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Prevalence of Virulence Genes Associated with Pathogenic *Escherichia coli* Strains Isolated from Domestically Harvested Rainwater during Low- and High-Rainfall Periods

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The possible health risks associated with the consumption of harvested rainwater remains one of the major obstacles hampering its large-scale implementation in water limited countries such as South Africa. Rainwater tank samples collected on eight occasions during the low- and high-rainfall periods (March to August 2012) in Kleinmond, South Africa, were monitored for the presence of virulence genes associated with *Escherichia coli*. The identity of presumptive *E. coli* isolates in rainwater samples collected from 10 domestic rainwater harvesting (DRWH) tanks throughout the sampling period was confirmed through universal 16S rRNA PCR with subsequent sequencing and phylogenetic analysis. Species-specific primers were also used to routinely screen for the virulent genes, *aggR*, *stx*, *eae*, and *ipaH* found in enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroinvasive *E. coli*, respectively, in the rainwater samples. Of the 92 *E. coli* strains isolated from the rainwater using culture based techniques, 6% were presumptively positively identified as *E. coli* O157:H7 using 16S rRNA. Furthermore, virulent pathogenic *E. coli* genes were detected in 3% (EPEC and EHEC) and 16% (EAEC) of the 80 rainwater samples collected during the sampling period from the 10 DRWH tanks. This study thus contributes valuable information to the limited data available regarding the ongoing prevalence of virulent pathotypes of *E. coli* in harvested rainwater during a longitudinal study in a high-population-density, periurban setting.

Rainwater harvesting is practiced worldwide and refers to the collection and storage of rainwater runoff for domestic and agricultural use (1–3). Domestic rainwater harvesting (DRWH) can also potentially serve as a safe and inexpensive water supply for households and worldwide, countries such as Australia, Greece, and Bermuda are making extensive use of this water source (4–6). However, possible health risks associated with the consumption of harvested rainwater remains one of the major obstacles hampering the large-scale implementation of DRWH, since microbial and chemical contaminants have previously been detected in rainwater tanks (7–9).

Depending on the atmospheric pollution, the harvesting method and the storage of rainwater, the quality of harvested rainwater may fluctuate and be compromised due to various pollutants, for example, bird or animal droppings (10). Whereas an increase in the use of stored rainwater is generally observed during the high-rainfall seasons, it should be noted that correspondingly increased levels of microbial pollution are also experienced during rainy seasons as large numbers of microorganisms are washed from various point- and nonpoint pollution sites, such as rooftops. Water then acts as an inert carrier of the pathogenic microorganisms, such as protozoa, helminths, viruses, and bacteria, and humans can become infected with diseases such as diarrhea, skin irritations, typhoid, and respiratory disorders from the microbially contaminated water sources (11). *Escherichia coli*, a general indicator of water quality, can, however, also be pathogenic and is divided into five classes—enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC)—based on the specific virulence genes present. All of the strains are associated with watery diarrhea, but some strains are associated with bloody diarrhea (EHEC), vomiting (ETEC), and fever (ETEC and EIEC) (12).

The frequency of detecting *E. coli* strains during low- and high-rainfall seasons in DRWH tanks in Kleinmond, a coastal town in South Africa, was monitored. The detection of the virulence genes associated with the four pathogenic *E. coli* types (EAEC, EHEC, EPEC, and EIEC) was examined in particular. Spearman rank-order correlations were also determined between *E. coli*, rainfall, temperature, and pH and between chemical compounds, e.g., metal ions.

MATERIALS AND METHODS

Sample collection and general rainwater analysis. The Department of Science and Technology commissioned the Council of Science and Industrial Research (CSIR) to investigate technologies that will improve the sustainability and quality of low-income subsidized housing in South Africa (13). Consequently, 411 pilot-scale houses (40 m^2) were constructed in Kleinmond, Western Cape, with each of the houses provided with a DRWH tank. From a cluster of 411 houses, 10 houses were selected for sampling rainwater during the study period (March to August 2012) and for the *E. coli* enumeration and identification. In addition, 29 houses (including the 10 for *E. coli* analysis) were selected for the statistical correlation studies. The vertical rainwater tanks, made of polyethylene, had a capacity of 2,000 liters. There were no obstacles obstructing the roofs, i.e.,

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TABLE 1 Primer sequences used in this study for the identification and detection of *E. coli*[a]

E. coli strain	Primer		Target gene	Product size (bp)
	Name	Seqeunce (5′–3′)		
EAEC	AggRKs1	GTATACACAAAAGAAGGAAGC	*aggR*	254
	AggRkas2	ACAGAATCGTCAGCATCAGC		
EHEC	VTcomU	GAGCGAAATAATTTATATGTG	*stx*	518
	Vtcomd	TGATGATGGCAATTCAGTAT		
EPEC	SK1	CCCGAATTCGGCACAAGCATAAGC	*eae*	881
	SK2	CCCGGATCCGTCTCGCCAGTATTCG		
EIEC	IpaIII	GTTCCTTGACCGCCTTTCCGATACCGTC	*ipaH*	619
	IpaIV	GCCGGTCAGCCACCCTCTGAGAGTAC		

[a] See reference 24.

trees or electrical power lines, and no first flush diverters were installed to eliminate the first flush of debris from the roof surface into the tanks. Sampling was initially conducted every 3 weeks (March to May 2012) and thereafter 1 to 4 days after a rain event (June to August 2012).

For microbial and chemical analysis water samples were collected in 2-liter sterile polypropylene bottles that were rinsed with tap water and sterilized with 70% ethanol. After collection, the samples were stored on ice to maintain a temperature below 4°C during transportation. In total, eight sampling sessions were conducted with data on the total rainfall recorded for each month obtained from the South African Weather Services (Pretoria, South Africa; personal communication).

Membrane filtration was also used to enumerate *E. coli*, and the procedure was performed in duplicate within 4 h of sampling. For sampling sessions 1 and 2, undiluted samples were filtered. From sampling session 3 a 1:4 dilution was made of each sample in duplicate. The method consisted of filtering 100 ml (a 25-ml rainwater sample plus 75 ml of sterile-distilled water) of each sample through a sterile GN-6 Metricel S-Pack membrane disc filter (Pall Life Sciences, Ann Arbor, MI) with a pore size of 0.45 μm and a diameter of 47 mm. The filters were then incubated on m-Endo Agar (Merck) at 35 ± 2°C for 18 to 24 h (15). The membrane filtration results were utilized for the enumeration of *E. coli* and selection of isolates.

For each of the DRWH tanks, an undiluted and a diluted (10^{-1}) rainwater sample were spread plated onto membrane lactose glucuronide agar (MLGA [Oxoid]; 35 ± 2°C for 18 to 24 h) to isolate *E. coli*. ChromoCult coliform agar (CCA [Merck]; Biolab, Wadeville, Gauteng) was used to obtain *E. coli* numbers, and the plates were also incubated at 35 ± 2°C for 18 to 24 h. The CCA counts were used for statistical purposes only, whereas isolates of *E. coli* were obtained from CCA and MLGA for the further selection of *E. coli*.

The temperature and pH of the rainwater at the sampling locations were measured using a hand-held mercury thermometer and color-fixed indicator sticks with a pH range of 0 to 14 (Albet, Barcelona, Spain). The concentrations of metals such as aluminum (Al), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), copper (Cu), and zinc (Zn), among others, were determined for the first sampling. Metal concentrations were determined using nitric acid digestion and inductively coupled plasma atomic emission spectrometry. All chemical analyses were performed at the Central Analytical Facility, Stellenbosch University. High-performance ion chromatography was used to determine the concentration of anions, such as chloride (Cl), nitrate (NO_3), and sulfate (SO_4), during the first sampling.

Molecular analysis of *E. coli*. (i) Isolation of *E. coli* and genomic DNA extractions. Typical *E. coli* isolates from 10 randomly selected rainwater tanks (within the cluster of 29 tanks monitored) were selected from ChromoCult coliform agar, m-Endo agar, and MLGA agar, which specifically selects for the growth of *E. coli* and suppresses the growth of other enteric species. These isolates were then subjected to the indole, methyl red, Voges-Proskauer, and citrate test (IMViC test) for the further selection of *E. coli* strains (16).

After purification and IMViC test analysis, single colonies obtained from nutrient agar were inoculated into Luria-Bertani broth (Merck) and were grown at 37°C for 12 h. Cells were harvested from 2 ml of the cell suspension by centrifuging at 16,000 × *g* for 10 min and discarding the supernatant. Genomic DNA was then isolated according to the manufacturer's instructions using the ZR soil microbe DNA miniprep kit (Zymo Research).

(ii) Universal 16S rRNA identification of *E. coli* isolates. With the use of conventional PCR, presumptive positive *E. coli* isolated from rainwater samples were identified. The universal 16S rRNA primers Fdd2 (5′-CCG GATCCGTCGACAGAGTTTGATCITGGCTCAG-3′) and Rpp2 (5′-CC AAGCTTCTAGACGGITACCTTGTTACGACTT-3′) and the PCR conditions adapted from Rawlings (17) were used to amplify the 16S rRNA conserved region (1,600 bp) of each isolate. The PCR conditions were optimized by increasing the annealing temperature from 54 to 59°C.

Phylogenetic trees of the results obtained for the 16S rRNA *E. coli* sequences were constructed to observe whether nonpathogenic and pathogenic strains of *E. coli* clustered together and how this varied between certain sampling sessions (18). Most similar type species with 97% similarity (<3% diversity) to the sequences of isolates were designated as the same species. The 16S rRNA sequences were aligned using CLUSTAL X (19, 20). The evolutionary distances for each 16S rRNA were also calculated by the neighbor-joining method with maximum composite likelihood model by 1,000 replicates and phylogenetic trees were created by using MEGA (21). All positions containing gaps and missing data were eliminated from the data set using complete deletion option.

(iii) Screening for pathogenic *E. coli* genes in rainwater samples. In addition to extracting DNA from each presumptive *E. coli* isolate, total DNA was also extracted from rainwater samples collected from 10 tanks. To extract total DNA from the water samples, a modified version of the boiling method proposed by Watterworth et al. (22) was used. Eight hundred milliliters of each sample was filtered through a sterile GN-6 Metricel S-Pack membrane disc filter (Pall Life Sciences) with a pore size of 0.45 μm and a diameter of 47 mm. The filters were then processed for DNA extraction as outlined in Ndlovu et al. (23).

The species-specific primers adopted from Toma et al. (24) that were used for the detection of the pathogenic *E. coli* strains (EPEC, EIEC, EHEC, and EAEC) in the rainwater samples are indicated in Table 1. All of the positive-control strains (EPEC B170, EIEC ATCC 43892, EHEC O157:H7, and EAEC 3591-87) utilized in the present study were obtained from the Cape Peninsula University of Technology, Bellville, South Africa. The PCR conditions and reagents utilized were adapted from the Ndlovu et al. (23) protocol as outlined in Table 2. The PCR conditions were also optimized by increasing the annealing temperature from 52 to 54°C.

TABLE 2 PCR conditions and reagents used to detect pathogenic *E. coli* in DNA extracted from water samples[a]

PCR reagent (concn)	Volume (μl)				Final concn
	EAEC	EHEC	EPEC	EIEC	
Buffer (5×)	12	12	12	12	1×
$MgCl_2$ (25 mM)	6	6	6	6	2.5 mM
AggRKs1 (10 μM)	1				0.16 μM
AggRkas2(10 μM)	1				
VTcomU (10 μM)		1.5			0.25 μM
Vtcomd (10 μM)		1.5			
SK1 (10 μM)			0.75		0.125 μM
SK2 (10 μM)			0.75		
IpaIII (10 μM)				0.6	0.1 μM
IpaIV (10 μM)				0.6	
Deoxynucleoside triphosphate (10 mM)	1.2	1.2	1.2	1.2	0.2 mM
GoTaq polymerase (5 U/μl)	0.5	0.5	0.5	0.5	2.5 U
Template DNA	5	5	5	5	
Distilled H_2O	33.3	32.3	33.8	34.1	
Final vol	60	60	60	60	

[a] Based on reference 23. The PCR conditions were as follows: stage 1, initial denaturing at 95°C for 2 min; stage 2, denaturing at 95°C for 1 min, primer annealing at 54°C for 1 min, and elongation at 72°C for 1 min; and stage 3, final elongation step at 72°C for 10 min.

Statistical analysis. The data obtained from the microbial and physico-chemical analysis of the collected rainwater samples was assessed by using the statistical software package Statistica version 11.0 (Stat Soft, Inc., Tulsa, OK). In each data set, analysis of the residuals revealed that the data were not normally distributed, which pointed to the requirement for the Spearman rank order correlation as a nonparametric correlation technique to test the significance of the data set. In this test, a restricted maximum-likelihood solution (REML) with type III decomposition was performed on all data recorded to establish whether there was variation between sampling sessions. Once it was established that variation was indeed present, variance estimation, precision, and comparison (VEPACK) analysis was performed. However, the data for pH, temperature, and average rainfall were set as fixed variables, and time and sample were set as grouping variables. The data pairs that showed significant differences were subsequently further analyzed using the least-squares difference (LSD) test and probabilities for *post hoc* pairwise comparisons. In all hypothesis tests, a significant level of 5% was used as the standard (25). In all tests, a P value of <0.05 was considered statistically significant.

RESULTS

Prevalence of *E. coli* during low- and high-rainfall periods. On average, the membrane filtration *E. coli* counts for the 10 DRWH tanks ranged from 0 (for isolated tanks) to 2.5×10^2 CFU 100 ml^{-1} over the entire sampling period. Throughout samplings 1 to 4, representing the low-rainfall period (16.8 mm in March to 30.6 mm in May), numerous tanks had no *E. coli* present, with 44% of the DRWH tanks sampled exceeding the drinking water guidelines as stipulated by the Department of Water Affairs (DWAF) (26) and the Australian Drinking Water Guidelines (ADWG) (27). In comparison, for samplings 5 to 8, where higher-rainfall events (74.7 mm in June to 198.1 mm in August) were recorded, 79% of the tanks sampled had *E. coli* counts significantly ($P < 0.05$) exceeding the standards. Overall, for all of the rainwater samples collected from the domestic rainwater harvesting tanks (1 to 29) for samplings 1 to 8, utilizing membrane filtration, 62% of the *E. coli* counts recorded exceeded the recommended values as stipulated by the DWAF (26) and the ADWG (27).

Identification of *E. coli* isolates based on 16S rRNA analysis. Of the 170 presumptive positive *E. coli* plate isolates identified throughout the sampling period from 10 DRWH tanks, 71% (121 strains) yielded a positive IMViC analysis and exhibited *E. coli* characteristics. The identity of these presumptive *E. coli* strains was then confirmed through universal 16S rRNA PCR with subsequent sequencing. Sequencing revealed that 76% (92 strains) of the IMViC-positive isolates were *E. coli* strains, while the remaining isolates belonged to the genera *Enterobacter*, *Serratia*, *Shigella*, and *Proteus*.

The phylogeny of the representative organisms according to GenBank for samplings 1, 3, and 6 (greater *E. coli* strain diversity identified during these sampling times) were analyzed using the neighbor-joining algorithm in CLUSTAL X (the phylogenetic tree for only sampling 3 is presented). Among the 92 *E. coli* isolates that were identified using GenBank, 4% were positively identified as ETEC isolates, which contain the heat-stable toxin (ST1). These presumptively positive ETEC isolates were identified predominantly in samplings 1 and 3. In addition, 6% of the total *E. coli* isolates were identified as *E. coli* O157:H7 (samplings 1, 2, and 3).

Three ETEC strains were identified during sampling 1 (results not shown), with two of the strains clustering together with a 99% statistical support. The *E. coli* strains most frequently isolated and identified during samplings 2 (results not shown) and 3 (Fig. 1)

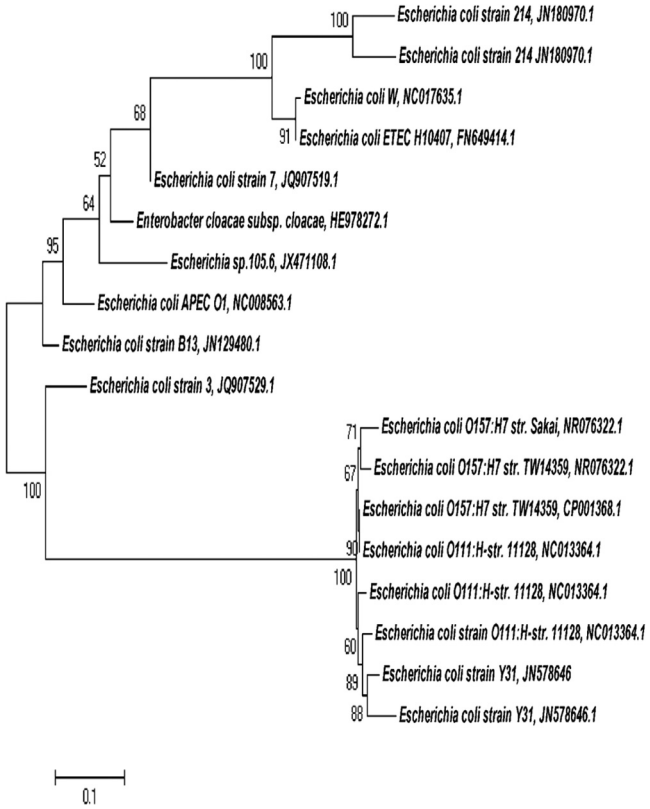

FIG 1 Unrooted phylogenetic tree of organisms isolated during sampling 3. The tree of isolates was constructed using the neighbor-joining algorithm of CLUSTAL X. Bootstrap values are shown at the nodes, with the accession numbers indicated after the strain name.

TABLE 3 Presence of *E. coli* harboring toxin genes in rainwater samples

Sampling	% of rainwater tanks contaminated with *E. coli* toxin gene(s)	Sample(s) positive for[a]: EPEC *eae*	EIEC *ipaH*	EHEC *stx*	EAEC *aggR*
4	10	ND	ND	ND	9
6	20	5	ND	ND	4
7	50	ND	ND	ND	2, 6, 8, 7, 10
8	90	1	ND	9, 10	1, 2, 3, 5, 6, 10

[a] ND, not detected. The sampling tank numbers where particular genes were isolated are indicated.

were the *E. coli* O157:H7 strains WAB1892 and TW14359, respectively, and *E. coli* O111:H− strain 11128 (sampling 3). From Fig. 1, it is clear that these two strains—*E. coli* O157:H7 strain TW14359 and *E. coli* O111:H− strain 11128—cluster together. *E. coli* O111:H− and O157:H7 are both main serotypes that produce Shiga toxins, which could explain their homology (28). *E. coli* O111:H− was also isolated during samplings 6, 7, and 8 (results not shown). From the 16S rRNA PCR analysis, the majority of the pathogenic strains of *E. coli* were isolated during samplings 1, 2, and 3, where the temperatures were higher and the total rainfall was low (16.8 mm in March to 30.6 mm in May). In addition, an avian isolate, *E. coli* APECO1, was identified during the third sampling period. The dominant strain identified in sampling 6 was *E. coli* DSM 1103 (data not shown). The results indicated that the two genera, *Shigella* and *Escherichia*, also grouped together, and this could be attributed to the fact that they are closely genetically related (29).

Presence of pathogenic *E. coli* genes in rainwater samples. Species-specific primers were used to screen for the virulent genes, *aggR*, *stx*, *eae*, and *ipaH* found in EAEC, EHEC, EPEC, and EIEC) respectively, in the rainwater samples collected from the 10 DRWH tanks. These PCR products were sequenced to confirm the amplification of the gene, and BLAST results that showed a homology of ≥98% was confirmed as the amplification of the correct gene. The virulence genes that were amplified in the various tanks throughout the sampling period are presented in Tables 1 and 3 and, as indicated, no pathogenic *E. coli* genes were detected during sampling sessions 1, 2, 3, and 5.

In the present study, the *aggR* gene, associated with the EAEC strain, was detected in 10% of the rainwater tanks during samplings 4 and 6, respectively (Table 3). Detection of the *aggR* gene was also confirmed in 50 and 60% of the DRWH tanks during sampling sessions 7 and 8. The occurrence of EPEC (intimin gene [*eae*]) and EHEC (Shiga toxin gene [*stx*]) was much lower than that of EAEC throughout the sampling period but was confirmed in samplings 6 and 8 (Table 3) to be 10% for EPEC (sampling 6) and 20% for EHEC (sampling 8).

Spearman rank-order correlations between *E. coli* and physicochemical properties of rainwater. For the statistical correlation studies, the results for 29 houses (including the 10 for *E. coli* analysis) were analyzed. Significant correlations ($P < 0.00$) were noted between *E. coli* counts, utilizing the spread plate technique (CCA) and the following parameters: *E. coli* counts utilizing membrane filtration ($r = -0.21$) (m-Endo) and rainfall ($r = -0.36$). It should also be noted that in the present study the pH ($r = -0.655$) and temperature ($r = -0.705$) of the rainwater samples showed a significant negative correlation to the average rainfall recorded. After analyzing all of the data, we found that the REML and the fixed-effect tests showed significant variation ($P = 0.00$, $F = 8.968$) for *E. coli* counts (utilizing the spread plate technique) among the eight sampling sessions. The LSD test then showed that the highest mean difference ($2{,}465.52 \pm 449.86$; $P = 0.00$) was recorded between sampling sessions 1 and 7, whereas the lowest mean difference, with the least amount of variation, was recorded between sampling sessions 2 and 6 (68.97 ± 449.8644; $P = 0.878$).

DISCUSSION

E. coli is included as a specific indicator organism of the level of fecal pollution from warm-blooded animals (4, 30), and the presence of *E. coli* in harvested rainwater samples was thus monitored in the low- and high-rainfall seasons in DRWH tanks in Kleinmond, South Africa. Enumeration techniques indicated that the *E. coli* counts recorded exceeded the recommended value of 0 CFU 100 ml^{-1} (26, 27) in 62% of the rainwater samples collected throughout the study period. In comparison to the low-rainfall season, a significant increase ($P < 0.05$) in the numbers of *E. coli* were also observed in harvested rainwater samples collected during the high-rainfall season (samplings 5 [74.7 mm] to 8 [198.1 mm]). The percentages of *E. coli* detected in similar studies conducted on the microbial quality of rainwater also varied from 33% (9) to 63% (31) to 79% (32) of the samples. However, these studies were performed 1 to 4 days after a rain event and, to our knowledge, only the study performed by Sazakli et al. (4) investigated the effect of seasonal variation on the quality of harvested rainwater, with the highest *E. coli* numbers recorded in autumn, which is the start of the rainfall season. A negative correlation was also recorded between pH and temperature and rainfall, which implies that *E. coli* numbers increased during samplings 5 to 8 when the rainfall was high and the pH (samplings 5 [pH 5.4] to 8 [pH 5.1]) and temperature (sampling five [14.8°C] to eight [13.3°C]) were low.

Ninety-two *E. coli* strains were subsequently isolated by culture techniques from the 10 rainwater tanks sampled in the Kleinmond Housing Scheme during the study period with a preliminary identification performed using 16S rRNA analyses. Nonpathogenic and pathogenic *E. coli* strains, including *E. coli* ETEC H10407, *E. coli* O157:H7, and the avian isolate *E. coli* APECO1, were isolated from numerous DRWH samples during the study. The ETEC strain identified predominantly in samplings 1 and 3 causes watery diarrhea, and in a few cases it can also cause vomiting and fever. Infants and travelers in underdeveloped countries are the most susceptible to this *E. coli* strain (33). *E. coli* O157:H7, isolated during samplings 2 and 3, is the predominant serotype of EHEC that produces the Shiga-like toxin that is responsible for causing watery and subsequent bloody diarrhea in humans. In addition, the avian isolate *E. coli* APECO1, isolated during sampling 3, most likely originated from bird feces and may contain many virulent genes belonging to extraintestinal pathogenic *E. coli*.

The whole DNA isolated from the harvested rainwater samples directly was then screened for the presence of the virulence genes, *aggR*, *stx*, *eae*, and *ipaH* associated with EAEC, EHEC, EPEC, and EIEC, respectively. The most prevalent gene detected during the study was *aggR*, which is associated with EAEC strains. This virulence gene was detected during samplings 4, 6, 7, and 8 at 10, 10, 50, and 60%, respectively. The presence of EAEC harboring the *aggR* gene in these tanks can have a severe impact on the health of children and adults. In addition, although the occurrence of EPEC

(intimin gene [*eae*]) and EHEC (Shiga toxin gene [*stx*]) was much lower than EAEC throughout the sampling period, the presence of these genes were also confirmed in samplings 6 and 8 (Table 3) at 10%, respectively, for EPEC and at 20% for EHEC (sampling 8). The detection of the intimin *eae* gene in EPEC could indicate the presence of Shiga-toxin producing *E. coli*, since this gene is found in more than one pathotype (34). The ingestion of EPEC, however, causes watery diarrhea that is associated with vomiting and low fever (12), whereas EHEC represents one of the most pathogenic *E. coli* groups that have the ability to cause bloody diarrhea, with little or no fever. If the disease is left untreated, it can lead to hemorrhagic colitis and can progress to hemolytic-uremic syndrome, affecting the kidney and liver. The virulence gene *ipaH*, associated with EIEC strains, was not detected in any of the rainwater samples collected during the sampling period.

It is important to note that during samplings 1, 2, 3, and 5 no virulence genes were detected in the rainwater tanks. Ten percent of the rainwater samples collected from the tanks during sampling 4 were contaminated with virulence genes, whereas sampling 8 had the highest number of tanks (90%) contaminated. Overall, during samplings 4, 6, 7, and 8, we found that 10, 20, 50, and 90%, respectively, of the tanks were contaminated with pathogenic *E. coli* toxin genes. It was also evident that the virulent genes were most often detected during sampling 8, where the highest average rainfall of 198 mm was recorded. Upon comparison, these results contradict the 16S rRNA analysis, where pathogenic *E. coli* (O157:H7 and ETEC) were most frequently identified from culturable *E. coli* strains during samplings 1 to 3. It can thus clearly be seen that 16S rRNA may not be not suitable for distinguishing between strains in a species, as was also confirmed by Lukjancenko et al. (29). The results of the present study also clearly indicate that the *E. coli* plate counts and the presence of *E. coli* virulence genes were lower in the low-rainfall season (samplings 1 to 4) and significantly increased ($P < 0.05$) in the high-rainfall season (samplings 5 to 8). The feces of warm-blooded animals could serve as a possible source of *E. coli* contamination in the rainwater tanks, since warm-blooded animals have been shown to carry a high number of pathogenic *E. coli* strains in their intestines (35). Ahmed et al. (36) conducted a study in Southeast Queensland, Australia, and successfully isolated *E. coli* species with identical biochemical phenotype profiles from rainwater tanks, as well as from bird and possum feces found on roof surfaces where the tanks were installed. Other sources of rainwater contamination include "leaf debris and organic material washed into the tank, animals, insects, and birds that have drowned in the water and breeding mosquitoes" (37). A recent study conducted in Singapore confirmed the presence of pathogenic microorganisms, including *E. coli*, in airborne particulate matter, which can serve as another source of contamination (38). A gravel road also runs along the outside of the settlement, and with cars passing by on a regular basis, dust could be disturbed and settle on the roof surface, resulting in contamination.

Although studied serotypes of *E. coli* are well recognized as being of zoonotic origin, these *E. coli* strains have been shown to infect humans and are therefore a health risk if present in a water source. For example, EHEC strains are known to be of zoonotic origin, with animals such as cattle being the reservoir for human infections (39). Therefore, even though microbial source tracking with the use of sewage-associated markers such as *Bacteroides* sp. strain HF183 (40), could be used to determine whether fecal contamination is of human origin, the focus of the present study was the detection of pathogenic *E. coli* serotypes present in harvested rainwater that could have potential health risks. Additional studies have also been conducted that focused on the detection of bacterial pathogens associated with human diseases, such as *Salmonella* and *Shigella* spp. in the harvested rainwater collected from DRWH in Kleinmond, South Africa.

In conclusion, *E. coli* counts, the isolation and identification of *E. coli* strains, and the detection of virulence genes associated with EAEC, EHEC, and EPEC strains in rainwater samples clearly indicate that the roof harvested rainwater is not suitable for potable purposes, with limited domestic application, since this water source could be associated with public health risks and human disease. Similar observations were made worldwide, where it was concluded that harvested rainwater is not suitable for drinking purposes without prior treatment (4, 7, 8, 41). In addition, rain allows pathogens from animal droppings and other organic debris to be flushed into the tanks via the gutters and, as *E. coli* counts and toxin genes were increased during the higher-rainfall period, the feces of birds, insects, and mammals could have filtered from the roof tops into the rainwater tank, which would have resulted in fecal contamination of the water source. However, harvested rainwater is an important, alternative water source that could be utilized if the technology is applied in the correct manner and if the rainwater is treated before it is used for drinking and certain domestic purposes. Therefore, promoting the correct use and maintenance of DRWH tanks could improve the microbial and chemical quality of the harvested rainwater (42; P. H. Dobrowsky, D. Mannel, M. De Kwaadsteniet, H. Prozesky, W. Khan, and T. E. Cloete, unpublished data). Future research is thus focusing on the implementation of point-of-use systems, such as nanofiltration, solar pasteurization, etc., for the treatment of harvested rainwater sources. In addition, solar pasteurization and filtration systems are currently being optimized and analyzed at the pilot plant-scale level, and the efficiency and durability of these systems in improving the microbial quality of harvested rainwater are being investigated.

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REFERENCES

1. **Gould J.** 1999. Contributions relating to rainwater harvesting: thematic review IV.3. Assessment of water supply options. World Commission on Dams. http://www.scribd.com/doc/34611820/Contributions-Relating-to-Rainwater-Harvesting.
2. **Mwenge Kahinda JM, Lillie ESB, Taigbenu AE, Taute M, Boroto RJ.** 2008. Developing suitability maps for rainwater harvesting in South Africa. Phys. Chem. Earth **33:**788–799. http://dx.doi.org/10.1016/j.pce.2008.06.047.
3. **Helmreich B, Horn H.** 2009. Opportunities in rainwater harvesting. Desalination **248:**118–124. http://dx.doi.org/10.1016/j.desal.2008.05.046.
4. **Sazakli E, Alexopoulos A, Leotsinidis M.** 2007. Rainwater harvesting, quality assessment and utilization in Kefalonia Island, Greece. Water Res. **41:**2039–2047. http://dx.doi.org/10.1016/j.watres.2007.01.037.
5. **Ahmed W, Gardner T, Toze S.** 2011. Microbiological quality of roof-harvested rainwater and health risks: a review. J. Environ. Qual. **40:**13–21. http://dx.doi.org/10.2134/jeq2010.0345.

6. **Lévesque B, Pereg D, Watkinson E, Maguire JS, Bissonnette L, Gingras S, Rouja P, Bergeron MG, Dewailly E.** 2008. Assessment of microbiological quality of drinking water from household tanks in Bermuda. Can. J. Microbiol. **54:**495–500. http://dx.doi.org/10.1139/W08-038.
7. **Yaziz MI, Gunting H, Sapari N, Ghazali AW.** 1989. Variations in rainwater quality from roof catchments. Water Res. **23:**761–765. http://dx.doi.org/10.1016/0043-1354(89)90211-X.
8. **Zhu K, Zhang L, Hart W, Liu M, Chen H.** 2004. Quality issues in harvested rainwater in arid and semi-arid Loess Plateau of northern China. J. Arid Environ. **57:**487–505. http://dx.doi.org/10.1016/S0140-1963(03)00118-6.
9. **Spinks J, Phillips S, Robinson P, Van Buynder P.** 2006. Bushfires and tank rainwater quality: a cause for concern? J. Water Health **4:**21–28.
10. **Pathak N, Heijnen H.** 2006. Health and hygiene aspects of rainwater for drinking, p 363–369. 32nd WEDC International Conference, Colombo, Sri Lanka.
11. **Kong RYC, Mak MMH, Wu RSS.** 2009. DNA technologies for monitoring waterborne pathogens: a revolution in water pollution monitoring. Ocean Coastal Manag. **52:**355–358. http://dx.doi.org/10.1016/j.ocecoaman.2009.04.011.
12. **Todar K.** 2008. Pathogenic *Escherichia coli*. *In* Todar K (ed), Todar's online textbook of bacteriology. Department of Bacteriology, University of Wisconsin, Madison, WI. http://www.textbookofbacteriology.net/e.coli_4.html.
13. **CSIR.** 2010. CSIR expertise and technology employed for improving low income subsidized housing, p 24. *In* Sustainable implementation, monitoring, and evaluation. National Survey Delivery Support, April 2011. CSIR, Pretoria, South Africa.
14. Reference deleted.
15. **U.S. Environmental Protection Agency.** 2009. Analytical methods approved for drinking water compliance monitoring under the total coliform rule: national primary drinking water regulations. U.S. Environmental Protection Agency Office of Water, Washington, DC.
16. **Harley JP, Prescott ML.** 1993. Laboratory exercises in microbiology. William C. Brown Publishers, New York, NY.
17. **Rawlings DE.** 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferooxidans*, *Thiobacillus thiooxidans*, and *Leptospirillum ferooxidans* strains in leaching environments, p 9–17. *In* Jerez CA, Vargas T, Toledo H, Wiertz JV (ed), Biohydrometallurgical processing. University of Chile Press, Santiago, Chile.
18. **Lee J, Lee CS, Hugunin KM, Maute CJ, Dysko RC.** 2010. Bacteria from drinking water supply and their fate in gastrointestinal tracts of germ-free mice: a phylogenetic comparison study. Water Res. **44:**5050–5058. http://dx.doi.org/10.1016/j.watres.2010.07.027.
19. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgens DG.** 2007. CLUSTAL W and CLUSTAL X version 2.0. Bioinformatics **23:**2947–2948. http://dx.doi.org/10.1093/bioinformatics/btm404.
20. **Tamura K, Nei M, Kumar S.** 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. **101:**11030–11035. http://dx.doi.org/10.1073/pnas.0404206101.
21. **Tamura K, Dudley J, Nei M, Kumar S.** 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599. http://dx.doi.org/10.1093/molbev/msm092.
22. **Watterworth L, Topp E, Schraft H, Leung KT.** 2005. Multiplex PCR-DNA probe assay for the detection of pathogenic *Escherichia coli*. J. Microbiol. Methods **60:**93–105. http://dx.doi.org/10.1016/j.mimet.2004.08.016.
23. **Ndlovu TM, Le Roux M, Khan W, Khan S.** 2013. Comparison of diagnostic tools and molecular based techniques for the rapid identification of *Escherichia coli* and coliforms in contaminated river water. M.S. thesis. Cape Peninsula University of Technology, Cape Town, South Africa.
24. **Toma C, Lu Y, Higa N, Nakasone N, Chinen I, Baschkier A, Rivas M, Iwanaga M.** 2003. Multiplex PCR assay for identification of human diarrheagenic *Escherichia coli*. J. Clin. Microbiol. **41:**2669–2671. http://dx.doi.org/10.1128/JCM.41.6.2669-2671.2003.
25. **Dunn OJ, Clark VA.** 1974. Applied statistics: analysis of variance and regression, 2nd ed. John Wiley & Sons, London, United Kingdom.
26. **Department of Water Affairs and Forestry.** 1996. South African water quality guidelines, 2nd ed, vol 1. Domestic water use. CSIR Environmental Services, Pretoria, South Africa.
27. **NHMRC/NRMMC.** 2011. Australian drinking water guidelines paper. 6. National water quality management strategy. National Health and Medical Research Council/National Resource Management Ministerial Council, Commonwealth of Australia, Canberra, Australia.
28. **Mainil J.** 1999. Shiga/verocytotoxins and Shiga/verotoxigenic *Escherichia coli* in animals. Vet. Res. **30:**235–257.
29. **Lukjancenko O, Wassenaar TM, Ussery DW.** 2010. Comparison of 61 sequenced *Escherichia coli* Genomes. Microb. Ecol. **60:**708–720. http://dx.doi.org/10.1007/s00248-010-9717-3.
30. **Rompré A, Servais P, Baudart J, De-Roubin MR, Laurent P.** 2002. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J. Microbiol. Methods **49:**31–54. http://dx.doi.org/10.1016/S0167-7012(01)00351-7.
31. **Ahmed W, Hodgers L, Sidhu JPS, Toze S.** 2012. Fecal indicators and zoonotic pathogens in household drinking taps fed from rainwater tanks in Southeast Queensland, Australia. Appl. Environ. Microbiol. **78:**219–226. http://dx.doi.org/10.1128/AEM.06554-11.
32. **Albrechtsen HJ.** 2002. Microbiological investigations of rainwater and graywater collected for toilet flushing. Water Sci. Technol. **46:**311–316.
33. **Tobias J, Vutukuru SR.** 2012. Simple and rapid multiplex PCR for identification of the main human diarrheagenic *Escherichia coli*. Microbiol. Res. **167:**564–570. http://dx.doi.org/10.1016/j.micres.2011.11.006.
34. **Ahmed W, Hodgers L, Masters N, Sidhu JPS, Katouli M, Toze S.** 2011. Occurrence of intestinal and extraintestinal virulence genes in *Escherichia coli* isolates from rainwater tanks in Southeast Queensland, Australia. Appl. Environ. Microbiol. **77:**7394–7400. http://dx.doi.org/10.1128/AEM.06047-11.
35. **Ishii N, Nakahigashi K, Baba T, Robert M, Soga T, Kanai A, Tomita M.** 2007. Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. Science **316:**593–597. http://dx.doi.org/10.1126/science.1132067.
36. **Ahmed W, Sidhu JPS, Toze S.** 2012. An attempt to identify the likely sources of *Escherichia coli* harboring toxin genes in rainwater tanks. Environ. Sci. Technol. **46:**5193–5197. http://dx.doi.org/10.1021/es300292y.
37. **Worm J, Van Hattum T.** 2006. Rainwater harvesting for domestic use. Agromisa Foundation/Digigrafi, Wageningen, Netherlands.
38. **Kaushik R, Balasubramanian R, De La Cruz AA.** 2012. Influence of air quality on the composition of microbial pathogens in fresh rainwater. Appl. Environ. Microbiol. **78:**2813–2818. http://dx.doi.org/10.1128/AEM.07695-11.
39. **Caprioli A, Morabito S, Brugere H, Oswald E.** 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Res. **36:**289–311. http://dx.doi.org/10.1051/vetres:2005002.
40. **Sidhu JPS, Ahmed W, Gernjak W, Aryal R, McGarthy D, Palmer A, Kolotelo P, Toze S.** 2013. Sewage pollution in urban stormwater runoff as evident from the widespread presence of multiple microbial and chemical source tracking markers. Sci. Total Environ. **463:**488–496.
41. **Nevondo TS, Cloete TE.** 1999. Bacterial and chemical quality of water supply in the Dertig village settlement. Water S. Africa **25:**215–220.
42. **Mannel D, Prozesky H.** 2012. Domestic rainwater harvesting: survey of perceptions of users in Kleinmond, project K5_2124_3. Water Research Commission, Pretoria, South Africa.