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Prevalence and Persistence of *Escherichia coli* Strains with Uropathogenic Virulence Characteristics in Sewage Treatment Plants[▽]

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We investigated the prevalence and persistence of *Escherichia coli* strains in four sewage treatment plants (STPs) in a subtropical region of Queensland, Australia. In all, 264 *E. coli* strains were typed using a high-resolution biochemical fingerprinting method and grouped into either a single or a common biochemical phenotype (S-BPT and C-BPT, respectively). These strains were also tested for their phylogenetic groups and 12 virulence genes associated with intestinal and extraintestinal *E. coli* strains. Comparison of BPTs at various treatment stages indicated that certain BPTs were found in two or all treatment stages. These BPTs constituted the highest proportion of *E. coli* strains in each STP and belonged mainly to phylogenetic group B2 and, to a lesser extent, group D. No virulence genes associated with intestinal *E. coli* were found among the strains, but 157 (59.5%) strains belonging to 14 C-BPTs carried one or more virulence genes associated with uropathogenic strains. Of these, 120 (76.4%) strains belonged to seven persistent C-BPTs and were found in all four STPs. Our results indicate that certain clonal groups of *E. coli* with virulence characteristics of uropathogenic strains can survive the treatment processes of STPs. These strains were common to all STPs and constituted the highest proportion of the strains in different treatment tanks of each STP.

Community sewage treatment plants (STPs) receive waste from diverse sources, including residential, industrial, and recreational facilities (31). Waste generated from these facilities contains the liquid and fecal discharges of humans and animals, household wastes, industry-specific materials, and storm water runoff (31). These materials are treated through primary, secondary, and tertiary sedimentation processes (18). Following these processes, effluent is normally clear and thus often recycled for nonpotable use (20), with excess water released into receiving waterways. However, due to possible malfunctions or poor management of wastewater systems (1), effluent containing pathogenic bacteria can be discharged into receiving waterways (11, 34). It has been speculated that waters contaminated with feces are a great risk to human health, as they are likely to contain human-specific enteric pathogens, including *Salmonella* spp. (30), *Shigella* spp. (10), enteroviruses (12), hepatitis A virus (13), and pathogenic *Escherichia coli* (30).

E. coli, while widely used as an indicator bacterium (30, 35), can actually be pathogenic and be responsible for both intestinal and extraintestinal diseases (16). Intestinal pathogenic strains of *E. coli* are rarely encountered in the fecal flora of healthy hosts. Extraintestinal pathogenic *E. coli* (ExPEC) strains commonly cause infections of any organ or anatomical site (28). The ability of these pathogenic bacteria to cause disease is due to their acquisition of specialized virulence factors, which commensal *E. coli* strains typically lack. These specialized virulence factors allow them to cause a broad spectrum of diseases (17, 28), such as gastroenteritis (34), diarrhea (16), urinary tract infections and meningitis (29), and soft tissue infections and bacteremia (28). *E. coli* strains belong to four main phylogenetic groups (A, B1, B2, and D) (2), with pathogenic strains belonging mostly to phylogenetic group B2 and, to a lesser extent, group D. Another phylogenetic group (group E) has also been identified; however, it is uncommon and is not widely used (5).

Presently, chlorination is an extremely widespread practice aimed at reducing the pathogen load in the final effluent to levels low enough to ensure that the organisms will not cause disease when the wastewater is discharged (31). Despite this, some pathogenic strains of *E. coli* may survive to become a significant public health risk (14, 35). The aim of this study was to investigate the presence and survival of these pathogenic *E. coli* strains during the treatment processes of four community STPs with different capacities in South East Queensland, Australia.

MATERIALS AND METHODS

Sewage treatment plants and sample collection. Four STPs that employ the activated sludge process were sampled between May 2006 and October 2007. Samples were collected in 50-ml centrifuge tubes from secondary sedimentation effluent, plant effluent, and final lagoon effluent from STPs 1 to 3 and from secondary sedimentation effluent (composite stages 1 to 5), two plant effluent stages (stages 1 to 3 and 5), and final lagoon effluent from STP 4. These STPs service populations equivalent to 60,000 (STP 1), 300,000 (STP 2), 100,000 (STP 3), and 170,000 (STP 4). The average times for water to pass through the treatment process, i.e., the hydraulic residence times, varied among the STPs and

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ranged between 20 and 30 h. Samples were transferred on ice to the laboratory, where they were processed within 24 h of collection by dilution (if necessary) and filtration through 0.45-μm membrane filters (Millipore, Bedford, MA) and placed on m-FC (Oxoid, Basingstoke, United Kingdom) agar plates. The plates were then incubated at 44.5°C for 24 h, and from each sample, up to 28 colonies (where possible) suspected of being *E. coli* were saved in nutrient broth (Oxoid) containing 20% (vol/vol) glycerol (Pronalys) and stored at −80°C for further analysis. In all, 367 isolates were saved.

DNA extraction and *E. coli* confirmation. Chromosomal DNA was extracted using a genomic DNA extraction kit (blood/bacterial/cultured cells) (Real Biotech Corporation, Taiwan) and 100 miniprep kits, with minor adaptations. Briefly, cells were harvested from 3-h nutrient broth (Oxoid) cultures, centrifuged for 1 min at 14,000 rpm, resuspended in 200 μl of GT buffer (Real Biotech Corporation), and lysed with 200 μl of GB buffer (Real Biotech) for 10 min. Cells were treated with 200 μl of absolute ethanol (96 to 100%), washed in a wash buffer twice, and centrifuged at 8,000 rpm for 2 min. The GD column (Real Biotech) was dried using a 14,000-rpm centrifugation step for 3 min. DNA was eluted using 100 μl of elution buffer and centrifuged at 14,000 rpm for 60 s to elute the purified DNA. The purified DNA was stored at −20°C.

Strains were confirmed as *E. coli* by PCR amplification of the universal stress protein (*uspA*) gene according to Chen and Griffiths (4). The reaction mixture included a master mix of 1,730 μl filter-sterilized, autoclaved Milli-Q water, 250 μl 10× PCR buffer (Bioline), 100 μl deoxynucleoside triphosphate (dNTP; 10 mM) (Fisher Biotech), 150 μl $MgCl_2$ (50 mM) (Bioline), 25 μl of forward and reverse *uspA* primers (50 μM) (Invitrogen), 20 μl *Taq* polymerase (Bioline), and 2.0 μl of purified bacterial DNA. The PCR was performed under the following conditions: denaturation for 5 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C; and a final extension step of 5 min at 72°C.

The primer sequences used were (forward) 5′-CCGATACGCTGCCAATCA GT-3′ and (reverse) 5′-ACGCAGACCGTAGGCCAGAT-3′, which are specific to the *uspA* gene expressed in *E. coli* K-12 strain W3110 (24), generating an 884-bp fragment. Amplified PCR products were electrophoresed on 2% agarose (Amresco, Astral Scientific) gels in 0.6× Tris base-EDTA (TBE) buffer and subsequently stained with ethidium bromide. In all, 265 strains were confirmed as *E. coli* using this method. These strains were then typed using a biochemical fingerprinting method and tested for their phylogenetic groups.

Biochemical fingerprinting. A biochemical fingerprinting method (PhPlate system; PhPlate AB, Stockholm, Sweden) was used to type *E. coli* strains. In this study we used PhP-RE plates, specifically designed to type *E. coli* strains. Briefly, *E. coli* colonies were suspended in the first well of each row, containing 325 μl of growth medium, comprised of 0.011% (wt/vol) bromothymol blue and 1% (wt/vol) proteose peptone (Bactus AB, Blackaby Diagnostics). Aliquots of 25 μl of bacterial suspensions were transferred into each of the other 11 wells, containing 150 μl of growth medium. Plates were then incubated at 37°C and read at intervals of 7, 24, and 48 h. Images of plates at corresponding times were scanned using an HP Scanjet 4890 scanner. After the final reading of plate images, the mean of the absorbance values from all individual readings was calculated for each reagent, creating the biochemical fingerprint for each isolate (19). Similarity among the isolates was calculated as a correlation coefficient, and strains showing an identity (ID) level of >0.965 were regarded as identical and assigned to the same biochemical phenotype (BPT). The ID level of the system was established based on the reproducibility of results after 60 isolates were tested in duplicate. Isolates showing similarity to each other above the ID level were regarded as identical and assigned to the same BPT. BPTs with more than one isolate were termed common BPTs (C-BPTs), and those with a single isolate were termed single BPTs (S-BPTs). Diversity among the isolates was calculated using Simpson's index of diversity.

All data, including calculations of correlations and coefficients, were handled and diversity indices were determined using PhPlate software version 4002 (PhPlate AB, Stockholm, Sweden).

Phylogenetic grouping and testing for virulence genes. All confirmed *E. coli* strains were tested for phylogenetic groups using multiplex PCR with the *chuA* and *yjaA* genes and the DNA fragment TSPE4.C2 according to Clermont et al. (5).

Representative C-BPTs (n = 25) from all four STPs were tested for the presence of 12 virulence genes associated with intestinal and extraintestinal *E. coli* strains using a series of three multiplex and five uniplex PCR sets as previously described (3). The virulence genes included *papC*, *papAH*, *papEF*, *iroN* from *E. coli* ($iroN_{E.\ coli}$), *cnf1*, *hlyA*, *eltA*, *estII*, *ipaH*, *eaeA*, stx_1, and stx_2. The PCR protocol for the genes *papC*, $iroN_{E.\ coli}$, *cnf1*, *papAH*, *papEF*, and *hlyA* was modified to the following conditions: 25 cycles of denaturation for 30 s at 94°C, 25 cycles of 30 s at 63°C, 25 cycles of 3 min at 68°C (1 min for $iroN_{E.\ coli}$ and the *cnf1* primers), and a final extension step of 10 min at 72°C. The uniplex-PCR mixture for the *papC*, *hlyA*, $iroN_{E.\ coli}$, and *cnf1* virulence genes consisted of 2.5 μl 10× reaction buffer (Bioline) (3 μl for $iroN_{E.\ coli}$), 1.25 μl 50 mM $MgCl_2$ (Bioline) (1.0 μl for *papC* and $iroN_{E.\ coli}$), 5.0 μl 2 mM dNTPs (Fisher Biotech) (2.5 μl for *papC*), 0.3 μl of each primer (Invitrogen) from a 50-pM/μl stock solution, 0.15 μl *Taq* polymerase (Bioline), 2.0 μl DNA, and sterile Milli-Q to make the final volume 30 μl for *cnf1* and $iroN_{E.\ coli}$ and 25 μl for *hlyA* and *papC*. The reaction mixture for the multiplex procedure for the *papAH* and *papEF* genes had a final volume of 30 μl and consisted of 15.35 μl sterile Milli-Q, 5.0 μl 10× reaction buffer, 1.5 μl 50 mM $MgCl_2$, 5.0 μl 2 mM dNTPs, and 0.3 μl of primers from a 50-pM/μl stock solution. All PCR products were separated electrophoretically as described above.

Statistical analysis. The chi-square test (χ^2) was used to determine the significance of differences between the mean numbers of strains of different BPTs and diversity found in different tanks and STPs.

RESULTS

Of the 367 *E. coli*-like colonies tested, 264 were confirmed as *E. coli* using the *uspA* gene. The percentages of strains confirmed to be *E. coli* in all STPs varied from 72% to 83%.

Biochemical fingerprinting of these isolates showed the presence of both common and single BPTs (C- and S-BPTs, respectively) in each STP and also within different treatment tanks of each plant. Within each STP, *E. coli* strains from the secondary sedimentation tanks, plant effluent tanks, and final lagoon effluent were compared to each other. It was found that some strains originally found in the secondary sedimentation tank were also present in the plant effluent and final lagoon effluent; therefore, these strains were regarded as persistent strains. Of the 162 strains tested in STP 1, 49 (30.2%) belonged to two C-BPTs (i.e., C4 and C5) and were found in all three treatment tanks (Table 1). Some strains were also found in two treatment tanks, and although their numbers were small, they were considered less persistent, mainly due to the fact that they were inconsistently found in two consecutive tanks (Table 1). Phylogenetic grouping of all C-BPTs found in STP 1 (104 isolates) showed that they belonged to either group B2 (n = 56) or group D (n = 48) (Table 1). Similar results were found in other STPs, which contained 7 (STP 2), 2 (STP 3), and 3 (STP 4) C-BPTs. The most common C-BPTs in these STPs consisted of 20 (19%), 10 (29%), and 21 (32%) isolates, respectively.

In all, 42 C-BPTs representing 214 isolates and 50 S-BPTs were identified among the four STPs. Of these, 212 (80%) belonged to phylogenetic groups B2 and D (Table 2). The remaining 20% of isolates that belonged to other phylogenetic groups were spread among 18 BPTs (Table 2). Comparison of these BPTs showed that 23 C-BPTs were found in more than one STP, with 62.4% belonging to phylogenetic group B2 (data not shown).

Representative strains belonging to C-BPTs in all four STPs (n = 25) were tested for the presence of 12 virulence genes found in *E. coli* strains causing intestinal (i.e., *eltA*, *estII*, *ipaH*, *eaeA*, stx_1, and stx_2) or extraintestinal (i.e., *papC*, *papAH*, *papEF*, $iroN_{E.\ coli}$, *cnf1*, *hlyA*) infections. None of the strains carried virulence genes associated with intestinal infections. However, 157 (59.5%) isolates of 14 C-BPTs carried one or more of the virulence genes associated with uropathogenic *E. coli* strains (Table 3). Of these, 120 (76.4%) strains belonged to seven persistent C-BPTs and were found in all four STPs (data not shown).

TABLE 1. Prevalences of C- and S-BPTs and their phylogenetic groups among 162 *Escherichia coli* isolates obtained from three different treatment tanks in STP 1[a]

BPT(s)	No. of isolates found in:			Total no. (%) of isolates in the STP population	Phylogenetic group (no. of isolates)
	Secondary sedimentation tank (n = 77)	Plant effluent tank (n = 31)	Final lagoon effluent (n = 54)		
C1	5		1	6 (3.7)	B2 (6)
C2	2			2 (1.2)	B2 (2)
C3	2		1	3 (1.8)	B2 (3)
C4	6	4	5	15 (9.2)	D (15)
C5	19	3	12	34 (21.0)	B2 (34)
C6	3			3 (1.8)	D (3)
C7	2			2 (1.2)	B2 (2)
C8	6		1	7 (4.3)	D (7)
C9	2			2 (1.2)	D (2)
C10	2			2 (1.2)	D (2)
C11		2		2 (1.2)	B2 (2)
C12		2		2 (1.2)	D (2)
C13		2		2 (1.2)	B2 (2)
C14	1	3		4 (2.5)	D (4)
C15			4	4 (2.5)	B2 (4)
C16			4	4 (2.5)	B2 (4)
C17		1	2	4 (2.5)	B2 (3)
C18			2	2 (1.2)	D (2)
C19		1	1	2 (1.2)	D (2)
C20		1	1	2 (1.2)	D (2)
S1–S7	7			7 (4.3)	A (7)
S8–S11	4			4 (2.5)	B2 (4)
S12–S27	16			16 (9.9)	D (16)
S27–S38		12		12 (7.4)	NT
S39–S58			20	20 (12.3)	NT
DI	0.956	0.970	0.947		

[a] All C- and S-BPTs were numbered consecutively. NT, not tested; DI, diversity index.

DISCUSSION

To our knowledge, this is the first study that investigates the occurrence and persistence of pathogenic *E. coli* strains in different treatment tanks of STPs. Of the 367 *E. coli*-like colonies tested in four STPs, only 264 were confirmed as *E. coli* on the basis of the *uspA* gene. Identification of *E. coli* hardly causes any problems in microbiological laboratories. An additional criterion used for identification of *E. coli* was the test with cellobiose, which is a reagent of the 11 tests in the PhP-RE plates used in this study. Strains that were negative by this test during the biochemical fingerprinting of the isolates were not considered *E. coli.* In our study, three isolates that were *E. coli* as determined by a positive cellobiose test were negative for the *uspA* gene but positive for phylogenetic grouping. To be consistent with identification criteria for *E. coli*, these strains were not included in the study. This, however, may indicate that the sequences of primers used to detect the *uspA* gene may not efficiently represent all groups of *E. coli* strains and, thus, that we may have underestimated the number of *E. coli* strains in our samples.

TABLE 2. Number of C- and S-BPTs of 264 *Escherichia coli* strains found in all four STPs and their distribution among different phylogenetic groups

Location	No. of BPTs (no. of isolates) of phylogenetic group:				Total no. (no. of isolates) of:	
	A	B1	B2	D	C-BPTs	S-BPTs[a]
STP 1	7 (7)		14 (66)	25 (57)	20 (103)	27
STP 2	3 (8)		11 (45)	9 (21)	14 (65)	9
STP 3	1 (1)	2 (11)	2 (2)	3 (7)	4 (17)	4
STP 4	4 (4)	1 (21)	2 (3)	7 (11)	4 (29)	10
Total	15 (20)	3 (32)	29 (116)[b]	44 (96)[c]	42 (215)	50
% of isolates	7.6	12.1	43.9	36.4	81.4	18.9
% of BPTs	16.5	3.2	31.9	48.4	46.2	54.3

[a] Only the numbers of S-BPTs tested for phylogenetic grouping are presented.
[b] $P < 0.0001$ for the number of isolates belonging to B2 versus the number belonging to both A and B1.
[c] $P < 0.0001$ for D versus A and B1 combined.

TABLE 3. Prevalence of virulence genes among *Escherichia coli* strains belonging to seven C-BPTs and persistent BPTs found in all four STPs[a]

STP (no. of isolates of C-BPTs)	No. (%) of isolates with indicated virulence gene					
	papAH	*papEF*	*papC*	*hlyA*	*cnf1*	$iroN_{E.\ coli}$
STP 1 (68)	2 (2.9)	12 (17.6)	2 (2.9)	52 (76.5)	18 (26.5)	44 (64.7)
STP 2 (49)[b]				26 (53.9)		45 (91.8)
STP 3 (13)	3 (23.1)	3 (23.1)	3 (23.1)	13 (100)	3 (23.1)	13 (100)
STP 4 (27)	3 (11.1)			25 (92.6)		27 (100)
Total (157)	8 (5.1)	15 (9.5)	5 (3.2)	116 (73.9)	21 (13.4)	129 (82.2)

[a] Some isolates were positive for multiple virulence genes.
[b] Three isolates were not tested.

The prevalence of *E. coli* in receiving waterways can be due to an array of sources, including domestic and/or wild animals, malfunctioning septic systems, industrial outlets, combined sewer overflows, and wastewater effluents, as well as ineffectively controlled treatment stages and, to an extent, the persistence of bacteria after disinfection (1). The net outcome of the STP treatment process is recycled water with a reduced number of bacteria and, ideally, no pathogens (15). In a typical STP, the bacterial count is normally reduced by 90% (27, 35); however, studies by Harwood et al. (15) and Kay et al. (18) have shown that approximately 67% of the initial number of total coliforms present in the primary and/or secondary sedimentation effluent can still be detected in disinfected tertiary-effluent samples (15, 18). Moreover, organisms with high initial concentrations (in influent) can still be retained at detectable levels in disinfected effluents.

In our study, the number of *E. coli* strains sampled from each treatment tank varied among the STPs, and despite the fact that some STPs (e.g., STP 1) were sampled more extensively than others, only those *E. coli* strains that were more prevalent in the secondary sedimentation tanks were detected in the final lagoon effluent of each STP. Interestingly, most strains of C-BPTs found in a final lagoon effluent were also found in other STPs, indicating that strains belonging to these BPTs either persist in STPs due to their higher numbers or have a better ability to survive the treatment processes, including disinfection, or they exhibit a combination of both traits. An alternative explanation is that some BPT patterns may be common to several *E. coli* strains. It has been shown that while there is a dramatic reduction in the number of *E. coli* organisms during the treatment processes of STPs, up to 10^2 CFU/ml may enter receiving waterways after the disinfection process (26). Other studies also indicate that the sewage treatment process reduces the number of pathogens insufficiently (32), with further treatment and disinfection required to render the

water safe for release. In the present study, we found that the majority of the surviving *E. coli* strains belonged to phylogenetic groups B2 and D. The association of the phylogenetic groups of *E. coli* with diseases at specific sites of the human body has been widely reported and allows for separation of commensal (A and B1) and pathogenic (B2 and D) strains (2, 6). In our study, the efficacy of water treatment to remove the bacterial pathogens responsible for a range of waterborne diseases was indexed by the presence or absence of these *E. coli* strains (7). It has to be noted that the prevalences of pathogens in different phylogenetic groups may vary in different geographical regions and could possibly alter over time (9). Regardless, phylogenetic grouping of *E. coli* strains can be used as a simple tool to identify whether the strains isolated from surface waters are pathogenic or not.

A comparison of the C-BPTs found in all four STPs indicated that 37.4% of the strains were also present in other STPs and at higher proportions than others. Interestingly, all C-BPTs (except one) contained strains belonging to phylogenetic group B2.

Our search for the presence of virulence genes associated with intestinal *E. coli* proved negative, but strains of 14 C-BPTs harbored one or more virulence genes associated with extraintestinal *E. coli*. The high prevalence of strains with uropathogenic virulence genes and belonging to the same C-BPTs in all STPs indicates that these strains may have a better ability to survive the treatment process. Some of the strains belonging to phylogenetic groups A and B1 also showed BPTs identical to those of strains belonging to B2 and D groups. These strains, however, generally had a lower prevalence than strains belonging to the B2 and D groups in the same BPTs.

Community STPs normally serve somewhere between hundreds of thousands to millions of residents (25). In these STPs, the populations of *E. coli* strains can be quite diverse, depending on a variety of factors, including the health or hygiene status of the population (8), the geographical region/environment (6, 9), and the host diet (8). Additionally, the niche of the sewage and sludge in these STPs provides an excellent environment for the growth of *E. coli* and other bacteria due to the presence of variable mixtures of bacteria and nutrients from organic matter (21). These environments may therefore become a "hot spot" (23) for the genetic exchange between certain bacterial species. An example of such is the transfer of the *chuA* gene, used for phylogenetic grouping, between *E. coli* O157:H7 and *Shigella dysenteriae* strains (33). The fact that some *E. coli* strains of the same BPTs belonged to different phylogenetic groups may be due to the loss or horizontal transfer of genes used for the classification of *E. coli* into different phylogenetic groups (22).

In conclusion, our findings indicate that *E. coli* strains carrying uropathogenic virulence genes belonging to phylogenetic groups B2 and D can survive all treatment processes of STPs, with these particular strains establishing dominance in other STPs. This suggests an enhanced ability for these strains to persist the treatment processes of STPs. From a public health point of view, the presence of these strains in the final lagoon effluent of STPs increases the likelihood of their release in surface waters, presenting a significant risk, and should therefore be considered in risk management regimes.

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