

Genomic analysis of trimethoprim-resistant extraintestinal pathogenic *Escherichia coli* and recurrent urinary tract infections

Dmitriy Li¹, Cameron J. Reid¹, Timothy Kudinha^{2,3}, Veronica M. Jarocki^{1,*} and Steven P. Djordjevic^{1,*}

Abstract

Urinary tract infections (UTIs) are the most common bacterial infections requiring medical attention and a leading justification for antibiotic prescription. Trimethoprim is prescribed empirically for uncomplicated cases. UTIs are primarily caused by extraintestinal pathogenic *Escherichia coli* (ExPEC) and ExPEC strains play a central role in disseminating antimicrobial-resistance genes worldwide. Here, we describe the whole-genome sequences of trimethoprim-resistant ExPEC and/or ExPEC from recurrent UTIs (67 in total) from patients attending a regional Australian hospital from 2006 to 2008. Twenty-three sequence types (STs) were observed, with ST131 predominating (28%), then ST69 and ST73 (both 7%). Co-occurrence of trimethoprim-resistance genes with genes conferring resistance to extended-spectrum β -lactams, heavy metals and quaternary ammonium ions was a feature of the ExPEC described here. Seven trimethoprim-resistance genes were identified, most commonly *dfrA17* (38%) and *dfrA12* (18%). An uncommon *dfrB4* variant was also observed. Two *bla*_{CTX-M} variants were identified – *bla*_{CTX-M-15} (16%) and *bla*_{CTX-M-14} (10%). The former was always associated with *dfrA12*, the latter with *dfrA17*, and all *bla*_{CTX-M} genes co-occurred with chromate-resistance gene *chrA*. Eighteen class 1 integron structures were characterized, and *chrA* featured in eight structures; *dfrA* genes featured in seventeen. ST131 H30Rx isolates possessed distinct antimicrobial gene profiles comprising *aac(3)-IIa*, *aac(6)-Ib-cr*, *aph(3')-Ia*, *aadA2*, *bla*_{CTX-M-15}, *bla*_{OXA-1} and *dfrA12*. The most common virulence-associated genes (VAGs) were *fimH*, *fyuA*, *irp2* and *sitA* (all 91%). Virulence profile clustering showed ST131 H30 isolates carried similar VAGs to ST73, ST405, ST550 and ST1193 isolates. The sole ST131 H27 isolate carried molecular predictors of enteroaggregative *E. coli*/ExPEC hybrid strains (*aatA*, *aggR*, *fyuA*). Seven isolates (10%) carried VAGs suggesting ColV plasmid carriage. Finally, SNP analysis of serial UTI patients experiencing worsening sequelae demonstrated a high proportion of point mutations in virulence factors.

DATA SUMMARY

The 67 draft genomes from the whole-genome sequencing described here have been submitted to the National Center for Biotechnology Information with the accession numbers SAMN14547713 to SAMN14547779, under BioProject PRJNA623470.

INTRODUCTION

Urinary tract infections (UTIs) are the most common bacterial infections to require medical attention and incur an estimated economic burden of ~\$2 billion per annum (£1.51 billion;

\$1=£0.76) in the USA alone [1]. In the UK, in 2013–2014, the National Health Service spent £434 million in treating 184000 patients with unplanned admissions relating to UTIs [2]. In Australia, UTIs caused 69823 hospitalizations in 2017–2018, resulting in 234455 inpatient days and treatment costs as high as \$AU6400 (£3500) per patient [3, 4]. Additionally, UTIs are the leading rationale behind antibiotic prescription by general practitioners [5].

The most common aetiological agent of UTIs are extraintestinal pathogenic *Escherichia coli* (ExPEC), responsible for ~75–95% of cases [6]. Epidemiological studies utilizing multi-locus sequence typing (MLST) indicate that certain pandemic

Received 17 May 2020; Accepted 28 October 2020; Published 18 November 2020

Author affiliations: ¹Three Institute, University of Technology Sydney, Ultimo, NSW 2007, Australia; ²NSW Health Pathology, Microbiology, Orange Hospital, Orange, NSW 2800, Australia; ³School of Biomedical Sciences, Charles Sturt University, Orange, NSW 2800, Australia.

***Correspondence:** Veronica M. Jarocki, veronica.jarocki@uts.edu.au; Steven P. Djordjevic, steven.djordjevic@uts.edu.au

Keywords: antimicrobial resistance; class 1 integrons; extraintestinal pathogenic *Escherichia coli*; hospital; ST131; urinary tract infection.

Abbreviations: AMR, antimicrobial resistance; ARG, antimicrobial gene; CC, clonal complex; EAEC, enteroaggregative *Escherichia coli*; ESBL, extended-spectrum β -lactamase; ExPEC, extraintestinal pathogenic *Escherichia coli*; IS, insertion sequence; MDR, multidrug resistant; MDS, multidimensional scaling; MGE, mobile genetic element; MLST, multilocus sequence typing; NSW, New South Wales; PAI, pathogenicity island; ST, sequence type; UTI, urinary tract infection; VAG, virulence-associated gene; WGS, whole-genome sequencing.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Nine supplementary data items are available with the online version of this article.

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sequence types (STs) account for more than 50% of all ExPEC infections worldwide. These ExPEC lineages are ST131, ST69 (also known as 'clonal group A'), ST10, ST405, ST38, ST95, ST73 and ST127 [7, 8].

ExPEC can enter the intestinal tract via contact with poultry, companion animals, the environment and through sexual contact [9]. Additionally, travellers are at higher risk of ingesting ExPEC strains [10]. The subsequent introduction of ExPEC into the urinary tract via the urethra can occur due to host behaviours, physiological abnormalities or medical interventions, such as catheterization [6]. After contaminating the urethra, ExPEC can ascend to infect the bladder (cystitis), the kidney (pyelonephritis) or enter the bloodstream (urosepsis) with potentially dire outcomes as ExPEC-associated sepsis has a mortality rate of up to 30% [11]. Indeed, each year at least 1.7 million adults in the USA develop sepsis, and 1 in 3 patients who die in hospital have sepsis [12, 13].

ExPEC possess an array of virulence-associated genes (VAGs) that enable ascension, colonization and persistence within a hostile and nutrient-deficient environment. These VAGs – commonly located on chromosomal pathogenicity islands (PAIs) or encoded on virulence plasmids – include uroepithelial adhesins, such as P-fimbriae (*pap* genes), S-fimbriae (*sfa* genes), F1C-fimbriae (*foc* genes), and Dr adhesins (*dra* genes, *afa* genes), toxins including secreted autotransporter toxin (*sat*) and cytotoxic necrotizing factor 1 (*cnf1*), capsule (*kpsM II*), and several iron-acquisition systems, such as aerobactin (*iuc* genes, *iutA*), salmochellin (*iro* genes) and yersiniabactin (*ybt* genes, *irp1*, *irp2*, *fyuA*) [14, 15]. ExPEC have also been associated with a greater abundance of bacteriocins, toxins that inhibit the growth of other *E. coli* [16]. These bacteriocins are frequently carried on virulence plasmids, such as ColV plasmids [17]. While no singular VAG can be attributed to ExPEC pathogenesis, a molecular definition of ExPEC has been proposed as being *E. coli* that contain at least two of the following VAGs: *papA* and/or *papC*, *sfa/foc*, *afa/draBC*, *kpsM II* and *iutA* [18]. Furthermore, the presence of certain VAGs have been associated with specific uro-clinical syndromes, such as P- and S-fimbriae, Dr adhesins, and the invasin *ibeA* with pyelonephritis [6], and yersiniabactin *irp2* and *fyuA* with sepsis [19]. *FyuA* is also directly linked to ExPEC biofilm formation in human urine [20], as is the salmochelin receptor *iroN* [21].

In Australia, severe pyelonephritis and sepsis are typically treated with intravenous gentamicin and amoxicillin, while cystitis is typically treated empirically with either nitrofurantoin or trimethoprim [22]. However, a 5 year study on antimicrobial resistance (AMR) in urinary *E. coli* from an Australian metropolitan hospital found significant increases in trimethoprim, nitrofurantoin, amoxicillin and gentamicin resistance, as well as significant extended-spectrum β -lactam resistance [23]. ExPEC, particularly ST131 strains, have been central to a worldwide increase in extended-spectrum β -lactamase (ESBL)-producing *E. coli*. The ST131 lineage consists of three major clades, each of which is strongly associated with specific *fimH* alleles. Strains from clade C typically carry *fimH30* and

Impact Statement

Extraintestinal pathogenic *Escherichia coli* (ExPEC) that cause urinary tract infections (UTIs) represent a significant disease burden and contribute greatly to the spread of antimicrobial resistance (AMR), both in Australia and worldwide. While whole-genome sequencing (WGS) provides the most precise means to track resistance and virulence mechanics, to date genomic analyses on UTI-associated ExPEC within Australian hospitals has been limited, particularly for those hospitals situated in regional and remote areas. In this study, ExPEC isolates were taken from patients suffering from cystitis, pyelonephritis and urosepsis attending a Western New South Wales Hospital between 2006 and 2008. We used WGS to investigate diversity, virulence, class 1 integron and AMR gene carriage, and SNPs that occurred in multiple isolates derived from the same patients. By doing so, we provide a baseline for future studies tracking the evolution of ExPEC AMR and virulence potential in Australian UTI-associated ExPEC populations, and particularly the evolution of ExPEC resistant to first-line UTI treatment. This work adds to the national picture of ExPEC, and additionally provides hospital-specific information that may inform future policy making and practices.

are currently the most prominent subtype identified in human infections [24]. Clade C is further divided into two subclades – C1 and C2. C1 mostly comprises strains with *gyrA* and *parC* SNPs conferring fluoroquinolone resistance, designated H30R, while the latter consists of strains with both fluoroquinolone resistance SNPs and carriage of plasmid-associated *bla*_{CTX-M} genes, designated H30Rx [25–27]. *E. coli* ST131 first emerged in the literature in the early 2000s and has been linked to a 300% increase in USA hospital admissions due to ESBL-producing *E. coli* in the following decade [28, 29]. The global rapid increase in ESBL-producing *E. coli* has had serious knock-on effects, driving carbapenem prescription and in turn promoting the spread of potentially untreatable carbapenemase-producing *E. coli* [30].

Like all *E. coli*, ExPEC have highly flexible genomes and a proclivity to capture and disseminate genes through horizontal gene transfer [31]. Horizontal gene transfer can occur via plasmid conjugation, phage transduction and via small mobile genetic elements (MGEs), such as transposons, insertion sequences (IS) and integrons, allowing for inter- and intra-species movement of genetic information [32]. As observed with the global dissemination of *bla*_{CTX-M}, a single MGE capture event can have worldwide repercussions [30].

AMR surveillance of ExPEC populations within high selective pressure environments, such as hospitals, could provide meaningful insights and inform future policy making and practices. In addition to the aforementioned 5 year study on AMR within ExPEC isolated in an Australian hospital, a

recent Australian government report also stated that *E. coli* resistances to ESBLs and fluoroquinolones are climbing [4]. While these reports highlight national trends, they tend to focus on hospitals situated in metropolitan areas, while more remote and rural areas get neglected. Furthermore, genomic surveillance of antimicrobial genes (ARGs) and VAGs within Australian hospital *E. coli* populations is currently limited.

Here, we used whole-genome sequencing (WGS) to characterize 67 ExPEC strains from patients with UTIs collected in a large Western New South Wales (NSW) hospital catering to regional, remote and rural Australian communities. The genomes were typed using Clermont phylogrouping, e-serotyping and MLST, and screened for the presence of ARGs, VAGs, PAIs and plasmid replicons, and several AMR associated class 1 and class 2 integrons found in this collection were characterized. Furthermore, isolates from recurrent infections, including those with a subsequently more severe clinical sequelae, were interrogated for SNPs leading to point mutations.

METHODS

Sample collection and selection criteria for WGS

Urine specimens were collected by clinical staff members of participating health-care centres using a standardized protocol. Semi-quantitative culture was performed on horse blood, MacConkey and chromogenic agars, followed by conventional identification. Isolates were stored in 50% (v/v) glycerol in trypticase soy broth at -70°C .

Phenotypic resistance testing

The isolates were tested for susceptibility to 14 antibiotics as per the disc diffusion method specified by the Clinical and Laboratory Standards Institute (CLSI) [33], using Neo-Sensitabs discs (Rosco). The antibiotics tested were (disc content) amikacin (30 μg), amoxicillin–clavulanate (60 μg), ampicillin (25 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), ciprofloxacin (10 μg), gentamicin (10 μg), imipenem (10 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), tetracycline (30 μg) and trimethoprim–sulfamethoxazole (5 μg). The double-disc diffusion test was used to detect the production of ESBLs [34].

DNA isolation and WGS

DNA extraction and WGS were performed as described previously [35]. Briefly, DNA was extracted using the ISOLATE II genomic DNA kit (BioLine) and stored at -20°C . Short-read sequencing was performed using an Illumina HiSeq 2500 v4 sequencer in rapid PE150 mode.

Genome assemblies

Raw reads were used to assemble draft genome sequences via Shovill software using default settings (<https://github.com/tseemann/shovill>). Assemblies underwent quality control using assembly-stats software (<https://github.com/sanger-pathogens/assembly-stats>). Assembly statistics for this

collection are available in Data S1 (available with the online version of this article).

Phylogenetic analysis

A maximum-likelihood phylogenetic tree of this collection was reconstructed using IQ-TREE 2 (<http://www.iqtree.org/>) [36] and a SNP-based phylogenetic tree was reconstructed using snplord (<https://github.com/maxcummins/pipelord/snplord/tree/master/snplord>), an automated pipeline that utilizes snippy (<https://github.com/tseemann/snippy>), Gubbins [37] (<https://github.com/sanger-pathogens/gubbins>) and SNP-sites (<https://github.com/sanger-pathogens/snp-sites>). The SNP-based tree of the entire collection was built using 76.0% (3525942 bp/4639675 bp) of the reference genome K12 MG1655 (GCA_000005845.2) and consisted of 35038 SNPs (141270 SNPs before recombination filtering). Trimethoprim-sensitive ExPEC isolates ERR434278, ERR434751, ERR434270, ERR434273 and ERR434271 [38], and trimethoprim-sensitive enteroaggregative *E. coli* (EAEC) isolates SRR5470250, SRR5024242 and SRR3574247 [39], all representing the major STs in this collection, were added to both the phylogenetic analysis and gene screening as controls. An additional SNP-based ST131 only tree was built using 92.03% (4831235 bp/5249449 bp) of reference genome ST131 EC958 (GCA_000285655.3) to identify SNP sites. Recombination filtering reduced 7460 SNPs to 386 SNPs. The ST131 SNP-based tree was reconstructed using IQ-TREE 2 and a recombination filtered alignment of 478 bp produced by Gubbins. All trees were visualized using the Interactive Tree of Life (iTOL) v4 online-based software [40] (<https://itol.embl.de/>). The ExPEC pangenome was calculated using Roary v3.11.2 [41] and visualized using Phandango [42]. Clonal samples from serial patients were removed from this analysis (total of 57 isolates used) to prevent deflation of the core genome.

Serial patient SNP analysis

The snplord pipeline was run on isolates originating from a single patient using the isolate with the earliest isolation date as a reference in each instance. SNPs called by this pipeline were checked manually in .gbk files generated by Prokka [43] using SnapGene. Gubbins output was used to determine which SNPs were the result of homologous recombination.

Gene screening

Isolate STs, serogroups and phylogroups were determined *in silico* using MLST v2.0 [44] (<https://cge.cbs.dtu.dk/services/MLST/>), SerotypeFinder v2.0 [45] (<https://cge.cbs.dtu.dk/services/SerotypeFinder/>) and ClermontTyping [46] (<http://enterobase.warwick.ac.uk/>), respectively. ST131 isolates additionally underwent FimH typing using FimTyper [47] (<https://cge.cbs.dtu.dk/services/FimTyper/>). The ARIBA read-mapping tool [48] was used to screen for ARGs, plasmid replicons and VAGs using the following reference databases: ResFinder [49] (<https://cge.cbs.dtu.dk/services/ResFinder/>), PlasmidFinder [50] (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) and VirulenceFinder [51] (<https://cge.cbs.dtu.dk/services/>)

VirulenceFinder/). However, as VirulenceFinder screens for only one *papA* allele, an additional BLASTN search was also performed using 14 *papA* alleles [52]. An additional custom database with AMR-associated ISSs, class 1 and 2 integrases, and additional ExPEC-associated VAGs was also utilized and can be accessed at https://github.com/CJREID/custom_DBs. ARIBA data were processed using a bespoke script accessible at <https://github.com/maxlcummins/pipelord/tree/master/ariбалord> and visualized using the following R packages: ggplot2 v3.3.0 (<https://github.com/tidyverse/ggplot2>) and ggtree v2.2.1 [53] (<https://github.com/YuLab-SMU/ggtree>).

To infer ColV plasmid and PAI carriage, short reads from each isolate were mapped to the ColV reference plasmid pCERC4 (KU578032), PAI I_{CFT073} (AE014075.1, start 3406225 bp, end 3469205 bp), PAI II₅₃₆ (AJ494981), PAI III₅₃₆ (AF301153) and PAI IV₅₂₆/HPI (high pathogenicity island) using the Burrows–Wheeler Aligner (BWA) v0.7.17 [54] and converted to BAM file format using SAMtools v0.1.18 [55]. A bespoke script, available at <https://github.com/maxlcummins/pipelord/tree/master/plasmidlord>, was used to produce a histogram of read-depth as a function of reference coordinate and clustered based on their Euclidean distances, and used to generate a heatmap.

BLASTN [56] was used to determine whether integrons characterized in this collection are present in other genomes available in public databases, and associated metadata were pulled from GenBank [56] and Enterobase [57]. Integron B structure comparison was achieved using EasyFig [58]. All gene schematics were generated using SnapGene (www.snapgene.com/).

Statistical analysis

R software v4.0.2 was used for the reconstruction of correlation heat maps and classical (metric) multidimensional scaling (MDS) analysis, using the ggplot2 v3.3.0 (<https://ggplot2.tidyverse.org/>) package for visualization, and the ggcorrplot v0.1.3 (<https://github.com/kassambara/ggcorrplot>)

package for correlation calculation. Two MDS analyses were performed, one for identified virulence genes and one for AMR genes, using the standard R package functions dist and cmdscale in conjunction with a presence/absence matrix (with one for presence and zero for absence).

RESULTS AND DISCUSSION

Genomic surveillance using WGS can provide high-resolution data on ARG presence, persistence, evolution and potential for horizontal transfer [59], as well as inform on population diversity and VAG carriage, and provide in-depth analyses of SNP-mediated mutations. Yet to date, genomic analyses on UTI-associated ExPEC within Australian hospitals has been limited. Here, we provide a retrospective WGS study on ExPEC resistant to first-line trimethoprim treatment and/or isolates collected from serial UTIs between 2006 to 2008 from a regional hospital in Western NSW, Australia. We aimed to provide a baseline for future studies tracking the evolution of ExPEC AMR and virulence potential in Australian UTI-associated ExPEC populations by reporting on ARG, VAG and MGE carriage, characterizing 18 class 1 integrons structures identified in this collection and identifying SNPs occurring in recurrent UTI isolates.

Demographic and clinical characteristics

From clinical samples collected between 2006 and 2008, 76 isolates were selected for genome sequencing due to phenotypic trimethoprim resistance and/or originating from a serial UTI patient. After quality-control measures, the final collection comprised 67 *E. coli* draft genomes (mean contig size of 23477 bp, with a mean number of contigs of 234) originating from 37 samples taken from patients with cystitis (55%), 23 samples from patients with pyelonephritis (34%) and 7 samples from patients with urosepsis (10%) (Fig. 1a). Recurrent UTIs are common, particularly in women, with 27% reporting a recurrence within 6 months [53]. In this

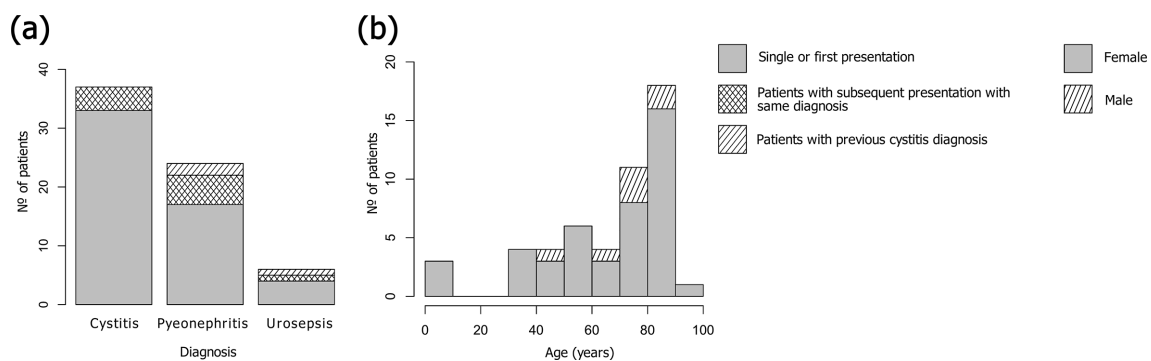


Fig. 1. Sample population and clinical characteristics. (a) Diagnoses of patients presenting with UTIs over the study period. For the patients with multiple samples taken for the same diagnosis (cross-hatched stripes): for cystitis, the mean number of days between collections was 126 days; for pyelonephritis, 63 days; and for urosepsis, 68 days. For patients with a previous cystitis diagnosis and subsequent pyelonephritis or urosepsis diagnoses (sloped stripes): the mean length between collections was 174 days, and 181 days for urosepsis ($n=1$). (b) Age and biological sex of patients. Females are represented by grey, males by shading.

collection, 12 out of 51 patients were sampled more than once due to multiple, or ongoing, UTIs during the study period.

Women are more likely to contract UTIs than men at a ratio of 8:1, and one in three women will experience at least one UTI necessitating antibiotics by age 24 [60]. The risk of UTIs tends to increase with age in both sexes [61]; however, the prevalence of UTIs in women over the age of 65 is double that of the overall population [62]. This collection is reflective of UTI epidemiology with more isolates derived from female patients (44 vs 7; 86 vs 14%) and with patients' ages ranging from 13 months to 91 years, the majority (64%) being above 66 years. The mean age for female patients was 66 years, and 74 years for male patients (Fig. 1b). No patient was identified as pregnant.

Phylogenetic analysis

While ExPEC are phylogenetically diverse, a recent systematic review and meta-analysis of 217 studies flagged a handful of

globally dominant STs, the most prominent being ST131 (phylogroup B2), followed by ST69 (D), ST10 (A), ST405 (D), ST38 (D), ST95 (B2), ST648 (D) and ST73 (B2) [7]. Consequently, B2 and D constitute the phylogroups most frequently associated with ExPEC infections. To ascertain the evolutionary relationships between samples in this collection, a maximum-likelihood phylogenetic tree was reconstructed using the complete genome of *E. coli* strain K12-MG1655 as a reference (Fig. 2a). The tree demonstrates clear clustering based on phylogroup and ST (Fig. 2a). The collection comprised 23 STs, and despite selecting for trimethoprim resistance, the collection followed global trends with ST131 (O25:H4) being the most common ($n=19$; 28%), followed by ST69 (ONT:H18 and O11:H18) and ST73 (O22:H1, O25:H1, O6:H1, and ONT:H1) with five isolates each. Isolates from phylogroup B2 were the most prevalent ($n=38$; 56%), and the least prevalent were from B1 ($n=3$; 4%).

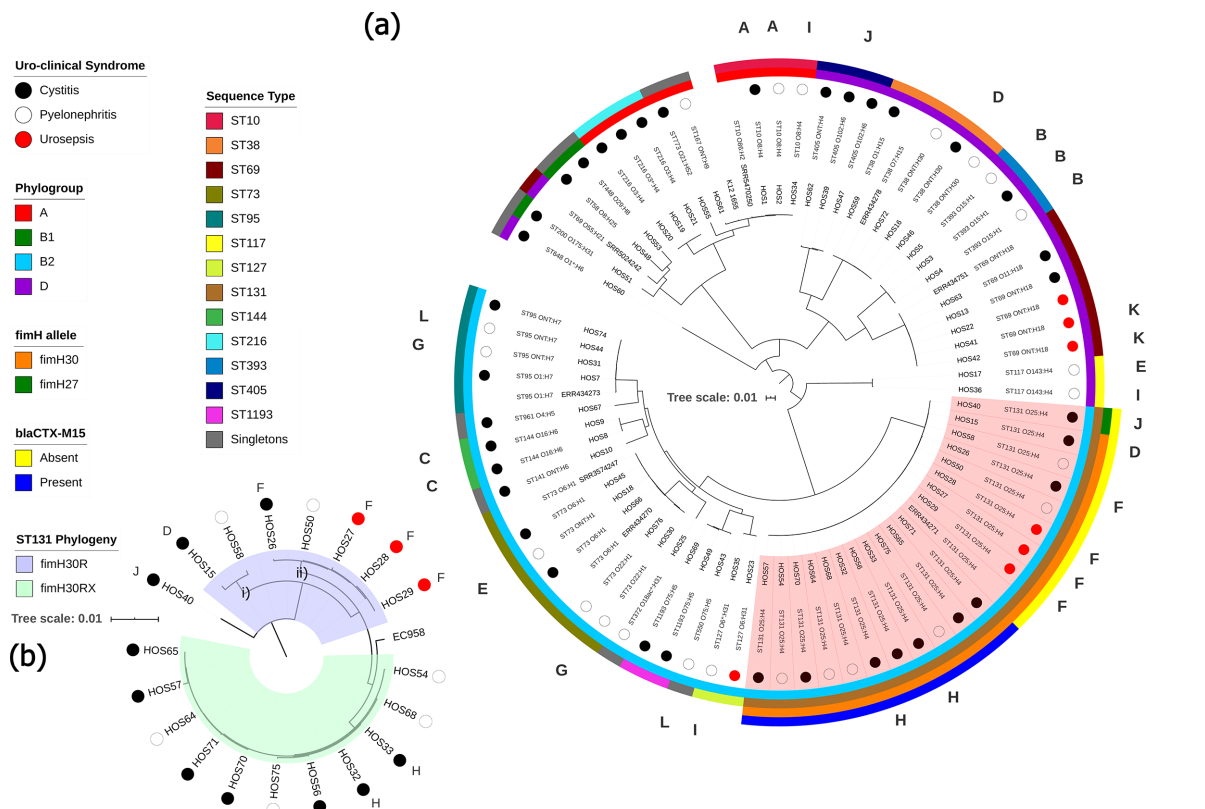


Fig. 2. Maximum-likelihood phylogenetic trees showing genetic relatedness of ExPEC strains. Tree scale bars represent number of substitutions per site of alignment. (a) Mid-point rooted maximum-likelihood phylogenetic tree, inferred using iq-TREE 2 and K12-MG1655 as a reference, containing the 67 ExPEC isolates sequenced in this study. Coloured circles represent uro-clinical syndrome (black, cystitis; white, pyelonephritis; red, urosepsis). The inner-most ring represents the phylogroup (A, red; B1, green; B2, light blue; D, purple). The next ring represents ST. The two outermost rings apply to ST131 isolates only (marked in red shaded area), the inner ring highlights *fimH* alleles (orange, *fimH30*; green, H27) and outer ring shows the presence or absence of *bla*_{CTX-M15} (blue, present; yellow, absent). Letters around the perimeter represent multiple isolates taken from a single patient, one letter per patient. (b) SNP-based phylogenetic tree, inferred using iq-TREE 2, resolving ST131 isolates into clades. H30R, blue shaded area; H30Rx, green shaded area. Trimethoprim-susceptible ExPEC isolates ERR434278, ERR434751, ERR434270, ERR434271, and trimethoprim-susceptible EAEC isolates SRR5470250, SRR5024242 and SRR3574247 were added as controls.

The fact that ST131 isolates, which are associated with ESBL resistance, outnumbered ST69 isolates, which are associated with trimethoprim resistance [63], is reflective of the high number of UTIs caused by ST131, particularly in association with pyelonephritis, within this regional NSW community [64–66]. To resolve the ST131 isolates into clades, we screened for *fimH* alleles in conjunction with a separate SNP-based phylogenetic tree (Fig. 2b). Only one ST131 isolate, HOS40, carried H27; thus, it belonged to clade B, a clade associated with animal to human transmission [67]. All other ST131 isolates carried H30, thereby belonging to the globally dominant clade C [24]. Of the ST131 H30 isolates, 7 (37%) were H30R (clade C1) and 11 (61%) were ESBL *bla*_{CTX-M-15}-associated H30Rx (clade C2) [25]. SNP analyses showed that the H27 isolate differed from H30 by a mean of 277 SNPs, and that H30R isolates differed from H30Rx by a mean of 93 SNPs. The H30Rx isolates originated from different patients, situated in seven different postcodes (~300 km between the two most distant postcodes), caused either cystitis or pyelonephritis, and were isolated over a period of 569 days. Yet despite these differences, the H30Rx isolates differed by only seven SNPs on average (across 99.6% of the reference H30Rx HOS54 genome), indicating a persistent clone in this community. Conversely, H30R isolates differed by a mean of 58 SNPs, due to the presence of two distinct branches (Fig. 2b; i and ii), and the SNP difference between H30R and H30Rx branches was 140. The pangenome for this collection consisted of 14091 genes making up a core genome of 2886

genes and an accessory genome of 11205 genes, leading to a total pangenome length equal to 11.32 Mb, and can be viewed in Data S2.

Phenotypic resistance

ExPEC have been a driving force behind a worldwide increase in ESBL-producing *Enterobacteriaceae* [68]. Furthermore, ExPEC isolates have been reported as carrying transmittable resistances to carbapenem and colistin [69, 70], both being last-line antibiotics for multidrug-resistant (MDR) Gram-negative bacteria. The World Health Organization (WHO) has flagged increasing cases of MDR bacteria worldwide as one of the most serious public-health threats and deemed surveillance as a strategic priority of the Global Action Plan on AMR [71]. Despite trimethoprim remaining a first-line empirical treatment option for UTIs, resistance in uropathogens is increasing worldwide. In Australia, resistance to trimethoprim in *E. coli* has increased from 16.6% in 2004 [72] to 31.2% in 2017 [73]. These figures and trends are similar to those in other developed countries, though lower than in developing countries where trimethoprim resistance in UTI-associated ExPEC ranges from 54 to 82% [74]. Isolates were tested against 11 antibiotics (Fig. 3). The mean number of antibiotics an isolate was resistant to was five, with two isolates (HOS56 and HOS70, both ST131 H30Rx) being resistant to 10/11 tested (all but the carbapenem imipenem). The most-common resistance phenotype, aside from trimethoprim

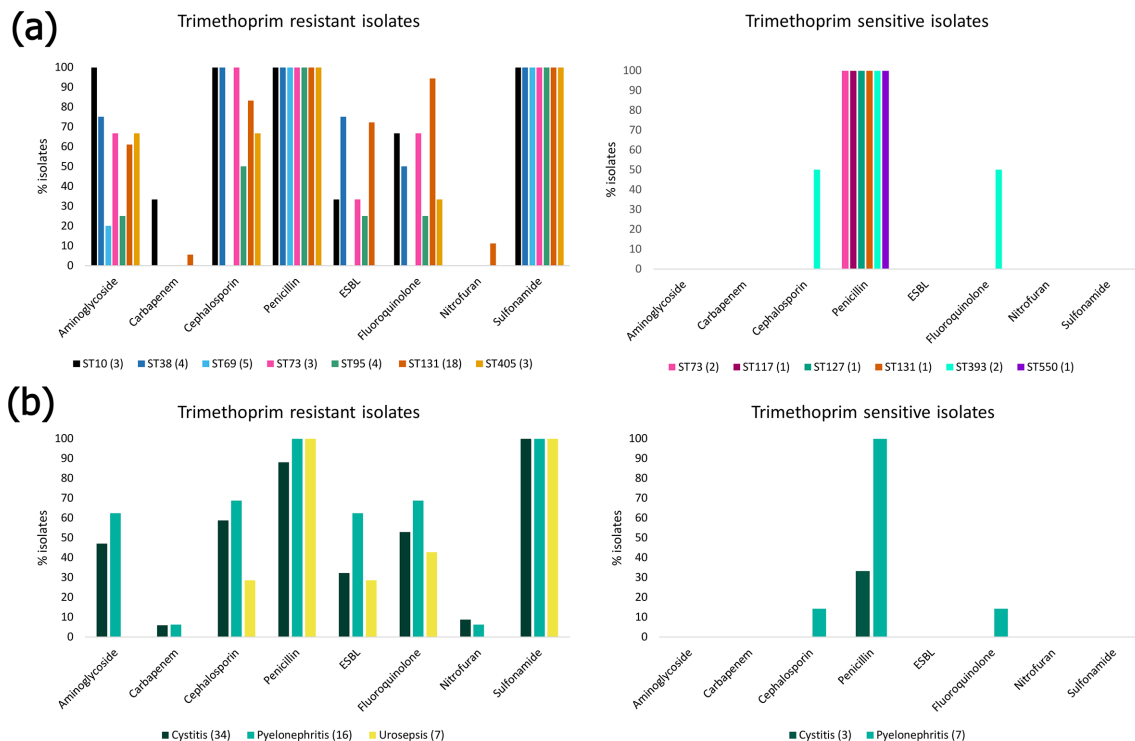


Fig. 3. Phenotypic resistance of ExPEC isolates. (a) Phenotypic resistance profiles by ST. For trimethoprim-resistant isolates, only STs with three or more isolates were included. Full phenotypic resistance profiles can be viewed in Data S3. (b) Resistance phenotypes by uro-clinical syndrome.

and trimethoprim/sulphonamide, was ampicillin ($n=50$; 75%). High levels of ampicillin resistance in UTIs have been described worldwide, leading to governing bodies recommending its disuse [4]. The least common phenotypic resistances were to imipenem ($n=2$; 3%) and the anaerobic DNA inhibitor nitrofurantoin ($n=4$; 6%). This hopeful observation indicated that both a first and a last-line treatment option for UTIs remained largely efficacious, at least in the past, though low levels of nitrofurantoin and imipenem resistance continue to be reported in UTI isolates currently [75, 76]. Antibiotic resistance varied by ST (Fig. 3a) and uro-clinical syndrome. Isolates originating from patients with cystitis and pyelonephritis shared similar phenotypic resistance profiles; however, isolates from urosepsis patients tended to be more sensitive and no isolate was resistant to the aminoglycoside gentamicin, the cephalosporin cefotaxime, nitrofurantoin or imipenem (Fig. 3b). A high co-occurrence (>30%) of trimethoprim and ESBL resistance was recently reported in the USA [77]. Furthermore, Mulder *et al.* [78] demonstrated that previous use of extended-spectrum β -lactams in patients with UTIs was significantly associated with trimethoprim resistance. These data are reflective of treatment practices and suggests a step-wise acquisition of resistances to antimicrobials over time, having become resistant to first-line treatment and then increasingly to ESBLs.

Genetic screening

This collection was screened for ARGs, ISs associated with AMR, class 1 and 2 integrases (*intI1*, *intI2*), heavy-metal-resistance genes, VAGs and plasmid replicons using ARIBA

(Fig. 4). A full list of identified genes and associated metadata for each isolate is available in Data S3.

ARGs

AMR in uropathogens complicates the treatment of UTIs. An advantage of WGS in AMR surveillance is the level of detail and precision gleaned from identifying specific ARG alleles, and co-resistances not tested on standard antibiotic panels, including disinfectant and heavy-metal-resistance genes [59]. Here, we identified a total of 40 ARGs (Fig. 4), with the majority of isolates ($n=56$; 84%) carrying at least three ARGs.

The most common mechanism for acquiring trimethoprim resistance is through the acquisition of *dfr* genes [79]. There are currently more than 40 identified *dfr* variants [80]. These are often associated with MGEs, such as plasmids and transposons, and are almost exclusively observed as resistance gene cassettes within the variable regions of class 1 and 2 integrons in human [81], animal [35, 82, 83] and environmental [84] *E. coli* isolates, resulting in their rapid spread. Seven trimethoprim-resistance genes were identified in this collection, the most common being *dfrA17* ($n=26$; 38%), followed by *dfrA12* ($n=12$; 18%). Several ESBL genes were also detected, the most common being of the *bla*_{CTX-M} type ($n=18$, 26%). *bla*_{CTX-M} progenitor genes are thought to have originated from the chromosomes of soil bacteria [85] and their subsequent capture by predominately IncF family plasmids has led to their global dissemination in both clinical and non-clinical settings [30]. IncF family replicons were the most prevalent replicons detected in this collection, the foremost

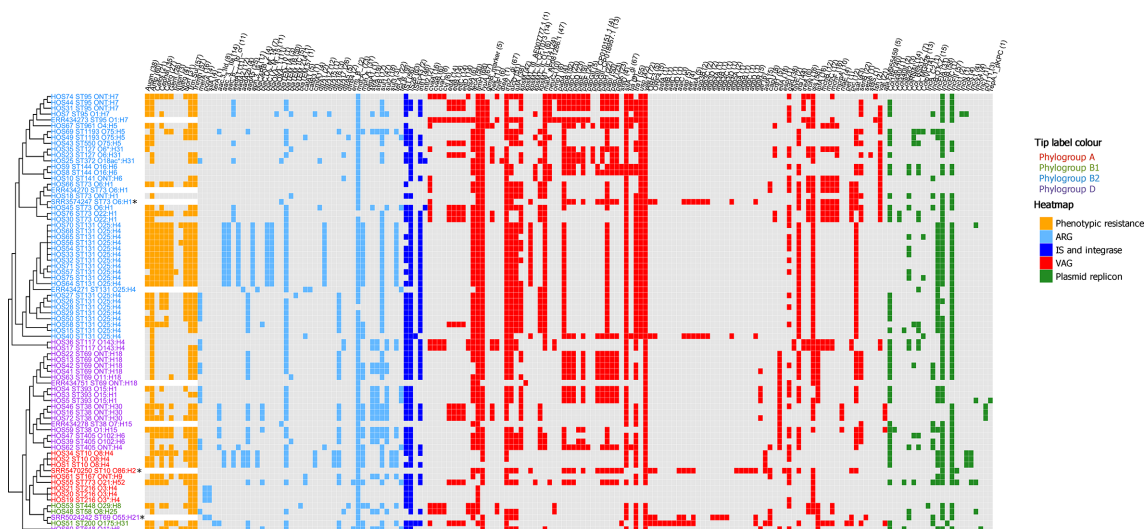


Fig. 4. Genotypic profile of 73 ExPEC isolates. Tip label colour corresponds to phylogroups (red, A; green, B1; teal, B2; purple, D). The adjacent heatmap represents phenotypic resistances and intact genes present in each isolate (orange, phenotypic resistance; light blue, ARG; dark blue, IS and class 1 and 2 integrase (*intI1*, *intI2*); red, VAG; green, plasmid replicon). Along the top of the heatmap, the numbers in brackets after gene names represent the total number of isolates with trait present). Trimethoprim-susceptible ExPEC isolates ERR434278, ERR434751, ERR434270, ERR434273 and ERR434271 and trimethoprim-susceptible EAEC isolates SRR5470250, SRR5024242 and SRR3574247 (marked by asterisks) from other studies were added as controls. The tree on the left side is the maximum-likelihood tree seen in Fig. 2 (reference K12 MG1655 omitted) presented in cladogram form.

being IncFIB ($n=53$; 79%), followed by IncFII ($n=51$; 76%). Additionally, IncFIA replicons were detected in 30 isolates (44%). While bla_{CTX-M} ESBLs comprise a heterogeneous family of enzymes, the $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ variants are currently most prevalent worldwide and are strongly associated with ST131 [30], though other STs have also contributed to their spread [86]. In this historic collection, $bla_{CTX-M-15}$ ($n=11$; 16%) and $bla_{CTX-M-14}$ ($n=7$; 10%) were the only two bla_{CTX-M} variants identified. All $bla_{CTX-M-15}$ genes were carried solely by ST131 H30Rx isolates (Fig. 2a). Conversely, the $bla_{CTX-M-14}$ genes were present in four ST38 isolates, the sole examples of ST648 and ST773 and an H30R ST131 isolate. UTI isolates carrying bla_{CTX-M} genes have previously been noted exhibiting high co-resistance to trimethoprim [77]. In this collection, all isolates carrying $bla_{CTX-M-15}$ also carried $dfrA12$, and all isolates carrying $bla_{CTX-M-14}$ co-occurred with $dfrA17$. Three additional ESBL genes were identified, bla_{OXA-10} , $bla_{TEM-214}$ [87] and bla_{TEM-57} [88], occurring in one ST95 isolate, one ST131 H30R isolate and one ST448 isolate, respectively.

In addition to trimethoprim- and ESBL-resistance genes, other common ARGs identified were β -lactamase gene bla_{TEM-1B} ($n=49$; 73%), sulphonamide-resistance gene $sul1$ ($n=46$; 69%), aminoglycoside-resistance gene $aadA5$ ($n=25$; 37%), tetracycline-resistance gene $tetA$ ($n=22$;

30%) and macrolide-resistance gene $mphA$ ($n=31$; 46%). Fluoroquinolone-resistance genes $aac(6)-Ib-cr$ ($n=11$; 16%) and $qepA1$ ($n=1$; ST448) were also detected. Isolates belonging to phylogroup D contained the most ARGs on average (nine per isolate), consistent with reports stating that an MDR profile in ExPEC is most associated with this phylogroup [89–91]. However, three ST10 isolates of phylogroup A carried the highest number of ARGs overall ($n=14$). An MDS analysis demonstrated that ST131 H30Rx isolates possessed the most distinct ARG profiles (Fig. 5, red area), which could be attributed to the strong correlation of $aac(3)-IIa$, $aac(6)-Ib-cr$, $aph(3')-Ia$, $aadA2$, $bla_{CTX-M-15}$, bla_{OXA-1} and $dfrA12$ in this ST and clade (Data S4). Notably, all the trimethoprim-sensitive ExPEC and EAEC isolates used as controls in this study clustered together with four trimethoprim-sensitive isolates from this collection (Fig. 5, blue area), with the exception of one control ExPEC ST131 isolate (Fig. 5, red arrow).

Heavy-metal- and detergent/disinfectant-resistance genes are often carried by the same MGEs harbouring ARGs, sparking concern and growing evidence that selective pressures induced by their extensive use in industry, agriculture and health-care facilities, as well as via contamination, can co-select for AMR [92–94]. The hospital wherein our isolates originated caters for a catchment area significantly associated with agriculture,

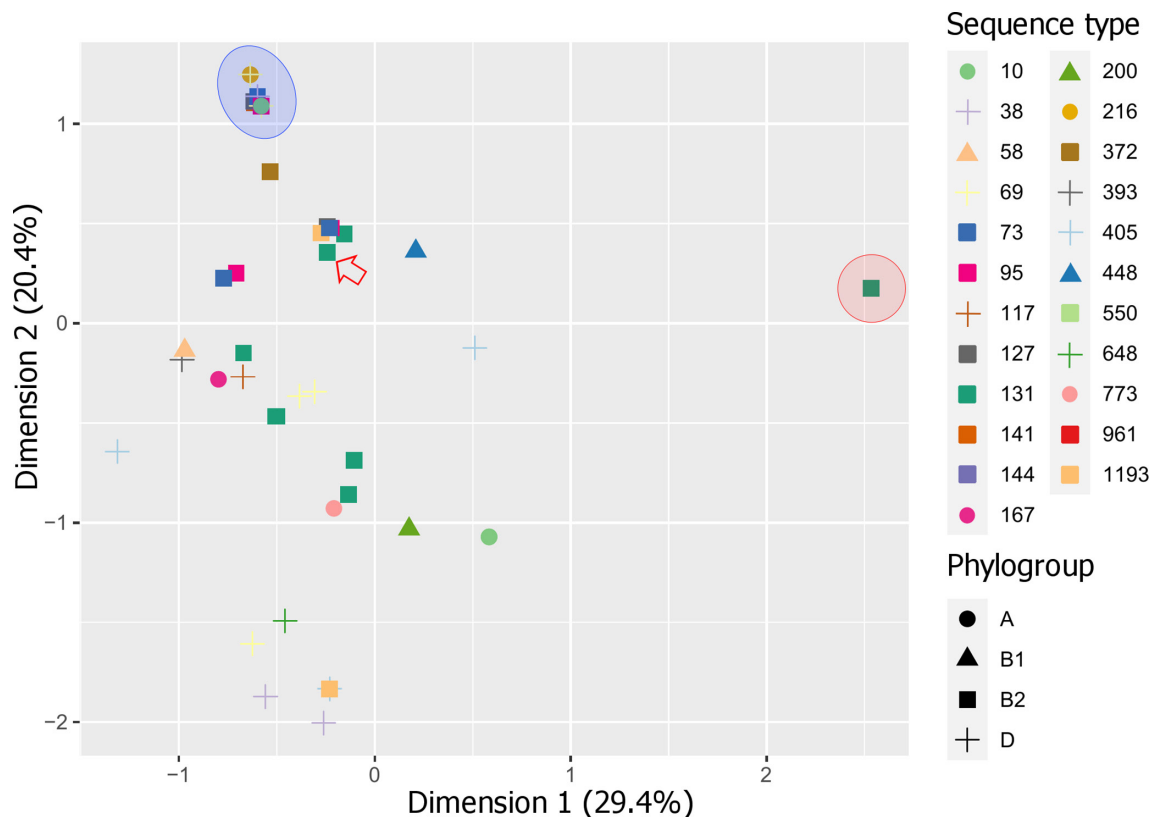


Fig. 5. MDS analysis of ARGs detected in the ExPEC collection. Red area, ST131 H30Rx cluster (11 isolates share the same coordinate); blue area, trimethoprim-sensitive control isolates clustered with four trimethoprim-sensitive isolates from this collection, with the exception of one control ST131 isolate (red arrow). The percentage of total explained variation for each dimension is indicated in parentheses after each axis label.

including food animals (bovine, sheep, pig and poultry), and various crops, such as fruits and nuts [95]. Thus, the isolates were screened, and two detergent-resistance genes – *qacEΔ1* ($n=47$; 70%) and *qacH* ($n=3$; 4%) – conferring resistance to quaternary ammonium compounds, and six heavy-metal-resistance genes were identified. All isolates carried *zntA*, which confers resistance to zinc, lead and cadmium. We also identified *chrA* ($n=32$; 48%), corresponding to chromium resistance, and *merA* ($n=16$; 24%), which confers mercury resistance. Silver- and copper-resistance genes *silA* and *pcoA* were present in only three isolates (4%; all ST216) and tellurium-resistance gene *terA* was observed in a single ST200. Heavy-metal-resistance genes, including *zntA*, *merA*, *pcoA* and *chrA*, are known to be significantly associated with detergent resistance genes, and *sul*, *tet*, *bla_{TEM}*, *bla_{SHV}* and *bla_{CTX}* variants [93]. Furthermore, chromium resistance has been positively correlated with relative *bla_{CTX-M}* and *bla_{OXA}* gene abundance [96]. Here, 100% of isolates carrying *bla_{CTX-M}* genes also carried *chrA*, and 91% of isolates with *bla_{OXA}* also harboured *chrA*.

Class 1 and 2 integrons

The presence of class 1 or 2 integrons is considered a reliable proxy for an MDR genotype [97]. Class 1 integrons are more prevalent than class 2 integrons [97] and typically contain two conserved regions – 5'-CS and 3'-CS – flanking a variable gene cassette. The *intI1* integrase gene, responsible for capturing, removing and rearranging genes within the variable cassette, is located in the 5'-CS region. The 3'-CS region typically contains a truncated but functional *qacEΔ1* gene fused to a *sul1* gene [98]. Class 1 and 2 integrons are mobilized and spread by MGEs, such as transposons and conjugative plasmids. Additionally, the insertion element IS26 is renowned for its ability to capture and assemble ARGs in complex resistance regions [32] and alter the structure of class 1 integrons [35, 98]. Here, IS26 was identified in 63 (94%) isolates, including all 45 (67%) that carried *intI1*.

Class 1 and 2 integron loci are often rich in repetitive sequences, posing challenges to assembling complete structures using short-read sequencing data. Despite this limitation, we resolved 18 class 1 integron structures, including ARGs and MGEs found downstream of the typical 3'-CS region (Fig. 6a) and 1 class 2 integron (Fig. 6b). BLASTN and GenBank were used to determine whether these integrons had been previously deposited into public databases (Table 1). In general, the integrons from this collection are also found in ExPEC-associated STs, most commonly ST131 and ST617 [of ST10 clonal complex (CC)]. They are most frequently seen on IncF plasmids of varying plasmid multilocus sequence types (pMLSTs), in a range of hosts, including humans, dogs and gulls, and from a range of geographical locations, including Europe, Asia, North America and Australia (Table 1). The most common genes located in the variable gene cassette were *dfrA17* and *aadA5* (Fig. 6a).

Integron *k* was the most common structure in this collection, present in 12 isolates (from ST131 and ST405), and carried *dfrA12* (trimethoprim), *aadA2* (aminoglycoside), *qacEΔ1*

(quaternary ammonium compounds), *sul1* (sulphonamide) and *mphA* (macrolide) resistance genes. The same integron was described in an ST131 H30Rx isolate taken from a French patient a year after contracting pyelonephritis while travelling in Nepal [99]. The integron *k* structure also carried the aforementioned chromium resistance gene *chrA*, which in turn was also present in seven other integron structures (*d*, *e*, *f*, *g*, *l*, *m* and *r*). Class 1 integrons are often translocated via mercury-resistance transposons carrying genes of the *mer* operon [81], and such transposons were present in integrons *i*, *j* and *n* structures. Integrons *n* and *r* also carried a recently characterized *dfrB4* gene conferring high-level resistance to trimethoprim. Both isolates that carry these integrons (ST1193 and ST448) predate the first report of this gene in a clinical sample (UTI) in 2017 [100]. Additionally, integron *r* carried an uncommon plasmid-mediated fluoroquinolone-resistance gene, *qepA*. Integron *b* was the only structure to carry an ESBL gene (*bla_{OXA-10}*) and carried additional genotypic resistance genes for trimethoprim (*dfrA15*), chloramphenicol (*cmlA1*), aminoglycosides (*aadA1*) and sulphonamides (*sul1*). An identical structure to integron *b* could not be found in public databases; however, a similar structure was observed chromosomally in *Pseudomonas aeruginosa* strain SP4527 (ST357) isolated from a patient with bacteraemia in India, in 2016 (Fig. 6b). Integron *c* consists of a short *dfrA5*-IS26 configuration that has been detected in, and is identical to, *E. coli* strains sourced from cattle located on several NSW properties [98, 101], and in commensal *E. coli* from Australian pigs [35]. Furthermore, the signature has been used to track ColV plasmids carrying a complex resistance locus in patients with UTI and urosepsis in Australia in *E. coli* ST58 [102, 103]. These studies highlight how plasmids that play an important role in carrying intestinal pathogenic *E. coli* virulence genes [101] and extraintestinal pathogenic virulence gene cargo [102, 103] can be tracked by the presence of this unique signatures. Moreover, it shows that transposon belonging to the Tn3 family of mercury-resistance transposons can mobilize complex resistance loci on diverse plasmid backbones. Integron *l* has been described within a larger salmonella genomic island 1 (SGI1) structure in *Proteus mirabilis* [104]. The integron *a* structure carried the sole example of a *sul3* gene, which has been associated with Australian *E. coli* isolates from pig farms [35, 83]. This integron, found in our ST10 isolates, also uniquely carried *dfrA16*, *bla_{CARB-2}* and *qacH*. Integron *a* has been located on IncFIA and IncI plasmids in CC10 (ST617) *E. coli* isolates originating from both human clinical samples from Germany and the USA, a broiler chicken sample from Belgium (Table 1), as well as farmed red deer from Spain [105]. Incidentally, the patients from whom these isolates originated resided in proximity to two red deer farms. Lastly, the class 2 integrase *intI2* gene was identified in one ST372 and one ST73 isolate (Fig. 6b). The *intI2* from this class of integron is inactive, impeding its ability to modify gene cassettes. Thus, class 2 integron cassettes are highly conserved [97]. The *dfrA1-sat2-aadA1* cassette found in this collection represents the most common

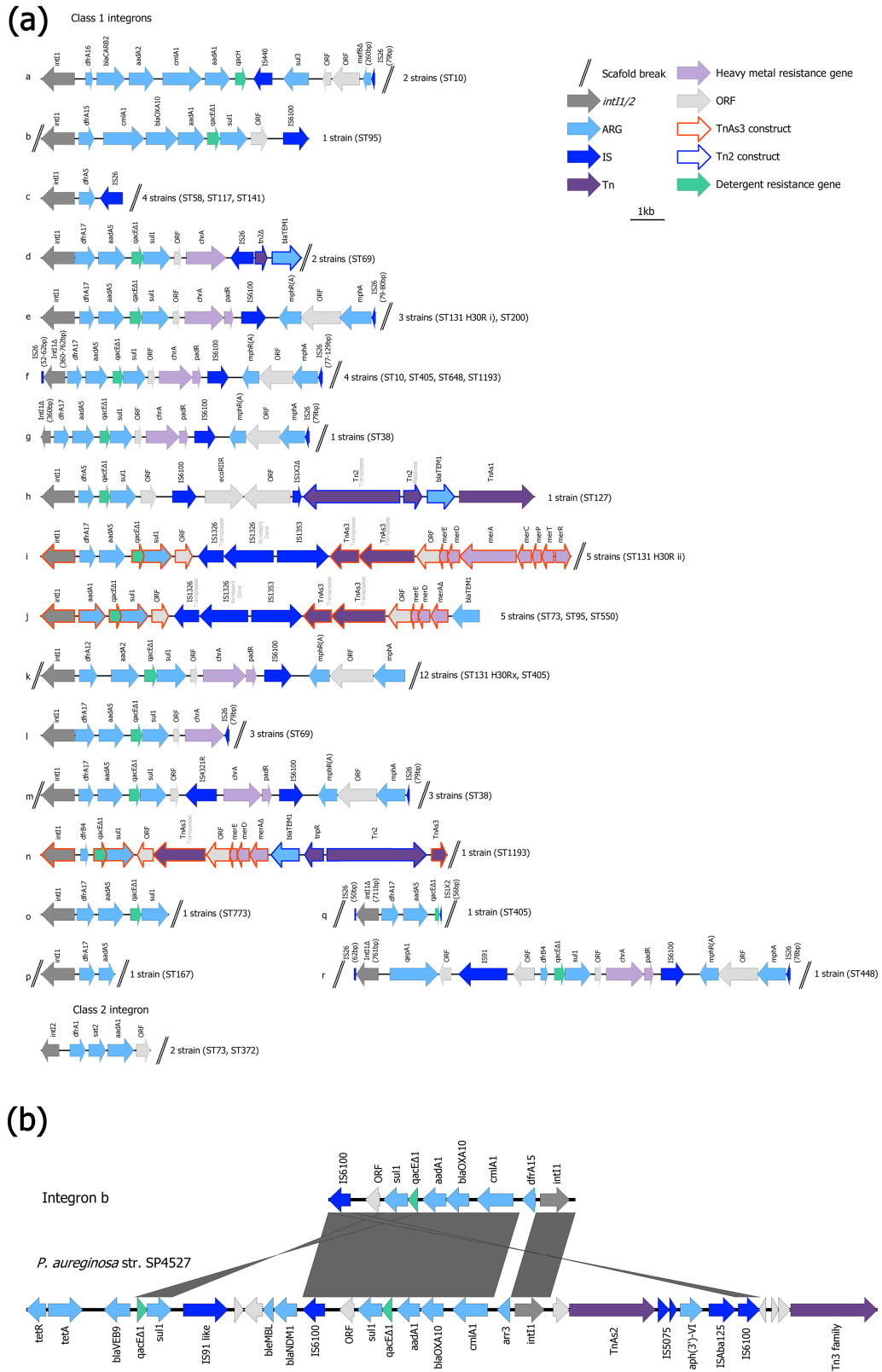


Fig. 6. Class 1 and class 2 integron structures. (a) Class 1 (a–r) and class 2 integrons structures identified in this collection. ST131 H30R i and ii refer to the two H30R subclades resolved by the SNP-based phylogeny presented in Fig. 2(b). (b) EasyFig comparison of integron b and the chromosome-associated integron structure from *Pseudomonas aeruginosa* strain SP4527.

Table 1. Summary of BLAST hits to integrons found in this collection

Rows in bold indicate integrons with no 100% matches. The closest integron match is provided

Integron	Accession no.	Coverage; Identity	Integron location	Inc group (pMLST)	Isolation date	Species (ST)/ [STs from this collection]	Host	Host disease	Host location
a	MK070495.1	100%; 100%	Plasmid	I1 (CC-3)	2013	<i>E. coli</i> (NA)/[ST10]	Chicken	NA	Belgium
	CP018105.1	100%; 100%	Plasmid	F (F-:A18:B-)	2016	<i>E. coli</i> (ST617)	Human	NA	Germany
b	CP034409.1	93%; 100%	Chromosome	-	2016	<i>P. aeruginosa</i> (ST357)/[ST95]	Human	Sepsis	India
c	CP026940.1	100%; 100%	Plasmid	F (F2:A-B1)	2010	<i>E. coli</i> (ST58)/[ST58, ST117, ST141]	Cow	Mastitis	France
d	CP041523.1	100%; 100%	Plasmid	F (F29:A-B10)	2014	<i>E. coli</i> (ST69)/[ST69]	Human	Sepsis	USA
e	CP041032.1	100%; 100%	Plasmid	F (F31:A4:B1)	2018	<i>E. coli</i> (ST410)/[ST131, ST200]	Dog	Cellulitis	Portugal
	CP041338.1	100%; 100%	Plasmid	F (F36:A1:B20)	2008	<i>E. coli</i> (ST131)	Human	NA	Sweden
	LR595886.1	100%; 100%	Plasmid	F (F2:A1:B-)	2014	<i>E. coli</i> (NA)	Human	Healthy	UK
f	MK295830.1	100%; 100%	Plasmid	F (F1:A6:B20)	2007	<i>E. coli</i> (ST131)/[ST10, ST405, ST648, ST1193]	Human	NA	Israel
	MK295829.1	100%; 100%	Plasmid	F (F1:A6:B20)	2015	<i>E. coli</i> (ST131)	Dog	NA	Israel
	MK295827.1	100%; 100%	Plasmid	F (F1:A6:B20)	2015	<i>E. coli</i> (ST131)	Human	NA	Israel
g	MK295830.1	100%; 100%	Plasmid	F (F1:A6:B20)	2007	<i>E. coli</i> (ST131)/[ST38]	Human	NA	Israel
h	CP021734.1	84%; 100%	Plasmid	N (A2:N5:J-)	NA	<i>E. coli</i> (ST617)/[ST127]	NA	NA	NA
i	CP041557.1	100%; 100%	Plasmid	F (F2:A1:B-)	NA	<i>E. coli</i> (ST131)/[ST131]	Human	NA	USA
	CP024721.1	100%; 100%	Plasmid	F (F2:A1:B1)	2015	<i>E. coli</i> (ST131)	Yak	NA	China
	CP024718.1	100%; 100%	Plasmid	F (F2:A1:B1)	2015	<i>E. coli</i> (ST131)	Yak	NA	China
j	CP021289.1	100%; 100%	Plasmid	F (F51:A-B10)	2010	<i>E. coli</i> (ST95)/[ST73, ST95, ST550]	Human	Sepsis	Australia
k*	CP023845.1	100%; 100%	Plasmid	F (F22:A1:B20)	2009	<i>E. coli</i> (ST131)/[ST131, ST405]	Human	Post UTI	Sweden
	LC056386.1	100%; 100%	Contig	-	2013	<i>E. coli</i> (NA)	Environment	NA	India
	CP028322.1	100%; 100%	Plasmid	F (F73:A-B-)	2000	<i>E. coli</i> (ST73)	Human	NA	USA
	CP012632.1	100%; 100%	Plasmid	F (F2:A-B-)	2008	<i>E. coli</i> (ST95)	Human	Sepsis	USA
l	CP041523.1	100%; 100%	Plasmid	F (F29:A-B10)	2014	<i>E. coli</i> (ST69)/[ST69]	Human	Sepsis	USA
m	CP032145.1	100%; 100%	Chromosome	-	2015	<i>E. coli</i> (ST38)/[ST38]	Gull	NA	Turkey
	CP040263.1	100%; 100%	Chromosome	-	2014	<i>E. coli</i> (ST38)	Mollusc	NA	Norway
	CP040390.1	100%; 100%	Chromosome	-	2016	<i>E. coli</i> (ST38)	Gull	NA	USA
n	CP016546.1	97%; 99.97%	Chromosome	-	2015	<i>E. coli</i> (ST359)/[ST1193]	Human	Healthy	Netherlands
o	CP019014.1	100%; 100%	Plasmid	F (F2:A1:B-)	2013	<i>E. coli</i> (ST131)/[ST773]	Human	NA	USA
p	CP041393.1	100%; 100%	Plasmid	F (F36:A4:B-)	2018	<i>E. coli</i> (ST617)/[ST167]	Dog	NA	USA
	MG764548.1	100%; 100%	Plasmid	F (F33:A4:B-)	NA	<i>E. coli</i> (NA)	NA	NA	China
	CP019076.1	100%; 100%	Plasmid	F (F36:A4:B-)	2013	<i>E. coli</i> (ST617)	Human	NA	China
	CP021737.1	100%; 100%	Plasmid	F (F36:A4:B-)	NA	<i>E. coli</i> (ST617)	NA	NA	NA
	KU043115.1	100%; 100%	Plasmid	F (F36:A4:B-)	2013	<i>E. coli</i> (ST617)	Human	UTI	China
q	CP023854.1	100%; 99%	Plasmid	F (F48:A1-B49)	2009	<i>E. coli</i> (NA)/[ST405]	Human	UTI	Sweden

Continued

Table 1. Continued

Integron	Accession no.	Coverage; Identity	Integron location	Inc group (pMLST)	Isolation date	Species (ST)/ [STs from this collection]	Host	Host disease	Host location
r	CP014320.1	100%; 99%	Plasmid	F (F36:A-B32)	2007	<i>E. coli</i> (ST131)/[ST448]	Human	UTI	USA

NA, Not Applicable.

*Only 100% matches from *E. coli* are shown.

array, and is particularly prevalent in UTI-associated *Proteus* species [106].

VAGs

ExPEC employ a range of VAGs that enable ascension, colonization and persistence within the urinary tract. Like ARGs, many VAGs are also carried on MGEs, including plasmids as well as chromosomally situated PAIs [15]. Thus, the virulence potential of ExPEC strains is constantly evolving and tracking VAGs improves understanding of ExPEC pathogenesis. Using the VirulenceFinder database, a total of 84 VAGs were identified in this collection, with the number of VAGs per isolate spanning from 2 to 36, with a mean of 20 and a median of 19.

To combat the iron-scarcity encountered in the urinary tract, ExPEC have acquired a number of siderophore and ferrous iron uptake systems to scavenge Fe³⁺ and Fe²⁺, which are vital for growth, persistence and establishing full virulence [15]. ExPEC-associated iron-acquisition systems include yersinia-bactin (*ybt*, *irp*, *fyuA*), salmochelin (*iro*), aerobactin (*iuc*, *iut*), ferric citrate transport system (*fec*) and the Sit ferrous iron utilization system (*sit*). In this study, the most common iron-acquisition genes were *fyuA*, *irp2* and *sitA* (all *n*=61, 91%), followed by *iucD* and *fecA* (both *n*=45; 67%). The high carriage of *fyuA* is consistent with previous reports tracking VAGs in ExPEC [14, 107, 108]. While there are contradicting reports regarding *fyuA* and mortality [107, 109, 110], immunization with FyuA has been shown to protect against pyelonephritis in mice [111].

Several adhesins and fimbriae demonstrate specificity for binding uroepithelium. Of these, the type 1 fimbriae and P-fimbriae are most prevalent in UTI-associated ExPEC [15]. Type 1 fimbriae bind uroepithelial associated α -D-mannosylated proteins via fimbrial adhesin H (*fimH*). Thus, the *fimH* gene plays a pivotal role in ExPEC urothelial adhesion [15]. P-fimbriae (*pap* genes) bind α -D-galactopyranosol(1-4)- β -D-galactopyranoside-containing receptors found in the upper urinary tract and, therefore, have been associated with pyelonephritis [112]. Here, the most common adhesins identified were *fimH* (*n*=61; 91%), *papB* (*n*=47; 70%) and *papI* (*n*=45; 67%). However, other P-fimbriae operon genes were less common, such as *papC*, *papD*, *papJ* and *papK* (each *n*=20; 30%), *papF* (*n*=21; 31%), *papH* and *papG* (both *n*=19; 28%), and *papA* (*n*=17; 25%).

In addition to factors facilitating adhesion and growth, ExPEC express toxins contributing to host tissue damage, as well as immune evasion molecules [113]. Regarding toxins, several serine protease autotransporters of *Enterobacteriaceae*

(SPATE) genes were identified including *sat* (secreted autotransporter toxin) (*n*=39; 58%), which has been shown to compromise gap junctions in uroepithelium [112], *vat* (*n*=18; 27%) and *pic* (*n*=8, 12%). Enterotoxins *senB* (*n*=21; 31%) and *astA* (*n*=4; 6%) were also observed. Regarding immune evasion, VAGs encoding increased serum survival protein (*iss*), VirB5-like protein TraT (*traT*) and outer-membrane protein T (*ompT*) were identified in 52 (78%), 50 (75%) and 5 (7%) isolates, respectively.

Despite studies reporting various VAGs linked to pyelonephritis and sepsis [15, 114, 115], we found no single gene nor plasmid replicon strongly associated with a particular pathology (correlation heat map in Data S5), nor was there a significant difference between the number of VAGs present and uro-clinical syndrome (Fig. 7a). The discrepancy with the literature may have arisen due to sampling based on trimethoprim resistance and/or the relatively small sample size, limiting statistical power. Nevertheless, an MDS analysis showed some clustering of STs based on virulence profiles (Fig. 7b). Notably, isolates formed two distinct diagonal groups, which could be attributed to the distribution of *pap* genes with *papC*, *papD*, *papJ* and *papK* present in all isolates within the distal group and absent in all isolates in the top group. Other *pap* genes were also more prevalent in the bottom group including *papGII* (0% top, 78% bottom), *papGIII* (0, 17%), *papH* (0, 96%), *papE* (4%, 0%) and *papF* (4, 96%). Also of note was that the ST131 H30 isolates clustered with two emerging pathogen STs, ST405 [116] and ST1193 [117], as well as an ST550 isolate [same CC as ST1193 – CC14] and one ST73 isolate that deviated from other ST73 isolates (Fig. 7b, red area). The ST131 H30 isolates fell under virotype C [*afa* operon (-), *iroN* (-), *ibeA* (-), *sat* (+)], which is the most represented ST131 virotype internationally and associated with a higher frequency of infection [118]. The other isolates within this cluster could also be categorized as virotype C. The only ST131 H27 isolate did not cluster with its ST and carried the molecular predictors of EAEC/ExPEC hybrid strains (*aata*, *aggR*, *fyuA*), as did one ST200 isolate. The EAEC isolates sourced from outside this collection (Fig. 7b, blue arrows) all deviated from ExPEC isolates of the same ST; however, they did not form a separate cluster and each carried ExPEC-associated genes including *fyuA* (Fig. 4). These EAEC isolates were not previously described as hybrid strains and originated from cases of diarrhoeal disease in England [40]. However, hybrid EAEC/ExPEC strains are known to cause UTI outbreaks [119, 120]. EAEC strains carry most of their virulence cargo on plasmids and are renowned for prolific biofilm formation [121]. Indeed, EAEC/ExPEC hybrid strains

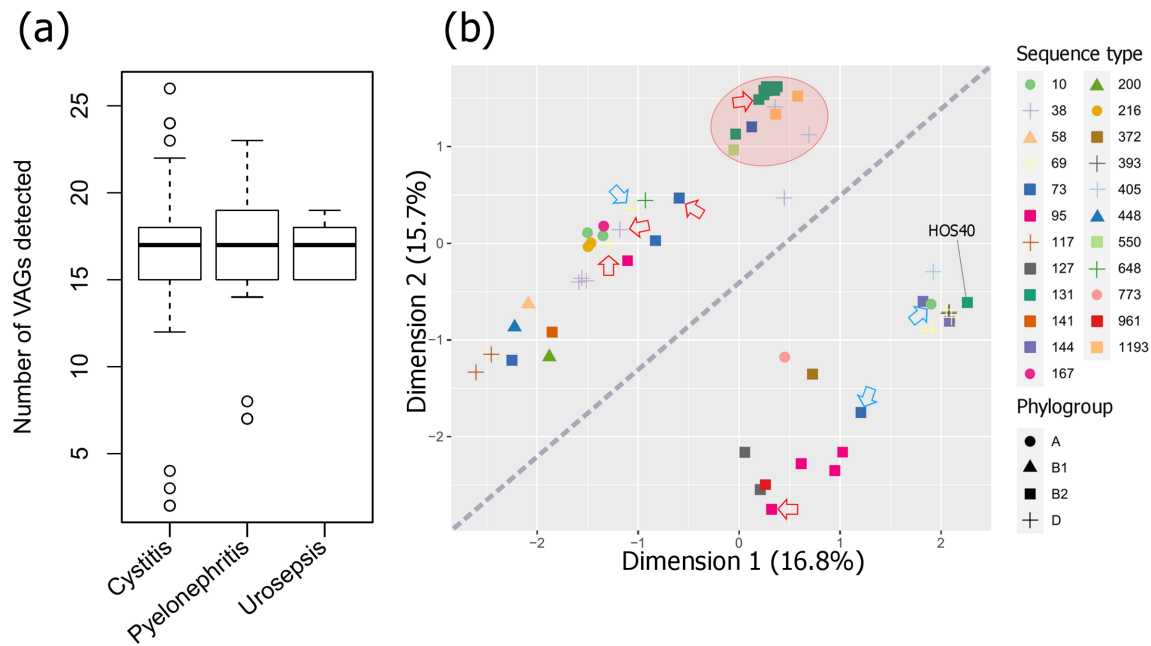


Fig. 7. Statistical analysis of VAGs detected in the ExPEC collection. (a) Box plot of uro-clinical syndrome versus the number of VAGs. Genes in operons *cvaABC(i)*, *eitABCD*, *papABCDEFGHJK*, *aafABCD*, *aggABCDR* and *mchBCF* were counted as one if at least one gene was present. (b) MDS analysis on detected VAGs. Additional ExPEC isolates sourced from outside this collection are indicated by red arrows, additional EAEC isolates are indicated by blue arrows. The diagonal line splits isolates into a *pap* gene rich group (below the line) and a *pap* gene poor group (above the line). The red area highlights the ST131 H30 isolates cluster with two emerging pathogen STs, ST405, ST1193, as well as an ST550 isolate and one ST73 isolate. The percentage of total explained variation for each dimension is indicated in parentheses after each axis label.

have shown significantly higher levels of biofilm formation, as well as enhanced adhesion to uroepithelium cells, compared to non-hybrids [122].

PAIs

The PAI marker genes *malX* and *usp* were seen in 44 (66%) and 38 (57%) isolates, respectively. The acquisition of PAIs via horizontal transfer can vastly contribute to the evolution of *E. coli* pathogens as they contain potent combinations of VAGs, including the aforementioned P-fimbriae, salmochelin, aerobactin and yersiniabactin operons, as well as shigella-fimbriae (S-fimbriae, *sfa*) and haemolysin toxin (*hly*) operons [123, 124]. As such, to indicate the presence of ExPEC-associated PAIs, we used short-reads derived from each isolate mapped to reference sequences PAI I_{CFT073}, PAI II₅₃₆ and PAI III₅₃₆. PAI IV₅₃₆ [also known as high pathogenicity island (HPI)] was also screened for; however, possibly due to allelic differences, the read-depth was relatively lower for this PAI (Data S6) so BLAST was used in conjunction with assembled draft genomes to indicate its presence. While all isolates had at least partial hits to genes and gene clusters within PAI I_{CFT073}, PAI II₅₃₆ and PAI III₅₃₆, most demonstrated extensive deletions of regions, and few had near intact examples (Fig. 8). The most represented VAG operon was aerobactin in PAI I_{CFT073} and the least were S-fimbriae in PAI III₅₃₆. However, PAI IV₅₃₆ – which consists of the yersiniabactin operon (*ybt* genes, *irp1*, *irp2*, *fyuA*; total 11 genes), a P4-like integrase and

an uncharacterized protein YeeI nestled between tRNA-Ser and tRNA-Asn – was identified over >95% length at >98% identity in 58 isolates (Data S7). In addition to being a potent siderophore system, yersiniabactin can protect against copper toxicity, redox-based phagocytosis and is a prerequisite for sepsis in some *E. coli* strains [19].

ColV plasmid markers

ColV plasmids are considered a defining feature of APEC (avian pathogenic *E. coli*) [125]; however, they have also been associated with human ExPEC strains [102, 126]. While the expression of colicin V may benefit ExPEC strains by reducing competition for nutrients [127], ColV plasmids are intriguing in that they share high sequence homology to PAIs, and it is theorized that they and other plasmids and phages are the progenitors of ExPEC-associated PAIs [123]. As ColV plasmids are heterogeneous in their VAGs and pMLST, Liu *et al.* [67] defined ColV plasmids as having at least one gene from four or more of the following: (i) *cvaABC* and *cvi* (ColV operon); (ii) *sitABCD* (ferrous iron utilization system); (iii) *hlyF* (regulator of outer membrane vesicle biogenesis) and *ompT*; (iv) *iucABCD* and *iutA* (aerobactin operon); (v) *iroB-CDEN* (salmochelin operon); and (vi) *etsABC* (novel ABC transport system). In this study, ARIBA gene screening identified seven isolates (10%) meeting this criterion – HOS66 (ST73, cystitis), HOS45 (ST73, cystitis), HOS74 (ST95, cystitis), HOS17 (ST117, pyelonephritis), HOS36 (ST117,

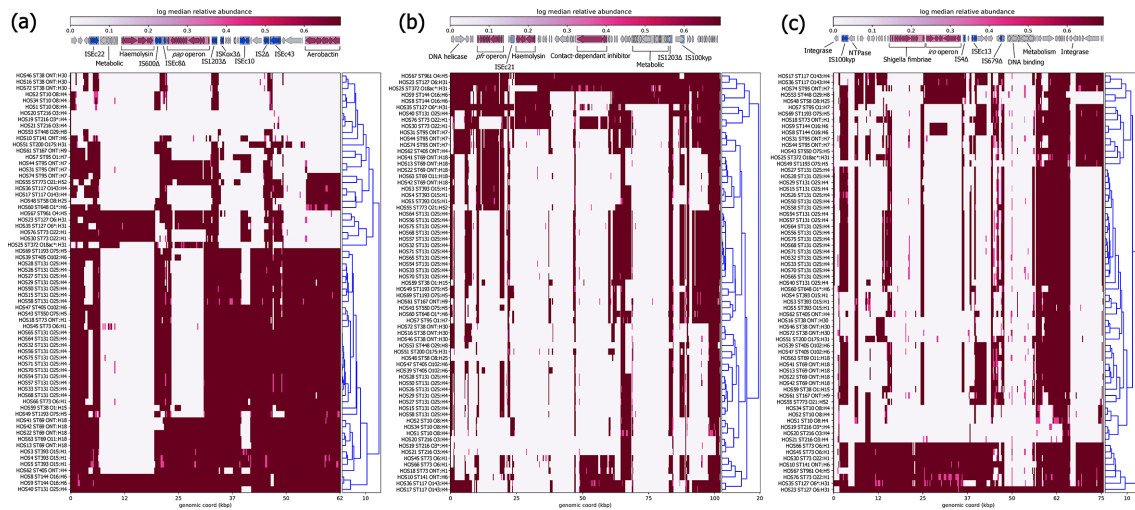


Fig. 8. Mapping of short-reads indicating the presence of ExPEC-associated PAIs. Clustering of rows is based on the similarity between isolate PAI coverage profiles. (a) Schematic and heatmap for PAI I_{CFT072} coverage. (b) Schematic and heatmap for PAI II₅₃₆ coverage. (c) Schematic and heatmap for PAI III₅₃₆ coverage. Trees alongside the heatmaps were reconstructed by hierarchical clustering using Euclidean agglomeration method.

pyelonephritis), HOS48 (ST58, cystitis) and HOS53 (ST448, cystitis). Three of these isolates (HOS17, HOS36 and HOS48) also carried the aforementioned integron *c* (*dfrA5-IS26*) that is used to track ColV plasmids containing complex AMR regions. Future long-read sequencing studies are needed to provide deeper insight into these initial observations (plasmid short-read mapping is provided in Data S8).

SNP analysis of serial patient isolates

Phenotypic diversification via adaptive evolution is driven by horizontal gene transfer and by mutation [128]. A single SNP-mediated point mutation can lead to an amino acid replacement with significant adaptive effects, as witnessed in single amino acid replacements in *parC/E* and *gyrA* genes causing fluoroquinolone resistance [24, 25], and in *fimH*, which increases binding to uroepithelium and biofilm formation [129]. Such adaptive mutations can increase bacterial fitness and provide rapid adaption to niche-specific conditions [128]. ExPEC are exposed to a variety of niches in addition to their primary reservoir, the intestinal tract, and as a result may be under greater selective pressure for adaptive mutations. Indeed, UTI-associated ExPEC are known to have significantly higher mutation rates compared to other *E. coli* pathotypes [130]. While studies have been conducted comparing SNP-mediated point mutations in UTI and faecal isolates [131], relatively little is known about the SNPs that occur in recurrent UTIs, particularly when subsequently more serious sequelae occur. In this study, 13 patients had more than one presentation of symptomatic UTI during the study period (indicated by letters in Fig. 2). Six patients had subsequent UTIs caused by the same ST, and seven patients had different STs. Here, we focus on the patients who had multiple infections with the same ST (Fig. 9), and on SNP-mediated mutations with a higher probability of inducing functional changes

as predicted by the BLOSUM62 substitution matrix [132]. A catalogue of all SNPs can be viewed in Data S9.

Patient A was a 37-year-old female who in the first instance presented with cystitis with an ST10 isolate (HOS1) as the causative agent and then pyelonephritis (HOS2; ST10) 63 days later. The ST10 isolates had a difference of 29 SNPs, with most occurring in genes involved in DNA processing, transport and virulence (Fig. 9). However, mutations with negative BLOSUM62 scores (indicating a higher probability that the original function is not conserved) occurred most frequently in VAGs and DNA processing. Potentially increasing the significance of the observed mutations, another patient, a 69-year-old male living in the same postcode, presented with pyelonephritis 16 days after patient A's second presentation. The causative agent was also an ST10 (HOS34), which shared 71% ($n=24$) of the same SNPs found in HOS2. Both HOS2 and HOS34 had the same SNPs causing non-synonymous amino acid substitutions with negative BLOSUM scores in the following genes: *irp1* (D2717A), *ybtS* (P184A), *astA* (G103V), *aroC* (G152E), *rnhB* (R98L), *hrpB* (G644E), *traC* (G644W), *lsrA* (E296G) and *recC* (S794C). Interestingly, *recC* was previously shown to be under positive selective pressure for adaptive mutations [130]. Though not present in HOS34, HOS2 possessed a SNP causing A432D (negative BLOSUM62 score) in DNA-repair protein Mfd (mutation-frequency-decline). Mfd promotes mutagenesis and accelerates AMR development to multiple antibiotic classes [133]. Fascinatingly, point mutations produce Mfd variants that express greater activity than wild-type [134].

Patient B was a 21-year-old-female who first presented with cystitis (HOS3) then pyelonephritis 284 days later (HOS4, 13 SNP difference), then pyelonephritis again 128 days later (HOS5, 19 SNP difference from HOS3). The causative agent in

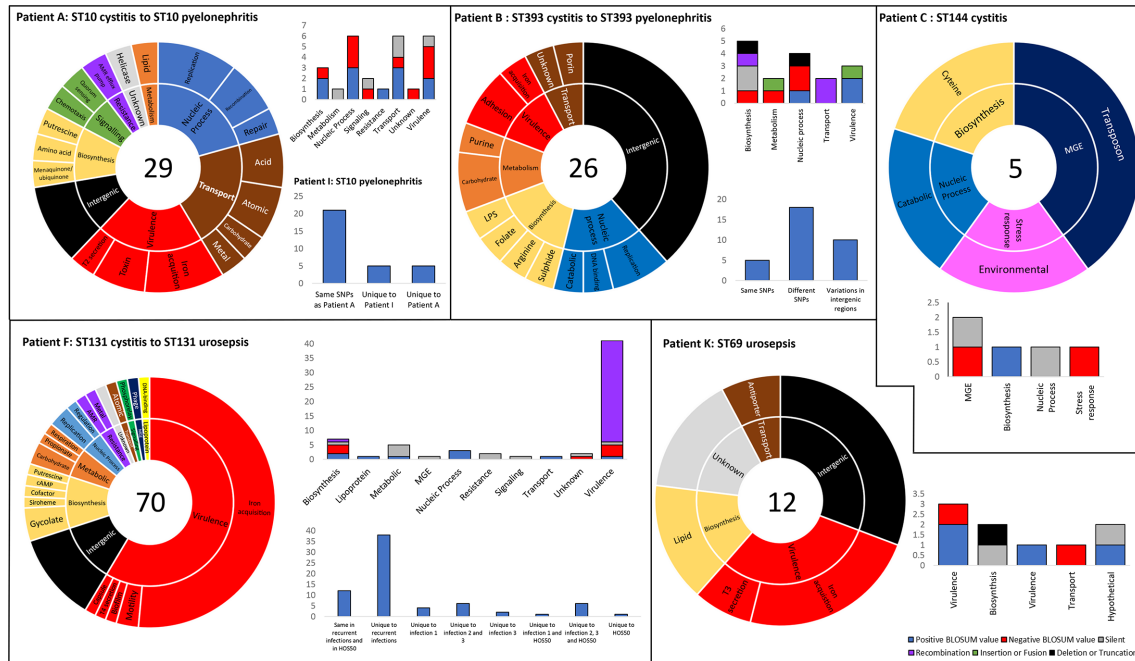


Fig. 9. Serial patient isolates – SNP distribution and consequence to amino acid sequence. Each panel represents a UTI patient from whom multiple isolates of the same ST were derived. Sunburst graphs represent the distribution of SNPs within functional groups. The number in the centre represents the total number of unique SNPs (accumulative if more than one recurrent infection occurred). Stacked column graphs represent the consequence of SNPs to protein sequence. Color legend for the stacked columns located bottom right corner. Unstacked column graphs represent the distribution of specific SNPs in recurrent infections from the same patient, and in the case of patient A and F, two different patients.

each episode was ST393; however, unlike the high percentage of conserved SNPs in ST10 pyelonephritis isolates, HOS4 and HOS5 ST393 only shared 23% of all SNPs against the HOS3 reference, possibly due to the longer period between isolations, and only shared two negative BLOSUM62 score amino acid substitutions in chromosome partition protein MukF (F401V and T394H). Most SNPs occurred in intergenic regions, which may be significant as these regions often contain riboswitches, promoters, terminators and regulator binding sites, and SNPs can cause significant phenotypic changes [135].

Patient C was a 64-year-old female with cystitis and two samples were taken on the same day. Both isolates (HOS8 and HOS9) were ST144 and differed by five SNPs. While no SNPs occurred in VAGs (Fig. 9), two mutations with potential for functional change were identified in transposase TnpB (G11R) and environmental stress protein Ves (G7R). Conversely, in patient H, a 67-year-old male who presented with cystitis (HOS33; ST131 H30Rx) and then cystitis again a week later (HOS34; ST131 H30Rx), the two causative agents were indistinguishable.

Patient F was a 72-year-old female with cystitis followed by urosepsis 181 days later (HOS26; ST131 H30R; 53 SNPs or 20 SNPs without recombination), followed by urosepsis 101 days later (HOS27; ST131 H30R; 61 or 27 SNPs) and then a subsequent sample was taken 3 days later (HOS28; ST131

H30R; 62 or 26 SNPs). The vast majority of SNPs were a result of recombination, which is 50–100 times more likely to occur than mutations [136], and occurred in aerobactin biosynthesis protein *iucC* and iron transport system genes *sitABC*. However, three point mutations with potential change to function were identified in all urosepsis isolates: R18L in capsule protein KpsM, R268L in molybdoenzyme biosynthesis protein MoeA and M58R in molybdoenzyme chaperon protein YcdY. Intriguingly, HOS50 (also ST131 H30R), originating from a 50-year-old patient presenting with pyelonephritis 21 days prior to patient F's initial presentation, also possessed these three precise amino acid substitutions produced by the same SNPs. These identical substitutions may be indicative of a shared source, particularly as both patients resided in the same postcode, or may be examples of patho-adaptive mutations that increase fitness in the upper urinary tract. However, no recombination events were detected in HOS50.

Lastly, patient K was a 2-year-old female who presented with urosepsis (HOS41; ST69) and subsequent urosepsis 100 days later (HOS42; ST69; 13 SNPs). Similar to isolates derived from patient B, most SNPs occurred within intergenic regions (Fig. 9); however, R133L in yersiniabactin protein *ybtT* and R337S in K^+/H^+ antiporter CvrA may have incurred functional changes. Future characterization of all these SNP-mediated mutations is required to ascertain any functional changes.

Funding information

This work was funded by the Australian Centre for Genomic Epidemiological Microbiology (Ausgem), a collaborative partnership between the NSW Department of Primary Industries and the University of Technology Sydney.

Acknowledgements

The authors would like to acknowledge Max L. Cummins from the University of Technology Sydney for his bioinformatic pipelines and assistance in their use.

Author contributions

D. L. contributor roles: data curation, formal analysis, investigation, methodology, software, validation, visualization. C. J. R. contributor roles: formal analysis, methodology, software. T. K. contributor roles: data curation, resources. V. M. J. contributor roles: investigation, methodology, project administration, supervision, validation, writing – original draft. S. P. D. contributor roles: conceptualization, funding acquisition, project administration, supervision. All authors had a role in writing – review and editing.

Conflicts of interest

The authors declare that there are no conflicts of interest

Ethical statement

The project was approved by Charles Sturt University and Sydney West Area Health Service research ethics committees. Since clinical information for patients with UTIs was provided anonymously by clinicians, patient consent was not required.

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