiol. **76:**7382–7391.
4. **Ahmed, W., T. Gardner, and S. Toze.** 2011. Microbiological quality of roof-harvested rainwater and health risks: a review. J. Environ. Qual. **40:**13–21.
5. **Anastasi, E. M., et al.** 2010. Prevalence and persistence of *Escherichia coli* strains with uropathogenic virulence characteristics in sewage treatment plants. Appl. Environ. Microbiol. **76:**5882–5886.
6. **Anderson, K. L., J. E. Whitlock, and V. J. Harwood.** 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Appl. Environ. Microbiol. **71:**3041–3048.
7. **Bertin, Y., K. Boukhors, N. Pradel, V. Livrelli, and C. Martin.** 2001. stx_2 subtyping of Shiga toxin-producing *Escherichia coli* isolated from cattle in France: detection of a new stx_2 subtype and correlation with additional virulence factors. J. Clin. Microbiol. **39:**3060–3065.
8. **Bielaszewska, M., et al.** 2008. Shiga toxin-negative attaching and effacing *Escherichia coli*: distinct clinical associations with bacterial phylogeny and virulence traits and inferred in-host pathogen evolution. Clin. Infect. Dis. **47:**208–217.
9. **Boerling, P., S. A. McEwen, J. B. Wilson, R. P. Gyles, and C. L. Gyles.** 1999. Association between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. **37:**497–503.
10. **Byappanahalli, M. N., R. L. Whitman, D. A. Shivley, M. J. Sadowskyand, S. Ishii.** 2006. Population structure, persistence, and seasonality of autochthonous *Escherichia coli* in temperate, coastal forest soil from a Great Lakes watershed. Environ. Microbiol. **8:**504–513.
11. **Chapman, T. A., et al.** 2006. Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. Appl. Environ. Microbiol. **72:**4782–4795.
12. **Clermont, O., S. Bonacorsi, and E. Bingen.** 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl. Environ. Microbiol. **66:**4555–4558.
13. **Davies, C. M., J. A. Long, M. Donald, and N. J. Ashbolt.** 1995. Survival of fecal microorganisms in marine and freshwater sediments. Appl. Environ. Microbiol. **61:**1888–1896.
14. **de Louvois, J.** 1994. Acute bacterial meningitis in the newborn. J. Antimicrob. Chemother. **34**(Suppl. A)**:**61–67.
15. **Desmarais, T. R., H. M. Solo-Gabriele, and C. J. Palmer.** 2002. Influence of soil on fecal indicator organisms in a tidally influenced subtropical environment. Appl. Environ. Microbiol. **68:**1165–1172.
16. **Despins, C., K. Farahbakhsh, and C. Leidl.** 2009. Assessment of rainwater quality from rainwater harvesting systems in Ontario, Canada. J. Water Supply Res. Technol. AQUA **58:**117–134.
17. **Escobar-Páramo, P., et al.** 2004. Large-scale population structure of human commensal *Escherichia coli* isolates. Appl. Environ. Microbiol. **70:**5698–5700.
18. **Evans, C. A., P. J. Coombes, and R. H. Dunstan.** 2006. Wind, rain and bacteria: the effect of weather or the microbial composition of roof-harvested rainwater. Water Res. **40:**37–44.
19. **Ewers, C., T. Janssen, S. Kiessling, H. C. Phillip, and L. H. Vieler.** 2005. Rapid detection of virulence-associated genes in avian pathogenic *Escherichia coli* by multiplex polymerase chain reaction. Avian Dis. **49:**269–273.
20. **Frahm, E., and U. Obst.** 2003. Application of the fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. J. Microbiol. Methods **52:**123–131.
21. **Germon, P., et al.** 2005. *ibeA*, a virulence factor of avian pathogenic *E. coli*. Microbiology **151:**1179–1186.
22. **Gilmore, M. S., and J. J. Ferretti.** 2003. The thin line between gut commensal and pathogen. Science **299:**1999–2002.
23. **Hamelin, K., et al.** 2006. A virulence and antimicrobial resistance DNA microarray detects a high frequency of virulence genes in *Escherichia coli* from Great Lakes recreational waters. Appl. Environ. Microbiol. **72:**4200–4206.
24. **Hamelin, K., et al.** 2007. Occurrence of virulence and antimicrobial resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within St. Clair River and Detroit River areas. Appl. Environ. Microbiol. **73:**477–484.
25. **Hamilton, M. J., A. Z. Hadi, J. F. Griffith, S. Ishii, and M. J. Sadowsky.** 2010. Large scale analysis of virulence genes in *Escherichia coli* strains isolated from Avalon Bay, CA. Water Res. **44:**5463–5473.
26. **Hartl, D. L., and D. E. Dykhuizen.** 1984. The population genetics of *Escherichia coli*. Annu. Rev. Genet. **18:**31–68.
27. **Hörman, A., et al.** 2004. *Campylobacter* spp., *Giardia* spp., *Cryptosporidum* spp., noroviruses, and indicator organisms in surface water in Southwestern Finland, 2000-2001. Appl. Environ. Microbiol. **70:**87–95.
28. **Hughes, L. A., et al.** 2009. Risk factors for the occurrence of *Escherichia coli* virulence genes *eae*, stx_1 and stx_2 in wild bird populations. Epidemiol. Infect. **137:**1574–1582.
29. **Ishii, S., D. L. Hansen, R. E. Hicks, and M. J. Sadowsky.** 2007. Beach sand and sediments are temporal sinks and sources of *Escherichia coli* in Lake Superior. Environ. Sci. Technol. **41:**2203–2209.
30. **Ishii, S., K. P. Meyer, and M. J. Sadowsky.** 2007. Relationship between phylogenetic groups, genotypic cultures, and virulence gene profiles of *Escherichia coli* strains from diverse human and animal sources. Appl. Environ. Microbiol. **73:**5703–5710.
31. **Johnson, J. R., and A. L. Stell.** 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis. **181:**261–272.
32. **Johnson, J. R., and T. A. Russo.** 2002. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E. coli*". J. Lab. Clin. Med. **139:**155–162.
33. **Johnson, J. R., and E. Oswald.** 2002. Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in The Netherlands. J. Infect. Dis. **185:**774–784.
34. **Johnson, J. R., M. A. Kuskowski, T. T. O'Bryan, and J. N. Maslow.** 2002. Epidemiological correlates of virulence genotype and phylogenetic background among *Escherichia coli* blood isolates from adults with diverse sources bacteremia. J. Infect. Dis. **185:**1439–1447.
35. **Kaper, J. B., J. P. Nataro, and H. L. T. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140.
36. **Law, D.** 2000. Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. J. Appl. Microbiol. **88:**729–745.
37. **Mead, P. S., and P. M. Griffin.** 1998. *Escherichia coli* O157:H7. Lancet **352:**1207–1212.
38. **Mühldorfer, I., et al.** 1996. Characterization of *Escherichia coli* strains isolated from environmental water habitats and from stool samples of healthy volunteers. Res. Microbiol. **147:**625–635.
39. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11:**142–201.
40. **Natural Resource Management Ministerial Council, Environment Protection and Heritage Council, and National Health and Medical Research Council.** 2008. Australian guidelines for water recycling: managing health and environmental risks (phase 2). Augmentation of drinking water supplies. National Environment Protection Council, Canberra, Australia. http://www.ephc.gov.au/taxonomy/term/39.
41. **Orskov, I., and F. Orskov.** 1985. *Escherichia coli* in extra-intestinal infections. J. Hyg. (Lond.) **95:**551–575.
42. **Paton, A. W., and J. C. Paton.** 2002. Direct detection and characterization of

Shiga toxigenic *Escherichia coli* by multiplex PCR for stx_1, stx_2, *eaeA*, *ehxA*, and *saa*. J. Clin. Microbiol. **40:**271–274.
43. **Philips, C. A.** 1999. The epidemiology, detection and control of *Escherichia coli* O157. J. Sci. Food Agric. **79:**1367–1381.
44. **Pinfold, J. V., N. J. Horan, W. Wirojanagud, and D. Mara.** 1993. The bacteriological quality of rainjar water in rural northeast Thailand. Water Res. **27:**297–302.
45. **Qadri, F., A-M. Svennerholm, A. S. G. Faruque, and R. B. Sack.** 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. Clin. Microbiol. Rev. **18:**465–483.
46. **Ram, S., P. Vajpayee, and R. Shanker.** 2007. Prevalence of multi-antimicrobial agent resistant Shiga toxin and enterotoxin producing *Escherichia coli* in surface waters of river Ganga. Environ. Sci. Technol. **41:**7383–7388.
47. **Russo, T. A., and J. R. Johnson.** 2000. A proposal for an inclusive designation for extraintestinal pathogenic *Escherichia coli*: ExPEC. J. Infect. Dis. **181:**1753–1754.
48. **Savill, M. G., et al.** 2001. Enumeration of Campylobacter in New Zealand recreational and drinking waters. J. Appl. Microbiol. **91:**38–46.
49. **Sazakli, E., A. Alexopolous, and M. Leotsinidis.** 2007. Rainwater harvesting quality assessment and utilization in Kefalonia Island, Greece. Water Res. **41:**2039–2047.
50. **Silva, V. L., J. R. Nicoli, T. C. Nascimento, and C. G. Diniz.** 2009. Diarrheagenic *Escherichia coli* strains recovered from urban pigeons (*Columba livia*) in Brazil and their antimicrobial susceptibility patterns. Curr. Microbiol. **59:**302–308.
51. **Simmons, G., V. Hope, G. Lewis, J. Whitmore, and G. Wanzhen.** 2001. Contamination of potable roof-harvested rainwater in Auckland, New Zealand. Water Res. **35:**1518–1524.
52. **Spinks, J., S. Phillips, P. Robinson, and P. Van Buynder.** 2006. Bushfires and tank rainwater quality: a cause for concern? J. Water Health **4:**21–28.
53. **Uba, B. N., and O. Aghogho.** 2000. Rainwater quality from different roof-catchments in the Port Harcourt District, Rivers State, Nigeria. J. Water Supply Res. Technol. AQUA **49:**281–288.
54. **U.S. Environmental Protection Agency.** 2002. Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC). EPA/821/R-02/023. U.S. Environmental Protection Agency Office of Water, Washington, DC.
55. **WHO.** 2004. Guidelines for drinking water quality, 3rd ed. World Health Organization, Geneva, Switzerland.