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Genetic evaluation of ESBL-producing *Escherichia coli* urinary isolates in Otago, New Zealand

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Objectives: The incidence of infections with ESBL-producing *Escherichia coli* (ESBL-Ec) in New Zealand is increasing. ESBL-Ec most commonly cause urinary tract infections and are seen in both community and hospitalized patients. The reason for the increasing incidence of ESBL-Ec infections is unknown.

Methods: In this study, 65 urinary ESBL-Ec isolates from the Otago region in 2015 were fully genetically characterized to understand the mechanisms of transmission. The ESBL gene, *E. coli* STs, plasmid types and genetic context (e.g. insertion sequences) of ESBL genes were determined by a combination of whole genome and plasmid sequencing. The phylogenetic relationships of the isolates were compared with ESBL-Ec isolates sequenced as part of the 2016 nationwide survey.

Results: Significant diversity of *E. coli* strains, plasmids, and the genetic context of ESBL genes was seen. However, there was evidence of common mobile genetic elements in unrelated ESBL-Ec.

Conclusions: Multiple introductions of ESBL resistance genes or resistant bacterial strains with limited horizontal transmission of mobile genetic elements accounts for the increased incidence of ESBL-Ec in this low prevalence area. Future studies should investigate modes of transmission of ESBL-Ec in the Otago region.

Introduction

Escherichia coli is the cause of the majority of urinary tract infections (UTIs) worldwide.[1] With one in five women experiencing UTI during their life, UTIs are among the most prevalent bacterial infections.[2] The emergence of ESBL-producing *E. coli* (ESBL-Ec) has presented a major challenge in the treatment of UTIs.[2] ESBLs hydrolyse and inactivate most β-lactam antibiotics, including penicillins, extended-spectrum cephalosporins and monobactams.[3] Further, ESBLs, including but not limited to TEM, SHV, OXA and CTX-M, are typically encoded on large plasmids that frequently carry additional antimicrobial resistance genes, resulting in phenotypic MDR and limited treatment options for infected patients.[3] As a result, there is a high risk of failure of empirical treatment in ESBL-Ec infection, which can result in increased mortality and therapy costs.[1] The increasing prevalence of ESBL-Ec infection worldwide has been recognized as an urgent threat by WHO.[1]

Though the overall prevalence of ESBL-Ec resistance in New Zealand is low, a doubling in occurrence was observed between 2007 and 2016.[4] The Otago region, in the South Island, which has one of the lowest incidences of ESBL-Ec in New Zealand, has seen the number of isolates from routine diagnostic urine specimens from both community and hospitalized patients double between 2012 (40) and 2015 (81). It is unknown whether this increase is due to clonal expansion, increased transmission in the community or healthcare facilities, or due to introductions from outside of New Zealand.

Previous antibiotic use, recurrent UTIs, and prolonged hospitalization have been identified as risk factors for carriage of resistant pathogens and commensals.[1,5] Globally identified sources of ESBL-Ec include food, wildlife and aquatic environments, including freshwater and wastewater.[6] Those immigrating or returning from international travel in high prevalence countries have high rates of carriage of ESBL-Ec (up to 75%) and are an apparent source of introduction within communities.[7] Evidence indicates community transmission is the primary mode of ESBL-Ec spread worldwide.[7] While transmission of successful strains of ESBL-Ec, such as ST131, is well documented, transmission of mobile genetic elements (MGEs), such as plasmids, encoding ESBL genes between different bacterial strains and species is an alternative mechanism of transmission.[8] To understand whether the increasing incidence of ESBL-Ec in Otago, New Zealand is due to the clonal spread of one (or a

small number of) strains or ESBL-encoding MGEs, the genetic relationship between ESBL-Ec strains was determined using WGS and isolation and sequencing of plasmids.

Methods

Clinical isolates

ESBL-Ec isolated from urine samples, from community or hospitalized patients, submitted to Southern Community Laboratories (SCL) between February 2015 and January 2016 were included in the study; there were no changes in laboratory surveillance practices during the study period. Isolated bacteria were identified by MALDI-TOF MS (Biotyper; Bruker Daltonics, Billerica, MA, USA) and antimicrobial susceptibility testing performed by the disc diffusion method according to EUCAST guidelines.[9] Cefpodoxime-resistant isolates were assessed for ESBL production by the combination disc test method.[10] All ESBL-producing isolates from patients at first presentation were routinely cryopreserved at −80°C; only non-duplicate unique patient isolates were included in the study. Following thawing, isolate identification, antimicrobial susceptibility and ESBL production were confirmed prior to sequencing.

Illumina NextSeq WGS data analysis

DNA was extracted from overnight cultures using the NucleoSpin Tissue kit (Macherey-Nagel, Bethlehem, PA, USA), per the manufacturer's instructions. WGS was performed at the Microbiological Diagnostic Unit Public Health Laboratory (MDU PHL), University of Melbourne. Nextera library preparation was performed prior to sequencing on an Illumina NextSeq instrument (San Diego, CA, USA). WGS data of 300 ESBL-Ec isolates submitted to the Institute of Environmental Sciences Research (ESR) from hospital and community laboratories in New Zealand in August 2016 as part of a national survey (BioProject PRJNA531554) were also included in this study.[4] The Nullarbor bioinformatic pipeline was used to determine the read quality, species, ST, resistance profile and phylogenetic relationship of isolates from the WGS data.[11] The *E. coli* ST131 strain EC958 (GenBank: HG941718.1) was used as the reference genome.

Genetic clustering of isolates was assessed with fastbaps.[12] Clermont phylotype of isolates was determined *in silico* with ClermonTyper.[13,14] The FimH type of isolates was determined *in silico* with CHTyper.[15]

ESBL plasmid isolation

Plasmids encoding resistance to extended-spectrum cephalosporins were isolated by transfer to an extended-spectrum cephalosporin-susceptible, sodium azide-resistant recipient laboratory strain, *E. coli* J53, using filter mating conjugation.[16,17] Thirty microlitres of 1:1 donor to recipient bacteria mixture was pipetted onto 0.45 μm pore MF-Millipore mixed cellulose ester Membrane Filters (Merck KGaA, Darmstadt, Germany) on selective LB agar containing 250 mg/L sodium azide and 1.5 mg/L cefotaxime and incubated overnight at 37 °C. For controls, 30 μL of either *E. coli* J53 or the test isolate alone were pipetted onto membrane filters.

Where conjugation was unsuccessful, plasmids were isolated by transformation. Plasmids were isolated from donor strains using a modified alkaline lysis method suitable for large plasmids.[18] Plasmid DNA quality was assessed using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Electrocompetent *E. coli* ST18 and DH10B strains were prepared in-house using three washes of 10% glycerol.[19] Electroporation was performed on 40 μL (8×10^9 cells) of electrocompetent cells and 1 μL (40–80 ng) of plasmid DNA using the Gene Pulser Xcell Electroporation system (Bio-Rad Laboratories Inc., Hercules, CA, USA) with standard settings (0.1 cm, 1.8 kV, 200 Ω, 25 mF). Electrocompetent *E. coli* DH10B cells (Thermo Fisher Scientific) were used for the majority of experiments due to their competence in hosting large plasmids in comparison to *E. coli* ST18 cells, which achieved low rates of successful transformation in initial experiments (3/6). Glycerol stocks (50% glycerol in LB broth) of the successful transformants and transconjugants were stored at -80°C.

Successful conjugation or transformation was confirmed by PCR (Robust Kapa2G Hotstart PCR kit, Kapa Biosystems, Wilmington, MA, USA) and gel electrophoresis (100 V, 1 h), as per manufacturer's instructions. DNA was extracted from bacterial colonies using the boiling method.[20] PCR conditions and primers described by Dallenne *et al.*[20] were used to confirm the presence of ESBL genes in transformants and transconjugants. Primers and methods described by Kuhnert *et al.*[21] were used to confirm the strain of putative transconjugants as *E. coli* J53.

MinION plasmid analysis

Plasmids from transconjugants and transformants were isolated using a modified alkaline lysis method.[18] The quality of twice-cleaned plasmids (Agencourt AMPure XP beads, Beckman Coulter, High Wycombe, UK) was assessed using a Nanodrop (Thermo Fisher Scientific) and gel electrophoresis (0.8% LMP agar, 70 V, 5–50 min). DNA concentration was determined by Qubit Fluorometer (Invitrogen). DNA libraries were prepared using the Rapid Barcoding Sequencing kit (SQK-RBK004) (Oxford Nanopore Technologies Ltd, Oxford, UK) and MinION sequencing was performed using R9.4 (FLO-MIN106) flow cells.[22] Oxford Nanopore Technologies MinKNOW software (versions 0.45.2.6–2.34.3) was used to collect raw sequencing data.

Reads were basecalled using ONT programs MinKNOW (versions 2.0–2.1) for live basecalling or Albacore (version 2.3.0) for post-run basecalling. Porechop (version 0.2.3) was used to demultiplex and remove adaptor sequences.[23] EPI2ME (versions 2.5.2–3.9.3) (Oxford Nanopore Technologies) was used to assess the read quality of each sample.

Hybrid assembly

Unicycler was used to perform hybrid assembly of all plasmids.[24] Any hybrid assembly that did not yield a complete plasmid with Unicycler alone was repeated following filtering of the WGS Illumina data with Bowtie 2 to include only those reads that matched the relevant plasmid as sequenced by the MinION.[25] miniasm or sed (on any hybrid assemblies incomplete with miniasm) were used to generate a long-read only assembly from the MinION reads in order to make a reference index for Bowtie 2.[26] The '–very-sensitive local' pre-set was used to account for the higher error rate in the MinION reads.

Analyses on completed plasmids

Following successful plasmid assembly, the resulting graph (.fastg output) was searched and annotated using Bandage.[27] Plasmids were typed *in silico* using PlasmidFinder and pMLST.[28] ISfinder was used to identify insertion sequences surrounding ESBL genes.[29] NCBI BLAST, EPI2ME, ResFinder (version 3.1) and CARD antimicrobial resistance online were used to determine the presence of antibiotic resistance genes.[30–34]

Statistical analysis

The χ^2 test, *post hoc* binomial test and sign test were used for statistical analyses. A two-tailed $P < 0.05$ was considered significant. All analyses were performed using the statistical software SPSS (build 1.0.0.1246; SPSS Inc., Chicago, IL, USA) and Excel Analyse-it for Microsoft Excel (version 2.20, 2009; Analyse-it Software Ltd, Leeds, UK).

Results

Clinical isolates

Of 81 non-duplicate unique patient ESBL-Ec isolated from urine during the study period, 65 (80.2%) were characterized in this

study. Thirteen (16.0%) were missing from storage, though no systematic bias was apparent. Three (3.7%) cryopreserved isolates were found not to be ESBL producers. Most isolates were from a community source (50/65, 76.9%). The median patient age was 66.9 years (range 4.7 to 100.6 years); 34/65 (52.3%) were ≥65 years old. Fifty-six (83.6%) patients were female.

ESBL and other β-lactamase genes

There was a dominance of $bla_{CTX\text{-}M}$ ESBL genes (56/65, 86.2%) (Table 1), the majority of which (30/56, 53.6%) were found in conjunction with bla_{TEM} and bla_{OXA} β-lactamase genes not known to have an ESBL phenotype.[26–28] In addition, there was one $bla_{CMY\text{-}2}$ AmpC resistance gene found in conjunction with $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1B}$. The ESBL gene $bla_{SHV\text{-}12}$ was found in the majority of non-CTX-M isolates (8/9). There was no significant difference between the proportions of $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ ($P > 0.05$), the predominant $bla_{CTX\text{-}M}$ types identified. Five isolates encoding $bla_{CTX\text{-}M\text{-}55}$ were also identified, two of which also encoded $bla_{CTX\text{-}M\text{-}14}$.

Other resistance genes

The resistance genes for other antibiotic classes with ≥90% sequence coverage are illustrated in Table S1 and the Nullarbor output file (available as Supplementary data at *JAC-AMR* Online). Notably, a partial (61%) *mcr-1* gene sequence (encoding colistin resistance) was only found in one isolate.

Table 1. ESBL and other β-lactamase genes detected

β-Lactamase-encoding genes	Number	%
Total $bla_{CTX\text{-}M}$	56	86.2
total group 9	35	53.8
total $bla_{CTX\text{-}M\text{-}14}$	17	26.1
$bla_{CTX\text{-}M\text{-}14}$	7	10.8
$bla_{CTX\text{-}M\text{-}14}/bla_{TEM\text{-}1B}$	10	15.4
total $bla_{CTX\text{-}M\text{-}27}$	18	27.7
$bla_{CTX\text{-}M\text{-}27}$	14	21.5
$bla_{CTX\text{-}M\text{-}27}/bla_{TEM\text{-}1B}$	2	3.1
$bla_{CTX\text{-}M\text{-}27}/bla_{OXA\text{-}1}$	2	3.1
total group 1	19	29.2
total $bla_{CTX\text{-}M\text{-}15}$	14	21.5
$bla_{CTX\text{-}M\text{-}15}$	4	6.2
$bla_{CTX\text{-}M\text{-}15}/bla_{OXA\text{-}1}$	4	6.2
$bla_{CTX\text{-}M\text{-}15}/bla_{OXA\text{-}1}/bla_{TEM\text{-}1B}$	4	6.2
$bla_{CTX\text{-}M\text{-}15}/bla_{TEM\text{-}1B}$	2	3.1
total $bla_{CTX\text{-}M\text{-}55}$	5	7.7
$bla_{CTX\text{-}M\text{-}55}$	2	3.1
$bla_{CTX\text{-}M\text{-}55}/bla_{TEM\text{-}1B}$	3	4.6
total group 1 + group 9	2	3.1
$bla_{CTX\text{-}M\text{-}14}/bla_{CTX\text{-}M\text{-}55}$	2	3.1
Total $bla_{SHV}/bla_{TEM}/bla_{OXA}$ only	9	13.8
$bla_{TEM\text{-}1B}$	1	1.5
$bla_{SHV\text{-}12}$	1	1.5
$bla_{SHV\text{-}12}/bla_{TEM\text{-}1B}$	5	7.7
$bla_{SHV\text{-}12}/bla_{TEM\text{-}1A}/bla_{OXA\text{-}9}$	2	3.1

Of the 65 isolates, resistance genes for aminoglycosides were found in 39 (60.0%), macrolides in 35 (53.8%), sulfonamides in 39 (60.0%), tetracyclines in 39 (60.0%) and trimethoprim in 40 (61.5%) isolates. Less commonly identified resistance genes included those to chloramphenicol in 12 (18.4%), fosfomycin in 3 (4.6%), lincosamides in 1 (1.5%) and both quinolones and aminoglycosides in 13 (20.0%). Genes encoding resistance to three or more classes of antibiotics were found in 41 (63.1%) isolates. This phenomenon was observed more frequently in isolates carrying $bla_{CTX\text{-}M}$ ESBLs (38/56, 67.9%) than those carrying $bla_{TEM\text{-}1B}/bla_{OXA\text{-}9}/bla_{SHV\text{-}12}$ ESBLs (3/9, 33.3%) ($P < 0.05$). The incidence of this phenomenon did not differ greatly between different $bla_{CTX\text{-}M}$ types ($bla_{CTX\text{-}M\text{-}14}$, 11/17 [64.7%]; $bla_{CTX\text{-}M\text{-}15}$, 9/14 [64.3%]; $bla_{CTX\text{-}M\text{-}27}$, 14/18 [77.8%]).

Phylogenetic relationship and MLST

There were five clusters evident in the core genome phylogeny tree, while the pan-genome phylogeny tree showed three clusters (Figure 1 and Figure S1). The most common Clermont phylogenetic groups were B2 and D (38/65, 58.5% and 15/65, 23.1%, respectively). The Achtman MLSTs of this set of isolates (Figure 1) showed a high degree of diversity with 23 different STs, but the most common ST was ST131 (27/65, 41.5%).[35] A statistically significant association of ST131 with $bla_{CTX\text{-}M\text{-}27}$ was observed ($P < 0.01$). The *H*30 clone was the most common *fimH* allele (20/65, 30.8%), with 13 of these belonging to the subclone *H*30R (fluoroquinolone resistance).

The core genome phylogeny and MLSTs of the 65 Otago isolates were examined in the context of the ESBL-Ec from the nationwide 2016 ESR survey of ESBL-producing Enterobacteriaceae (Figure 2).[35] Over the 1 month survey period, 521 non-duplicate ESBL-producing Enterobacteriaceae were isolated, of which 386 (74.1%) were *E. coli*; of the ESBL-Ec, 47.4% were from patients ≥65 years old and 68.1% were from community patients.[35] WGS data were available on 300 (77.7%) of the ESBL-Ec. Considering both datasets, ST131 was dominant, representing 54.2% (198/365) of isolates; an ST could not be determined in 11 (3.0%). ST131 frequency among Otago isolates (27/65, 41.5%) was lower than among ESR isolates (171/300, 57.0%). ST38 was the second most frequently occurring ST among Otago isolates (7/65, 10.8%) and ESR isolates (21/300, 7.0%). ST1193 (20/300, 6.7%) and ST12 (10/292, 3.4%) were the next most commonly occurring STs within the set of ESR isolates, however, they were less common among the Otago isolates (ST1193: 1/65, 1.5% and ST12: 1/65, 1.5% respectively). In contrast, ST405 was more common amongst the Otago isolates (5/65, 7.7%) than the ESR isolates (7/300, 2.3%). ST69 was equally represented in both sets of isolates at 3.1% of Otago isolates and 3.3% of ESR isolates. The ESR isolates showed more diversity with 37 different ST; 22 of these were not represented in the Otago isolates. Of the Otago isolates, six STs were not represented within ESR isolates (ST14, 297, 448, 636, 744 and 4204).

MinION sequencing

ESBL-encoding plasmids were able to be isolated from 44 of 65 (67.7%) ESBL-Ec isolates (Table S2). Of these, 32 (72.7%) were isolated by conjugation and 12 (27.2%) by transformation.

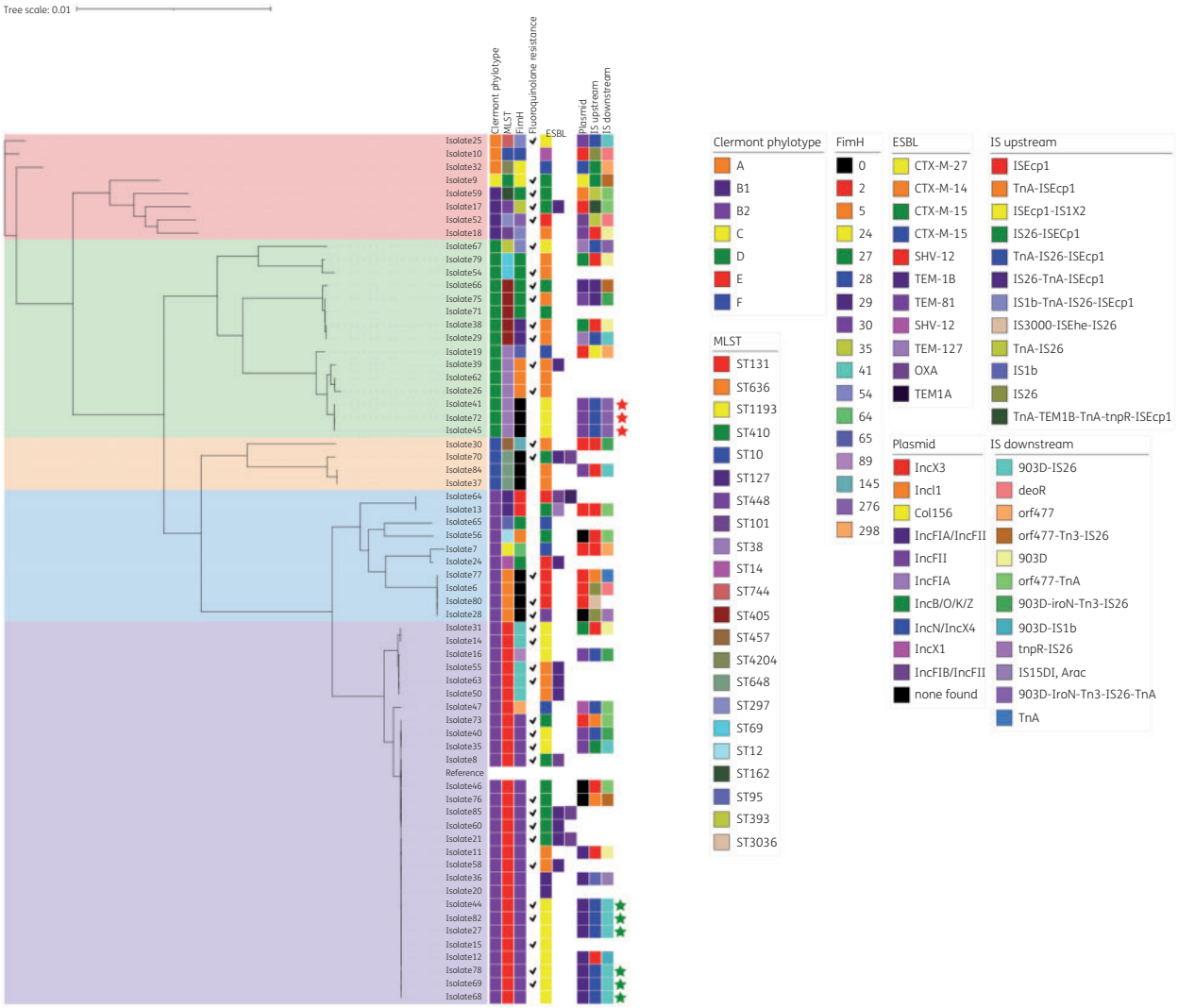

Figure 1. Core genome SNP phylogenetic tree annotated with ESBL genes and genetic context. Identical isolates in terms of gene, IS, plasmid incompatibility type, Clermont type, *fimH* subclone and MLST are marked with the same colour star. The five clusters determined with fastbaps (version 1.0.4) are highlighted. Produced by FastTree (version 2.1.10 Double precision [No SSE3], Open MP [64 threads]) with SNPs from Snippy-core (version 3.2-dev) using a reference genome of ST131 ESBL-Ec (GenBank: HG941718.1). Core SNP alignment has 65 taxa and 209 001 bp. Core SNP density was 209 001 SNPs across 5 109 767 bp in the reference genome. The scale bar indicates the number of substitutions per site. Figure generated using iTOL online (version 6.0).

The EPI2ME quality analysis of the reads of all four MinION runs showed an average read length of 12 247 bp, quality score of 10.048, read number of 3044.61 and yield of 33.2 Mbp. Most plasmids (40/44, 90.9%) were able to be completed by hybrid assembly; 16 required the Bowtie 2 pre-filter. However, sufficient information could still be obtained from three of four of these non-circularized plasmids to characterize the plasmid type and insertion sequences around the ESBL gene. Conversely, plasmid type was unable to be identified with PlasmidFinder in three other isolates despite completed, circularized plasmids.

β-Lactamase gene type of isolated plasmids

WGS identified all β-lactamase genes present in the isolates. With the MinION data, only the plasmids that were able to be conjugated or transformed *in vitro* were sequenced. Not all β-lactamases from the original isolates were present on the isolated plasmids (Table 2 and Table S2). The β-lactamases that were not transferred are also shown. Of the nine isolates that contained only one or more of TEM/SHV/OXA, a plasmid was able to be isolated from six (66.7%). Of 56 CTX-M-encoding isolates, 38 (67.9%) ESBL-encoding plasmids were able to be isolated. While an ESBL-encoding plasmid could be isolated from most CTX-M-27-encoding isolates (16/18, 88.9%), it was only possible in 6/17 (42%) CTX-M-14-encoding isolates. Of the isolates encoding a group 1 CTX-M, a plasmid could be isolated from 8/14 (57.1%) CTX-M-15-encoding and 4/5 (80%) CTX-M-55-encoding isolates. Notably, a $bla_{\text{TEM-1B}}$ gene was transferred from one isolate (1/16, 6.25%) wherein $bla_{\text{CTX-M-14}}$ co-occurred but was not isolated. A plasmid carrying both $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-1B}}$ was able to be

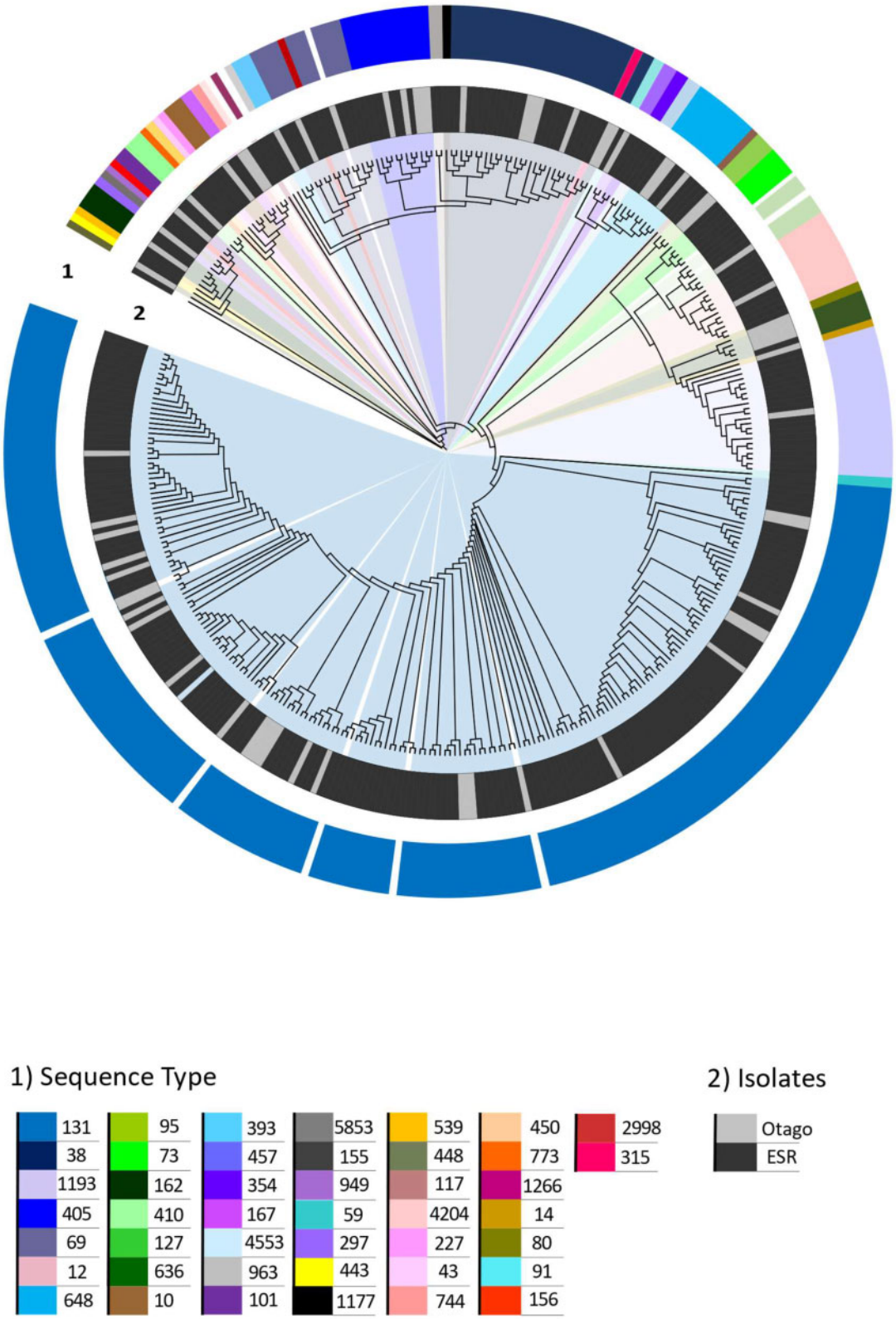

Figure 2. Core genome SNP phylogenetic tree annotated with ST and isolate source. Produced by FastTree (version 2.1.10, double precision [No SSE3]) with SNPs from Snippy-core (version 4.6.0) using the reference genome ST131 ESBL-Ec (GenBank: HG941718.1). Core SNP alignment has 365 taxa and 154 607 bp. Core SNP density was 154 607 SNPs across 5 109 767 bp in the reference genome. Figure generated using iTOL (version 6.3).

Table 2. β-Lactamase genes encoded by isolated plasmids

β-Lactamase gene encoded by isolated plasmids (MinION)	β-Lactamase genes present in original isolate (Illumina WGS)	Number
$bla_{CTX\text{-}M\text{-}14}$[a]	$bla_{CTX\text{-}M\text{-}14}$	3
	$bla_{CTX\text{-}M\text{-}14}/bla_{CTX\text{-}M\text{-}55}$	2
	$bla_{CTX\text{-}M\text{-}14}/bla_{TEM\text{-}1B}$	3
$bla_{CTX\text{-}M\text{-}15}$[a]	$bla_{CTX\text{-}M\text{-}15}$	3
	$bla_{CTX\text{-}M\text{-}15}/bla_{OXA\text{-}1}$	3
	$bla_{CTX\text{-}M\text{-}15}/bla_{OXA\text{-}1}/bla_{TEM\text{-}1B}$	1
$bla_{CTX\text{-}M\text{-}15}$[a]/$bla_{TEM\text{-}1B}$[b]	$bla_{CTX\text{-}M\text{-}15}/bla_{CMY}/bla_{TEM\text{-}1B}$	1
$bla_{CTX\text{-}M\text{-}15}$[a]/$bla_{TEM\text{-}127}$[b, c]	$bla_{OXA\text{-}9}/bla_{SHV\text{-}12}/bla_{TEM\text{-}1A}$	1
$bla_{CTX\text{-}M\text{-}27}$[a]	$bla_{CTX\text{-}M\text{-}27}$	12
	$bla_{CTX\text{-}M\text{-}27}/bla_{OXA\text{-}1}$	2
	$bla_{CTX\text{-}M\text{-}27}/bla_{TEM\text{-}1B}$	2
$bla_{CTX\text{-}M\text{-}55}$[a]	$bla_{CTX\text{-}M\text{-}55}$	1
	$bla_{CTX\text{-}M\text{-}55}/bla_{TEM\text{-}1B}$	3
$bla_{SHV\text{-}12}$[a]	$bla_{SHV\text{-}12}$	1
	$bla_{SHV\text{-}12}/bla_{TEM\text{-}1B}$	3
$bla_{SHV\text{-}183}$[d, c]	$bla_{CTX\text{-}M\text{-}14}/bla_{TEM\text{-}1B}$	1
$bla_{TEM\text{-}81}$[e]	$bla_{SHV\text{-}12}/bla_{TEM\text{-}1B}$	1
$bla_{TEM\text{-}1B}$[b]	$bla_{CTX\text{-}M\text{-}14}/bla_{TEM\text{-}1B}$	1

[a]ESBL phenotype.
[b]Broad-spectrum β-lactamase phenotype.
[d]Phenotype undefined.
[c]Excluded from further analyses.
[e]Inhibitor resistant β-lactamase phenotype.

isolated. In the two isolates in which $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}55}$ co-occurred, a plasmid encoding only $bla_{CTX\text{-}M\text{-}14}$ was isolated.

There were two cases of incongruence. One plasmid isolated was found to carry a $bla_{SHV\text{-}183}$ ESBL gene, despite this gene not being identified in the Illumina WGS results (which identified $bla_{CTX\text{-}M\text{-}14}$ and $bla_{TEM\text{-}1B}$). In another instance, a plasmid that carried $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}127}$ was identified from an isolate which, according to the Illumina WGS results, only carried $bla_{OXA\text{-}9}/bla_{SHV\text{-}12}/bla_{TEM\text{-}1A}$ β-lactamase genes. In both cases the anomalous genes were found in the raw MinION reads using the EPI2ME resistance workflow. The genes were absent in both the filtered and raw Illumina reads (CARD and CGE ResFinder). Furthermore, no β-lactamase gene was present in both sets of Bowtie 2-filtered Illumina sequence. The most likely explanation is carryover contamination of barcodes from previous libraries despite an intervening wash step, which has been observed in other studies.[36,37] Though the same barcodes were not used for isolates containing the respective incongruent gene in these experiments, the same flow cell had been used for other experiments with other *E. coli* isolates. For this reason, these two plasmids were excluded from later analyses.

Genetic context of ESBL genes

Multireplicon ESBL-encoding plasmids were common in this set of isolates, with IncFIA/IncFII being one of the two most common incompatibility types in this set of plasmids, alongside IncFII (both $n = 10$, 22.7%) (Figure 1). The plasmid incompatibility type of four of the isolates was unable to be identified.

IS*Ecp1* occurred upstream (5′) of all $bla_{CTX\text{-}M}$ genes. There was an exclusive association of downstream *orf477* with group 1 and IS*903D* with group 9 CTX-M groups. There was a varying presence of IS*26* and Tn*3* both upstream and downstream of ESBL genes, plus a varying presence of *iroN*-Tn*3*-IS*26* downstream of IS*903D*. The IS*Ecp1*-IS*26* element was associated with $bla_{CTX\text{-}M\text{-}27}$ (14/16, 87.5%). All four isolates carrying $bla_{SHV\text{-}12}$ had different insertion sequences surrounding the gene. The genetic context of ESBL genes in different isolates is summarized in Table S2 and six examples are shown in Figure 3.[38]

Overall considerable genetic diversity was seen with isolates differing in at least one of the categories of MLST, plasmid type and genetic context (Figure 1). Only in the isolates carrying $bla_{CTX\text{-}M\text{-}27}$ were notable clones observed. Clusters of three ST38/IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26*, Tn*A* isolates and six ST131/IncFIA-IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, IS*26* isolates were identified. There were also two ST131/IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26* isolates seen. The remaining isolates carrying $bla_{CTX\text{-}M\text{-}27}$ differed in MLST, Clermont type, *fimH* subclone, plasmid incompatibility type and/or insertion sequences.

There was, however, evidence of common MGEs in unrelated ESBL-Ec (Figure 1 and Table S2). An IncI plasmid encoding Tn*A*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}15}$-*orf477*, Tn*A* was found in two isolates (59 and 73) with different STs (ST162 and ST131 respectively). Similarly, an untypeable plasmid encoding IS*Ecp1*- $bla_{CTX\text{-}M\text{-}15}$-*orf477*, Tn*A* was found in two isolates (46 and 56) of different STs (ST131 and ST12 respectively). There was also evidence of the same ESBL gene in the same genetic context but on different plasmids in isolates with different STs. IS*Ecp1*-$bla_{CTX\text{-}M\text{-}14}$-IS*903D* was found in four isolates (11, 18, 38 and 79) of different STs (ST131, ST101, ST405 and ST69, respectively) on three different plasmid types (IncFIA/IncFII, IncFII, IncB/O/K/Z and IncB/O/K/Z, respectively). Similarly, in addition to the six ST131/IncFIA-IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, IS*26* isolates, Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, IS*26* was also found on an IncFII plasmid in a ST744 isolate. In addition to the three ST38/IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26*, Tn*A* isolates, Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26*, Tn*A* was also found on an IncFIA plasmid in a ST393 isolate. In contrast, all the $bla_{CTX\text{-}M\text{-}55}$, bla_{SHV} and bla_{TEM} differed in their genetic context.

Discussion

In this study we sequenced 80.2% of the ESBL-Ec isolates from urine samples collected in the Otago region of New Zealand over a 12 month period. While there was some evidence of common ESBL-Ec clones and MGEs, overall there was considerable genetic diversity between isolates, plasmids and the genetic context of the ESBL genes. This is consistent with multiple introductions from outside the region with limited clonal transmission of resistant strains, plasmids and MGEs.

The dominance of $bla_{CTX\text{-}M}$ ESBL genes, IncF plasmids, ST131 and the *H*30 subgroup is in agreement with what has previously been reported worldwide and in New Zealand.[4,39–41] The 2016 ESR survey of New Zealand (68.1%) and this study (76.9%) had a similar proportion of community isolates.[4] There was a higher prevalence of $bla_{TEM}/bla_{SHV}/bla_{OXA}$ genes compared with nationwide reports, which may be due to the low overall prevalence of ESBL-Ec

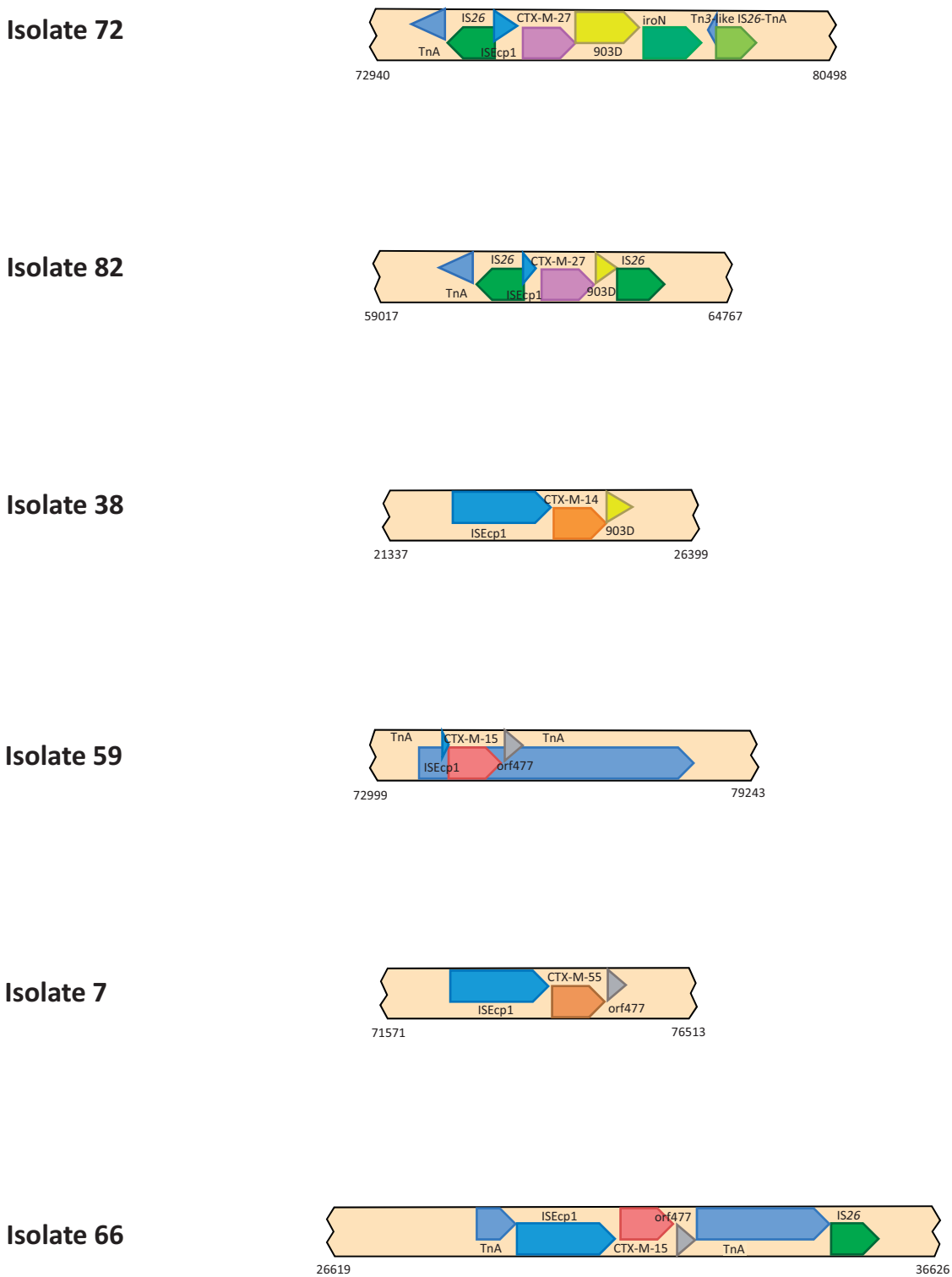

Figure 3. Maps of insertion sequences surrounding the ESBL genes of six representative isolates. Produced using Simple Synteny (version 1.3.2). Isolate 72 (Tn*A*-IS*26*-IS*Ecp1*/*903D*-*iroN*-Tn*3*-IS*26*-Tn*A*) shares the same genetic context as isolates 41, 45 and 67. Isolate 82 (Tn*A*-IS*26*-IS*Ecp1*/*903D*-IS*26*) shares the same genetic context as isolates 25, 27, 29, 44, 68, 69 and 78. Isolate 38 (IS*Ecp1*/*903D*) shares the same genetic context as isolates 11, 18, 31 and 79. Isolate 59 (Tn*A*-IS*Ecp1*/*orf477*-Tn*A*) shares the same genetic context as isolate 73. Isolate 7 and isolate 66 are unique in genetic context.

in the Otago area.[3,4,42] The statistically equal proportions of $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ is in contrast to most countries (including Portugal, Italy, USA, Mexico, Bolivia, India and South East Asia), where $bla_{CTX\text{-}M\text{-}15}$ increased and became dominant from 2009–16, but in line with some countries (Israel, Spain, Canada, United Kingdom, Egypt, China, Vietnam, Turkey, France, Germany, Korea and Japan) where the proportion of $bla_{CTX\text{-}M\text{-}15}$ decreased compared with pre-2007 in favour of $bla_{CTX\text{-}M\text{-}27}$, although not necessarily overtaking $bla_{CTX\text{-}M\text{-}15}$.[43,44] An association between $bla_{CTX\text{-}M\text{-}27}$ and ST131 has been reported in countries where $bla_{CTX\text{-}M\text{-}27}$ incidence is increasing or is greater than $bla_{CTX\text{-}M\text{-}15}$, and where there is a dominance of ST131.[43–51]

The dominant Clermont phylogenetic groups in this study (B2 and D) have been associated with pathogenic urinary *E. coli* strains.[52] ST131 has been implicated in the rise in ESBL resistance worldwide, and is particularly important in UTIs due to their increased biofilm formation ability and common MDR to fluoroquinolones.[39,53] Future studies to determine the presence of *H*30Rx or *H*30R1 subclones and any associations to $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}27}$, as has been described in previous studies, would be useful for comparison.[4,40,53,54]

A total of 21/65 (32.3%) plasmids were not able to be isolated. Both conjugation and transformation were used for isolation of the ESBL-encoding plasmid. The conjugation method better demonstrates the transferability of these plasmids in the environment, as opposed to electroporation. The large size of the plasmids (40–150 kb) may have affected transferability under laboratory conditions.[19] Alternatively, the resistance gene may have been located on the chromosome in a transposon. A plasmid was able to be isolated from isolates of nearly all MLST types (except two). All MLST types carried transferable plasmids, and none in particular were associated with highly transmissible plasmids. Further optimization of conjugation techniques and electroporation conditions might allow isolation of more plasmids.

The specificity of downstream insertion sequences to $bla_{CTX\text{-}M}$ groups has been previously observed.[43,55–62] The IS*Ecp1*-IS*26* element was associated with $bla_{CTX\text{-}M\text{-}27}$, which has been observed in a previous large-scale study in Japan, though that study did not assess for the presence of Tn*A*, which occurred upstream of the IS*26*-IS*Ecp1* element in most $bla_{CTX\text{-}M\text{-}27}$ isolates (14/16, 87.5%) in Otago.[40] IS*3000* only appeared upstream of bla_{SHV} ESBL genes, though it has been observed upstream of group 9 $bla_{CTX\text{-}M}$ ESBL genes in a previous study.[55]

The high genetic diversity and limited number of identical isolates in terms of gene, IS, plasmid incompatibility type and MLST (three ST38/IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26*, Tn*A* isolates, six ST131/IncFIA-IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, IS*26* isolates and two ST131/IncFII/Tn*A*, IS*26*, IS*Ecp1*-$bla_{CTX\text{-}M\text{-}27}$-IS*903D*, *iroN*, Tn*3*, IS*26*) indicates multiple introductions into the Otago population with more limited evidence of horizontal transmission.[63,64] These introductions could be from numerous sources. Misuse of antibiotics in humans or agriculture could constitute selection pressures for the mobilization of antibiotic resistance genes.[65–67] Recent studies have shown that the low antibiotic concentrations that have been found in environmental residues (such as hospital and wastewater effluent) are sufficient for the positive selection of ESBL resistance.[65–67] Many of the ESBL genes and associated sequence and plasmids types found in this study have been commonly isolated from animal and environmental sources in international studies.[43,46,49,55,68–72] Future epidemiological studies investigating the presence of ESBL genes in the environment, animals and faecal carriage in asymptomatic humans will be important for the elucidation of potential reservoirs, identification of person-to-person contact, or common exposure to potential sources. Future studies should also collect travel and migration history from patients. The finding that the increase in ESBL-Ec UTI incidence in Otago is not due to the transmission of highly virulent clones is important to avoid inappropriate outbreak management.[67]

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Transparency declarations

None to declare.

Supplementary data

Table S1 and S2, Figure S1 and the Nullarbor report are available as Supplementary data at *JAC-AMR* Online.

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