Antimicrobial resistance and distribution of *sul* genes and integron-associated *intI* genes among uropathogenic *Escherichia coli* in Queensland, Australia

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We studied 137 uropathogenic *Escherichia coli* (UPEC) isolates from hospitalized adult patients (Queensland, Australia) for their resistance to 17 antimicrobial agents using the calibrated dichotomous sensitivity method and the presence of class I, II and III integron-associated integrase (*intI*) genes, including functional class II *intI2*, as well as the presence of *sul1*, *sul2* and *sul3* genes, using PCR. Randomly amplified polymorphic DNA PCR, a high-resolution biochemical-fingerprinting method (PhP) and phylogenetic grouping were also used to identify the clonality of the sulphonamide-resistant isolates. One hundred and twenty (87.6 %) isolates were resistant to one or more of the tested antimicrobial drugs, with the highest resistance (70.1 %) observed against sulphafurazole (96 isolates). Of these, 84 (87.5 %) contained one or more *sul* alleles, with *sul1* being the most common allele [occurring in 69 (72 %) isolates]. Only 38 of 69 (55.1 %) strains carrying the *sul1* gene were positive for class I integrase. Our results indicate a high prevalence of sulphafurazole-resistant UPEC strains belonging to different clones among patients with urinary tract infection in Queensland, Australia. We also conclude that these strains carry predominantly a *sul1* gene that is not commonly associated with the presence of class I integrase, indicating that it may be carried on either a bacterial chromosome or other genetic elements.

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INTRODUCTION

Urinary tract infection (UTI), although one of the most easily treatable diseases, has been reported to be the most common hospital-acquired infection, affecting mainly women, children and the elderly (Foxman & Brown, 2003; Gupta *et al.*, 2001; Russo & Johnson, 2003; Tartof *et al.*, 2007). *Escherichia coli* is the primary aetiological agent, accounting for 75–90 % cases of UTI (Gupta *et al.*, 2001; Kaper *et al.*, 2004; Nicolle, 2002). The acquisition of resistance genes by horizontal transfer is currently thought to play a major role in the development of multi-drug-resistant (MDR) strains because a substantial proportion of the resistance genes are located on conjugative plasmids, transposons, insertion sequences and integrons (Chang *et al.*, 2011; Fierer & Guiney, 1999; Moritz & Hergenrother, 2006). Integrons are mobile DNA elements with the ability to integrate and express gene cassettes by site-specific

recombination (Mazel, 2006; Rowe-Magnus & Mazel, 2002). Site-specific recombination is catalysed by an integrase that is encoded within the conserved 5' region of integrons. Several classes of integrase have been described with those classes of integrons, i.e. class I integrase (*intI1*) defines class I integrons (Collis *et al.*, 2002; Nield *et al.*, 2001).

With the standard treatment for UTI being a combination of trimethoprim and sulphamethoxazole (Grape *et al.*, 2003), the sulphonamides are classified as a highly important antimicrobial agent for treatment of *E. coli* infections, and the presence of sulphonamide resistance can lead to treatment failure in cases of UTI. In *E. coli*, sulphonamide resistance often correlates with the presence of dihydropteroate synthase (DHPS) genes in integrons (Huovinen *et al.*, 1995; Sköld, 2000). Currently, three different types of DHPS gene (*sul1*, *sul2* and *sul3*) responsible for sulphonamide resistance have been characterized. The *sul1* gene is found exclusively on large conjugative plasmids and on class I integron at the 3' end (Hall & Collis, 1998; Trobos *et al.*, 2009), whilst *sul2* is usually located on plasmids belonging to the IncQ family or another type represented by pB1 (van Treeck *et al.*, 1981). Although a link between the presence of

Abbreviations: CDS, calibrated dichotomous sensitivity; DHPS, dihydropteroate synthase; MDR, multi-drug-resistant; RAPD, randomly amplified polymorphic DNA; UPEC, uropathogenic *Escherichia coli*; UTI, urinary tract infection.

Table 1. Distribution of antimicrobial drug-resistance patterns and the presence of integron-associated *intI* genes among 137 uropathogenic *E. coli* strains

AMC, Amoxicillin–clavulanic acid; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CEF, cephalothin; CPD, cefpodoxime; CTT, cefotetan; CTX, cefotaxime; FEP, cefepime; GEN 10, gentamicin 10 µg; GEN 200, gentamicin 200 µg; IMI, imipenem; NAL, nalixidic acid; NIT, nitrofurantoin; SF, sulphafurazole; TET, tetracycline; TMP, trimethoprim; TZP, piperacillin–tazobactam.

Resistance	No. of isolates	No. of <i>intI</i> ⁺ isolates	Phylogenetic group(s)
None	17	2*	B2 (15)*, D (1)*, A (1)
Single antimicrobial	31	1	
AMP	5	0	B2 (4), D (1)
CEF	9	0	B2 (5), D (3), B1 (1)
SF	14	1*	B2 (11), D (1)*, A (1)
NAL	1	0	B2
CTT	1	0	B2
TMP	1	0	B2
Two antimicrobials	26	9	
AMP/SF	9	4	B2 (9)
CEF/SF	8	1	B2 (8)
TET/SF	2	1	B2 (2)
TMP/SF	2	2	B2 (2)
GEN10/SF	1	1	B2
GEN10/CEF	1	0	B2
AMP/CEF	2	0	B2 (2)
CTT/SF	1	0	B1
Three antimicrobials	24	12	
AMP/CEF/SF	15	5	B2 (15)
AMP/TMP/SF	3	3	B2 (1), D (2)
AMP/TET/SF	1	0	B2
AMP/IMI/SF	1	1	B2
AMP/TMP/CTT	1	1	D
AMP/TZP/SF	1	1	B2
TET/TMP/SF	2	1	B2 (2)
Four antimicrobials	19	11	
AMP/TMP/TET/SF	2	1*	B2 (1)*, A (1)
AMP/GEN10/CEF/SF	1	1	B2
AMP/TMP/CEF/SF	6	6	B2 (5), D (1)
AMP/CPD/CEF/SF	1	0	B2 (1)
AMP/CEF/TET/SF	2	0	B2 (2)
AMP/AMC/CEF/SF	1	0	B2
AMP/AMC/TZP/CEF	1	0	B2
TMP/CEF/TET/SF	1	1	D
TMP/GEN10/TET/CEF	2	2	B2, D
TMP/GEN10/SF/NIT	1	0	D
ATM/NIT/CEF/SF	1	0	D
Five antimicrobials	11	11	
AMP/TMP/CEF/TET/SF	7	7	B2 (2), D (5)
AMP/AMC/CEF/TMP/SF	1	1	D
AMP/TMP/CEF/NAL/SF	1	1	B2
TMP/CEF/TET/SF/NIT	1	1	B1
TMP/NAL/TET/CEF/SF	1	1	B2
Six antimicrobials	2	2	
AMP/TMP/NAL/TET/CEF/SF	1	1	B2
AMP/AMC/TMP/TZP/CEF/SF	1	1	D
Eight antimicrobials	4	4	
AMP/GEN10/TMP/CEF/NIT/NAL/TET/SF	1	1	D
AMP/AMC/TZP/GEN200/TMP/CEF/NAL/SF	1	1	B2
AMP/CEF/TMP/CPD/CTX/ATM/FEP/SF	2	2	B2 (2)
Nine antimicrobials	1	1	

Table 1. cont.

Resistance	No. of isolates	No. of <i>intI</i> ⁺ isolates	Phylogenetic group(s)
AMP/AMC/CEF/TMP/CPD/TZP/NAL/TET/SF	1	1	B2
Eleven antimicrobials	1	0	
AMP/AMC/TMP/CAZ/CPD/ATM/FEP/CTT/			
CEF/TET/SF	1	0	B2
Twelve antimicrobials	1	1	
AMP/AMC/TMP/GEN10/CAZ/CTX/CPD/ATM/	1	1	B2
CTT/CEF/TET/SF			

*No. of strains belonging to different phylogenetic groups.

the *sul3* gene and non-classic class I integrons has been proposed (Antunes *et al.*, 2007), the structure of plasmids carrying *sul3* has not been well-described (Wu *et al.*, 2010). Furthermore, while *sul3* has rarely been found, both *sul1* and *sul2* are highly prevalent (Blahna *et al.*, 2006; Ho *et al.*, 2009; Trobos *et al.*, 2009).

The aims of this study were to investigate the pattern of antimicrobial resistance, the prevalence of sulphonamide-resistant strains and finally the association of *sul* genes with *intI* genes among uropathogenic *E. coli* (UPEC) strains isolated from hospitalized patients with UTI in Queensland, Australia.

METHODS

Sample collection. In total, 137 *E. coli* strains from hospitalized adult patients (one isolate per patient) with acute uncomplicated symptomatic UTIs were collected from three metropolitan hospitals, namely Princess Alexandra Hospital, Mater Hospital and Royal Brisbane and Women's Hospital, in Brisbane, Queensland, Australia, between May 2006 and October 2007. Criteria for symptomatic UTI included dysuria, and urgency and frequency of micturition (Mabbett *et al.*, 2009). Patients with UTI due to catheterization and those yielding mixed infection were excluded from the study. All isolates were tested for purity on MacConkey agar no. 3 and confirmed as *E. coli* using the highly specific *E. coli* universal stress protein (*uspA*) gene. Chromosomal DNA was extracted by using a Real Biotech Corporation (RBC) Genomic DNA Extraction kit (Blood/Bacterial/Cultured Cells) and an 884 bp fragment of the *uspA* gene was amplified as described by Chen & Griffiths (1998). The DNA-amplification reaction mixture (25 µl) contained 17.3 µl MilliQ water, 2.5 µl 10× PCR buffer (Bioline), 1 µl dNTPs (10 mM) (Fisher Biotech), 1.5 µl MgCl₂ (50 mM) (Bioline), 0.25 µl of each *uspA* primer from a 50 pmol µl⁻¹ stock solution (Invitrogen), 0.2 µl *Taq* DNA polymerase (Bioline) and 2.0 µl purified DNA template. Amplification was carried out in a thermal cycler and runs included a 5 min initial denaturation step at 95 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, and a 5 min final extension step at 72 °C. Amplified PCR products were electrophoresed on 2% agarose (Amresco) in 0.6× TBE (TrisBase EDTA) buffer, stained with ethidium bromide and viewed under UV light using a Gel Doc system (Bio-Rad). DNA was then stored at -20 °C in elution buffer for further use and the strains were stored on tryptone soya broth with 15% glycerol at -80 °C for further analysis.

Antimicrobial resistance. All isolates were tested for their resistance to 17 commonly used antimicrobial drugs according to the calibrated dichotomous sensitivity (CDS) method (Bell *et al.*, 2006). The antimicrobials tested were ampicillin (25 µg), amoxicillin-clavulanic acid (60 µg), cefotaxime (5 µg), ceftazidime (10 µg), cefotetan (30 µg), imipenem (10 µg), aztreonam (30 µg), piperacillin-tazobactam (55 µg), cefepime (10 µg), cefpodoxime (10 µg), trimethoprim (5 µg), gentamicin (10 and 200 µg), nalidixic acid (30 µg), cephalothin (30 µg), nitrofurantoin (300 µg), sulphafurazole (300 µg) and tetracycline (30 µg) (a less commonly used drug). All susceptibility tests were calibrated for the chosen antimicrobial drugs using *E. coli* strain ATCC 25922 for quality control. The CDS method reports antimicrobial drug susceptibilities as either 'susceptible' or 'resistant' and therefore the results were not expressed as MIC values, and instead interpreted only as susceptible or resistant. Isolates with resistance to two or more antimicrobial drugs were regarded as MDR (Bartoloni *et al.*, 2004).

Phylogenetic grouping. All isolates were tested to determine their phylogenetic groups using PCR according to the method described by Clermont *et al.* (2000). The primer pairs used were ChuA-F, 5'-GACGAACCAACGGTCAGGAT-3', and ChuA-R, 5'-TGCCGCCAG-TACCAAAGACA-3'; YjaA-F, 5'-TGAAGTGTCAAGAGACGCTG-3', and YjaA-R, 5'-ATGGAGAATGCGTTCCTCAAC-3'; and TspE4.C2-F, 5'-GAGTAATGTCGGGGCATTCA-3', and TspE4.C2-R, 5'-CGC-GCCAAACAAAGTATTACG-3'; these generated fragments of 279, 211 and 152 bp, respectively. Gel electrophoresis was run on 2% agarose in 0.6× TBE buffer, stained with ethidium bromide and viewed as described above.

Typing of isolates. All sulphonamide-resistant isolates were typed by using the randomly amplified polymorphic DNA (RAPD) PCR method and Phene Plate (PhP) fingerprinting. RAPD-PCR was performed with the PB1 (5'-ACACGCACACGGAAGAA-3') primer according to Ramos *et al.* (2010). The PCR was carried out in a 50 µl volume containing 5 µl 10× PCR buffer (Bioline), 3.8 µl MgCl₂ (50 mM) (Bioline), 1 µl (10 mM) dNTPs (Fisher Biotech), 5 µl (20 pmol µl⁻¹) primer PB1 (Invitrogen), 0.4 µl *Taq* DNA polymerase (Bioline) and 1.5 µl purified DNA template. PCRs were amplified as described previously (Ramos *et al.*, 2010) and products were size-separated by electrophoresis in 2.2% agarose with ethidium bromide at 90 V for 180 min. Banding patterns were compared visually and divided into similarity groups (RAPD types).

PhP fingerprinting was done by using high-resolution biochemical-fingerprinting PhP-RE plates (PhPlate AB), which were developed specifically to type *E. coli* strains (Landgren *et al.*, 2005). Inoculation of the plates was done according to the instructions of the manufacturer and the plates were incubated at 37 °C. The rate of each reaction was evaluated by measuring the *A*₆₂₀ in each well after 7, 24 and 48 h

incubation using a digital scanner. After the final scan, PhPlate software v. 4002 (PhPlate AB) was used to create A_{620} data from the scanned plate images and the mean A_{620} in each well over the three readings was calculated, yielding the biochemical fingerprint for each isolate. The biochemical fingerprints of the isolates were compared pairwise and the similarity between each pair of strains was calculated as the similarity coefficient and clustered by UPGMA (Saeedi *et al.*, 2005; Sneath & Sokal, 1973). Isolates showing similarity coefficients to each other that were higher than the default identity level of the software (0.975) were regarded as identical and assigned to the same biochemical phenotype. All data handling, including calculations of correlations and coefficients, as well as clustering, was performed using the PhPlate software v. 4002 (PhPlate AB). Strains were regarded as a common clonal group if they had the same RAPD-PCR pattern, PhP type and phylogenetic group; otherwise, they were referred to as a single clone.

Identification of the *sul1*, *sul2* and *sul3* genes. All *E. coli* isolates resistant to sulphafurazole were tested for the presence of the *sul1*, *sul2* and *sul3* genes. Multiplex PCR amplification of *sul1* and *sul2* was performed in a 50 µl volume with 5 µl 10× PCR buffer (Bioline), 2 µl (50 mM) $MgCl_2$, 5 µl (2 mM) dNTPs, 0.5 µl (10 pmol µl⁻¹) each primer, 2 µl template DNA and 0.5 µl *Taq* polymerase (Bioline). Conditions of these PCRs were initial denaturation at 94 °C for 5 min, followed by 36 cycles of 94 °C for 15 s, 67 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 7 min. Amplification of the *sul1* and *sul2* genes was performed with the *sul1*-F, *sul1*-R, *sul2*-F and *sul2*-R primers described by Kern *et al.* (2002), which generate fragments of 433 and 293 bp for the *sul1* and *sul2* genes, respectively. Primers and amplification conditions for the *sul3* gene were as described by Hammerum *et al.* (2006). The PCR was performed in a 25 µl reaction containing 3 µl 10× PCR buffer, 1 µl 50 mM $MgCl_2$, 2.5 µl 2 mM dNTPs, 0.15 µl *Taq* polymerase (Bioline), 2 µl DNA and 0.5 µl 10 pmol µl⁻¹ primers (*sul3*-F, 5'-GAGCAAGATTTTGGGAATCG-3', and *sul3*-R, 5'-CTAACCTAGGGCTTTGGATAT-3'), which generate a 750 bp fragment. Thermal cycler conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 53 °C for 40 s and 72 °C for 1 min. A final extension was performed at 72 °C for 5 min.

Amplified *sul1*, *sul2* and *sul3* PCR products were electrophoresed on 2% agarose in 0.6× TBE buffer, stained with ethidium bromide and

viewed under UV light using a Bio-Rad Gel Doc system, as described above. Positive controls included *E. coli* DH5 α containing plasmids R388 and pSRC15, harbouring the *sul1* and *sul2* genes, respectively, and a clinical *E. coli* strain (22.1 R1) containing the *sul3* gene, all kindly provided by Ruth Hall from the School of Molecular Bioscience, University of Sydney. MilliQ water served as a negative control.

Detection of integron-associated *intI* genes. All *E. coli* strains were tested for the presence of different classes of integron-associated integrases by using a multiplex PCR as described by Dillon *et al.* (2005). The PCR protocol for the *intI* genes was modified to the following conditions: after initial denaturation at 94 °C for 10 min, the reaction mixture was run through 25 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 30 s and extension at 72 °C for 1 min. Cycling was followed by a final extension incubation of 10 min at 72 °C. Negative and positive controls were included in each reaction. Positive controls were *E. coli* cells containing vectors with inserts possessing integrons of a single class only. All of the positive controls came from a subclone of those described by Long *et al.* (2009). These included *E. coli* uB1637, containing plasmid R388 carrying *Int3*, a class I integron, and *E. coli* DH5 α , containing pMAQ612 carrying *Tn7* (class II) or pSMB731 (class III). After amplification, PCR products were separated electrophoretically and viewed as described above. The selected primers for detection of all three classes of integrases were *Int1F*, 5'-CAGTG-GACATAAGCCTGTTC-3', and *Int1R*, 5'-CCCCAGGCATAGACT-GTA-3', for the *intI1* gene; *Int2F*, 5'-CACGGATATGCGACAA-AAAGGT-3', and *Int2R*, 5'-GATGACAACGAGTGACGAAATG-3', for the *intI2* gene; and *Int3F*, 5'-GCCTCCGGCAGCGACTTTCAG-3', and *Int3R*, 5'-ACGGATCTGCCAACCTGACT-3', for the *intI3* gene, which generate fragments of 160, 788 and 979 bp, respectively.

E. coli strains that harboured class II integrases were tested further for functionality of the protein encoded by the *intI2* gene. The primer sets for detection of this gene were HS913, 5'-CGCCTAATCCCAGCA-ATAAAAT-3', specific for truncated version of *intI2*; HS914, 5'-GCGCCTAATCCCAGTAATTAAC-3', specific for functional version of *intI2*; and HS502, 5'-GTAGCAAACGAGTGACGAAATG-3', as a complementary primer for both. The PCR protocol to detect the

Table 2. Association between phylogenetic groups and antimicrobial drug resistance among 137 *E. coli* strains isolated from patients with UTI

Significant differences were only observed between phylogenetic groups B2 and D.

Antimicrobial drug	No. of strains	No. (%) of strains in phylogenetic group:				P-value*
		A	B1	B2	D	
Trimethoprim						
Susceptible	96	2	2	84 (78.5)	8 (33)	<0.001
Resistant	41	1	1	23 (21.5)	16 (67)	
Tetracycline						
Susceptible	111	2	2	92 (86)	15 (62.5)	0.007
Resistant	26	1	1	15 (14)	9 (37.5)	
Gentamicin 10 µg						
Susceptible	128	3	3	102 (95.3)	20 (83.3)	0.036
Resistant	9	0	0	5 (4.7)	4 (16.7)	
Nitrofurantoin						
Susceptible	133	3	2	107 (100)	21 (87.5)	<0.001
Resistant	4	0	1	0	3 (12.5)	

*P-values (by Fisher's exact test) are shown when <0.05.

Fig. 1. Association between phylogenetic groups and the presence of integron-associated *intI* genes among 137 *E. coli* strains isolated from patients with UTI. Comparison between different phylogenetic groups showed a significant difference between strains in phylogenetic groups B2 (filled bars) and D (empty bars) only.

functionality of the *intI2* gene has been described by Márquez *et al.* (2008a).

Statistical analysis. Differences in the prevalence of *intI* genes between phylogenetic groups, as well as between antimicrobial drug-resistant strains in hospitals, were assessed by using χ^2 and Fisher's exact tests. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Antimicrobial-resistance patterns of the isolates varied among the hospitals. In all, 119 (88.2 %) isolates were resistant to one or more of the 17 tested antimicrobial drugs, with the highest resistance found against sulphafurazole (70.1 %), ampicillin (50.4 %), cephalothin (50.4 %), trimethoprim (29.6 %) and tetracycline (17.8 %) (Table 1). Thirteen (9.5 %) isolates were resistant to all of the above five antimicrobial drugs; four patterns, i.e. AMP/SF, AMP/CEF/SF, AMP/TMP/CEF/SF and AMP/TMP/CEF/TET/SF, found among the strains from all hospitals.

The majority (78.1 %) of the isolates belonged to phylogenetic group B2, whereas 17.5 % belonged to phylogenetic group D. The remaining 4.4 % of isolates belonged to phylogenetic groups A and B1. With the exception of one, all isolates belonging to phylogenetic group D, and 92 of 107 (85.7 %) isolates belonging to phylogenetic group B2 were resistant to at least one of the tested antimicrobial drugs (Table 1). Strains resistant to trimethoprim, tetracycline, gentamicin and nitrofurantoin were found to be significantly more associated with phylogenetic group D than B2 (Table 2).

In all, 54 (39.4 %) isolates were positive for the presence of integrases, with 47 (34.3 %) isolates being positive for class I integron-associated integrase, while seven (5.1 %) isolates were positive for the *intI2* gene. Three (2.2 %) isolates carried both class I and II integrases. Class III integrase was

Table 3. Prevalence of antimicrobial drug resistance among 137 uropathogenic *E. coli* strains with and without integron-associated *intI* genes

S, Susceptible; R, resistant; NS, not significant.

Antimicrobial drug	<i>intI</i> ⁺ isolates (n=54)		<i>intI</i> ⁻ isolates (n=83)		<i>P</i> -value*
	S (%)	R (%)	S (%)	R (%)	
Trimethoprim	18 (33.7)	36 (66.7)	78 (94.0)	5 (6.0)	<0.001
Sulphafurazole	5 (9.3)	49 (90.7)	36 (43.4)	47 (56.6)	<0.001
Ampicillin	14 (25.9)	40 (74.1)	53 (63.9)	30 (36.1)	<0.001
Cephalothin	19 (35.2)	35 (64.8)	47 (56.6)	36 (43.4)	0.014
Tetracycline	35 (64.8)	19 (35.2)	76 (91.6)	7 (8.4)	<0.001
Imipenem	53 (98.1)	1 (1.9)	83 (100)	0 (0.0)	NS
Cefotetan	52 (96.3)	2 (3.7)	80 (96.4)	3 (3.6)	NS
Piperacillin-tazobactam	50 (92.6)	4 (7.4)	82 (98.8)	1 (1.2)	NS
Gentamicin (10 and 200 µg)	47 (87.0)	7 (13.0)	81 (97.6)	2 (2.4)	0.015
Nitrofurantoin	52 (96.3)	2 (3.7)	81 (97.6)	2 (2.4)	NS
Amoxicillin-clavulanic acid	49 (90.7)	5 (9.3)	80 (96.4)	3 (3.6)	NS
Nalixidic acid	48 (88.9)	6 (11.1)	82 (98.8)	1 (1.2)	0.010
Aztreonam	53 (98.1)	1 (1.9)	81 (97.6)	2 (2.4)	NS
Cefpodoxime	50 (92.6)	4 (7.4)	81 (97.6)	2 (2.4)	NS
Ceftazidime	51 (94.4)	3 (5.6)	82 (98.8)	1 (1.2)	NS
Cefotaxime	51 (94.4)	3 (5.6)	83 (100)	0 (0.0)	0.030
Cefepime	52 (96.3)	2 (3.7)	82 (98.8)	1 (1.2)	NS

**P*-values (by Fisher's exact test) are shown when <0.05.

Table 4. *sul* and *intI* gene distribution among sulphonamide-resistant isolates

Values are no. of PCR-positive isolates.

Integrase	None	<i>sul1</i> only	<i>sul2</i> only	<i>sul1</i> + <i>sul2</i>	<i>sul1</i> + <i>sul3</i>	<i>sul2</i> + <i>sul3</i>	<i>sul1</i> + <i>sul2</i> + <i>sul3</i>	Total
None	12	8	2	20	0	3	2	47
<i>intI1</i>	0	8	10	26	1	0	0	45
<i>intI2</i>	0	0	0	1	0	0	0	1
<i>intI1</i> + <i>intI2</i>	0	2	0	0	1	0	0	3
Total	12	18	12	47	2	3	2	96

not detected among the isolates. Functionality testing of the protein encoded by the *intI2* gene of all seven strains harbouring class II integrases showed that these proteins were non-functional. Comparison of the isolates belonging to different phylogenetic groups showed that strains belonging to group D carried significantly ($P<0.001$) more *intI* genes than those belonging to phylogenetic group B2 (Fig. 1).

All except two isolates ($n=52$) carrying *intI* genes were resistant to at least one antimicrobial drug (Table 1). The prevalence of MDR isolates among the integrase-positive strains (51 of 54) was significantly ($P<0.001$) higher than that found among the integrase-negative strains (38 of 83). Furthermore, resistance to trimethoprim, sulphafurazole, ampicillin, cephalothin, tetracycline, gentamicin, nalixidic acid or cefotaxime was significantly more common among isolates harbouring *intI* genes than among those that did not (Table 3).

Of the 137 isolates tested, 96 (70%) were resistant to sulphafurazole. In view of the high prevalence of sulphonamide-resistant strains in our collection, these strains were tested further for three allelic variations of the *sul* gene (*sul1*,

sul2 and *sul3*). Table 4 shows the distribution of these allelic genes among both integron-positive and integron-negative strains. Typing of sulphafurazole-resistant isolates showed that the strains belonged to 11 common and 33 single clonal groups. Within each common clonal group, there existed different antimicrobial drug-resistance patterns, *intI* genes and *sul* alleles (Table 5). Of these, 84 (87.5%) carried one or more *sul* alleles, with *sul1* being the most common allele found in 69 (71.8%) isolates. *sul2* and *sul3* were found in 64 (66.6%) and seven (7.2%) isolates, respectively.

Whilst all integrase-positive isolates carried at least one *sul* gene, 42% of isolates carrying *sul* alleles did not show the presence of any *intI* genes. Furthermore, 10 isolates carrying class I integrase showed the presence of only the *sul2* gene. Statistical analysis also confirmed the lack of a relationship between the presence of class I integrase and the *sul1* allele (Table 6). Similar results were found between the phylogenetic group of the strains and the presence of *sul* alleles ($P=0.126$). However, strains harbouring the *sul2* gene showed a significantly higher prevalence of resistance to ampicillin ($P<0.016$), trimethoprim ($P=0.018$) and cephalothin ($P=0.03$) (Fig. 2). Similar results were found

Table 5. Distribution of 96 sulphonamide-resistant *E. coli* strains carrying *intI* genes among different common (CTs) and single clonal (STs) types

Type	No. of isolates	No. of strains positive for <i>intI</i> genes		Phylogenetic group(s)
		<i>intI1</i>	<i>intI2</i>	
CTs				
1	5	4	0	B2
2	2	0	0	B2
3	6	2	0	B2
4	9	2	0	B2
5	19	11*	1*	B2
6	4	1*	1*	B2
7	5	3	0	B2
8	7	7*	1*	D
9	2	0	0	B2
10	2	1	0	B2
11	2	0	0	B2
STs	33	17	1	B2 (21), D (8), A (2), B1 (2)

*Strains showing the presence of both class I and II integrases.

Table 6. Association between class I integron-associated integrase and presence of the *sulI* gene

Values are no. (%) of isolates.

	<i>sulI</i> ⁻	<i>sulI</i> ⁺	Total	P-value
<i>intI1</i> ⁻	17 (63)	31 (44.9)	48	0.112
<i>intI1</i> ⁺	10 (37)	38 (55.1)	48	
Total	27	69	96	

with the *sulI* gene, except that strains carrying this gene were significantly resistant to only ampicillin (57 % of ampicillin-sensitive strains carried the *sulI* gene, as opposed to 80 % of ampicillin-resistant strains) ($P=0.015$).

DISCUSSION

Sulphonamides have been used alone or in combination with trimethoprim for treatment of UTIs (Ang *et al.*, 2009; Warren *et al.*, 1999), which explains the commonly reported high levels of resistance to these antimicrobial agents among *E. coli* strains (Blahna *et al.*, 2006; Ho *et al.*, 2009). In our study, we found a high level of resistance to these antimicrobial agents. However, we also found that >61 % of our sulphafurazole-resistant strains were susceptible to trimethoprim, which is markedly different from prevalences reported elsewhere (Bean *et al.*, 2005; Blahna *et al.*, 2006).

Integron distribution in UPEC has been the subject of several studies (Chang *et al.*, 2007; Farshad *et al.*, 2008; Rao *et al.*, 2006; Rijavec *et al.*, 2006). These studies have established a strong association between the presence of integrons and antimicrobial resistance, to both multiple and single drugs. *E. coli* carrying several antibiotic-resistance genes may act as a donor of those resistance genes to other pathogenic *E. coli* and even other species. As reported by Leverstein-van Hall *et al.* (2002, 2003), integrons may be a major culprit for the dissemination of antimicrobial resistance, and their prevalence has ranged from 16.6 to 59.0 % in clinical *E. coli* isolates (Farshad *et al.*, 2008; Márquez *et al.*, 2008b; Mathai *et al.*, 2004; Solberg

et al., 2006; Yu *et al.*, 2003). According to our study, 39.4 % of UTI-causing *E. coli* possessed class I and II integrases, which is in line with other reports. Our results, however, indicated that the presence of *intI* genes was associated strongly with the MDR property. We also found that two isolates carrying either *intI1* or *intI2* genes were susceptible to all antimicrobial drugs tested in our study. It must be noted, however, that the detection of *intI* genes does not always correlate with the presence of functional integron.

In this study, although the majority of the isolates (78.1 %) belonged to phylogenetic group B2, we found a strong association between the presence of the integron-associated *intI* gene and phylogenetic group D, which constituted only 18 % of the isolates. A previous study on *E. coli* collected from patients with asymptomatic bacteriuria, cystitis and pyelonephritis from one of the three hospitals of this study also indicated a similar distribution of these phylogenetic groups among their isolates (Mabbett *et al.*, 2009), although the prevalence of virulence genes varied among different pathotypes. In our study, we did not investigate the prevalence of virulence genes among our isolates, as the aims of the study were different.

Class II integrons are normally found in 4–20 % of UPEC strains (Castro *et al.*, 2005). In these strains, the class II integrase is inactive, due to the presence of a premature in-frame stop codon (Hansson *et al.*, 2002). Barlow & Gobius (2006) reported an *E. coli* strain in which the stop codon had been replaced by a glutamine codon and suggested that the associated Int2 protein is functional, and concluded that such functional integron genes may represent an additional mechanism by which resistance-gene cassettes could be mobilized in pathogens. Márquez *et al.* (2008a) developed a set of primers that were able to differentiate functional from non-functional class II integrase. In our study, we used this set of primers to identify the functionality of our strains to mobilize the resistance gene, and we detected the presence of both functional and non-functional proteins encoded by our PCR products. Whilst sequencing of these PCR products (309 bp amplicon for functional and 308 bp amplicon for non-functional) did not fully confirm the presence or absence of functional

Fig. 2. Association between the presence of the *sul2* gene and resistance to antimicrobial drugs. Only antimicrobial drugs with a significant association are shown. White bars, absence of *sul2* gene; filled bars, presence of *sul2* gene; S, susceptible; R, resistant.

intI2 genes, full sequencing of the 788 bp *intI2* amplicon indicated that these strains in fact did not carry a functional class II integrase. We therefore recommend that, to avoid any false-positive results, all strains showing positive results for functional class II integrase using primer sets HS914/HS502 should be tested by sequencing the 788 bp *intI2* amplicon.

Although the pattern of gene-frequency distribution in most published data is *sul2>sul1>sul3* (Blahna *et al.*, 2006; Grape *et al.*, 2003; Infante *et al.*, 2005; Kern *et al.*, 2002), our results indicated that this pattern for UPEC isolates in Queensland, Australia, was *sul1>sul2>sul3*. The relationship between integron class I and the sulphonamide-resistance gene (*sul1*) as a conserved segment of an integron class I component has frequently been pointed out before (Ho *et al.*, 2009; Kern *et al.*, 2002; Sköld, 2000). However, in our study we found that 45 % of isolates carrying the *sul1* allele did not show a positive PCR result for the presence of class I integrase; also, 10 strains of 96 carrying *intI1* were not positive for the presence of the *sul1* gene, which is of interest because these strains were *sul2*-positive. The *sul1* gene is a conserved region for class I integron, although it has been referred to as a semi-conserved segment (Grape *et al.*, 2005). In our study, we found that majority of strains carrying the *sul1* gene belonged to diverse clonal groups. Interestingly, these strains did not carry class I integrase. In contrast to that, we found that 10 sulphafurazole-resistant strains carrying the integron-associated *intI1* gene did not carry the *sul1* gene. These findings suggest either that class I integrons in our isolates have lost the *sul1* gene region or that this gene is carried on another genetic context in these strains. Similar findings have been reported by others (Grape *et al.*, 2005; Infante *et al.*, 2005; Kern *et al.*, 2002). Furthermore, we also found that about 13 % of isolates that were resistant to sulphafurazole did not harbour any *sul* alleles. This might be because of the limitations of our disc-diffusion method or the presence of other *sul* alleles in those strains responsible for sulphonamide resistance. Nonetheless, this figure is much higher than those reported elsewhere (Frank *et al.*, 2007; Grape *et al.*, 2005; Kern *et al.*, 2002).

The loss and gain of mobile genetic elements is an important mechanism that disseminates new traits rapidly to recipient micro-organisms. For instance, acquisition of resistance genes by horizontal transfer is the main role in the development of MDR strains (Chang *et al.*, 2011; Moritz & Hergenrother, 2006). Naturally, the recipient micro-organism would be able to colonize a new environment when selective pressures select for variants that can survive these pressures (Croxen & Finlay, 2010). From an evolutionary point of view, the emergence of a new pathotype of *E. coli* could be due to multiple horizontal gene transfer that exposes these bacteria to new selective pressures. Such selective pressures may eventually select for more virulent organisms that disseminate to become an epidemic clone, such as those seen with enterohaemorrhagic and enteroinvasive *E. coli* (Wirth *et al.*, 2006). In our study, the high diversity found among sulphafurazole-resistant strains

indicated that the majority of strains carrying the *sul* gene were not members of the same clone and the high prevalence of these strains among patients with UTI is not due to the dissemination of a single or a few clones of UPEC in these three hospitals. The presence of different antimicrobial drug-resistance patterns and phylogenetic groups among these isolates further supports this conclusion.

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