

Co-resistance to quaternary ammonium compounds in extended-spectrum beta-lactamase-producing *Escherichia coli*

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Received: 06-05-2020, **Accepted:** 17-08-2020, **Published online:** 15-09-2020

doi: [www.doi.org/10.14202/IJOH.2020.134-142](https://doi.org/10.14202/IJOH.2020.134-142) **How to cite this article:** Sütterlin S, Heydecke A, Tano E (2020) Co-resistance to quaternary ammonium compounds in extended-spectrum beta-lactamase-producing *Escherichia coli*, *Int. J. One Health*, 6(2): 134-142.

Abstract

Background and Aim: Extended-spectrum β -lactamases (ESBL) in *Escherichia coli* constitutes one of the major threats to modern medicine, and the increasing pollution with quaternary ammonium compounds (QACs) has been suspected to contribute to the spread of ESBL-producing bacteria. The aim of the study was to investigate ESBL_A and ESBL_{M-C}-producing *E. coli* isolates for their co-resistance to QACs and their phylogeny isolated from a Swedish University Hospital.

Materials and Methods: Co-resistance in *E. coli* with production of ESBL enzymes of the type *bla*_{CTX-M} (n=23) was compared to *E. coli* producing AmpC type ESBL enzymes *bla*_{CMY} and *bla*_{DHA} (n=27). All isolates were tested for susceptibility to antibiotics and QACs, and high-quality whole-genome sequences were analyzed for resistance determinants.

Results: The plasmid-borne small multidrug resistance (SMR) efflux pump *sugE(p)* was solely present in *bla*_{CMY}-producing *E. coli* (n=9), within the same genetic environment *bla*_{CMY}-*blc*-*sugE(p)*. Other small multidrug efflux pumps were found without association for ESBL-types: *emrE* (n=5) and the truncated *qacEΔ1* (n=18).

Conclusion: Co-resistance of ESBL enzymes and SMR efflux pumps in *E. coli* was common and might indicate that other substances than antibiotics contribute to the spread and emergence of antibiotic resistance.

Keywords: biocides, *Escherichia coli*, extended-spectrum β -lactamases, quaternary ammonium compounds.

Introduction

Antimicrobial resistance is an urgent global threat to public health with a high disease burden to humans [1]. Since the 2000s, public awareness has increased regarding the worrisome rapid spread of antibiotic resistance, and many efforts have been taken to slow this development down. Actions have been taken to combat well-known factors that drive antibiotic resistance, such as limit the overuse of antibiotics and improve hygiene in medical care, animal husbandry, and the community [2].

However, despite all efforts, the emergence and distribution of extended-spectrum β -lactamases (ESBL) in *Escherichia coli* and other *Enterobacteriaceae* that cause resistance to the 3rd-generation cephalosporins ESBL-producing *E. coli* continue, and therefore, other potential driving factors like the extensive use of biocides have been discussed. Concerns regarding the potential risk of biocidal substances have been raised early [3]. Especially, the intense use of substances that

belong to quaternary ammonium compounds (QACs) has frequently been stressed as a potential risk, especially as the effects of sublethal concentrations on bacterial populations are rather unknown [4,5]. They are heavily utilized as preservatives and fabric softeners and thus released in significant amounts into the water cycle [6]. Anthropogenic contamination of soils and water environments with QACs can significantly contribute to enrichment of mobile genetic elements involving resistance determinants to antibiotics and QACs [7,8].

A majority of the clinically recognized ESBL enzymes have been mobilized from chromosomes of bacteria populating soils and other wet environments. Some ESBL enzymes became well-adapted in *E. coli*, such as *bla*_{CTX-M} enzymes mobilized from *Kluyvera* spp., *bla*_{CMY} enzymes from *Citrobacter* spp., or *bla*_{DHA} from *Morganella morganii*, and they disseminate mainly through plasmids and certain bacterial clones [9,10]. Some of these plasmid-borne ESBL enzymes can be inhibited by beta-lactamase inhibitors, a fact that is used to classify ESBL enzymes in clinical contexts [11]. It distinguishes among others classical ESBL_A (like *bla*_{CTX-M}) that are inhibitable by beta-lactamase inhibitors and those that cannot be inhibited which are called miscellaneous ESBL_{M-C} (like *bla*_{CMY} or *bla*_{DHA}); the latter are also known as plasmid-borne AmpC beta-lactamases.

Co-resistance to *bla*_{CTX-M} and biocides has been documented for QACs, in bacterial isolates involved in

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hospital outbreaks and environment [12,13]. However, this is less well studied for ESBL_{M-C}. Against the background, that ESBL_{M-C}-producing *E. coli* have become an emerging problem, it seems needful to study the coresistance potential of QACs to ESBL_{M-C} [10,14].

Thus, the aim of the study was to investigate ESBL_A and ESBL_{M-C}-producing *E. coli* isolates for their coresistance to QACs and their phylogeny isolated from a Swedish University Hospital.

Materials and Methods

Ethical approval

After retrieval of relevant information from the referral, all isolates were anonymized; therefore, no ethical approval was necessary.

Bacterial isolates

This study comprises a total of 58 urinary tract isolates producing ESBL enzymes that were collected between 2011 and 2016 at Uppsala University Hospital. Urinary tract samples were cultured quantitatively on blood agar plates and cystine–lactose–electrolyte-deficient agar plates (Oxoid, UK), and species identification was done using standard laboratory procedures and automated species identifications systems Maldi TOF (Bruker Daltonics, USA). The study followed the recommendations of the Nordic Committee on Antimicrobial Susceptibility Testing (www.nordiccast.org) for diagnostics and classification of ESBL enzymes: All isolates with reduced susceptibility to cefpodoxime were further investigated using a synergy test that assesses the inhibition of cefotaxime, ceftazidime, or cefepime by clavulanic acid. Beta-lactamases that can be inhibited by clavulanic acid classified as classical ESBL (ESBL_A), and presumed enzymes that cannot be inhibited classified as suspicious plasmid-borne AmpC beta-lactamases (ESBL_{M-C}). Suspicious ESBL_{M-C} were further verified by polymerase chain reaction (PCR) for the presence of plasmid-borne enzymes of type *bla*_{CMY}, *bla*_{MOXM}, *bla*_{DHAM}, and *bla*_{ACCM} (for details see below). All isolates were frozen as glycerol stock at -80°C.

For the purpose of this study, *E. coli* isolates originating from urinary tract samples producing ESBL_A and ESBL_{M-C} were randomly chosen resulting in 30 isolates producing ESBL_A and 28 isolates producing ESBL_{M-C}. After excluding isolates originating from the same individual, 54 isolates were included in the study; thereafter, all isolates were anonymized.

PCRs to distinguish ESBL_{M-C}

Each PCR contained 1 µL template DNA, 1 µL of 10 µM each primer, and HotStarTaq Master Mix Kit (Qiagen, Germany) to a final volume of 25 µL. The primers used had the following sequences with the expected size of the amplification product in parenthesis: *bla*_{ACCM} forward 5'-AAC AGC CTC AGC AGC CGG TTA-3' and *bla*_{ACCM} reverse 5'-TTC GCC GCA ATC ATC CCT AGC-3' (346 bp), *bla*_{DHAM} forward 5'-AAC TTT CAC AGG TGT GCT GGG T-3'

and *bla*_{DHAM} reverse 5'-CCG TAC GCA TAC TGG CTT TGC-3' (405 bp), *bla*_{CITM} forward 5'-TGG CCA GAA CTG ACA GGC AAA-3' and *bla*_{CITM} reverse 5'-TTT CTC CTG AAC GTG GCT GGC-3' (462 bp), and *bla*_{MOXM} forward 5'-GCT GCT CAA GGA GCA CAG GAT-3' and *bla*_{MOXM} reverse 5'-CAC ATT GAC ATA GGT GTG GTG C-3' (520 bp) [15]. *Hafnia alvei* CCUG 45642 (*bla*_{ACC1M}), *M. morgani* CCUG 43604 (*bla*_{ADHA} M), *Citrobacter freundii* CCUG 43597 (*bla*_{ACT1M}), and *Aeromonas hydrophila* CCUG 30208 (*bla*_{AMOX} M) served as positive controls. The reaction mixtures were processed in a GeneAmp PCR System 9700 (Applied Biosystems, USA) with the following conditions: One cycle of 10 min at 94°C; 35 cycles of 60 s at 94°C, 60 s at 55°C, and 60 s at 72°C, respectively; one cycle of 8 min at 72°C. Some 5 µL of the PCR products were separated by gel electrophoresis on 1% agarose gel stained with GelRed (Biotium, USA) and compared with a molecular weight marker (Life Technologies, USA) after photographing in ultraviolet light.

Susceptibility to QACs and antibiotics

Antibiotics

Antibiotic susceptibility testing was performed using disk diffusion methodology as recommended by NORDICAST (www.nordiccast.org) and included the following antibiotics: Ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, mecillinam, cefadroxil, cephalexin, cefepime, cefotaxime, ceftazidime, ceftibuten, cefuroxime, ertapenem, meropenem, aztreonam, ciprofloxacin, gentamicin, tobramycin, tigecycline, nitrofurantoin, and trimethoprim. We categorized the isolates into susceptible, indeterminate, or resistant using the species-related breakpoints defined by NordicAST (version 7.0).

QACs

Susceptibility testing for biocides was performed by determining the minimal inhibition concentration (MIC) according to ISO 20776-1:2006 with the modification that ISO-Sensitest Broth (Oxoid, UK) was used. A microdilution assay with a final volume of 100 µL was used to determine the MIC for the following substances (concentration ranges in parenthesis): Benzalkonium chloride (BAC, 4-128 mg/L) and cetyltrimethylammonium bromide (CTAB; 4-128 mg/L) (all Sigma-Aldrich, USA). All isolates with elevated MIC values (BAC ≥64 mg/L and CTAB ≥64 mg/L) were retested in macrodilution format (1 mL). Stock solutions were prepared freshly and inoculated with bacteria within 2 h after a serial dilution in respective range. A final inoculate of 5×10⁵ CFU/mL was prepared from an overnight culture in 1.5 mL Luria-Bertani broth (Sigma-Aldrich, USA) in room atmosphere at 35°C. The MIC assays were incubated for 18-20 h in room atmosphere at 35°C and the MIC values were read as the lowest concentration yielding no visible growth. *E. coli* ATCC 25922, *Enterobacter*

cloacae CCUG 38138, and *Klebsiella pneumoniae* ATCC 700603 were used as control strains.

Whole-genome analysis

DNA preparation and whole-genome sequencing

One colony of each isolate was incubated in 2 mL LB broth (Sigma-Aldrich, USA) for 8 h at 37°C in room atmosphere. DNA preparation was done using a Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's recommendations for Gram-negative bacteria with the exception that DNA was rehydrated with 10 mM Tris-HCl (pH 8.0). The quality and quantity of the extracted DNA was controlled by gel electrophoresis, spectrophotometry (Nanodrop, Thermo Fisher), and Quant-iT dsDNA BR assay and a Qubit instrument (Invitrogen). After standardizing the DNA extracts, the samples were transferred to Oxford Genome Center for library preparation and whole-genome sequencing. Fragmented DNA was end-repaired, A-tailed, adapter-ligated, and amplified using Nextera DNA library Prep (Illumina, USA). Sequencing was done on an Illumina HiSeq4000 platform, generating 150 bp paired-end reads.

Sequence analysis

Paired-end reads of the isolates from all the datasets were assembled using VelvetOptimiser software (v2.2.4) with kmer lengths from 21 to 99 using default optimization functions. Species confirmation, determination of phylotypes, and multi-locus sequence typing were performed according to the seven gene Achtman scheme using the pipeline implemented in Enterobase [16]. A neighbor-joining tree was constructed for rMLST allele nucleotide sequences of the study isolates [17,18]. Concatenated sequences for the rMLST scheme were retrieved through BIGSDB, aligned with MAFFT (v7.271, <https://mafft.cbrc.jp/alignment/software/>) and the tree was calculated using PHYLIP (v3.695, <http://evolution.genetics.washington.edu/phylip.html>). Paralogous loci were excluded (BACT000060, BACT000065) resulting in 51 concatenated ribosomal loci for the rMLST. The dataset was then bootstrapped 500 times with phylip seqboot followed by calculations of distance matrices with phylip dnadist and neighbor-joining trees with phylip neighbor and a consensus tree using phylip consense. Illumina short reads were mapped to the antimicrobial resistance determinants database ARG-Annot (version 3) and a custom gene database for known biocide-related resistance genes using srst2 (v0.2.0, <https://github.com/katholt/srst2>; supplementary material Table-S1). The result was interpreted as positive when the minimum coverage was over 90%, maximum divergence under 10%, and a maximum number of mismatches per read of 10 (default settings). All findings from short-read mapping were confirmed using nucleotide BLAST on draft genomes, using a word size of 10, match/mismatch scores of 1/−2, the gap cost was linear, and the filter was set for low

complexity regions; results were considered positive when the identity of a hit was over 90% (<https://blast.ncbi.nlm.nih.gov/>). Replicon sites of suspected plasmids were typed using PlasmidFinder (www.genomicepidemiology.org, December 2019) and insertion sequences were determined using ISFinder (<https://www-is.biotoul.fr>, December 2019). All data have been submitted to the European Nucleotide Archives and are available under the project reference number PRJEB17631, Table-S2 in the supplemental material for individual accession numbers.

Statistical analysis

The strength of the association between phenotypic resistance and resistance determinants was calculated with R and to each group, ESBL_A and ESBL_{M-C} were determined with the odds ratio and 95% confidence intervals. Associations with $p < 0.05$ and a lower confidence interval > 1 were considered as statistically significant. Graphical illustrations of the results were produced using the package ggplot2 as implemented R (R Foundation for Statistical Computing, Vienna, Austria, <http://www.R-project.org/>, version 3.4.4, 2018). The phylogenetic tree with metadata was illustrated using iTOL version 5 [19].

Results

General comments on the datasets

Three isolates were excluded from further analysis; in one case because of production of solely *bla*_{SHV-12}, and could, therefore, not be assigned to the groups of interest; and in two further isolates, no ESBL determinants could be found. Thus, the resulting group sizes were $n=23$ for ESBL_A and $n=27$ for ESBL_{M-C}; and another isolate that had both ESBL_A and ESBL_{M-C} enzymes (*bla*_{CTX-M-14} and *bla*_{CMY-2}). Within the isolate group, ESBL_A enzymes of CTX-M-type were found: *bla*_{CTX-M-15} ($n=18$), *bla*_{CTX-M-27} ($n=3$), *bla*_{CTX-M-1} ($n=2$), *bla*_{CTX-M-14} ($n=1$), and *bla*_{CTX-M-8} ($n=1$). The isolates within group ESBL_{M-C} produced beta-lactamases of type *bla*_{CMY-2} ($n=21$), *bla*_{CMY-42} ($n=1$), and *bla*_{DHA-1} ($n=5$).

Sequence data

Species verification on sequence data using the Enterobase pipeline confirmed the purity of the whole-genome extracts and that all isolates belonged to the species *E. coli*. The average coverage of the high-quality short reads from all collections was 118 (SD of ± 74). Draft genomes were obtained for all included isolates resulting in a median contig number ($> 10,000$ bp size) of 55 (range 36-179), the median N50 value was 171,769 (range 48,359-322,359), the median total length of nucleotides assembled in the draft genome (> 0 bp) was 5,179,586 bp (range 4,677,941-9,341,633 bp).

Comparison of ESBL_A and ESBL_{M-C}

Phylogeny

All *E. coli* phylogroups were represented among the isolates; and the phylogroups were dominated by

certain clonal complexes that are frequently associated with extra-intestinal manifestations. Within phylogroup B2, the clonal complex of sequence type 131 (CC ST131) dominated (12/51; 24%), in phylogroup A, clonal complex ST10 (CC ST10) was most often found (8/51; 16%), and correspondingly in phylogroup C dominated clonal complex ST23 (5/51; 10%). For phylogroup D, both clonal complex ST69 (CC ST69) and ST38 (CC ST38) were represented with three (3/51, 6%) and four isolates (4/51, 8%), respectively. The odds that isolates producing ESBL_{M-C} were part of one of the phylogroups A, B1, C, F, or D were 5.1 (1.2-26.8, $p=0.01$); this trend was true for all of these phylogroups, however, only statistically significant for phylogroup A (odds ratio 8.9 [1.0-428.3], $p=0.03$). Hence, ESBL_A-producing isolates were associated to phylogroup B2 with an odds ratio of 5.1 (1.2-26.8, $p=0.01$) (Figure-1).

Susceptibility testing

Antibiotics

Resistance to the penicillin ampicillin was high (51/51; 100%), while the combination with beta-lactam inhibitors increased susceptibility for amoxicillin/clavulanic acid (41/51; 80%) and piperacillin/tazobactam (2/51; 4%). Low resistance rates were seen for cephalosporins: Cefotaxime (44/51; 86%), ceftazidime (41/51; 80%), ceftriaxone (43/51; 84%), cephalixin (45/51; 88%), cefepime (13/51; 25%), and ceftibuten (32/51; 63%). The resistance rates for aminoglycosides varied for respective substances: Tobramycin (19/51; 37%), gentamicin (11/51; 22%), and amikacin (1/51; 2%). Other commonly used antibiotics for urinary tract infections also had high resistance rates: Trimethoprim (31/51; 61%), ciprofloxacin (30/51; 59%), and nitrofurantoin (3/51; 6%). None of the isolates was resistant to carbapenems or mecillinam. ESBL_A isolates tended to have higher resistance rates compared to ESBL_{M-C} isolates regarding cefuroxime (23/23 (100%) ESBL_A versus 11/27 (41%)

ESBL_{M-C}, $p<0.005$) and aminoglycosides, especially tobramycin (16/23 [70%] ESBL_A vs. 3/27 [11%] ESBL_{M-C}, $p<0.005$). Conversely, ceftibuten had a higher resistance rate in ESBL_{M-C} isolates compared to ESBL_A (25/27 [92%] ESBL_{M-C} vs. 8/23 [35%] ESBL_A, $p<0.005$) (Figure-2).

QACs

The MIC ranges for BAC and for CTAB in all study isolates were 32-64 mg/L. For the reference strains, the following MIC ranges for BAC were found: *E. coli* (ATCC25922) 16-32 mg/L, *E. cloacae* (CCUG38136) 32-64 mg/L, and *K. pneumoniae* (ATCC700603) 32-64 mg/L; and accordingly, *E. coli* (ATCC25922) 16-64 mg/L, *E. cloacae* (CCUG38136) 32-128 mg/L, and *K. pneumoniae* (ATCC700603) 128-512 mg/L for CTAB. No difference was seen between both groups ESBL_A and ESBL_{M-C}.

Genetic resistance determinants

Antibiotic resistance determinants

Besides ESBL, other beta-lactamases were detected: *bla*_{OXA-1} that were only found in ESBL_A-producing isolates (15/23 [65%] ESBL_A vs. 0/27 [0%] ESBL_{M-C}, $p<0.005$); and TEM-1D beta-lactamases in 17 isolates, without a statistically significant distribution between the collections. The plasmid-borne quinolone resistance determinants *qnrB* were found in five isolates and only in ESBL_{M-C}-producing isolates, however, the association was not statistically significant. In contrast, the quinolone resistance determinant *aac(6')Ib-cr* was only found in ESBL_A isolates (14/23 [61%] ESBL_A vs. 0/27 [0%] ESBL_{M-C}, $p<0.005$). The macrolide 2' phosphotransferase *ImpH* was detected in 20 isolates and more often in ESBL_A-producing *E. coli* (14/23 [61%] ESBL_A vs. 6/27 [22%] ESBL_{M-C}, $p<0.004$). Aminoglycoside resistance determinants *aac* and *aad* were more often found in ESBL_A (19/23 [83%] ESBL_A vs. 6/27 [22%] ESBL_{M-C}, $p<0.005$). Furthermore, chloramphenicol resistance determinant *cat* was only found in isolates with ESBL_A production (15/23 [65%] ESBL_A vs. 0/27 [0%] ESBL_{M-C}, $p<0.005$) (Figure-2).

Biocidal resistance determinants

The plasmid-borne small multidrug efflux pump *sugE(p)* was only found in ESBL_{M-C} isolates (9/27 [33%] ESBL_{M-C} vs. 0/23 [0%] ESBL_A, $p=0.002$). All isolates positive to *sugE(p)* produced concomitantly *bla*_{CMY-2} and *bla*_{CMY-42}, and the isolates were distributed within nearly all phylogenetic groups. In all *sugE(p)* positive isolates, the gene was found in the same genetic environment *bla*_{CMY}-*blc*-*sugE* mostly with transposable insertion sequences. Four isolates had transposable elements that showed similarity to ISEcp1 (IS1380 family) and one had an ISSbo1 (IS91 family) that was assembled on a contig with an IncII replicon site. For two isolates, no insertion sequences were found, and in further two, it was an incomplete ISEcp1 (Figures-1 and 3). The

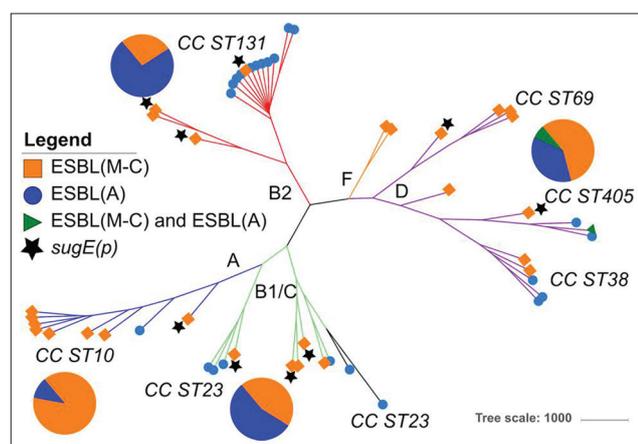


Figure-1: Network illustration for the study *Escherichia coli* isolates based on an rMLST neighbor-joining tree. Isolates with ESBL_A (blue dots), ESBL_{M-C} (orange rectangles), both ESBL_A and ESBL_{M-C} (green triangle), *sugE(p)* (black stars); phylogroups were illustrated by colored leaves: B2 (red), D (purple), A (blue), F (orange), B1/C (green).

multidrug efflux pump *emrE* was found in five isolates, and the truncated *qacEΔ1* was found in 18 isolates; no association was seen for ESBL-types.

The multidrug efflux pump determinants *acrABR*, *emrAB*, and *acrEF* (*envCD*), and the outer membrane channel *tolC* were present in all isolates.

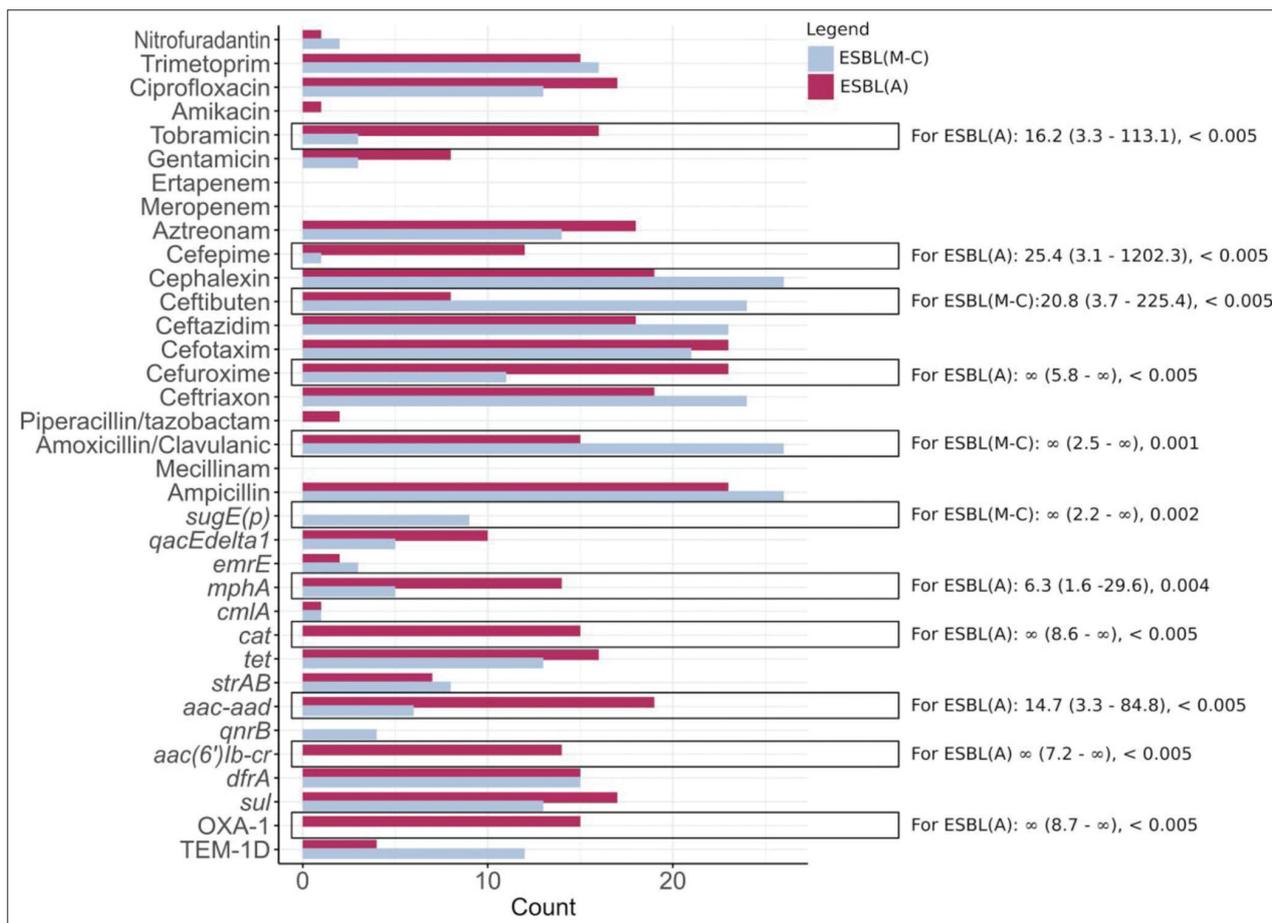


Figure-2: Comparison of ESBL_A and ESBL_{M-C} for their susceptibility to antibiotics and antimicrobial resistance determinants. Red staples ESBL_A, gray staples ESBL_{M-C}, boxes highlight antibiotic resistance or resistance determinants with statistically significant results (odds ratio [95% confidence interval], probability p).

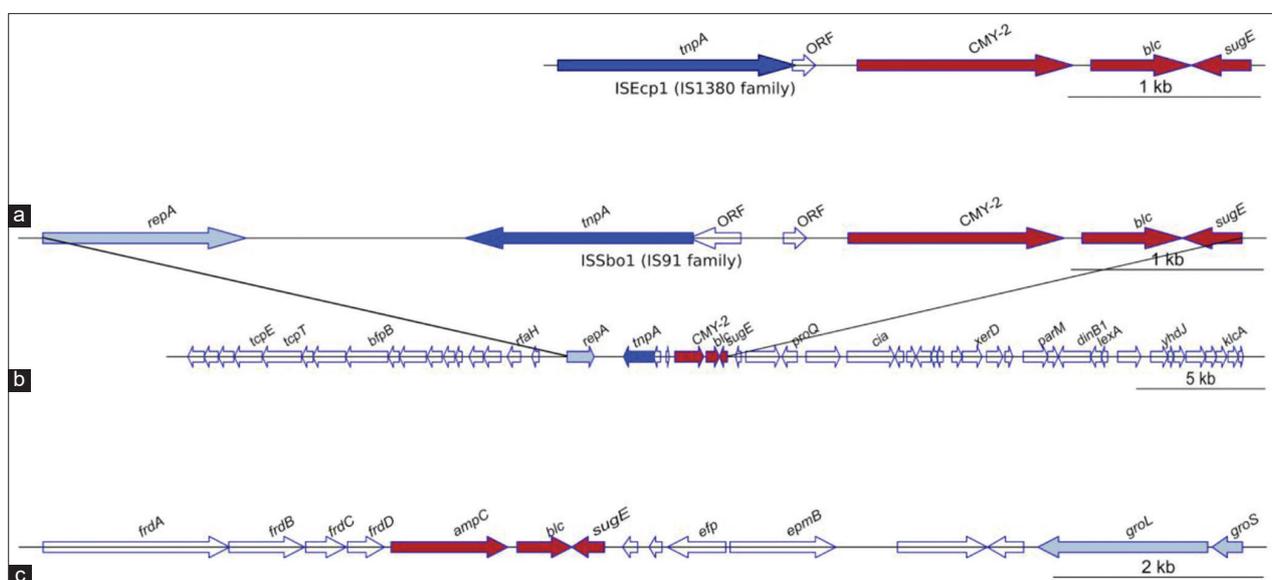


Figure-3: Representative gene synteny of *bla*_{CMY-2} with *sugE* for various isolates in the study. First diagram (a) shows *sugE(p)* and *bla*_{CMY-2} with its surrounding genes and insertions sequence ISEcp1 (IS1380 family), the second diagram (b) shows the assembly of IncI1 plasmid (below) with *bla*_{CMY-2}, *sugE(p)*, and insertions sequence ISSbo1 (IS91 family), and the third diagram shows a representative genetic environment of chromosomal *sugE*.

Discussion

The present study investigated coresistance to QACs in ESBL_A- and ESBL_{M-C}-producing *E. coli* isolates. None of the isolates showed increased tolerance to BAC or CTAB and compared to other studies, resistance determinants to QACs were rare. Nonetheless, ESBL_{M-C} was associated to the plasmid-borne small multidrug resistance (SMR) efflux pump *sugE(p)*, while ESBL_A was mainly associated with other antibiotic resistance determinants that confer resistance to macrolides (*mphA*), chloramphenicol (*cat*), aminoglycosides (*aac-aad*), quinolones *aac(6')Ib-cr*, and beta-lactamases *bla*_{OXA-1}. The different ESBL types were also more frequently harbored by isolates belonging to different phylogroups: ESBL_{M-C} was more often found in none-B2 phylogroups, especially in phylogroups A and D, while ESBL_A was frequently found in B2 phylogroup.

Major resistance mechanism to QACs in Gram-negative bacilli is mediated through efflux pumps, where SMR) proteins are known to confer resistance to a variety of QACs. The *sugE(p)* has been described to mediate tolerance to a range of antiseptics and other toxic lipophilic compounds [20]. None of the isolates in the study harboring plasmid-borne *sugE(p)* genes showed increased MIC values to CTAB or BAC. Chung and Saier [21] showed that overexpression of chromosomally encoded *sugE* determinant in *E. coli* does only confer phenotypic tolerance to a narrow spectrum of QACs including CTAB, which, in addition, might be induced by mutants in *sugE* leading to hypersensitivity to QACs. It is thereby possible that isolates need selective pressure by QAC exposure to express phenotypic resistance. Still, it is uncertain how additional *sugE* genes, gained through horizontal gene transfer, and might give additional benefit to the host organism. Indeed, our findings might be in line with the report of Kermani *et al.* [22], who found that the primal function of SMR proteins is guanidinium export, and only a limited portion of these proteins mediates multidrug efflux. QACs are heavily used as disinfectants in animal food production, and phenotypic resistance has been measured in exposed isolates from these environments. In contrast, clinical isolates might not be exposed to significant levels of QACs, as these compounds are rather toxic to humans. However, QAC-resistant isolates have been found in hospital environments and have been linked to spreading with significant mortality [12].

It has been suggested that plasmid-borne *sugE(p)* genes have been mobilized from *C. freundii*, together with *bla*_{CMY} and the outer membrane lipoprotein Blc, an event that has been hypothesized to happened at least 6 times [23]. However, only nine out of 22 *bla*_{CTX-M}-producing *E. coli* isolates showed the genetic structure *bla*_{CMY}-*blc-sugE(p)*, which was accompanied by transposable elements. These genetic elements have been found in a variety of

other *Enterobacteriaceae*, such as *Klebsiella oxytoca*, *Salmonella* spp., or *Shigella* spp. [24]. Curiously, the chromosomal environment of *sugE(c)* in *E. coli* compromises the same genetic structure with *ampC-blc-sugE*, however, no transposon-like element was found close by. Mobilization events are common in bacteria, and species that inhabited soils and wet environments have frequently been the source for antimicrobial resistance determinants causing huge problems in clinical situations [23]. Even though SMR proteins do not seem to transfer measurable tolerance to QACs tested here, they have been associated with increased mobilization and spread of antimicrobial resistance in polluted environments [8].

Once antibiotic resistance determinants have been acquired by human pathogens like *E. coli*, they can successfully spread by clones. *E. coli* belonging to ST131 have emerged during the 2000s as a pandemic, hypervirulent, and multiresistant clone [25]. Isolates that belong to *E. coli* ST131 do often produce *bla*_{CTX-M-15}, which has also been found in the present study: A majority of the *bla*_{CTX-M}-producing isolates belonged to ST131. In contrast, for *bla*_{CTX-M}-producing isolates from Uppsala University Hospital, no strong association to a certain clone was observed, the isolates were rather evenly distributed over all phylogenetic groups. So far, *bla*_{CMY} enzymes spread mostly polyclonally within *E. coli*, and extensive dissemination was rather linked to mobile genetic elements such as IncI1 plasmids and insertions sequence ISEcp1 [26,27]. For the present collection, *sugE(p)* was mainly found in the context of a transposable ISEcp1 element and in one case with an ISSbo1 element that was assembled on an IncI1 plasmid. Chiu *et al.* [28] showed that *blc* and *sugE(p)* might have a regulatory function for *bla*_{CMY}, leading to downregulation or upregulation, respectively.

Conclusion

Resistance determinants associated with SMR proteins that have been associated to QAC resistance were frequently found in ESBL-producing isolates, although no phenotypic tolerance could be detected. While the biological role of many proteins belonging to the SMR efflux pumps is not elusively clear, their wide spread might indicate other sources for selective pressure than antibiotics.

Authors' Contributions

SS, AH, and ET: Conception or design of the work SS and ET: Data collection SS and AH: Data analysis and interpretations. SS and AH: Drafting the article. AH and ET: Critical revision of the article. All authors read and approved the final manuscript.

Acknowledgments

Susanne Sütterlin holds a grant from Departmental funds Landstinget Uppsala län (ALF funds) (ALF 810901), and Anna Heydecke holds a

grant from the Center for Research and Development Gävleborg, Uppsala University, Gävle, Sweden. The generation of sequence data was financially supported by a grant from Afa Insurance, Sweden (Grant number 150411) and Alf-de-Ruvo Memorial Foundation, Sweden. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Competing Interests

The authors declare that they have no competing interests.

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Supplemental material

Table-S1: Summary over the nucleic acid sequences included in the sequence analyzes.

Genes	Function	Accession number	Source	References
<i>qac</i> Δ1	Quaternary ammonium compound efflux SMR transporter	AF205943, NG_048042	R751 (<i>E. coli</i>), pVS1 (<i>P. aeruginosa</i>)	[1,2]
<i>qacE</i>	Quaternary ammonium compound efflux SMR transporter	NG_048041	R75 (<i>E. aerogenes</i>)	[3]
<i>qacF</i>	Quaternary ammonium compound efflux (SMR type transporter)	NG_051881	pIP833 (<i>E. aerogenes</i>)	[4]
<i>sugE</i> (p)	Multidrug efflux pump (SMR type transporter)	AY339625, AY581205	p541 (<i>E. coli</i>), pIW759 (<i>S. enterica</i> subsp. <i>enterica</i> , serovar Typhimurium)	[5,6]
<i>emrE</i> (mrvC)	Multidrug efflux pump (SMR-type)	M62732	<i>E. coli</i>	[7,8]
<i>tolC</i>	Outer membrane channel	ENA X54049	<i>E. coli</i>	[9]
<i>acrR,A,B</i>	Multidrug efflux pump, RND family	U00734	<i>E. coli</i>	[10]
<i>acrEF</i> (<i>envCD</i>)	Multi efflux pump (RND family)	X57948	<i>E. coli</i>	[11]
<i>emrABR</i>	Multi efflux pump	M86657, NC_000913.3, U19993	<i>E. coli</i>	[12,13]

SMR – small multidrug resistance

Table-S2: Accession numbers of sequence raw data in ENA within project PRJEB17631 and its corresponding isolate ID.

ENA	Isolate ID	ENA	Isolate ID
ERR1718941	WTCHG_320308_269190	ERR1718939	WTCHG_320308_268190
ERR1718881	WTCHG_320308_238190	ERR1718943	WTCHG_320308_270190
ERR1718885	WTCHG_320308_240190	ERR1718945	WTCHG_320308_271190
ERR1718887	WTCHG_320308_241190	ERR1718947	WTCHG_320308_272190
ERR1718889	WTCHG_320308_242190	ERR1718949	WTCHG_320308_273190
ERR1718891	WTCHG_320308_243190	ERR1718951	WTCHG_320308_274190
ERR1718893	WTCHG_320308_244190	ERR1718953	WTCHG_320308_275190
ERR1718897	WTCHG_320308_246190	ERR1718955	WTCHG_320308_276190
ERR1718899	WTCHG_320308_247190	ERR1718957	WTCHG_320308_277190
ERR1718901	WTCHG_320308_248190	ERR1718959	WTCHG_320308_278190
ERR1718903	WTCHG_320308_249190	ERR1718961	WTCHG_320308_279190
ERR1718905	WTCHG_320308_250190	ERR1718963	WTCHG_320308_280190
ERR1718907	WTCHG_320308_251190	ERR1718965	WTCHG_320308_281190
ERR1718909	WTCHG_320308_252190	ERR1718969	WTCHG_320308_284190
ERR1718911	WTCHG_320308_253190	ERR1718971	WTCHG_320308_285190
ERR1718913	WTCHG_320308_254190	ERR1718973	WTCHG_320308_286190
ERR1718915	WTCHG_320308_255190	ERR1718975	WTCHG_320308_287190
ERR1718917	WTCHG_320308_256190	ERR1718977	WTCHG_320308_288190
ERR1718919	WTCHG_320308_257190	ERR1718983	WTCHG_320308_291190
ERR1718921	WTCHG_320308_258190	ERR1718985	WTCHG_320308_292190
ERR1718923	WTCHG_320308_259190	ERR1718987	WTCHG_320308_293190
ERR1718925	WTCHG_320308_261190	ERR1718991	WTCHG_320308_295190
ERR1718927	WTCHG_320308_262190	ERR1718993	WTCHG_320308_296190
ERR1718929	WTCHG_320308_263190	ERR1718997	WTCHG_320308_302190
ERR1718931	WTCHG_320308_264190	ERR1718999	WTCHG_320308_303190
ERR1718933	WTCHG_320308_265190		

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