# Antibiotic resistance mechanisms in *Enterobacteriaceae*

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*Enterobacteriaceae* species are important human pathogens while increasing number of antibiotic resistant strains are detected worldwide. The most common antibiotic resistance in *Enterobacteriaceae* is observed against beta-lactams, fluoroquinolones, aminoglycosides while recently resistance to polymyxins has also appeared. Beta-lactam resistance is mainly conferred by beta-lactamases, capable to hydrolyze beta-lactam antibiotics. The most important beta-lactamases are the cephalosporinases for example extended-spectrum beta-lactamases (ESBLs) and the carbapenemases for example metallo-beta-lactamases (MBLs), *Klebsiella pneumoniae* carbapenemases (KPCs) and oxacillinase OXA-48 enzymes. Other resistance mechanisms against beta-lactams are the outer membran permeability change and efflux pumps. Fluoroquinolone resistance is caused by aminoacid changes in gyrase and topoisomerase IV enzymes. However, recent studies confirmed the importance of plasmid-mediated quinolone resistance mechanisms including Qnr determinants, aminoglycoside-acetyltransferase(6')-Ib-cr enzyme and QepA, OqxAB efflux pumps. Aminoglycoside resistance is mainly explained by modifying enzymes inactivating the antibiotic by acetylation, by adenylation or by phosphorilation. Resistance to polymyxins develops by the modification of the target molecule, notably the addition of 4-amino-4-deoxy-l-arabinopyranose on the lipid A component of lipopolysaccharide. The genetic background of resistance mechanisms is diverse since they can be present on chromosomes, plasmids, integrons and transposones. This chapter gives an overview of beta-lactam, fluoroquinolone, aminoglycoside and polymyxin resistance mechanisms in *Enterobacteriaceae*.

**Keywords** beta-lactamases, fluoroquinolone resistance, aminoglycoside resistance, polymyxin resistance

## 1. Introduction

*Enterobacteriaceae* are Gram-negative, rod shaped 1-3 μm large bacteria. They are facultative anaerob and their natural host is the human and animal intestine, where they belong to the commensal microbial flora e.g.: *Escherichia coli*, *Klebsiella* spp., *Proteus* spp., *Morganella* spp., *Providentia* spp., *Enterobacter* spp., *Serratia* spp. These bacteria can be pathogens of urinary tract, respiratory tract, bloodstream and wounds. Obligate human intestinal *Enterobacteriaceae* pathogens include *Salmonella* spp., *Shigella* spp. and *Yersinia* spp. [1, 2, 3]. Infections caused by *Enterobacteriaceae* are treated with antibiotics, and the efficient agents are fluoroquinolones, beta-lactams and aminoglycosides. Fluoroquinolones (ciprofloxacin, norfloxacin and levofloxacin) are potent antibacterial agents, inhibiting the bacterial DNA synthesis by blocking gyrase and topoisomerase IV enzymes. Nalidixic acid is the basic molecule with quinolone ring while the addition of fluor and substituents on it resulted in fluoroquinolones. The beta-lactam antibiotics inhibit cell wall synthesis and the agents with antibacterial activity against enteric bacteria include amoxicillin, ampicillin, piperacillin, ticarcillin each alone or in combination with beta-lactamase inhibitors (clavulanic acid, sulbactam, tazobactam). Extended-spectrum cefalosporins (ceftazidime, cefotaxime, cefepime), carbapenems (imipenem, ertapenem, meropenem) and aztreonam are more potent beta-lactams. Aminoglycoside antibiotics (amikacin, gentamicin, netilmicin and tobramycin) block the protein syntheis of 30s ribosomal subunit while miscellaneous antibiotics namely, colistin (disrupts cell membrane), fosfomycin (inhibits the cell wall synthesis), nitrofurantoin (damages the DNA), trimethoprime and trimethoprime-sulfomethaxazol (inhibit the folic acid synthesis) have considerable antibacterial activity against enteric bacteria. *Enterobacteriaceae* can develop several mechanisms to avoid the inhibitory effect of antibiotics thus becoming resistant. Interpretation of a pathogen as clinically resistant is based on minimal inhibitory concentrations (MICs) of an antibiotic associated with the antimicrobial activity with a high likelyhood of therapeutic failure [1, 4, 5]. Nowadays, multi-drug resistant strains emerged, possessing several resistant mechanisms against different antibiotic groups. In this chapter an overview of resistance mechanisms against beta-lactams, fluoroquinolones, aminoglycosides and polymyxins will be given.

## 2. Resistance mechansims to beta-lactams

Resistance to beta-lactams in *Enterobacteriaceae* is mainly conffered by beta-lactamases. These enzymes inactivate beta-lactam antibiotics by hydrolysis. Two classifications of beta-lactamases are known, namely the Ambler and the Bush-Jacoby-Medeiros (Table 1). The Ambler classes are based on the amino acid homology, where they are clustered in four molecular classes namely, A, B, C and D. Molecular classes A, C, and D include the beta-lactamases with serine at their active site, whereas molecular class B stands for metallo-beta-lactamases (MBLs), enzymes with zinc molecule in the active-site. The Bush-Jacoby-Medeiros classification grouped the beta-lactamases in three major groups and 16 subgroups. This classification is based on the substrates and inhibitors of the enzymes [6, 7].

**Table 1** Beta-lactamases occuring in *Enterobacteriaceae*

| Bush-Jacoby-Medeiros classification | Ambler classification | Distinctive substrate | Inhibitor | Representative enzyme |
|---|---|---|---|---|
| 1 | C | cefalosporins | none | AmpC |
| 2b | A | penicillins, early cefalosporins | beta-lactamase inhibitors | TEM-1, TEM-2, TEM-13 SHV-1 |
| 2be | A | extended-spectrum cefalosporins and aztreonam | beta-lactamase inhibitors | TEM-3, SHV-2, PER, VEB, CTX-M-15 |
| 2d | D | cloxacillin | beta-lactamase inhibitors | OXA-1, OXA-10 |
| 2de | D | extended-spectrum cefalosporins | beta-lactamase inhibitors | OXA-11, OXA-15 |
| 2df | D | carbapenems | beta-lactamase inhibitors | OXA-23, OXA-48 |
| 2f | A | carbapenems | beta-lactamase inhibitors | KPC, IMI, SME, NMC |
| 3a | B | carbapenems | EDTA | MBL |

[6]

### 2.1. AmpC type beta-lactamases

AmpC beta-lactamases are mainly chromosomally encoded in *Enteorbacteriaceae* and they confer resistance to cephalothine, cefazoline, cefoxitin, most penicillins and to beta-lactamase inhibitor (clavulanic acid). Chromsomal AmpC enzymes are inducible and can be expressed at high levels by mutation in *ampD* leading to AmpC hyperinducibility or constitutive hyperproduction [8]. Overexpression confers resistance to extended-spectrum cephalosporins including cefotaxime, ceftazidime and ceftriaxone. AmpC enzymes located on transmissible plasmids are usually constitutively expressed and appear in bacteria lacking or poorly expressing a chromosomal AmpC gene, such as *E. coli*, *K. pneumoniae*, and *P. mirabilis*. AmpC enzymes encoded by both chromosomal and plasmid genes are capable to hydrolyze broad-spectrum cephalosporins more efficiently [9].

### 2.2. Extended spectrum beta-lactamases (ESBLs)

ESBLs are beta-lactamases capable of conferring bacterial resistance to the penicillins, early and extended-spectrum cephalosporins, and aztreonam (but not to cephamycins or carbapenems) by hydrolysis of these antibiotics, and are inhibited by beta-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam [6]. The most common ESBLs are SHV-, TEM-, CTX-M. Each of these enzyme derives from its own progenitor. Interestingly, SHVs are more prevalent in Europe, TEMs are dominantly present in the USA while the CTX-Ms are being increasingly detected worldwide [10].

The origine of SHV-1 (sulphydryl variable) is the chromosome of *Klebsiella* spp. and has only a narrow beta-lactam hydrolyzing activity conferring resistance to penicillin and ampicillin [6]. The aminoacid sequence of SHV-1 has two hot spots where a single aminoacid replacement extends its hydrolysis spectrum to early and extended spectrum cefalopsorins and aztreonam. These hot spots are aspartate in position 179 and glycin in position 238 [11]. Altogether 171 SHV-type beta-lactmases are known most of them are ESBLs [12]. They differ from SHV-1 maximum in five aminoacid positions. Interestingly, SHV-38 is a chromosomal SHV enzyme with ESBL and carbapenemase activity as it confers resistance to extended-spectrum cefalosporins and to imipenem too. Although it has a decreased activity against amoxicillin and cefalothin [13].

TEM-1 and TEM-2 (patient's name: Temoneira) are usually found in *E. coli* and both have hydrolytic activity mainly to ampicillin. TEM-3 has the activity of ESBL, and it differs from TEM-2 by two aminoacid substitutions [14]. The two hot spots in the aminoacid sequence determining ESBL activitiy are the arginine in position 164 and glycin in position 238. Both aminoacids change to serine extends the hydrolytic activity to ESBL. Over 200 type TEM beta-lactamases are known and the majority of them are ESBLs [12]. However, they also differ maximum in five positions from the progenitor TEM-1 or TEM-2. The TEM-type ESBLs are derivatives of TEM-1 while the TEM-2 analogous have only a broad-spectrum beta-lactamase effect. In TEM-1 the aminoacid changes in positions of 39, 69, 165, 182, 244, 261, 275, 276 determines the IRT (inhibitor resistant TEM) type beta-lactamases. These TEMs hydrolytic activity directly do not change but they are resistant to beta-lactamase inhibitors [15].

CTX-M beta-lactamases (cefotaximase-Munich) are derived from *Kluyvera* spp. where it is chromosomally coded. In *Enterobacteriaceae* usually *E. coli* and *Klebsiella* spp. carry the gene of this beta-lactamase on plasmids. These enzymes were named after hydrolytic activity of cefotaxime although their spectrum includes extended spectrum

 

cephalosporins and aztreonam. Altogether 140 CTX-M enzymes were identified and all of them are ESBLs [10, 12]. These enzymes are comprised in five sub-groups as CTX-M-1, -2, -8, -9 and -25 wheras the most dominant is CTX-M-15 which belongs to CTX-M-1 sub-group [16].

OXA beta-lactamases (Ambler class D and Bush-Jacoby-Medeiros group 2d) were named after their oxacillin-hydrolyzing abilities in fact they inactivate benzylpenicillin, cloxacillin and oxacillin [6]. They predominantly occur in *Pseudomonas aeruginosa* [17] but have been detected in many other gram-negative bacteria especially in *Enterobacteriaceae* [18]. OXA-1 and OXA-10 beta-lactamases have only a narrow hydrolytic spectrum however, other OXA beta-lactamases are ESBLs including OXA-11, -14, -15,-16, -28, -31, -35 and -45 as they confer resistance to cefotaxime, ceftazidime and aztreonam. Althogether 311 OXA-type beta-lactamses were discovered inlcuding narrow-spectrum and extended-spectrum-beta-lactmases. [12, 19].

PER (Pseudomonas extended resistance) beta-lactamase hydrolyzes penicillins and cephalosporins and inhibited by clavulanic acid. PER-1 was first detected in *Pseudomonas aeruginosa* [20] and later in *Salmonella* sp, and in *Acinetobacter* isolates as well [21, 22, 23, 24].

VEB (Vietnam extended-spectrum beta-lactamase) has 38% homology with PER. It confers resistance to ceftazidime, cefotaxime, and aztreonam while inhibited by clavulanic acid. The gene encoding VEB-1 was found to be plasmid mediated and such plasmids frequently carry non-beta-lactam resistance determinants [25].

The minor ESBLs include GES, BES, TLA, SFO and BEL as they are rarely identified and geographically localised [26].

### 2.3. Carbapenemases

Carbapenemases are beta-lactamases with a wide hydrolytic spectrum. These enzymes inactivate almost all hydrolyzable beta-lactams including the carbapenems as a unique, additional substrate [27]. Carbapenemases are among beta-lactamases from Ambler classe A, B and D [7].

In class A, the dominant carbapenemase is KPC (*Klebsiella pneumoniae* carbapenemase) which was mainly detected on plasmids of *K. pneumoniae* [28, 29]. In previous years, sporadic cases of KPC-producing *E. coli, Enterobacter cloacae, Serratia marcescens*, and *Citrobacter freundii* were detected [30]. Until today, KPC enzymes have 15 different amino-acid variants [12] and have hydrolytic activity on the extended-spectrum cephalosporins, carbapenems and aztreonam [6]. The IMI (imipenem hydrolyzing beta-lactamase), NMC (non-metallo-carbapenemase) and SME (*Serratia marcescens* enzyme) carbapenemases belong also to Ambler class A and 2f in Bush-Jacoby-Medeiros classification. These enzymes are chromosomal located in *Enterobacter* spp, and in *S. marcescens* while they are closely related to each other as IMI and NMC have 97% aminoacid similarity and they are homolog 70% to SME [31, 32]. All the three enzymes have a broad hydrolysis spectrum that includes the penicillins, early cephalosporins, aztreonam, and carbapenems [27, 33].

Ambler class B and Bush-Jacoby-Medeiros group 3a include the metallo-beta-lactamases (MBLs) all capable to hydrolyse not only the carbapenems but all the hydrolysable beta-lactams [6]. The mechanism of hydrolysis is based on the interaction of zinc ions in the enzyme's active site and this is the part inhibited by EDTA [27]. Non-fermentative bacteria as *Acinetobacter baumanii* and *Pseudomonas aeruginosa* inherit MBLs usually chromosomally, where their genes are incorporated in integrons as gene cassettes. Conservated sequences in the integrons enables for recombination crossover. In *Enterobacteriaceae* MBL genes are located on transferable plasmids thus disseminat by conjugation. The first transferable MBL was IMP metallo-beta-lactamase ("active on imipenem") detected in *Pseudomonas aerugonisa,* later on it was detected in *Enterobacteriaceae* [34, 35]. VIM (Verona integron-encoded metallo-beta-lactamase) was also first detected in *P. aeruginosa*, although it is widely disseminated in *Klebsiella* spp, and *E. coli*. The currently emerging MBL is the NDM (New Delhi metallo-beta-lactamase) detected in the chromosome of *Acinetobacter baumanii*, but detected in *Enterobacteriaceae* mainly in *Klebsiella* sp. and *E. coli* [36, 37, 38, 39].

OXA-48 is a class D carbapenemase that belongs to OXA-type beta-lactamases with a hydrolyzing spectrum including penicillins and carbapenems, but excluding extended-spectrum cephalosporins (ceftazidime, ceftriaxone) and aztreonam [40]. OXA-type beta-lactamases are dominant in *Acinetobacter* species, although OXA-48 has only been detected in *Enterobacteriaceae* isolates and mainly in *K. pneumoniae* and *E. coli* [41].

## 3. Resistance to fluoroquinolones

### 3.1. Chromosomal fluoroquinolone resistance determinants

Fluoroquinolone resistance in *Enterobactericeae* is traditionally explained by the mutations in the molecules targeted by fluoroquinolones namely, DNA gyrase and DNA topoisomerase IV. Each target enzyme's coding gene (namely *gyrA*, *gyrB* for DNA gyrase and *parC, parE* for DNA topoisomerase IV) has quinolone-resistance determining regions (QRDRs) [42] at which nucleic acid mutations leading to aminoacid substitutions can diminish quinolone binding. Generally, multiple mutations are required to achieve clinically important resistance in *Enterobacteriaceae* (Table 2). Another well-described resistance mechanism is the decreased intracellular drug accumulation by upregulation of native

efflux pumps. Several *Enterobacteriaceae* species own a chromosomal native AcrAB-TolC efflux pump belonging to the resistance-nodulation division (RND) family. AcrA is a membrane fusion protein, AcrB is an inner-membrane pump and TolC is an outer-membrane protein and they build up an efflux pump whose overexpression leads to fluoroquinolone resistance observed in *E. coli, Klebsiella* spp. and *Enterobacter* spp. [43, 44, 45].

Permeability of Gram-negative cell wall to fluoroquinolones was also described [46, 47]. These mechanisms of resistance are mutational, arising in an individual organism and then passing vertically to its surviving progeny. Neither of the above mentioned mechanism seems to transfer effectively on mobile genetic elements [48].

**Table 2** The MIC values with aminoacid substitutions in *E. coli* due to mutations in *gyrA*, *gyrB* and *parC*

| **Ciprofloxacin MIC (µg/ml)** | | ***gyrA*** | ***gyrB*** | ***parC*** |
|---|---|---|---|---|
| wild-type | 0.007 | Ser83… Asp87 | Lys447 | Ser80…Glu84 |
| | 0.06 | Ser83… Asp87 | Lys447 | Ser80…Glu84 |
| low-level resistance | 0.125 | Ser83… Asp87 | Lys447 | Ser80…Glu84 |
| | 0.25 | Ser83… Asp87 | Lys447 | Ser80…Glu84 |
| | 0.25 | Leu83…Asp87 | Lys447 | Ser80…Glu84 |
| breakpoint | 1 | Leu83…Asp87 | Lys447 | Arg80…Glu84 |
| high-level resistance | 2 | Leu83…Asp87 | Lys447 | Ile80…Val84 |
| | 4 | Leu83…Asp87 | Glu447 | Ser80…Lys84 |
| | 8 | Leu83…Tyr87 | Lys447 | Ser80…Lys84 |
| | 16 | Leu83…Asn87 | Lys447 | Arg80…Glu84 |
| | 32 | Leu83…Tyr87 | Lys447 | Ser80…Lys84 |
| | 64 | Leu83…Asn87 | Lys447 | Ile80…Glu84 |
| | 128 | Leu83…Tyr87 | Lys447 | Ile80…Lys84 |

[5, 49]

Low-level fluoroquinolone resistance by plasmid-mediated quinolone resistance determinants

Plasmid-mediated quinolone resistance determinants include Qnr determinants [50], aminoglycoside acetyltranferase(6')-Ib-cr variant enzyme [51] and efflux pumps: QepA and OqxAB [52, 53]. Each of these determinants can cause low-level fluoroquinolone resistance by increasing the ciprofloxacin MIC values above the wild-type but still below the resistance breakpoint. Low-level resistance is a unique state of bacterium cells where the mutation rate increases 100-times on chromosomal genes (*gyrA* and *parC*) thus facilititating the selection to higher level fluoroquinolone resistance [5, 50, 54, 55, 56].

The Qnr proteins belong to the pentapeptide-repeat family, which is defined by a tandem five aminoacid repeat with the recurrent motif [57]. Five lineages of Qnr determinants were identified and labeled as Qnr A, B, C, D and S. Chromosomal *qnr* genes were discovered as progenitors of their plasmidic analogues [58]. The progenitor of *qnrA* gene was discovered in an environmental bacterium *Shewanella algae,* while the source of *qnrB* determinants was found to be *Citrobacter* spp, and *qnrS* ancestor was detected in *Vibrio splendidus* [59, 60, 61]. QnrD is frequently detected on plasmids of the Proteeae tribe including *Proteus sp., Morganella sp.* and *Providentia sp.* [62, 63]. The function of Qnr protein is the protection of gyrase and topoisomerase IV enzymes from the fluoroquinolone action. Qnr proteins attach to the surface of the enzymes and minimise the opportunities of fluoroquinolone inhibition thus increasing the inhibitory concentration [48]. The effect of Qnr proteins is characterized by mutant preventive concentration (MPC), this is the lowest concentration where bacterial cells die without developing mutations on chromosomal genes. The Qnr positive strains can develop chromosomal mutations during therapy when exposed to ciprofloxacin concentration below MPC [64].

Aminoglycoside acetyltransferase (6')-Ib-cr [aac(6')-Ib-cr] variant is a modifying enzyme capable to inactivate ciprofloxacin and norfloxacin by N-acetylating them on the amino-nitrogen of the piperazynil substituent. This effect increases the resistance in the low-level resistance range and the MPC value as well leading chromosomal mutations during ciprofloxacin therapy [51]. *E. coli* frequently carries this resistant determinant, wherase the gene of aac(6')-Ib-cr variant is mostly identified as a gene cassette in integrons associated with beta-lactamase genes of OXA-1, CTX-M-15 and TEM-1 [65].

QepA efflux pump is a 511-aminoacid protein with 14-transmembrane segments, it belongs to the major facilitator superfamily. This pump confers low-level resistance to norfloxacin, ciprofloxacin while does not significantly affect the less hydrofilic agenst as levofloxacin, moxifloxacin or nalidixic acid. Its function is an active efflux provided by a proton motive force and inhibited by carbonyl cyanid m-chlorophenylhidrazone. This resistant determinant is found rare, but appears in *E. coli* [52].

OqxAB efflux pump belongs to the resistance nodulation division family. It has an OqxA membran fusion protein and an OqxB inner membrane pump with 12-transmembrane α-helicis while a TolC outer membrane protein is required

 

to the pump to function fully [53]. This is the first identified resistance determinant to olaquindix, a fluoroquinolone agent used as growth promoters in swine, while other substrates of this pump were detected as nalidixic acid, norfloxacin, ciprofloxacin and chloramphenicol [53, 66]. Its function is $H^+$ driven efflux and inhibited by carbonyl cyanid m-chlorophenylhidrazone. The prevalence of *oqxAB* is rare and was found in *E. coli* isolates from animal and environmental origine [66, 67].

## 4. Resistance to aminoglycosides

Resistance to aminoglycosides is mainly explained by the antibiotic modification. Aminoglycoside modifying enzymes catalyze the modification at $-OH$ or $-NH_2$ groups of the 2-deoxystreptamine nucleus or the sugar moieties and can be aminoglycoside acetyltransferases (AACs), aminoglycoside O-nucleotidyltranferases (ANTs), or aminoglycoside O-phosphotransferases (APHs). AACs catalyze the acetylation of $-NH_2$ groups in the aminogylcoside molecule using acetyl coenzym A as donor substrate. ANTs mediate inactivation of aminoglycosides by catalyzing the transfer of an AMP group from the donor substrate ATP to a hydroxil group in the aminoglycoside molecule [68]. Other well descriebed mechanisms are modification of target molecule due to the methylation of 16S rRNA by Arm and Rmt methyltransferases [69]. Efflux pump AcrD in *E. coli* can extrude amikacin, gentamicin, neomycin, kanamycin, and tobramycin from the bacterial cell [70]. The genes of these *aac*, *arm* and *rmt* are usually member of integrons where they are associated with beta-lactam and quinolone resistance determinants [71].

## 5. Resistance to polymyxins

*Enterobacteriaceae* strains can develop resistance to polymyxins due to the modification of lipopolysacharide (LPS) molecule. The modification of the LPS occurs with the addition of 4-amino-4-deoxy-l-arabinose (LAra4N) to a phosphate group in lipid A. This addition causes an absolute increase in lipid A charge, making the positively charged polymyxins inactive [72]. The biosynthesis of LAra4N is mediated by PmrA/PmrB and PhoP/PhoQ two component regulatory system. PmrD has a protective role by inhibiting PmrA dephosphorilation. While PmrA exerts negative feedback by repressing *pmrD* transcription [73, 74]. These mechanism are common in gram-negative bacteria and well described in *Salmonella enterica serotype thyphimurium*, but in *Yersinia pestis* PmrD is not present, in *E. coli* PhoP/PhoQ exists but does not activate PmrA/PmrB.

Further modifications of the bacterial outer membrane include the increased production of capsule polysaccharide (CPS) in *K. pneumoniae*. CPS limits the interaction of polymyxins with their target sites. Thus, upregulation of CPS production leads to increased polymyxin resistance [75]. Interestingly, *Proteus* spp, *Providentia* spp, and *Serratia* spp. are intrinsically resistant to polymyxins, wherase *P. mirabilis* owns 4-amino-4-deoxy-L-arabinopyranose on LPS phoshate thus conferring resistance [76].

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