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RESEARCH ARTICLE

Comparative prevalence of *Escherichia coli* carrying virulence genes and class 1 and 2 integrons in sub-tropical and cool temperate freshwater

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Abstract Aquatic environments are now recognized secondary habitat of potentially pathogenic *Escherichia coli*. In this study, PCR-based analyses were used to determine the phylogenetic composition and frequency of occurrence of eight clinically significant virulence genes (VGs) in *E. coli* isolates from sub-tropical Brisbane and cool temperate Tasmania freshwater in Australia. In Brisbane, non-commensal *E. coli* isolates belonging to the B2 and D phylogenetic group were dominant (72%). A significantly higher number ($P < 0.05$) of *E. coli* carrying VGs were detected in the sub-tropical freshwaters compared to the cool temperate water. Furthermore, diarrheagenic pathotype (EHEC) was also observed in the sub-tropical freshwater. The genes *east1* and *eaeA* were significantly more common ($P < 0.00001$) than other VGs. The *eaeA* gene which codes for intimin protein along with toxin genes *east1*, stx_1, stx_2, and LT1 were mostly detected in phylogenetic groups B2 and D. The ANOVA results also suggested a statistically significant difference ($P < 0.016$) between the VGs carried by phylogenetic groups B2 and D. Class 1 integrase (*intl1*) and class 2 integrase (*intl2*) genes were detected in 38 (24.83%) and 23 (15.03%) of *E. coli* isolates, respectively. The Gretna site (Tasmania) with known fecal input from bovine and ovine sources had the highest number of *E. coli* carrying *intl1* (29%) and *intl2* (13%) genes. In addition, class 2 integron was more commonly detected in the phylogenetic group B2. The results of this study highlight the need to better understand sources and reasons for the high prevalence of *E. coli* carrying clinically significant VGs in a sub-tropical environment and its public health implications.

Keywords Freshwater · *E. coli* · Virulence genes · Integrons · *intl*1 and *intl*2 genes

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Introduction

Escherichia coli are generally viewed as part of the commensal microbial flora of the vertebrate gut, and therefore, traditionally used as indicators when testing water for fecal pollution in aquatic environments (Tenaillon et al. 2010). Land use factors such as wastewater treatment plant discharge, sewer overflow, storm water discharges, water runoff from pastures used for intensive animal farming, and animal manure-treated agricultural fields all provide source and pathways to deliver fecal pathogens to freshwater (Sidhu et al. 2013, 2014). This could lead to higher human health risks especially in areas where contact with such waters is likely such as swimming, boating, and areas where rural populations often access untreated freshwaters for various uses including drinking water. *E. coli*-mediated disease outbreaks related to exposure to contaminated freshwater are well documented (Olsen et al. 2002; Shelton et al. 2006). Despite the significant disease burden linked to pathogenic *E. coli*, factors underpinning the distribution, prevalence, survival, and environment transport of pathogenic *E. coli* remain poorly understood (Bridge et al. 2010).

Escherichia coli strains can be categorized in four main phylogenetic groups, A, B1, B2, and D on the basis of

combination of the three genetic markers *chuA*, *yjaA*, and DNA fragment TspE4.C2 (Clermont et al. 2000), although some strains may also belong to additional groups C, E, and F (Clermont et al. 2013; Tenaillon et al. 2010). Extra-intestinal pathogenic *E. coli* (ExPEC) causing urinary tract infections, meningitis, and neonatal septicemia predominantly belong to group B2 and, to a lesser extent, to group D (Picard et al. 1999), whereas, intestinal diarrheagenic pathotypes (InPEC) and commensal strains belong to phylogenetic groups A and B1 (Johnson et al. 2001). *E. coli* strains belonging to B2 and D groups are reported to carry more virulence-associated genes compared to A and B1 group strains (Johnson et al. 2002).

Non-pathogenic bacteria can acquire antibiotic resistance and virulence genes (VGs) by horizontal or vertical gene transfer and through random DNA mutation (Barlow 2009). Gene transfer enables the exchange of genetic material located on mobile elements like plasmids and transposons among different strains or bacterial species (Frost et al. 2005). Integrons have been identified on these mobile elements which play an important role in the dissemination of antibiotic resistance genes (ARGs) as they carry determinants of site-specific recombination and an expression system, which integrates single or groups of mobile ARG cassettes (Hall and Collis 2006). Integrons have been associated with the presence of multiple ARGs in *E. coli* (Fluit and Schmitz 2004), and VGs could also be located in genetic mobile elements (Ochman et al. 2000); however, very little information is available on the relationship between the occurrence of VGs and the presence of integrons in *E. coli* isolates from aquatic environment.

To date, eight virulent *E. coli* pathotypes have been characterized and their unique mechanisms of pathogenesis were investigated (Croxen and Finlay 2010). These pathotypes are broadly classified by their ability to induce disease either within the gastrointestinal tract (diarrheagenic or InPEC) or in other niches of the body (extra-intestinal or ExPEC). Enterovirulent (or InPEC) strains can be classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), Shiga-toxigenic *E. coli* (STEC), enterohemorrhagic *E. coli* (EHEC) which is sub-set of STEC, enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DEAC) (Nataro and Kaper 1998). These pathotypes possess different VG combinations for the attachment and elaboration of enterotoxins and hemolysins (Bertin et al. 2001). Although each pathogenic *E. coli* strain possesses a highly diverse and idiosyncratic repertoire of VGs, many share similar mechanisms of virulence strategies including attachment and effacement, the production of toxins, capsule and siderophore synthesis, and cell invasion (Kaper et al. 2004).

Several factors have been linked to the environmental distribution of hazardous *E. coli* pathotypes including storm events, water type, land use (Masters et al. 2011), geography, and climate (Hamelin et al. 2006). While ETEC infections occur in high frequencies during all times of the year (Qadri et al. 2005), several studies have reported that ETEC infections from exposure to contaminated freshwater (both symptomatic and asymptomatic) are more prevalent during warm periods (Rao et al. 2003; Steinsland et al. 2002). In cold temperate climate, 70% of the *E. coli* strains from the river and freshwater around Munich, Germany, were commensal strains, classified as either phylogenetic B1 or A (Hoffmann et al. 2001). More recently, high prevalence of A and B1 phylogroups (up to ca. 70% together) has been reported in a temperate lagoon (Perini et al. 2015). Other studies from cold temperate climate Great Lakes (Ontario, Canada), Comox Lake (BC, Canada) and St. Clair River and Detroit River (Detroit, USA), and Rhine River (Germany) have also reported relatively low occurrence of *E. coli* carrying VGs and ARGs (Chandran and Mazumder 2015; Hamelin et al. 2006; Hamelin et al. 2007; Stange et al. 2016). Conversely, higher prevalence of VGs has been reported in *E. coli* isolates collected from the warmer water of Appalachia Bay (Florida, USA) (Parveen et al. 1997). A similar trend has been observed in the sub-tropical region of Brisbane, Australia, where InPEC VGs associated pathotypes (including ETEC) and ExPEC strains in various freshwaters have been observed in high frequencies (Anastasi et al. 2012; Sidhu et al. 2013). This suggests some influence of climatic conditions on the occurrence and survival of clinically significant *E. coli* pathotypes in the warmer environment.

Anthropogenic and animal sources are the major sources of *E. coli* release into the aquatic environment; therefore, the presence of virulent *E. coli* could be site specific. *E. coli* are generally considered as transient inhabitants of aquatic ecosystems (Edge and Hill 2005); however, they are also reported to survive and maintain populations in tropical ecosystems due to the availability of nutrients warm air, soil, and water temperatures (Jimenez et al. 1989; Winfield and Groisman 2003).

This study evaluated the profiles of *E. coli* strains isolated from freshwater in sub-tropical and cool temperate climate of two selected areas in Australia. Freshwater from three sites each in Brisbane (a sub-tropical climate) and Tasmania (a cool temperate climate) was examined for (i) the presence of *E. coli* possessing VGs associated with human pathology, (ii) distribution of *E. coli* phylogenetic groups, and (iii) presence of class 1 and 2 integrons and relationship between the occurrence of VGs and integrons.

Materials and methods

Sampling sites and sample collection

Freshwater samples were collected from three sites in Tasmania and three sites in Brisbane. A brief site description

along with GPS coordinates is provided in Table 1. Grab samples were collected 1 m from the shore and at a depth of about 0.5 m using a telescopic sampler. On each sampling occasion, two 500-mL water samples were collected in sterile glass bottles. In Brisbane, samples (n = 12) were collected from three sites (Milton, Enoggera Creek, and Mount Ommaney) between April and June 2013. Collected samples were transported to the laboratory in an esky on ice packs and processed within 6 h of collection. The water temperatures during the sampling period varied from 22 to 24 °C in Brisbane, and in Tasmania, it was between 15 and 20 °C. In Tasmania, water samples (n = 12) were collected at three sites (Gretna, Merton, and Tunbridge) between April and June 2013. Collected samples were shipped to Brisbane laboratory in an esky with ice packs and were processed within ~16 h after collection. The ambient water temperatures during the sampling period varied from 10 to 11 °C.

Isolation of *E. coli* from water samples

Membrane filtration method with ChromoCult coliform as selective agar (Merck, Germany) was used to capture and culture *E. coli* from the water samples (Sidhu et al. 2013). Briefly, 100 μL, 1 mL, and 10 mL water samples from Brisbane sites, 10 and 100 mL samples from Tasmanian sites, were filtered through 0.45-μm nitrocellulose (Millipore) filters (47 mm) in triplicate, placed on the plates, and incubated over a 24-h period at 37 °C to allow colonies to grow. Typical colonies showing dark blue to violet color were counted as *E. coli*. Two to three *E. coli* colonies were picked from the membranes and streaked onto the fresh ChromoCult medium. After this purification step, single colonies were picked from the plates and inoculated into 2-mL centrifuge tubes containing 1.5 mL nutrient broth (Oxoid) and incubated for 24 h at 37 °C in a shaking platform incubator at 100 rpm. All *E. coli* isolates were stored at −80 °C in nutrient broth and 15% (vol/vol) glycerol.

DNA extraction

To extract DNA, presumptive *E. coli* isolates were resuscitated overnight in 5 mL of nutrient broth at 37 °C. One milliliter of the cell culture from each isolate was centrifuged at 6000×g for 3 min. The resulting supernatants were removed, and the cell pellets were re-suspended by vortexing in 200 μL of sterile water. DNA was extracted from the cell pellets using the InstaGene matrix according to manufacturer's instructions (Bio-Rad Laboratories). *E. coli* isolates were confirmed by PCR amplification of the *uidA* gene as described previously (Sidhu et al. 2013).

PCR positive controls

E. coli ATCC 9637 was used as a positive control (*uidA* gene) in PCR assays to confirm presumptive *E .coli* strains. *E. coli* O157:H7 (ATCC 35150) was used as a positive control for *eaeA*, stx_1, and stx_2 genes. The *E. coli* strain belonging to serotype O138 of porcine origin was used as a positive control for heat-stable (ST) and heat-labile (LT) toxin genes. For the remaining target genes, pure cultures of clinical *E. coli* isolates containing target genes were used as positive controls.

Phylogenetic classification and screening for virulence genes and integrons

All confirmed *E. coli* isolates (n = 153) were assigned to four phylogenetic groups (A, B1, B2, or D) using a triplex PCR based on the presence or absence of three DNA fragments: *chuA*, *yjaA*, and TspE4C2, as previously described (Clermont et al. 2000). All *E. coli* isolates were screened for the presence of six diarrheagenic *E. coli* VGs by using previously published primer sets (Sidhu et al. 2013), Shiga toxin I and II producing genes stx_1 and stx_2 (López-Saucedo et al. 2003), intimin protein coating gene *eaeA* (Wang et al. 2002), type IV bundle-forming pili gene *bfp* and heat-labile toxin 1 gene *bfp* (Vidal et al. 2004), heat-stable toxin 1 producing gene ST (Guion et al. 2008) and heat-stable enterotoxin producing gene *east1* (Yamamoto and Nakazawa 1997), and transcriptional regulator gene *aggR* (Toma et al. 2003). The presence of class 1 and 2 integrons carrying the integrase genes *intl*1 (Kerrn et al. 2002) and *intl*2 (Orman et al. 2002), respectively, was also determined using previously published primers sets.

PCR reactions were performed on a Bio-Rad iQ5 thermocycler system (Bio-Rad Laboratories, California, USA), using SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, CA, USA). PCR amplification for phylogenetic classification, of VGs, *intl1*, and *intl2*, was performed in 20-μL reaction mixtures. Each 20-μL PCR reaction mixture contained 10 μL of EvaGreen® Supermix, 100–200 nM of each primer, Milli-Q water, and 3 μL of template DNA. Thermal cycling conditions for the detection of VGs were replicated from our previous study (Sidhu et al. 2013). Thermocycling parameters for the amplification of *chuA*, *yjaA*, and TspE4C2 were initial denaturing for 10 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, annealing at 55 °C for 30 s, and extension for 30 s depending on the product length at 72 °C followed by a final extension for 5 min at 72 °C. For the detection of *intl1* and *intl2*, annealing temperatures were 58 and 48 °C, respectively. For each PCR run, corresponding positive (target DNA) and negative (sterile water) controls were included. A melt curve analysis was performed after each PCR run to differentiate between actual products and primer dimers and to eliminate the possibility of false positive results. The melt curve was generated using

Table 1 Sampling site description and location in Brisbane and Tasmania, Australia

Site	GPS coordinates	Description
Brisbane		
Milton	27° 28′ 50.05″ S, 152° 59′ 53.84″ E	Storm water drain outlets from urban area, dilution effect due to large volume, and tidal influence; highly urbanized environment
Mt Ommaney	27° 31′ 46.20″ S, 152° 55′ 42.87″ E elevation 6 m	Urban, sampling river, multiple inputs, small amount of animal input from some farmlands so some agricultural input (horses), mostly urban, some small horticultural inputs, industrial potential, major waterway
The Gap	27° 26′ 44.12″ S, 152° 57′ 16.21″ E elevation 42 m	Highly urbanized Sampled from around bridge Selected due to being an urban stream, a lot of inputs from urban activity down from the reservoir, limited to no agricultural input
Tasmania		
Tunbridge	42° 8′ 23.44″ S, 147° 24′ 59.65″ E elevation 210 m	Small townships in the midlands of Tasmania with a population of 200. Primarily agricultural region the sampling site in Blackman River is surrounded by agricultural land
Gretna	42° 41′ 00.88″ S, 146° 56′ 11.51″ E elevation 35 m	A small township in the central highlands of Tasmania with a population of 250. Historically Derwent River (sampling site) had the highest *E. coli* detections (number and times of detection), indicating a potential upstream contamination mostly likely from grazing cows and sheep
Merton	42° 51′ 44.52″ S, 147° 13′ 38.63″ E elevation 261 m	Wellington Park water catchments are a source of 30% of Hobart's drinking water supply and are well protected, but there are increasing pressures on the catchments due to increasing tracking, hiking, and bike activities up on Mt Wellington

80 cycles of 10 s each starting at 55 °C and increasing at 0.5 °C intervals to a final temperature of 95 °C. The midpoint temperate (T_m) for each amplicon was determined using the iQ5 software (Bio-Rad).

Statistical analysis

Association between the prevalence of VGs and integrase genes (*intl1* and *intl2*) among phylogenetic groups and association between VGs and the presence of class 1 and 2 integrase genes were determined using χ^2or Fisher's exact test. Associations among genes were considered statistically significant when P values were <0.05. The Student's t test was performed to compare the significance of the difference between *E. coli* carrying VGs across sites; all tests were considered significant if the P value was <0.05. The difference in VG distribution among four phylogenetic groups across the six sites was determined by analysis of variance (ANOVA) on the pooled *E. coli* data from Brisbane and Tasmanian isolates with significance defined as $P < 0.05$. Statistical calculations were performed with Microsoft Excel and Statistica 10 (StatSoft).

Results

Identification and phylogenetic classification of *E. coli* The overall occurrence of culturable *E. coli* in the freshwater samples from Brisbane was relatively high (100–2000 cfu 100 mL^{-1} for each site). *E. coli* numbers in the water samples collected from two Tasmania sites (Merton and Tunbridge) were low (<10 cfu 100 mL^{-1}), whereas, the numbers in water samples collected from Gretna were higher (10–50 cfu 100 mL^{-1}). All 84 presumptive *E. coli* isolates, from the Brisbane samples, were confirmed as *E. coli* based on the presence of the *uidA* gene. Out of 70 *E. coli* isolates from Tasmania, 69 were confirmed as *E. coli*.

In this study, we were able to classify all confirmed *E. coli* isolates into A, B1, B2, and D phylogenetic groups (Fig. 1). In Brisbane isolates, the phylogenetic group B2 was the most prevalent (47%), followed by group D (25%), group A (17%), and group B1 (11%). Overall, potentially ExPEC strains B2 and D (72%) dominated over the commensal A (24%) strains. In Tasmanian isolates, the phylogenetic group B1 was the most prevalent (42.1%), followed by group B2 (23.1%), whereas, groups A and D had a similar frequency (17.4%). Overall, the

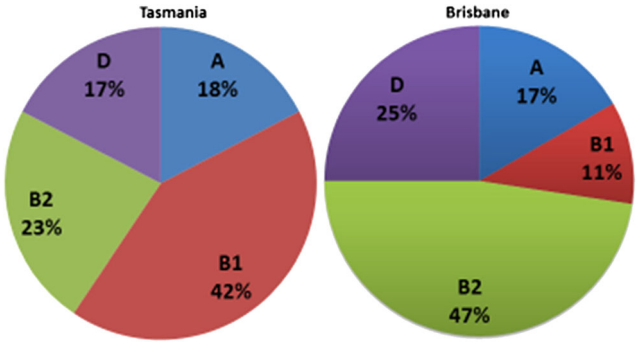

Fig. 1 Phylogenetic classification of *E. coli* isolates from Brisbane and Tasmania

commensal strains A and B1 (59.5%) dominated Tasmanian isolate population over the potentially ExPEC strains B2 and D (40.5%).

Distribution of virulence genes among *E. coli* isolates A significantly higher proportion ($P < 0.05$) of *E. coli* isolates from Brisbane (83%) carried VGs compared to Tasmania isolates (52%). The main EHEC virulence determinant gene, intimin gene (*eaeA*), was detected in 39 (25.49%) isolates followed by stx_1 in 11 (7.18%) and stx_2 in 9 (5.58%) isolates (Fig. 2 and Table 2). The main virulence determinants of ETEC, heat-stable toxin gene (ST1), were found to be more common (16.33%) than heat-labile toxin gene (LT1) which was detected in only three isolates (1.96%). The EAEC transcriptional regulator gene (*aggR*) and EaggEC heat-stable enterotoxin gene (*east1*) were detected in 10.45 and 36.60%, respectively.

Fifty percent of the *E. coli* isolates from Brisbane carried (40 out of 84) two or more VGs (Fig. 3). The most prevalent combination of genes was *eaeA* and *east1* (11 isolates), with both these genes observed to be in combination with two or three other clinically relevant VGs. Shiga toxin genes ($stx_{1\ and\ 2}$) were also detected in combination with *eaeA* and/or *east1*. This combination of genes which is typically carried by the EHEC pathotype was observed in at least 10 isolates. The main virulence determinant gene of EAEC pathotype (*east1 and* aggR) was carried by five isolates. Three isolates were detected with a combination of *east1* and *bfp* genes which put them in the EPEC pathotype category. A number of isolates also had combinations of genes from both EHEC and EAEC pathotypes.

In comparison *E. coli* isolates from Tasmania, only three of the eight VGs tested were detected (Table 2). The *east1* gene was the most commonly detected in 28 isolates (40.6%) followed by the *eaeA* gene which was detected in four isolates (5.8%). Multiple VGs were detected only in three isolates with two isolates that carried *east1* and *eaeA* and one isolate with *east1* and *bfp* gene combination. Two isolates carrying LT1 gene (heat-labile toxin 1) usually present in the ETEC pathotypes were also detected.

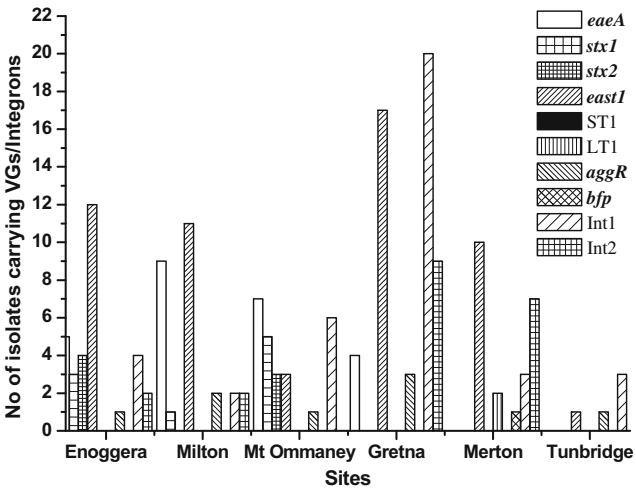

Fig. 2 Distribution of virulence genes and class 1 and 2 integrons across sites

Distribution of virulence genes among four phylogenetic groups The distribution of VGs among four *E. coli* phylogenetic groups is presented in Fig. 4. In general, isolates belonging to the phylogenetic groups B2 carried the highest number of VGs which was followed by the group D isolates. The *eaeA* gene which codes for intimin protein along with toxin genes *east1*, stx_1, stx_2, and LT1 were mostly detected in phylogenetic groups B2 and D. The ANOVA results also suggested a statistically significant difference ($P < 0.0016$) between the VGs carried by the phylogenetic groups B2 and D *E. coli*. Both *east1* and *eaeA* genes were significantly ($P < 0.00001$) more common as compared to other VGs in all phylogenetic groups.

Occurrence of integrons The presence of class 1 integron was observed in 12 (14.29%) isolates from Brisbane and 26 (37.68%) of isolates from Tasmania (Table 2). Overall, the difference in intI1 (37.28 vs 14.29%) and intI2 (23.18 vs 8.33%) carriage between Tasmania and Brisbane *E. coli* isolates sites was statistically significant ($P = 0.001$ and 0.013, respectively). The class 2 integron was observed in low frequencies in seven isolates (8.33%) from Brisbane and 16 (23.18%) of isolates from Tasmania. The *intl1* carrying *E. coli* mostly belonged to phylogenetic groups B1 17 (39%) and B2 14 (32%) phylogenetic groups, whereas, *intl2* was more common in the B2 15 (65%) phylogenetic group (Fig. 4). One site in Tasmania (Gretna) had the highest prevalence of *E. coli* carrying *intl1* (20) and *intl2* (9). The Merton site also had the second highest number of *E. coli* carrying *intl1* (three) and *intl2* gene (seven). In comparison, the Brisbane site, Milton, had the most *E. coli* carrying *intl1* (seven) and *intl2* gene (five). The frequencies of presence or absence of isolates carrying the *intl1* gene in four major phylogenetic groups were similar, with no statistically significant differences (ANOVA $P > 0.05$). However, the *intl2* gene was

Table 2 Occurrence of virulence genes (VGs), *intl1*, and *intl2* in *Escherichia coli* isolated from freshwater samples from Brisbane and Tasmania in Australia

Number of isolates	No. of *E. coli* carrying virulence gene and distribution (%)									
	eaeA[a]	*stx*$_1$	*stx*$_2$	LT1	ST1	*bfp*	*east1*	*aggR*	*intl1*	*intl2*
Brisbane (84)	35 (41.67)	11 (13.10)	9 (10.70)	1 (1.19)	25 (29.76)	7 (8.37)	28 (33.33)	12 (14.29)	12 (14.29)	7 (8.33)
Tasmania (69)	4 (5.8)	0 (0)	0 (0)	2 (2.9)	0 (0)	1 (1.50)	28 (40.6)	4 (5.79)	26 (37.68)	16 (23.18)
Total (153)	39 (25.49)	11 (7.18)	9 (5.88)	3 (1.96)	25 (16.33)	8 (5.22)	56 (36.60)	16 (10.45)	38 (24.83)	23 (15.03)

[a] Genes shared by more than one *E. coli* pathotype

significantly more likely to be absent in phylogenetic groups A and B1 ($P < 0.001$ and <0.0001, respectively). A comparison of total VGs carried by integron (*intl1* and/or *intl2* genes) positive and negative strains was carried out to determine if certain VGs were linked to the presence of integrons. The χ^2 or Fisher's exact test results revealed the statistically significant negative relationship between the presence of *Int1* and VGs *eaeA*, *east1*, and ST1 (Table S1). Similarly, negative relationship between the presence of *Int2* and *eaeA* gene was revealed.

Discussion

Escherichia coli with VGs in clinically significant combinations have been associated with significant intestinal and extra-intestinal human pathology (Kaper et al. 2004; Turner et al. 2006). In this study, significantly higher ($P < 0.05$) prevalence of single InPEC, VGs (*eaeA*, *stx*$_1$, *stx*$_2$, LT, ST, and *east1*) was observed in *E. coli* isolates from the Brisbane compared to Tasmania (Table 2). In particular, VGs *eaeA* (41.67%), ST1 (29.76%), *stx*$_1$ (13.10%), and *stx*$_2$ (10.70%) were highly prevalent in *E. coli* isolated from all Brisbane sites (Enoggera, Milton, and Mt Ommaney), whereas, *east1* (40.6%) *eaeA* gene (5.8%) was detected in the isolates from Tasmania.

The overall higher prevalence of *E. coli* carrying VGs from Brisbane locations compared to Tasmania locations could potentially be due to the urbanized nature of the former and predominantly rural nature of the later or alternatively prolonged survival of *E. coli* carrying VGs in warmer waters of Brisbane. Aquatic environments in highly urbanized environments tend to have more diverse sources of fecal input into freshwater; *E. coli* strains carrying VGs have been reported to be highly prevalent in polluted urban streams (Higgins et al. 2005). In a previous study from Brisbane, Australia, a similar pattern of occurrence of these three genes in *E. coli* isolates *eaeA* (56%), *stx*$_1$ (6%), and *stx*$_2$ (10%) was reported (Sidhu et al. 2013). In comparison, a much higher frequency of *eaeA* (93.3%), *stx*$_1$ (46.7%), and *stx*$_2$ (13.3%) genes has been reported in the total DNA extracted from water samples collected from Michigan and Indiana River passing through highly urbanized areas (Duris et al. 2009). This suggests that anthropogenic activities may be the sources of *E. coli* carrying clinically relevant VGs into freshwater.

In addition to the detection of four typical EHEC strains carrying *eaeA* gene along with *stx*$_1$, *stx*$_2$, or both (Boerlin et al. 1999) in Brisbane water, the high prevalence of *stx*$_1$/*stx*$_2$/*eaeA*

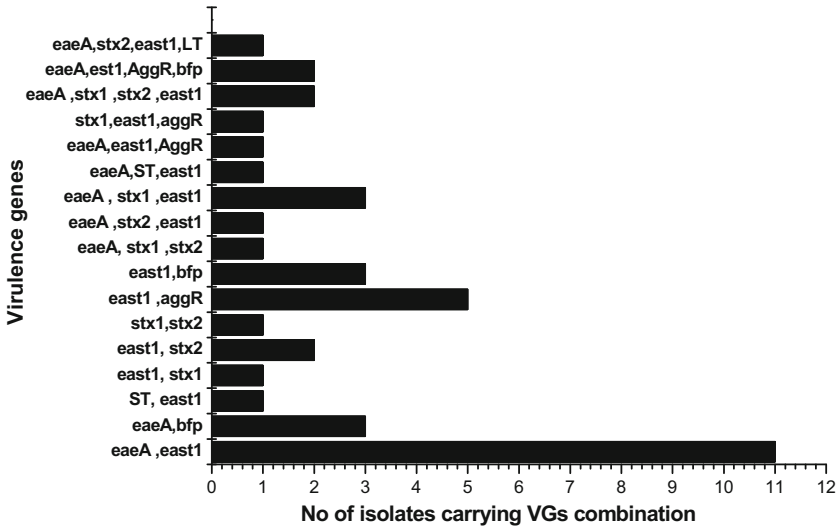

Fig. 3 *E. coli* isolates carrying combination of two or more VGs ($n = 40$) from Brisbane water samples

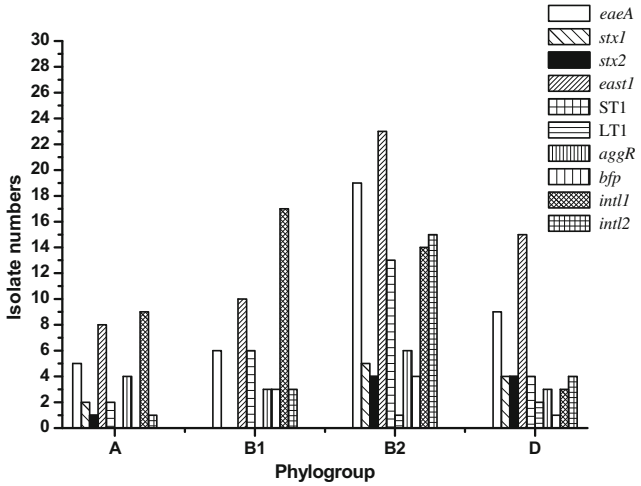

Fig. 4 Comparative distribution of virulence gene and class 1 and class 2 integrons in different *E. coli* phylogenetic groups across all sites

genes in other isolates was also observed. This suggests a high prevalence of atypical EHEC in freshwater which is in concurrence with the previously reported occurrence of a typical EHEC strains in surface water (Shelton et al. 2006). In contrast, in Tasmanian isolates, the prevalence of *eaeA* gene (6%) was low and none of the isolates carried stx_1 or stx_2 gene. High prevalence of EHEC pathotypes in sub-tropical climate including Brisbane (Sidhu et al. 2013), Bangladesh (Qadri et al. 2005), India (Khan et al. 2002), and China (Chen et al. 2011) has also been previously reported.

In this study, *E. coli* strains belonging to phylogenetic groups B2 and D were predominant in the freshwater from Brisbane (72%) but less common among Tasmanian isolates (40.5%). Although we have not tested for the prevalence of VGs defining ExPEC, the clones responsible for human extra-intestinal infections typically belong to the B2 and to a lesser extent the D phylogenetic group (Lecointre et al. 1998). Therefore, it is likely that a high proportion of our B2 and D group isolates from Brisbane may belong to ExPEC pathotype. In the previous studies, the high prevalence of ExPEC strains in sub-tropical freshwater has been reported (Hamelin et al. 2007; Masters et al. 2011). Conversely, a high prevalence of commensal strains (groups A and B1) in Tasmanian freshwater (59.5%) suggests a low prevalence of the ExPEC pathotype. A high prevalence (70%) of commensal strains *E. coli* belonging to the phylogenetic group B1 and A has been reported in the temperate river and freshwater around Munich, Germany (Hoffmann et al. 2001). Since both Brisbane (Australia) and Munich (Germany) are urbanized, the reasons for the high prevalence of non-commensal strains in sub-tropical water of the former are unknown. One possible explanation could be the difference in the adaptability of phylogenetic groups. *E. coli* belonging to the phylogenetic group B1 tend to be more environmentally adaptive (Berthe et al. 2013); therefore, it is possible that commensal *E. coli* strains are more adaptive and hence abundant in colder climate. The results of this study suggest a link between warmer climate and high prevalence of non-commensal *E. coli* carrying clinically important VGs which needs further investigation.

Heat-stable enterotoxin gene *east1* is associated with EAEC-mediated diarrhea in adults and children (Caprioli et al. 2005). The *east1* gene was the second most prevalent in the *E. coli* isolates from Brisbane (33.33%) and Tasmania (40.6%). The specific implications of the presence of *east1* in *E. coli* isolates, however, are not straightforward. While some studies have reported that *E. coli* carrying *east1* alone could cause diarrhea in subjects from Japan and Spain (Itoh et al. 1997; Viljanen et al. 1990), this gene has also been detected in commensal *E. coli* isolates (Ménard and Dubreuil 2002). For this reason, it was difficult to draw conclusions regarding the pathogenicity of *east1* carrying *E. coli* in this study. In Brisbane, 41.67% of the *E. coli* isolates carried either only the *eaeA* gene or in combination with the heat-stable enterotoxin gene *east1*. The intimin gene (*eaeA*) is reported to be the primary virulence factor of EPEC strains and known to cause infantile diarrhea even in the absence of other virulence or toxin genes (DeVinney et al. 1999). The results suggest that potential EPEC strains are more frequently detected in warm tropical water of Brisbane compared to colder Tasmanian water.

The proportion of *E. coli* isolates classified as the phylogenetic group A was relatively similar between Brisbane and Tasmania at 17 and 18%, respectively. In principle, strains belonging to the phylogenetic group A lack InPEC VG combinations given their commensal nature (Bonacorsi et al. 2000). This held true for all Tasmanian commensal phylogenetic group A isolates, with none of the isolate carrying any InPEC VGs. However, all Brisbane commensal phylogenetic group A isolates possessed *Shiga* toxin VGs (stx_1 or stx_2), with one isolate classified as an EHEC pathotype as this strain synchronously possessed *eaeA* and stx_2 VGs. Phylogenetic groups A and B1 predominate in the gut flora of vertebrates, and these strains must acquire virulence factors to become pathogenic (Duriez et al. 2001). In view of this, the current study suggests that Brisbane commensal phylogenetic group A strains have acquired VGs potentially via horizontal gene transfer (HGT).

Site-specific point and non-point sources of fecal pollution are expected to contribute towards the presence of *E. coli* carrying clinically relevant VGs and integrons. A high proportion of InPEC pathotype isolates was detected at the Milton site, located in the heavily urbanized area of Brisbane with close proximity to a storm water drain. This was potentially due to mobilization and transport of fecal contaminants from land to Brisbane River after the storm events (Sidhu et al. 2013). In comparison, the Merton site in Mt Wellington provides a significant proportion (30%) of Hobart's drinking water. The catchment area is well protected from point and non-

point of sources of pollution and had a very low prevalence of *E. coli* isolates carrying VGs and integrons.

The *E. coli* from Gretna (Tasmania) carried more VGs and integrons compared to all other sites including Brisbane sites (Fig. 2). The most likely sources of *E. coli* isolates carrying VGs at this site are livestock (bovine and ovine) in the pastures surrounding Derwent River. This observation is supported by the high prevalence of the B1 phylogenetic group *E. coli* (45%) in the Gretna isolates which are more likely to be carried by the vertebrate animals (Gordon and Cowling 2003). It is interesting to note that out of five *E. coli* isolates carrying both integrons, four came from this site. In a recent study from Poland, an association between integron carriage in coliform bacteria and colder water temperature has been reported (Koczura et al. 2015). Therefore, the site-specific source of fecal pollution along with potentially higher persistence of integron-positive bacteria in colder water may increase the prevalence of potentially pathogenic *E. coli*. This suggests that *E. coli* of animal origin may pose health risks to humans as animals are recognized sources of ExPEC strains (Belanger et al. 2011).

Antibiotic resistance gene cassettes are carried on integrons (Hall and Collis 2006) that are a highly efficient mechanism for the dissemination of ARGs among bacterial pathogens (Fluit and Schmitz 2004). The prevalence of class 1 integron-integrase gene (*intl1*) has been suggested as a good proxy for the presence of pollution from anthropogenic sources (Gillings et al. 2014). In this study, class 1 integron was twice as commonly detected (24.83%) compared to class 2 integron (15.03%). The observed high prevalence of *int*1 gene is most likely due to its wide distribution in *E. coli* and other gram-negative bacteria and its presence on plasmids that can be transferred to other bacteria (Hall and Collis 2006). In comparison, class 1 integrons were reported to be highly prevalent in the *E. coli* isolates from sediments (69%) and freshwater (71%) from Santa Ana River (Southern California, USA), while class 2 integrons were detected in low frequency in both sediments (15%) and freshwater (7%) (Ibekwe et al. 2011). Similarly, the high prevalence of class 1 integrons (41%) in *E. coli* isolates has also been reported from Minjiang River (China) (Chen et al. 2011).

Class 1 and 2 integrons are considered markers of multidrug resistance (MDR), and their presence in the bacterial genome, even regardless of the genetic content of the variable region, is associated with the MDR phenotype (Deng et al. 2015). High prevalence of *E. coli* carrying integrons along with the multiple VGs in Brisbane isolates shows high pathogenicity and antibiotic resistance potential. As a variable region, class 1 and 2 integrons may contain up to nine resistance genes in gene cassettes (Partridge et al. 2009). In this study, we have only focused on the *E. coli*; the presence of class 1, 2, 3, and 4 integrons in bacteria such as *Aeromonas* spp., *Pseudomonas* spp., and *Salmonella* spp. often detected in water remains unknown. The role of such integrons in the dissemination of antimicrobial resistance in the aquatic environment, require further investigation.

In this study, we also explored the association between the presence of *intl*1 and *intl*2 genes, VGs, and phylogenetic group affiliation. The frequencies of presence or absence of *intl1* gene in all four phylogenetic groups were statistically non-significant (χ^2 or Fisher's test, $P > 0.05$) in all four phylogenetic groups in warmer and cold waters. The results are in agreement with the previously reported non-association with the presence of *intl1* gene and phylogenetic groups in *E. coli* isolates from Warta River in Poland (Koczura et al. 2013). In contrast, class 1 integrons were reported to be significantly less prevalent in group A, *E. coli* isolated from freshwater and wastewater (Figueira et al. 2011). In this study, class 2 integron (*intl*2) was more commonly detected in the phylogenetic group B2 and was significantly less likely to be present in phylogenetic groups A and B1 isolates ($P < 0.01$). In contrast, class 2 integrons were reported to be more common in phylogenetic groups A1 and D2 *E. coli* isolates collected from freshwater and wastewater in northern Portugal (Figueira et al. 2011). This discrepancy is most likely due to the high mobility of transposons (Frost et al. 2005).

The results of this study suggested that warmer sub-tropical water harbors *E. coli* in high numbers which may carry clinically significant VGs. It is possible that favorable conditions for growth and proliferation (nutrients and warmer temperatures) may facilitate HGT, thereby increasing the presence of pathogenic *E. coli* in such environments. In a laboratory-scale study focused on the HGT in *E. coli*, transfer of $stx_{1/2}$ genes from pathogenic to commensal strains was found to be strongly influenced by high water temperature (37 °C) and strong UV irradiation (0.5 kJ/m^2) (Yue et al. 2012). Since water temperature and UV exposure are characteristically higher in tropical climates, this study potentially offers an explanation as to why *E. coli* carrying VGs may be more prevalent in the warmer waters. An alternative explanation could be the growth and proliferation of *E. coli* carrying VGs in the sub-tropical water due to the prevalence of nutrients and ideal growing temperature (Jimenez et al. 1989; Winfield and Groisman 2003). Further investigation is required to elucidate reasons for high prevalence of *E. coli* carrying multiple VGs in warmer subtropical climates.

Conclusions

The results of this study suggest that VGs and integron—bearing *E. coli* responsible for waterborne disease outbreaks (both InPEC and ExPEC pathotypes)—are comparatively more prevalent in the warmer climate which might pose a threat to public health. However, the reasons for this are not clear and need further investigation. In terms of public health

significance, site-specific anthropogenic sources of pollution are a major source of pathogenic *E. coli* into the aquatic environment. Therefore, there is a need for proper management of land use activities within the catchments that are used for the production of drinking water and recreational to limit human health risks.

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