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Pathogenic *Escherichia coli* found in sewage treatment plants and environmental waters

E. M. Anastasi [1], B. Matthews[2,3], H.M. Stratton[2,3], and M. Katouli[1,3]

School of Health and Sport Sciences, University of the Sunshine Coast, Maroochydore DC 4558, Queensland, Australia[1]; School of Biomolecular and Physical Sciences, Griffith University, Nathan Campus, Queensland, Australia[2]; and Smart Water Research Centre, Griffith University, Gold Coast Campus, Queensland, Australia[3].

Running title: Pathogenic *E. coli* in the environment

Corresponding author: Associate Professor Mohammad Katouli, Faculty of Science, Health and Education, University of the Sunshine Coast, Queensland, Maroochydore DC 4558, Australia.

Email: mkatouli@usc.edu.au

Phone: (+61) 7 5430 2845

Fax: (+61) 7 54302887

Abstract

We previously demonstrated that some *Escherichia coli* strains with uropathogenic properties survived treatment stages of sewage treatment plants (STPs), suggesting that they may be released into the environment. We investigated the presence of such strains in the surrounding environmental waters of four STPs from which these persistent strains were isolated. In all, 264 *E. coli* isolates were collected from 129 receiving water sites in a 20km radius surrounding STPs. We also included 93 *E. coli* strains collected from 18 animal species for comparison. Isolates were typed using a high resolution biochemical fingerprinting method (the PhPlate system), and grouped into common (C) types. One hundred and forty seven (56%) environmental isolates were identical to strains found in STPs' final effluents. Of these, 140 (95%) carried virulence genes (VGs) associated with intestinal pathogenic *E. coli* (IPEC) or uropathogenic *E. coli* (UPEC) and were found in a variety of sites within areas sampled. Of the remaining 117 environmental strains not identical to STP strains, 105 belonged to 18 C-types and 102 of them carried VGs found among IPEC or UPEC strains. These strains belonged mainly to phylogenetic groups A (A0 and A1) and B1and to a lesser extent $B2_2$, $B2_3$, D1 and D2. Eight out of 18 environmental C-types comprising 50 isolates also were identical to bird strains. The presence of a high percentage of environmental *E. coli* in waters near STPs carrying VGs associated with IPEC and UPEC suggest that they may have derived from STP effluents and other non-point sources.

Key words: Sewage treatment plant, *E. coli*, surface waters, intestinal pathogens

Introduction

It is generally accepted that conventional wastewater treatment reduces the numbers of enteric bacteria. Though the extent to which this occurs can vary extensively depending on the treatment process. Sewage treated through the activated sludge or other biological processes often still contain faecal bacteria (36) or pathogens. To minimize the risk of environmental release of faecal bacteria, effluents are disinfected using oxidative processes to destroy or deactivate these organisms (29, 41). Chlorine is the most commonly used disinfectant (45). Alternative processes such as ozonation and UV irradiation are currently used for disinfection in many countries (13, 21, 25, 29) or are under extensive review.

Poorly operated or inadequate disinfection processes can result in various microorganisms surviving or even multiplying in treated wastewater effluents (2, 17, 35), thus making their way into the environment. The occurrence of pathogenic microorganisms in environmental waters is an ongoing concern for public health officials and those in the water management area worldwide. Enteric pathogens in environmental surface waters mainly originate from effluent discharge from STPs. Additionally; surface and agricultural runoff (20) can play a significant role. Currently, the microbial quality of water is monitored by enumerating the levels of faecal indicator bacteria, (e.g. thermotolerant *Escherichia coli* and enterococci), to determine levels of faecal input and the possible presence of pathogens (14). However, some enteric pathogens such as viruses, protozoa, and some bacteria, have different survival rates as compared to faecal-bacterial indicators in aquatic environments (50). The existence of such strains harboring VGs has not been fully investigated within STPs and for the potential release into the surrounding environment.

Quantification of *E. coli* in surface waters also serves to evaluate the performance of an STP for microbial reduction. *E. coli* itself can be pathogenic, causing either intestinal (e.g. diarrhea) or extraintestinal (e.g. urinary tract) infections (32). We previously demonstrated that certain clonal groups of *E. coli* with uropathogenic properties, can persist throughout one or more treatment stages of STPs (3), suggesting that these clones carrying VGs may have an enhanced ability to survive the treatment processes, including disinfection. We postulated that these strains might eventually find their way and survive in surrounding environmental waters. The aim of the present study was to investigate this hypothesis by examining for the presence of these clonal groups of *E. coli* with VGs in receiving environmental waters surrounding selected STPs.

Materials and Methods

E. coli strains from STPs

Between May 2006 and October 2007, a total of 74 *E. coli* strains were collected from final lagoon effluent of four STPs in South East Queensland that all employ the activated sludge sewage treatment process. These strains were typed at the time of isolation using PhP-RE plates (3) and a representative was stored in nutrient broth containing 20% v/v glycerol (Pronalys) and kept at –80^{o}C for further analysis.

Collection of samples from environmental sites

During the same period, water samples were collected from 129 sites covering an overall 20km radius surrounding the four above-mentioned STPs (Table 1). The STPs discharged treated effluent to water ways included in the 20km radius. The sampling sites were downstream of STPs and received discharges from other tributaries or waterways. Water samples of one liter (1L) for each site were collected in sterile

polypropylene bottles with an air space of 2.5cm in each bottle and transported to the laboratory on ice for processing within a maximum of 24h of collection. The samples were filtered through 0.45μm membrane filters (Millipore, Bedford, Massachusetts, USA). Membrane filters were plated on m-FC (Oxoid, Basingstoke, UK) agar plates and incubated at 44.5°C for 24h. Quality control measures for the determination of *E.coli* colonies on m-FC agar were employed with reference cultures of *E. coli* (NCTC 9001) (positive), and *E. aerogenes* (NCTC 10006), *P.aeruginosa* and *S. aureus* (negative), and a spiked control.

Depending on the availability of colonies on m-FC agar up to four suspected *E. coli* colonies was isolated and stored in nutrient broth (Oxoid) containing 20% v/v glycerol and stored at –80°C for further analysis. DNA extraction of suspected colonies were performed (see below) and strains were confirmed to be *E. coli* by PCR amplification of the *E. coli*- specific universal stress protein (*usp*A) gene as described by Chen and Griffiths (1998) (11). Preparation of the PCR mastermix and the PCR amplification cycle has been described previously (3). At least one confirmed *E. coli* isolate was selected from each sample. In all, 264 confirmed isolates from environmental water sites were included in this study (Table 1).

Additionally a collection of 93 strains isolated from 18 animal species was added for comparison to environmental strains. These strains had been collected from different research institutes or animal facilities and were kept at –80°C at Griffith University, Nathan campus, Queensland (Table 1).

DNA Extraction

Chromosomal DNA extraction of all confirmed *E. coli* isolates was performed using the boiling method as described before (10). Briefly, this involved growing a single colony in 2mL Luria Bertani (LB) broth overnight at 37°C with gentle shaking. The culture was then

pelleted using centrifugation at 10,000 rpm, the supernatant removed and the pellet re-suspended in 200µL of sterile MilliQ water, followed by heat lysis at 100°C for 15 mins. Another centrifugation step, at 10,000 rpm was performed to collect DNA, in the supernatant, of which 150µL, was then transferred to a sterile 1.5mL Eppendorf microcentrifuge tube and stored at –20°C.

Phylogenetic grouping

E. coli strains were tested for their phylogenetic groups using the polymerase chain reaction (PCR) according to the method described by Clermont et al. (12). Preparation of the PCR mastermix and the PCR amplification cycle were performed as previously described (3). Previously tested *E. coli* strains isolated from hospitalized patients with urinary tract infections (i.e.RBH2), harboring all three genes necessary for phylogenetic traits were used as positive controls. These specific strains were confirmed by sequencing and have been used before (3). For a negative control, filtered MilliQ water was used. The primer pairs used were *chu*A.1 (5'-GACGAACCAACGGTCAGGAT-'3), and *chu*A.2 (5'-TGCCGCCAGTACCAAAGACA-'3), *yja*A.1 (5'-TGAAGTGTCAGGAGACGCTG-'3) and *yja*A.2 (5'-ATGGAGAATGCGTTCCTCAAC-'3) and TSPE4.C2.1 (5'- GAGTAATGTCGGGGCATTCA-'3) and TSPE4.C2.2 (5'-CGCGCCAACAAAGTATTACG-'3), at final concentrations of 20µM for each forward and reverse primer. These genes generate 279, 211, and 152 base pair (bp) fragments, respectively (12).

Typing of *E. coli* isolates

A high resolution biochemical fingerprinting method specifically developed for typing of *E. coli*, i.e. PhP-RE plates (PhPlate AB, Stockholm, Sweden) was used to type E. *coli* isolates. Briefly, *E. coli* colonies to be analyzed were suspended in the first well of each

row of the PhP-RE plates containing 325µL of growth medium (0.011% w/v bromothymol blue and 0.1% w/v proteose peptone). Aliquots of 25µL of these suspensions were transferred into each of the other 11 wells containing 150µL of growth medium. Substrates used in PhP-RE plates include cellulose (as a control), lactose, rhamnose, deoxyribose, sucrose, sorbose, tagatose, D-arbitol, Melbionate, Gal-lacton and ornithine. Plates were then incubated at 37°C and the metabolic rate of the inoculated bacteria was read at intervals of 7, 24 and 48 h by scanning images using a HP Scanjet 4890 scanner. After the final scan, the PhPlate software (PhPWin4.2) was used to create absorbance (A_{620}) data from all scanned images, generated from each of the interval readings (3). Two sets of controls were used for testing the isolates. The negative controls constituted PhP growth medium without bacteria. This control was used to normalize data after the final reading of each plate, according to the manufactures' instructions. For positive controls, *E. coli* strains (n=10) previously tested using the PhPlate system with known biochemical fingerprints were used. These strains were also tested in duplicate and the mean similarity between duplicate assays of all ten isolates minus two standard deviations was used for calculation of the identity level (ID). The mean absorbance value from all individual readings was calculated for each reagent, creating the biochemical fingerprint for each isolate (37). Similarity among the isolates was calculated as a correlation coefficient and clustered according to the un-weighted pair-group method using arithmetic averages (UPGMA) (43, 46) to yield a dendrogram. Isolates showing similarity to each other above the ID of >0.965 were regarded as identical and assigned to a common (C) type and those with one isolate were regarded as single (S) types.

Detection of virulence genes

All isolates were tested for the presence of 11 VGs associated with *E. coli* strains causing intestinal and extraintestinal infections. A series of three multiplex and five uniplex PCR sets were employed using an Eppendorf Mastercycler gradient thermocycler as originally described by Chapman et al. (10). The VGs examined included *pap*AH*, pap*EF *and pap*C*,* the siderophore gene iroN$_{E.coli,}$ and toxin genes *cnf*1, *hly*A*, elt*A, *est*II*, eae*A*, stx*$_1$ and *stx*$_2$. The primer sets for detection of these genes have been described before (10). The final concentration of each forward and reverse primer for each gene was 50µM. The PCR protocol for the genes, *pap*C*,* iroN$_{E.coli}$, *cnf1, pap*AH*,* *pap*EF and *hly*A were modified to the following conditions: denaturation for 4min at 95°C, 30s at 94°C for 25 cycles; 25 cycles of 30s at 63°C; 25 cycles of 3min at 68°C, with the exception of the reaction for iroN$_{E.coli}$ and *cnf*1, which had a 1min amplification step at 68°C, and a final extension step of 10min at 72°C. The multiplex PCR reaction volumes for *pap*C and *hly*A*, cnf*1and iroN$_{E.coli}$ VGs consisted of 2.5µL 10x reaction buffer (Bioline), 1.25µL (50mM) $MgCl_2$ (Bioline), 1.0µL (10mM) dNTPs (Fisher Biotech) 0.3µL of each primer (Invitrogen) (0.15µL and 0.9µL of each primer for *pap*C) from 50µM stock solution, 0.15µL *Taq* polymerase (Bioline), 2.0µL DNA and sterile MilliQ water to make the final volume to 30µL for *cnf*1, iroN$_{E.coli}$ and 25µL for *hly*A and *pap*C. The reaction volume for the multiplex procedure for the *pap*AH and, *pap*EF genes had a final volume of 30µL and consisted of 19.15µL sterile MilliQ water, 5.0µL 10x reaction buffer, 1.5µL (50mM) $MgCl_2$, 1.0µL (10mM) dNTPs, 0.3µL of primers from 50µM stock solution, 0.15µL Taq polymerase (Bioline), and 2.0µL DNA. Positive controls for genes *pap*AH, *pap*EF, *pap*C, *hly*A, *cnf*1 and iroN$_{E.coli}$, were *E. coli* strains RBH130 and RBH136 isolated from clinical settings as reported before (3) whilst *E. coli* strains 0149 and 0157:H7 were used as positive control for *est*II, *elt*A and *eae*A, *stx*$_1$ and *stx*$_2$ genes respectively. Each specific positive control for all PCR reactions, in this study were confirmed and sequenced. PCR products were separated electrophoretically for 90min at 100V on a

precast 2% agarose gel (AMRESCO, Astral Scientific) in 0.6x TrisBase EDTA (TBE) buffer. Gels were stained with ethidium bromide (0.1% w/v) and the Syngene camera with UV light was used to visualize each of the amplified PCR products at each of the following fragment sizes: 720bp (*pap*AH), 336bp (*pap*EF), 200bp (*pap*C), 1177bp (*hly*A), 498bp (*cnf*1), 665bp ($iroN_{E.coli}$), 696bp (*elt*A), 172bp (estII), 384bp (*eae*A), 255bp (stx_1) and 180bp (stx_2).

Results

Typing of the 264 environmental isolates showed the presence several strains with identical fingerprints. These strains were named as common (C) types. In all, 15 C-types (EC1-EC15) constituting 105 (40%) strains as well as 159 single (S) types. Three C-types i.e. EC1, EC7 and EC12 (see Supplementary figure 1) contained the highest number of isolates (n=50) (48%) and were mainly found in samples collected from lagoons, beaches and creeks respectively (Table 2). Among the C-types, 82 (78%) strains belonged to phylogenetic group B1 and all carried toxin gene *est*II alone or in combination with other VGs (Table 2). Strains with extraintestinal VGs were also found, but they constituted only (11%) of the EC-type isolates and they belonged to either phylogenetic group B1 (i.e. EC2) or group $B2_3$ (i.e.EC3) or D1 (i.e. EC5a and EC8) (Table 2). Some of the environmental strains had a high similarity (96%) to other C-types (e.g. EC7 and EC7a) and were regarded as subtypes of these C-types. Strains belonging to such subtypes however, had a different VG profile and in most cases they belonged to different phylogenetic groups. Typing of STP strains showed that they belonged to 17 C- and 12 S-types (Table 1). In all, 147 (56%) environmental strains were identical to four common (i.e. STP-C1 to STP-C4) and 12 single (i.e. STP-S1 to STP-S12) types (Table 3). Of these, three C-types (i.e. STP-C2 to SPT-C4) with 13

228 isolates and one S-type (i.e. STP-S6) carried VGs found among UPEC and they
229 belonged to phylogenetic groups A0 (n=2) and A1 (n=2) and D2 (n=10) (Table 3). Of the
230 remaining 117 environmental strains that were not identical to those of STPs, 102 (87%)
231 carried VGs associated with IPEC or UPEC. These strains belonged to phylogenetic
232 groups A (4 strains), B1 (104 strains), D (16 strains) and related genotypes (Table 3).
233 Typing of the 93 *E. coli* strains isolated from animal species showed that they belonged
234 to 15 C-types (i.e. AC-1 to AC-15) comprising 65% of isolates, and 33 single types.
235 Comparison of these strains with the environmental ones showed that eight out of the 18
236 environmental C-types (53 isolates) were identical to animal strains all of which were
237 from birds and all carried VGs associated with IPEC or UPEC (Table 4). Whilst identical
238 C-types in both groups belonged to the same phylogenetic groups, two out of the three
239 environmental subtypes (i.e. AC7a and EC5a) contained strains belonging to different
240 phylogenetic groups (Table 4). Only 22 of the 264 environmental isolates (8%) did not
241 carry any of the tested VGs.
242

243 ## Discussion

244 Although the presence of *E. coli* in natural waters has long been used as an indicator of
245 faecal pollution, there is growing body of evidence suggesting that there exists a
246 specialized subset of *E. coli* that can reproduce and persist in secondary environments
247 rather than being obligate intestinal flora in both tropical (6, 47) and temperate climates
248 (9, 22, 31, 39). In this study, we used the PhPlate system for typing of *E.coli*. Based on
249 this method we showed that 56% of *E. coli* strains harboring VGs found in the
250 environmental waters were identical to those found in the final effluent stage of STPs.
251 The fact that STPs are not planned specifically to remove pathogenic microorganisms
252 from wastewaters (36), has raised concern over the potential discharge of intestinal

253 pathogens into the environment and the significant public health risk this possesses (36).
254 We previously showed that *E. coli* strains with uropathogenic properties can survive all
255 stages of sewage treatment, including the disinfection stage (3). In the present study, we
256 found identical strains with uropathogenic properties in several sites. However, one STP
257 C-type (i.e. STP-C1) constituted the highest proportion of the environmental isolates and
258 was found in 78 sampling sites. Strains of this C-type when isolated from STP effluent
259 carried the $iroN_{E.coli}$ gene only but was shown to carry the *est*II gene when isolated from
260 the environmental samples. In fact, VG profile analyses of the environmental strains
261 showed that a high percentage of the isolates carried VGs associated with IPEC strains
262 with *est*II alone or in combination with *elt*A. We also found that some of the STP strains
263 carrying *pap*EF, *hlyA*, *pap*C genes when isolated from STP final effluent (i.e. EC8), also
264 carried the stx_2 gene. One possible explanation for this could be that environmental
265 isolates may acquire VGs when they are in the environment as postulated before (15,
266 19). Alternatively, this particular strain may have a lower abundance in STP effluent and
267 skipped our detection. It is possible that these strains only shared the same fingerprints
268 but were genetically different.
269 The PhP-RE plates used in this study for typing of *E. coli* strains assesses the kinetics of
270 11 highly discriminatory substrates in each plate. The system has been shown to be very
271 efficient in recognizing not only differences, but the similarities among the populations of
272 different bacterial groups (33) and has been shown to have the same discriminatory
273 power as molecular techniques, such as PFGE (43) and ERIC PCR (5). Under these
274 conditions this assay has been found to be a highly discriminatory typing tool for
275 differentiation of *E. coli* (33, 37), and comparable to other genotypic and phenotypic tools
276 commonly used in epidemiological and ecological studies such as microbial source
277 tracking (MST), making it unlikely that the methods would not discriminate between
278 genetically divergent strains (5, 7, 8, 24). Typing of the STP final effluent with this

method, showed that 84% (62/74 isolates) belonged to C-types, some of which were shared between two or more STP effluents. However, we also found 12 single STP isolates (S-types) in the final effluent of the STPs. These strains, despite their low prevalence in STPs were found to be also common in environmental waters at different sites. The environmental strains also had identical phylogenetic groups and mainly carried VGs associated with IPEC indicating that the prevalence of a specific strain in the environment may not be correlated with their high number in the final effluent of STPs from which these strains have been released but, on the ability of the strain to survive the environment as suggested before (49). It has to be noted that water samples were collected at only one occasion and thus the number of *E. coli* tested from these sites was too small (on average two *E. coli* strains from each site). Therefore it they may not be a true representative of the *E. coli* population at each sample site at various times.

Among the environmental strains, 117 did not show any similarity to common STP strains. These strains constituted 18 common types (105 isolates) and 78% of them belonged to the phylogenetic group B1. Like strains belonging to group A, this *E. coli* genotype is regarded as 'non-pathogenic' or commensal (12, 16) however, here, all (except three) carried VGs associated with IPEC strains. Previous works suggest that strains belonging to the B1 phylogenetic group are adapted to surviving and multiplying in the environment outside host enteric conditions (4, 22, 39, 49). Skurnik et al. (44) found that the strains belonging to phylogenetic groups A and to a lesser extent B1 may have the genetic background to emerge as an intestinal pathogen. Additionally, Hamelin et al. (26) showed that whilst most commensal *E. coli* isolates derived from phylogenetic groups A and B1, obligatory pathogens responsible for acute and severe diarrhea also belong to these phylogenetic groups. These results suggest that environmental *E. coli*

strains belonging to phylogenetic groups A and B1 should not be regarded merely as commensals as they may possess VGs associated with intestinal or extraintestinal strains. Contrary to this finding, Escobar-Páramo et al. (18) suggested that strains belonging to phylogenetic group A possess fewer VGs than other phylogenetic groups. These workers also found that strains belonging to the B1 group originating from wild and domestic animals possessed the *hly* gene, which is unusual, as B1 strains are normally devoid of VGs and none of the ECOR collection possesses *hly* (18). Our results also demonstrated that one of the environmental common types belonging to B1 (EC7) grouped with the bird strain AC2 carried the *hly* gene and also iroN$_{E.coli}$. However, other bird strains belonging to the B1 group did not carry the *hly* gene, though other VGs were presented (see Table 4). These data suggest that strains of animal origin may be one of the sources of pathogenic B1 isolates carrying *hly*. Due to limited size of the library of isolates from known animal sources used in the present study care should be taken when considering the host origin of the strains examined. Based on data obtained from this study as well as others, we postulate that either environmental selection pressures promote a subset of B1 strains harboring VGs, or phylogenetic grouping of the environmental strains may not adequately classify environmental strains of *E. coli.*

The majority of the environmental strains carrying *est*II and *elt*A genes also harbored genes such as *hly*A and iroN$_{E.coli}$ which are responsible for acquisition of iron and are commonly found among UPEC strains. Recently a number of studies have detected the presence of these and other VGs belonging to UPEC is surface and rain tank waters (1). Similarly, Masters et al. (38) found a high level of these and other VGs in all three water types. Other researchers have also shown the presence of pathogenic *E. coli* strains and their associated VGs originating from sewage and/or animal faeces in environmental waters (26-28, 40, 42). These studies support our findings that some, if not majority, of

331 the *E. coli* strains in environmental waters carry VGs associated with UPEC or IPEC.
332 Ahmed et al (2) showed that the PhP system can be successfully used for MST and
333 detection of leaky septic systems (2). We also used the same method for identification
334 of different C-types of *E.coli* surviving all stages of sewage treatment (3). These studies
335 support the usefulness of the PhP system for MST of *E.coli* in mixed environmental
336 samples. Using the PhP system, here we showed that STP effluent may be one of the
337 sources for environmental strains that carry VGs. In our study only 8% of the
338 environmental strains did not carry any of these VGs tested. The fact that 56% of the
339 environmental strains carried VGs belonging to the IPEC and UPEC pathotypes were
340 identical to those found in the final effluent of the STPs; suggesting that *E. coli* strains
341 surviving wastewater treatment processes can also survive the environment.
342 Some of the environmental strains belonging to the C-types EC5a, EC7a and EC12a,
343 were highly similar to three C-types and we regarded them as “subtypes”. Strains of
344 these subtypes however, in most cases had a different VG profile and two of them
345 belonged to different phylogenetic groups. It is of course possible that these strains
346 belonged to two different clonal groups with a very high similarity in their biochemical
347 fingerprint. Within each C-type there existed several phylogenetic groups with different
348 VG profiles indicating a lack of correlation between the biochemical fingerprinting,
349 phylogenetic grouping and the VG profiles of the strains. However, within strains
350 collected from each source, there was a relationship between the presence and
351 prevalence of certain C-types and phylogenetic groups. For instance, strains belonging
352 to the major C-type 1 in STPs (i.e. STP-C1) contained 78 strains (53% of the STP
353 isolates) and all belonged to phylogenetic group B1. A similar pattern was observed
354 among the environmental strains where major C-types EC1 (16 isolates) or EC7 (20
355 isolates) or EC12 (14 isolates) also belonged to phylogenetic group B1. This indicates
356 that strains with specific biochemical fingerprints and phylogenetic groups are more

successful in surviving natural waters. Strains of these phylogenetic groups/C-types however, carried different VG profiles indicating that the presence of VGs in such groups is independent of their phylogenetic group or biochemical fingerprints.

To identify the possible sources of environmental pathogenic strains not found in STPs effluent, we compared their biochemical fingerprints with 93 strains obtained from 18 animal species. Similar to the STP and environmental isolates, the majority of the animal strains also belonged to a few C-types, five of which were identical to the environmental strains and all of them belonged to birds and all carried VGs belonging to IPEC or UPEC. Although the exact species of birds was not identified in our animal collection, this finding suggests that birds could also account for a large portion of *E. coli* carrying VGs in the environment that have not originated from STPs.

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Table 1. *Escherichia coli* isolates tested from final effluent lagoon of four STPs, environmental sites and animal species. Strains with identical fingerprints were grouped into common (C) types. STP: Sewage treatment plant, S1-12: Single types 1 to 12.

STP final lagoon isolates	No. of isolates	Source of isolation (no. of sites)	No. of isolates per site	Animals	No. of isolates
STP-C1	2	Lagoon (4)	48	Birds	29
STP-C2	5	Parks (4)	21	Cattle	6
STP-C3	4	Beaches (20)	90	Chicken	5
STP-C4	11	Lakes (4)	4	Dingo	1
STP-C5	4	Flood channel (3)	5	Echidna	2
STP-C6	4	Natural waters (23)	23	Emu	5
STP-C7	8	Creeks (44)	44	Kangaroo	11
STP-C8	2	Other sites (non-specified)(26)	26	Koala	5
STP-C9	2	Environmental tank water (1)	3	Lizard	2
STP-C10	2			Mouse	1
STP-C11	3				
STP-C12	2			Pademelon	2
STP-C13	2			Pig	7
STP-C14	4			Possum	1
STP-C15	2			Python	2
STP-C16	2			Quoll	2
STP-C17	3			Sheep	5
S1-12	12			Wallaby	1
				Wombat	6
Total	74	Total	264	Total	93

Table 2. Pathogenic *E. coli* belonging to common (C) types found in environmental waters in a 20km radius of investigated STPs. Strains with identical fingerprints were grouped into common (C) types. Strains that were not identical but showed a high similarity (96%) to a C-type, were regarded as subtypes of that C-type and are identified with letter "a". PG: Phylogenetic groups, E: Environmental, VG: Virulence gene.

Environmental C-types (no. of isolates)	Source of isolation	PG	VG Combination
EC1 (16)	Settling lagoon	B1	*hly*A, *est*II
EC2 (3)	Settling lagoon	B1	$iroN_{E.coli}$, *est*II, *elt*A
EC3 (3)	Park	$B2_3$	*hly*A, stx_2
EC4 (6)	Park	B1	$iroN_{E.coli}$, *est*II
EC5 (2)	Beach	A1	*est*II, *elt*A
EC5a (2)	Beach	D1	$iroN_{E.coli}$, stx_2
EC6 (3)	Beach	A1	$iroN_{E.coli}$, *est*II, *elt*A
EC7 (20)	Beach	B1	$iroN_{E.coli}$, *hly*A, *est*II
EC7a (4)	Beach	A1	*est*II
EC8 (2)	Lagoon	D1	*papEF*, *hly*A, *pap*C, stx_2
EC9 (10)	Beach	B1	$iroN_{E.coli}$, *est*II, *elt*A
EC10 (2)	Natural waters	A0	$iroN_{E.coli}$, stx_1
EC11 (4)	Natural waters	B1	*est*II
EC12 (14)	Creeks	B1	$iroN_{E.coli}$, *est*II
EC12a (7)	Other sites	B1	*est*II
EC13 (2)	Creeks	B1	*est*II
EC14 (2)	Creeks	$B2_3$	*hly*A, stx_2
EC15 (3)	Flood channel	$B2_3$	-

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577 **Table 3.** Identical pathogenic *E. coli* found in the final effluent of four STPs and in
578 environmental waters. Strains with identical fingerprints were grouped into common (C)
579 types. PG: Phylogenetic groups, STP: Sewage treatment plant, VG: Virulence gene, S1-
580 S12: Single types 1-12.
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Final effluent types in STP1	Source of isolation	No. of Sites (No. of isolates)	PG	VG Combination
STP-C1	Lagoons, Parks, Beaches, Lake, Natural Waters, Creeks, Other sites (non-specified)	78 (78)	B1	*est*II
STP-C2	Lagoons, Beaches, Creeks	9 (9)	D2	*pap*EF, *hly*A, *cnf*1
STP-C3	Beaches	2 (2)	A0	$iroN_{E.coli}$, *hly*A, *cnf*1
STP-C4	Natural Waters	2 (2)	A1	$iroN_{E.coli}$, *cnf*1
STP-S1	Natural Water	1 (1)	D1	-
STP-S2	Parks, Natural Waters, Creeks, Other sites	6 (6)	D2	*est*II
STP-S3	Natural Water	1 (1)	A0	*est*II
STP-S4	Creek	1 (1)	D2	*est*II
STP-S5	Lagoons, Creeks, Parks, Other sites (non-specified)	26 (26)	B1	*est*II
STP-S6	Other sites (non-specified)	1 (1)	D2	$iroN_{E.coli}$, *cnf*1
STP-S7	Lagoon, Beach	2 (2)	A1	*est*II
STP-S8	Natural Waters	2 (2)	D1	-
STP-S9	Beach	1 (1)	A0	*elt*A, *est*II
STP-S10	Lagoons, Beaches, Natural Waters, Creek, Other sites	9 (9)	D1	*elt*A, *est*II
STP-S11	Beach, Natural Waters, Creeks	4 (4)	D1	-
STP-S12	Lagoons	2 (2)	D1	-

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Table 4. Identical pathogenic *E. coli* strains belonging to common (C) types found in animals (A) and environmental (E) waters. Strains with identical fingerprints were grouped into common (C) types. Strains that were not identical but showed a high similarity (96%) to a C-type, were regarded as subtypes of that C-type and are identified with letter "a". PG: Phylogenetic group, VG: Virulence gene.

Animal C-types (no. of isolates)	Animal species	Corresponding environmental C-types (no. of isolates)	Source of isolation	PG	VG Combination
AC1 (2)	Bird	EC14 (2)	Creeks	$B2_3$	*hly*A, stx_2
AC2 (2)	Bird	EC7 (20)	Beaches	B1	$iroN_{E.coli}$, *hly*A, *est*II
		EC7a (4)	Beaches	A1	*est*II
AC3(2)	Bird	EC5 (2)	Beaches	A1	*est*II, *elt*A
		EC5a (2)	Beaches	D1	$iroN_{E.coli}$, stx_2
AC4 (5)	Bird	EC8 (2)	Lagoon	D1	*pap*EF, *pap*C, *hly*A, stx_2
AC5 (2)	Bird	EC12 (14)	Creeks	B1	$iroN_{E.coli}$, *est*II
		EC12a (7)	Other sites	B1	*est*II