Antimicrobial resistance in Enterobacteriaceae : characterization and detection

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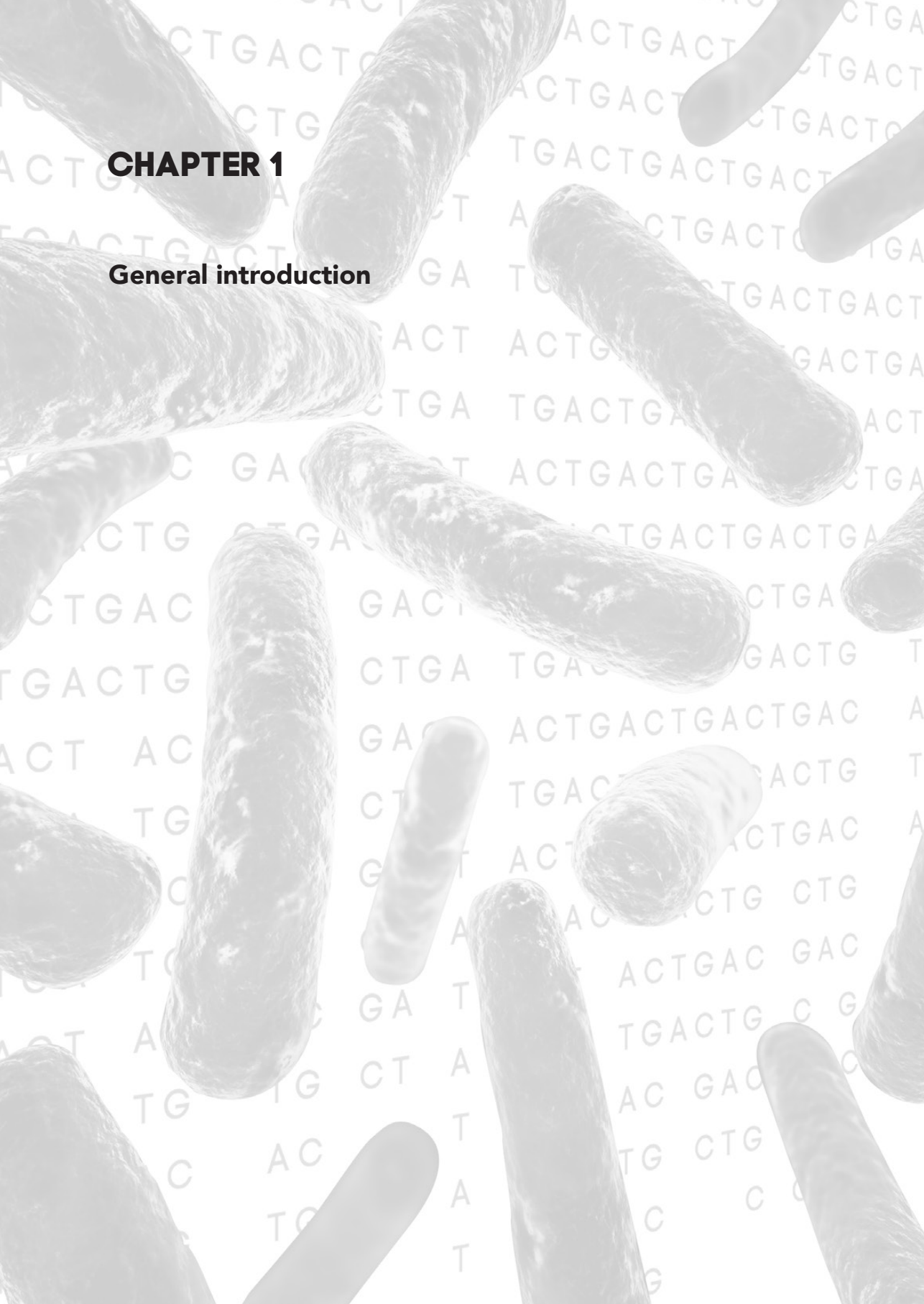

CHAPTER 1

General introduction

INTRODUCTION

Increasing rates of antimicrobial resistance have become a worldwide problem predominantly caused by Gram-negative bacteria, especially by the family of the *Enterobacteriaceae*. This family falls within the phylum Proteobacteria, class Gammaproteobacteria and order Enterobacteriales. The Gram-negative bacteria of the genera *Escherichia spp.*, *Klebsiella spp.*, *Enterobacter spp.*, *Serratia spp.*, and *Citrobacter spp.*, which are collectively called the coliforms, are members of the normal intestinal flora of humans and animals.

As opportunistic pathogens, *Enterobacteriaceae* have become one of the most important causes of nosocomial and community acquired infections. *Escherichia coli* is a frequent cause of urinary tract infections. *Klebsiella spp.* and *Enterobacter spp.* are important causes of nosocomial pneumonia. All members of the *Enterobacteriaceae* can cause bloodstream infections and intra-abdominal infections.[1,2] This may lead to secondary sepsis, a potentially fatal complication mediated by endotoxins.

ß-Lactams (mainly cephalosporins and carbapenems), fluoroquinolones and aminoglycosides constitute the main therapeutic choices to treat infections caused by these microorganisms.[1] However, increasing resistance rates to these compounds have been reported in Europe in the past years.[3,4] During the last two decades a worrisome trend has been the development of resistance to extended-spectrum cephalosporins, e.g. cefotaxime, ceftazidime and ceftriaxone.[5,6] Such resistance is most often due to the presence of extended-spectrum ß-lactamases (ESBLs), but may also be due to plasmid-mediated or chromosomally hyperproduced AmpC ß-lactamase.[7] Besides resistant to 3rd generation cephalosporins they are often resistant to different antibiotic families as fluoroquinolones, aminoglycosides and cotrimoxazole.[8] As a result, more patients need antimicrobial treatment using so-called 'last resort' agents. Carbapenems are considered as the best treatment options.[4,9] The use of carbapenems has led to the rapid selection of carbapenem-resistant *Enterobacteriaceae*.[10] Antimicrobial treatment options for these multidrug resistant infections are limited. Only a few antimicrobial agents (e.g. colistin, tigecycline, fosfomycin and amikacin) with an uncertain in vivo efficacy and/or reported toxicity are left to treat these infections.

1.2 Resistance trends in Europe

In Europe, antimicrobial resistance rates are provided by the European Antibiotic Resistance Surveillance System (EARS-Net). Overall, cephalosporin resistance has increased significantly across Europe from 2001 to 2008 with levels ranging from <1% up to 40% among invasive *E. coli* isolates. A significant increase between 2004 and 2008 is reported for France as well as the Netherlands (Antimicrobial resistance surveillance in Europe 2009; www.ecdc.europa.eu). In 2008, combined resistance of 3rd generation cephalosporins, fluoroquinolones and aminoglycosides is reported in 2-23% of the isolates from different European countries. No data are available for carbapenem resistance in *E. coli*.

Multidrug resistance to three classes of antibiotics (3rd generation cephalosporins, fluoroquinolones and aminoglycosides) is demonstrated in 14% of the *Klebsiella pneumoniae* isolates from invasive infections. Data on carbapenem resistance is available for 84% of the *K. pneumoniae* isolates. Their prevalence as reported in 2008 varies from as high as 39% (Greece) to low (<1% in Nordic countries).

1.3 Resistance trends in the Leiden University Medical Center

The Leiden University Medical Center (LUMC) in the Netherlands is a teaching hospital and is a highly specialized national center for bone-marrow and solid organ transplantation and for cardiovascular surgery.

Between 2003 and 2007, similar resistance trends are observed in the LUMC with an increase of ESBL producing *E. coli* (5%) and *K. pneumoniae* (9%) in 2008. The resistance rates to ciprofloxacin of *E. coli* and *K. pneumoniae* have risen from 8% to 14% and from < 1% to 7% respectively. Combined resistance of 3rd generation cephalosporins and aminoglycosides in 2008 is reported in 1.3% of the *E. coli* isolates and in 4.8% of the *K. pneumoniae* respectively. No carbapenem resistant isolates are reported.

1.4 Origin of resistance

Antimicrobial resistance is not a new phenomenon. Resistance genes have an ancient origin as demonstrated by the recent discovery of genes conferring resistance to ß-lactams, tetracycline, and glycopeptides in permafrost sediments dating from 30.000 years ago.[11] Similarly, multidrug resistant bacteria have been recently cultured from soil samples of an over 4 million year old cave in New Mexico that had been geographically isolated from the surface of the planet.[12] Resistance was found even to synthetic antibiotics that did not exist on earth until the 20th century. Although environmental reservoirs are well known as a possible source of antibiotic resistance genes detected in human pathogens, reports of antibiotic resistance genes from environmental bacteria with a high level of sequence similarity to those from human pathogens previously reported are limited. CTX-M-8 ß-lactamase in environmental bacteria of the genus *Kluyvera,*[13] or *qnrA*-like genes from marine and freshwater bacteria of the genus *Shewanella*[14] are examples of antibiotic resistance transferred from environmental bacteria to human pathogens. The environmental resistome is not only a reservoir of antibiotic resistance genes; it is also a generator of new mechanisms of resistance. The recently discovered carbapenem hydrolyzing New Delhi metallo-ß-lactamase is a good example of a new antibiotic resistance enzyme arising in the environment. Comparison of the genetic environments of bla_{NDM-1} in different bacterial species has identified the construction of the *aphA6-*bla_{NDM-1} chimera, which is likely to originate from *Acinetobacter baumannii*. [15]

1.5 Reservoirs of antimicrobial resistance

Livestock are an important reservoir of antimicrobial-resistant *Enterobacteriaceae*. Excessive use of antibiotics in the veterinary sector has contributed to the selection and spread of multidrug resistant *Enterobacteriacae*.[16] Although there is no complete evidence that agricultural use of antimicrobials is directly linked to development and dissemination of antibiotic resistance among human bacterial pathogens, different studies identified similarities in ESBL-producing *E. coli* isolates from chicken meat and humans according to mobile resistance elements, virulence genes and multilocus sequence typing.[17-19]

Besides livestock, companion animals may also be a source for antimicrobial resistance genes. Surveys in pets, cats and dogs in particular, have identified multidrug resistant *Enterobacteriaceae* in healthy pets as well as in clinical diagnostic samples.[20]

In addition, many different antimicrobial resistance genes to various antimicrobial classes have been detected in water environments.[21,22] River and lakes are examples of relevant putative reservoirs of multidrug resistant *Enterobacteriaceae*, since they collect surface waters from different origins, e.g. wastewater plants, water of urban or industrial effluents and agricultural activities, e.g. as found in Switzerland.[23] Similarly, multidrug resistant *Enterobacteriaceae* have been detected in tap and drinking water, as well as drain and sewage water in India,[24] household water supply in Dhaka, Bangladesh[25] and in untreated drinking water in Portugal.[26] These data implicate contaminated aquatic environments as a likely site for the exchange of antimicrobial resistance genes.

Bacteria colonizing the human intestine also play a relevant role in the development and spread of antimicrobial resistance in the environment. Many horizontal gene transfers occur among the intestinal flora. This process is influenced by antibiotic use, altered colonization in critically ill patients and ingestion of contaminated food or water. [27,28] Multidrug resistant bacteria can then subsequently be transmitted via the hands.[29] Recently, overseas travel as a risk factor for the acquisition of infections due to multidrug resistant *Enterobacteriaceae* has been described for infections due to quinolone-resistant *Salmonella spp.*,[30] quinolone-resistant *Shigella spp*,[31] and cotrimoxazole-resistant *E. coli*.[32,33]

Figure 1 shows the dissemination of antibiotics and subsequent resistance within agriculture, aquaculture, wastewater treatment and associated environments.

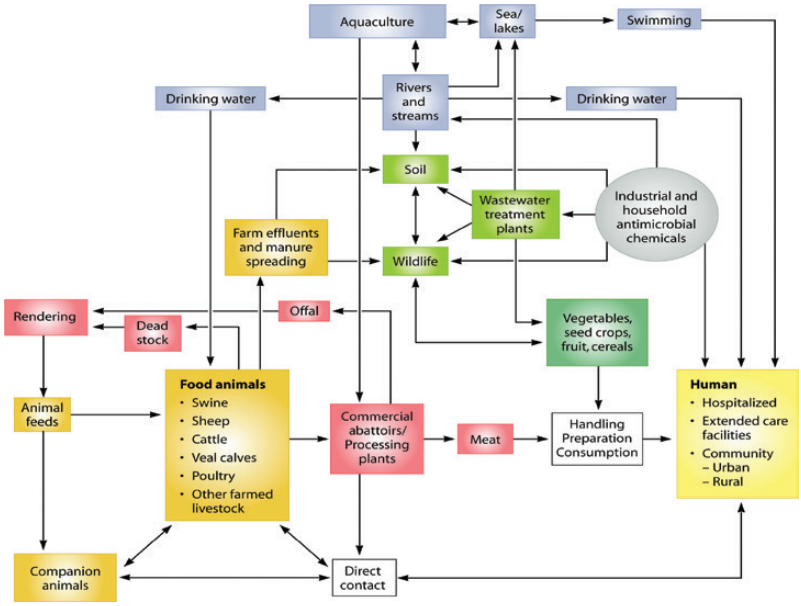

Figure 1. Dissemination of antibiotics and subsequent resistance within agriculture, aquaculture, wastewater treatment and associated environments. (adapted from Davies *et al.*)[34]

1.6 Genetics of resistance

Resistance to antimicrobial agents can either be intrinsic or acquired. Intrinsic resistance comprises all of the inherent properties, which is located on the chromosome of a particular species. *E. coli* naturally produces a chromosomal non-inducible AmpC ß-lactamase expressed at a low level, which is responsible for resistance to penicillin.[38] Other chromosomal resistance mechanisms include low permeability for a specific drug and/or due to the intrinsic presence of multidrug efflux pumps.

The second type is acquired resistance, in which a strain of an originally susceptible species becomes resistant. Acquired resistance mechanisms involve mutations in genes targeted by the antimicrobial agent or the transfer of resistance determinants borne on plasmids, bacteriophages, transposons and other mobile genetic elements.[34,39]

In general this exchange is accomplished through the processes of transformation, transduction or conjugation (Figure 2).[40]

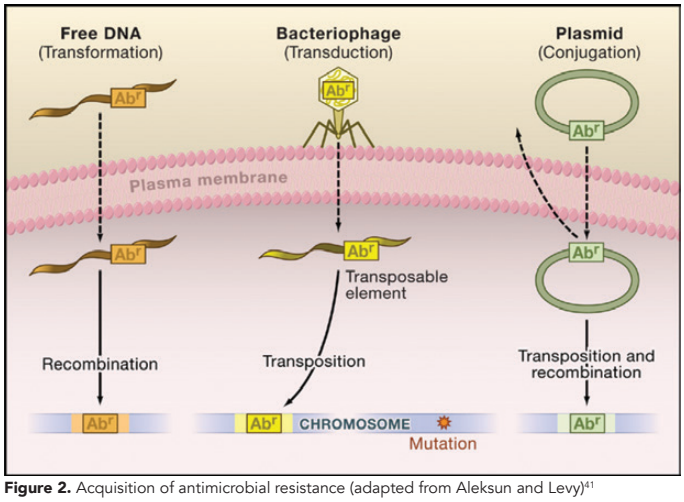

Figure 2. Acquisition of antimicrobial resistance (adapted from Aleksun and Levy)[41]

Plasmids contain genes for resistance; they replicate independently of the host's chromosome and can be distinguished by their origin of replication.
Multiple plasmids may be present within a single bacterium. Plasmids were and are the main vectors of antimicrobial resistance gene dissemination and exchange through 'horizontal gene transfer' with an extraordinary facility and lack of specificity between species.[42]

1.7 Antimicrobial drug targets

Most antimicrobial agents may be categorized according to their mechanism of action. The different antimicrobial drug targets in Gram-negative bacteria include the following:

***Bacterial cell wall**:* The ß-lactam agents (the largest family of antibiotics) act on the bacterial cell wall. They inhibit the cell wall synthesis by binding to the penicillin binding proteins and interfere with structural cross linking of peptidoglycans. The ß-lactam family includes penicillins and derivatives, cephalosporins, carbapenems, monobactams and ß-lactam inhibitors.

***Protein synthesis**:* Aminoglycosides interact with the conserved sequences of the 16S rRNA of the 30S subunit. They cause misreading and premature termination of translation of mRNA.

***Nucleic acid synthesis**:* The fluoroquinolone classes inhibit DNA replication by interacting with the DNA gyrase (*gyrase A* and *B*) and topoisomerase enzymes (*parC* and *parE*). Cotrimoxazole is composed of two antibacterial drugs, sulfamethoxazole and trimethoprim, which act synergistically by inhibiting purine synthesis at different steps of the metabolic pathway of folic acid. Sulfonamide inhibits *dihydropteroate synthetase* (DHPS), which catalyses the formation of dihydrofolate from para-aminobenzoic acid. In the subsequent step of the pathway, trimethoprim inhibits *dihydropholate reductase* (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate.

***Permeability of the bacterial cell membrane**:* Polymyxins, act on the cytoplasmic and outer membrane of gram negative bacteria. They bind to the phospholipids in the cytoplasmic membrane, causing loss of membrane integrity, leakage of cytoplasmic contents and finally cell death.

1.8 Main mechanisms of antimicrobial resistance in *Enterobacteriaceae*

Resistance to antimicrobial agents can be caused by four general mechanisms.[35-37]

1. Enzymatic modification
2. Target alteration
3. Decreased permeability of the bacterial membrane
4. Active efflux

Enterobacteriaceae are capable to combine all mechanisms mentioned above to achieve resistance to a single class or to multiple classes of antibiotics.
Resistance to ß-lactam antibiotics may be due to mutations in the penicillin-binding proteins (PBPs) or reduced permeability of the cell wall by mutations in outer membrane proteins (OMPs) and increased efflux.
The predominant mechanism of resistance to ß-lactams is the production of ß-lactamases. Two classification schemes exist for ß-lactamases: Ambler classes A-D, based on amino acid sequence homology and the Bush-Jacoby groups 1-4, based on substrate and susceptibility to the inhibitor clavulanic acid. Table 2 depicts and updated version of the functional classification scheme for ß-lactamases proposed initially by Bush in 1989,[43] expanded in 1995 by Bush-Jacoby and Medeiros,[44] and updated in 2009.[45] A complete update and overview of the individual ß-lactamases is available at www.lahey.org/studies.

The mechanisms of resistance to frequently used antibiotics are listed in Table 1 on the next page. An overview of the predominant resistance mechanisms are presented in more detail in the following sections.

Table 1. Resistance mechanisms and genes conferring resistance to the main antimicrobial classes in *Enterobacteriaceae*

Antimicrobial class	Antimicrobial Agent	Resistance mechanism	Most frequent resistant traits (enzymes/genes)	Location
Penicillins	Pencillin G Amoxicillin Piperacillin Ticarcilline	enzymatic modification decreased outer membrane permeability efflux	Penicillinases, Class I AmpC ß-lactamase	chromosome
Cephalosporins	Cefazolin Cefuroxim Cefoxitin Ceftazidime Cefotaxime Cefepime	enzymatic modification decreased outer membrane permeability efflux	Class I AmpC ß-lactamase	chromosome
Monobactams	Aztreonam	enzymatic modification decreased outer membrane permeability efflux	Class I AmpC ß-lactamase	chromosome
		enzymatic modification	ESBLs and AmpC*	plasmid
Quinolones	Nalixidic acid Ciprofloxacin Levofloxacin Gatifloxacin	target modification	*gyrA, gyrB, parC* and *parE*	chromosome
		decreased outer membrane permeability	*ompF, ompC, ompA, ompX* *marAB, soxRS*	
		efflux	*acrAB, tolC, mdfA, yhiV*	
		target protection	*qnr A-D*	plasmid
		enzymatic modification	*aac-(6')-Ib-cr*	
		efflux	*qepA, oqxAB*	
Carbapenems	Meropenem Imipenem Ertapenem Doripenem	decreased outer membrane permeability	*ompF, ompC, ompA* combined with the presence of plasmid-mediated ESBL and/or AmpC ß-lactamases	chromosome
		enzymatic modification	serine and metallo ß-lactamases**	plasmid
Anti-folate agents	Sulfonamide	target modification of enzyme dihydropteroate synthase (DHPS)	*folP*	chromosome
		enzymatic modification of target	*sul*	plasmid
	Trimethoprim	mutation in the enzyme dihydrofolate reductase (DHFR)	*folA*	chromosome
		enzymatic modification of enzyme dihydrofolate reductase	*dfrA*	plasmid
Aminoglycosides	Gentamicin Tobramycin Amikacin	efflux decreased outer membrane permeability	RND efflux pumps (*acrD*)	chromosome
		enzymatic modification	16S rRNA methylases: *armA, rmtA-C* nucleotidyltransferases (*ant*) phosphotransferases (*aph*) acetyltransferases (*aac*)	plasmid

* ESBL: extended-spectrum ß-lactamases: most prevalent genes are bla_{TEM}, bla_{SHV}, bla_{CTX-M}
AmpC: bla_{MOX}, bla_{FOX}, bla_{CMY}, bla_{DHA}, bla_{ACC}, $bla_{MIR/ACT}$

** Serine and metallo ß-lactamases: most -prevalent genes are bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}

***Extended spectrum ß-lactamases**:* The most important class A enzymes are the ESBLs. They confer resistance to penicillins, extended-spectrum cephalosporins and aztreonam, and are generally inhibited by ß-lactamase inhibitors (i.e. clavulanic acid, tazobactam, sulbactam). TEM, SHV and CTX-M are the three main types of ESBLs described. The earliest ESBLs, first identified in the 1980s, were mutants of the plasmid-borne parent enzymes TEM-1, TEM-2 and SHV-1 ß-lactamases.[7] Over time, these enzymes have undergone amino acid substitutions, which resulted in over 300 currently known TEM and SHV ESBL variants. A massive shift in the distribution of ESBLs has occurred since 2000 with the spread of CTX-M type ESBLs of which five subgroups (groups 1, 2, 8, 9 and 25) are circulating worldwide, both in nosocomial and in community settings. [46]

***AmpC ß-lactamases**:* Several *Enterobacteriaceae* possesses chromosomal genes encoding for class C AmpCs (c-AmpC; e.g. *Citrobacter freundii*, *Enterobacter* spp., *Serratia* spp., *Morganella* spp.). Induction of c-*ampC* expression is a complex mechanism involving the regulatory genes *ampR, ampD* and *ampG*. These ß-lactamases confer resistance to cephalosporins and ß-lactam/ß-lactamase inhibitor combinations. Also, *E. coli* possess a c-AmpC but its expression is influenced by mutations in the promoter and attenuator regions, which may result in constitutive hyperproduction of c-AmpC. In addition, genes encoding AmpC ß-lactamases have 'escaped' from the chromosomes of *Citrobacter* spp. and other genera to plasmids and are now circulating in various genera of the family *Enterobacteriaceae*. The enzymes they encode are called ACC, ACT, CMY, DHA, FOX, LAT, MIR and MOX, or 'plasmid-mediated AmpCs'.[47] Like ESBLs, AmpC ß-lactamases hydrolyze 3rd generation cephalosporins, but unlike ESBLs, they are also active against cephamycins.

***Carbapenemases**:* ß-lactamase*s* that hydrolyze ß-lactam antibiotics including carbapenems are carbapenemases, which are either chromosomally or plasmid-encoded. The most prevalent enzymes in *Enterobacteriaceae* are KPC, VIM, IMP, NDM-1 and OXA-48.[48] Carbapenem resistance can also evolve by porin loss, reducing drug entry in strains already containing bla_{AmpC} or ESBL-activity.[49] Most carbapenemase-producers are resistant to extended-spectrum (oxyimino) cephalosporins.[50] Isolates producing such enzymes have decreased susceptibility to carbapenems, but with some of these enzymes (OXA-48-like enzymes) the organisms may appear fully susceptible to cephalosporins *in vitro*.

Table 2. Classification schemes for bacterial ß-lactamases

Bush-Jacoby group (2009)	Bush-Jacoby-Medeiros group (1995)	Molecular class (subclass)	Distinctive substrate(s)	Inhibited by		Defining characteristic(s)	Representative enzyme(s)
				CA or TZB[a]	EDTA		
1	1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	NI[b]	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-ß-lactams	GC1, CMY-37
2a	2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-ß-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	NI	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-ß-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	NI	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4
2d	2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	NI	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-ß-lactams	OXA-11, OXA-15
2df	NI	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-ß-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	3	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
		B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	3	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1
NI	4	Unknown					

[a]CA, clavulanic acid; TZB, tazobactam.
[b]NI, not included

***Aminoglycosides**:* The major aminoglycoside resistance mechanism is based upon the presence of enzymes modifying the aminoglycoside molecule. These proteins are classified into three major classes according to the type of modification; AAC (*N*-acetyltransferases), ANT (*O*-nucleotidyltransferases) and APH (*O*-phosphotransferases). They are further divided into subclasses that are based on the site of modification and the spectrum of resistance within this class. Other known mechanisms of aminoglycoside resistance include decreased outer membrane permeability, active efflux and target alteration.[51] More recently, methylation of the aminoglycoside binding site by 16S rRNA methylases (*armA*, *rmtA-D*, *rmtF-G*, *npmA*) has been found to confer high-levels of resistance to all aminoglycosides except streptomycin.[52,53]

***Fluoroquinolones**:* Resistance to fluoroquinolones is usually mediated by chromosomal mutations in the quinolone resistance determining region (QRDR) that encodes DNA gyrase (*gyrA* and *gyrB*) and topoisomerase (*parC* and *parE*). [54] Nevertheless, low level resistance can also arise from the expression of plasmid-mediated quinolone resistance (PMQR) such as:

- *qnrA, -B, -C, -D, qnrS* that encode proteins protecting the DNA gyrase from the quinolone action.[55]
- aminoglycoside acetyltransferase encoded by the *aac(6')-Ib-cr* gene that also acetylates quinolones.[56]
- plasmid-mediated quinolone efflux pumps, *qepA* [57] and *oxqAB*.[58]

1.9 Porins and efflux pumps in antimicrobial drug resistance

***Outer membrane proteins**:* the outer membrane (OM) of Gram-negative bacteria constitutes the first permeability barrier that protects the cells against environmental stress. Simultaneously, it allows the selective uptake of essential nutrients and the secretion of metabolic waste products. The major route of entry for hydrophilic antibiotics through the OM is facilitated by water-filled diffusion channels formed by the outer membrane proteins (OMPs), called porins. Bacteria usually produce many porins; approximately 10^5 porin molecules are present in a single cell of *E. coli*. One of the bacterial strategies for drug resistance is to limit the intracellular access via modification of porins. It has been demonstrated that the expression

levels of the porins OmpC and OmpF, not only controls the permeability of the outer membrane to glucose and nitrogen uptake under nutrient limitation,[59] but may also be differentially regulated by the concentration of certain antibiotics in the environment.[60,61] One of the earliest examples is loss of the OmpF porin from *E. coli* in resistance to ß-lactams, which was found in 1981.[62]

OmpX is another OMP that is expressed in an inverse manner compared to the expression of OmpC and OmpF.[63,64] The expression of OmpX is upregulated instead of reduced upon exposure to e.g. fluoroquinolones.[51]

Mutations affecting the expression and/or function of the porins have a direct impact on the susceptibility to antimicrobials. These mutations can have different effects, such as (most commonly) porin loss, a modification of the size or conductance of the porin channel (Figure 3).

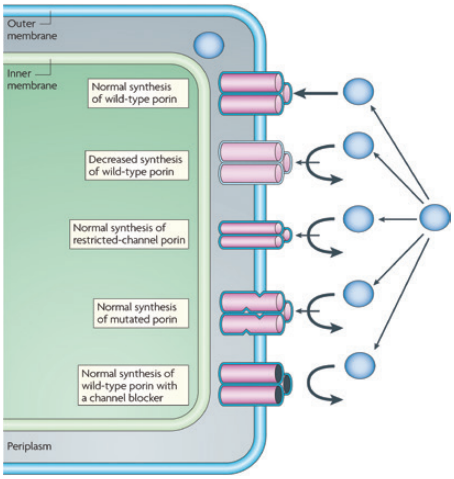

Figure 3. Multidrug resistance mechanisms associated with porin modification. (adapted from Pagès *et. al.*)[65] This figure shows the various resistance mechanisms that are associated with porin modification. The ß-lactam molecules and porin trimers are represented by blue circles and pink cylinders, respectively. The thickness of the straight arrows reflects the level of ß-lactam penetration through porin channels. The curved arrows illustrate the uptake failure that occurs with: a change (decrease) in the level of porin expression; an exchange in the type of porin that is expressed (restricted-channel porin); and mutation or modification that impairs the functional properties of a porin channel (mutated porin). The effect of pore-blocking molecules (black circles) is shown at the bottom of the figure.

Today, porins involved in antibiotic resistance have been identified in many bacterial species (Table 3). For example, carbapenem resistance in *K. pneumoniae* and *E. coli* may be caused by the presence of carbapenemases, but can also be caused by loss of OMPs combined with plasmid- mediated ß-lactamase.[66,67]

Table 3. Examples of porins related to antibiotic resistance in different species

Species	Porin	Antibiotic(s)
Escherichia coli	OmpC OmpF	ß-Lactams
Serratia marcescens	OmpF OmpC	ß-Lactams
Klebsiella pneumoniae	OmpK35 OmpK36	Cephalosporins, carbapenems, fluoroquinolones, and chloramphenicol Carbapenems
Klebsiella oxytoca	OmpK36	Carbapenems
Enterobacter cloacae	OmpF	Carbapenems
Enterobacter aerogenes	OmpC OmpF Omp36	Carbapenems Carbapenems Imipenem, cefepime and cefpirome

Another type of mutation includes those affecting regulatory proteins that control the expression of porin-encoding genes. For instance, the *ompB* operon, which contains the genes *ompR* and *envZ*, is known to regulate the expression of OmpC and OmpF in *E. coli*.[68] In addition to the *ompB* locus, many other proteins, like Rob, SoxS and MarA are known to participate in the regulation of the transcription of porin genes. Overall, mutations that lead to the loss, downregulation, or alterations of porins have a direct impact on antimicrobial susceptibility by limiting the rate at which an antimicrobial agent can enter the cell. Combined with secondary resistance mechanisms (e.g. ß-lactamases, target alteration, efflux) a bacterium can acquire high-level resistance.

Efflux pumps: In addition to the influx problem, several recent reports have shown a significant increase in the dissemination of *Enterobacteriaceae* with active antibiotic efflux.[69] This efflux occurs due to the activity of membrane transporters proteins, the so-called drug efflux pumps. There are essentially five families of chromosomally encoded bacterial efflux pumps: the resistance-nodulation-division (RND), major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) and small multidrug resistance (SMR) family. The RND efflux pumps, such as AcrAB and YhiV, and major facilitator family (MFS) pumps such as MdfA are

TolC-associated efflux pumps.[70] The overexpression of these efflux pump genes is related to antibiotic resistance in clinical *E. coli* isolates.[71,72]

In the past decade, plasmid-encoded efflux pumps have been identified. The plasmid-mediated quinolone resistance determinant *qepA* was recently detected in clinical isolates of *E. coli* from Belgium[73] and Japan.[57] Another plasmid-encoded RND efflux pump, *oxqAB*, has been identified in clinical isolates of *E. coli* and *K. pneumoniae* in Korea[58] and Spain.[74]

1.10 Rapid detection of antimicrobial resistance mechanisms

Microbiological laboratories play a central role in the combat against antimicrobial resistance trends by rapid and proper recognition of resistant bacteria. Currently, susceptibility testing in clinical microbial diagnostic laboratories is performed using standardized susceptibility testing methods such as disk diffusion or by the use of automated systems. Routine diagnostics end at this step, as it provides the required information for the treatment of a patient. With the emergence and growing complexity of antimicrobial drug resistance, it would be advantageous to rapidly characterize both phenotypic and molecular traits of *Enterobacteriaceae*. To obtain a better understanding of the molecular epidemiology and characteristics of resistant *Enterobacteriaceae* more information is required, e.g. the type of antimicrobial resistance mechanism, presence or absence of resistance genes and mutations conferring resistance. Besides the detection of resistance mechanism, typing of isolates and detection of virulence determinants provides crucial additional information. To fully understand the development and dissemination of resistance, it is not only important to genetically characterize the overall extent and spread of antibiotic resistance in outbreak settings, but particularly in endemic, clinical strains. The clinical diagnostic microbiology field should be aiming towards surveillance and early detection of resistance. In addition, comprehensive assessment of the overall abundance in antibiotic resistance in all bacteria is likely to provide valuable insights. These insights may help the development of newer antibiotics or other specific targets to control their spread.

Ideally, a rapid system should investigate all resistance traits in one single test and should provide easy to interpret results the same day the test is performed. Moreover, the assay should be able to identify specific variants of alleles that

encode for proteins with a different impact on the antibiotic resistance phenotype. Finally, the methodology should be easy to perform, relatively cheap, accurate and versatile enough to be regularly updated according to the evolution of the antibiotic resistance traits.

1.11 Molecular characterization of isolates

During the past decade, various novel applications focusing on the detection of antimicrobial resistance genes/mechanisms have been developed. Next to multiplex PCR, the introduction of microarrays has allowed the simultaneous identification of a large number of genes of interest in a single assay. Biophysical technologies, such as matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) also allows rapid identification of specific antibiotic resistance mechanisms. This includes the rapid detection of clinically important ß-lactamases, such as ESBLs and carbapenemases.[52,75-78] Despite these advances in MALDI-TOF MS technology, the possibilities are limited to the detection of resistance involving enzymatic hydrolysis, which still needs complex sample preparation of bacterial extracts and special skills for the interpretation of the spectra.
Advances in DNA sequencing technology have made it possible to sequence entire bacterial genomes. This technique has successfully been applied in genomic studies on typing and antimicrobial resistance in *Neisseria meningitides,*[79] MDR, *Mycobacterium tuberculosis,*[80] *Staphylococcus aureus*,[81] the German enterohemorrhagic *E. coli* O104:H4 outbreak strain,[82] and *Bacillus anthracis*.[83] A number of studies describing whole genome sequencing of clinical isolates in order to characterize the genetic determinants of antibiotic resistance have been described.[84-87]

PCR-based techniques: For antimicrobial-resistant bacteria carrying specific resistance genes, multiplex PCR detection methods are available. Rapid identification of clinically relevant antimicrobial resistance genes in *Enterobacteriaceae*, such as ESBLs,[88,89] plasmid-mediated AmpC,[90] plasmid-mediated quinolone resistance genes,[91,92] and carbapenemases[93] can be performed once a bacterial isolate is cultured. Quantitative reverse transcription PCR can be utilized for quantification of RNA expression levels of genes.

Microarray: The development of microarray based approaches has been another response to the challenge of rapid identification of multiple resistance mechanisms in a single isolate. Several microarrays for the genotyping of ß-lactamase genes have been developed. In a pilot study, we evaluated the ability of a commercially available microarray (Identibac AMR-ve™ Array Tube) to detect clinically important ß-lactamases from the ESBL families (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$), and resistance genes to aminoglycosides, fluoroquinolones and cotrimoxazole in thirty-seven clinical *K. pneumoniae* isolates. The microarray reliably detected and distinguished the most prevalent ESBL encoding enzymes from the five groups of the CTX-M family. There was 72-88% correlation between the phenotypic antimicrobial resistance and the microarray results for aminoglycosides and cotrimoxazole. For susceptible isolates, the overall agreement was between 70-93%. This microarray is a useful tool to complement phenotypic susceptibility testing and surveillance of antibiotic resistance determinants in a clinical laboratory.
Another commercially available microarray is launched by a Dutch company, CheckPoints B.V. This microarray is able to detect clinically relevant ESBLs, carbapenemases and AmpC genes. It provides definitive results within the same working day, allowing rapid implementation of isolation measures and appropriate antibiotic treatment. This company collaborates with various national and international research groups to evaluate and update their products.[94-100]

Whole genome sequencing: Direct detection of each of all resistance determinants in a single test requires technology with efficient multiplexing capabilities well beyond that of current PCR techniques and will most likely be based on whole genome sequencing (WGS). This technique is developing at incredible speed and application in clinical microbiology seems a matter of time. In theory, WGS technology provides complete characterization of clinical isolates with respect to resistance genes, their level of expression, mutations responsible for resistance, as well as the presence of virulence genes. Comparing isolates for epidemiological or evolutionary purposes is done in the same experiment. Thus, within one working day, all there is to know about a bacterial isolate should be available, both for diagnostic and research purposes. Although in its current form it may not be suited for routine testing yet, whole genome sequencing has demonstrated its utility in tracking outbreaks.[82,101-106]

1.12 Scope of the thesis

The aim of the research described in this thesis is to obtain more insight into the mechanisms of antimicrobial resistance in multidrug resistant *Enterobacteriaceae*, recovered at the Leiden University Medical Center, the Netherlands. This will be investigated using different phenotypic and molecular techniques. In addition, clonal relatedness among *E. coli* isolates will be characterized by AFLP and MLST to identify the sequence types present. Finally, a first step towards complete characterization of clinical isolates with respect to resistance genes, mutations responsible for resistance, the presence of virulence genes, as well as their sequence type will be made using whole genome sequencing. In this thesis genetic characterization of antimicrobial resistance mechanisms in *Enterobacteriaceae* will be performed using different techniques, which have been described in section 1.11:

- PCR and sequence analysis
- Quantitative reverse transcription PCR (qRT-PCR)
- Microarrays
- Whole genome sequencing (WGS)

In **Chapter 2** resistance mechanisms are characterized in a collection of clinical *Enterobacteriaceae* resistant to ciprofloxacin and cephalosporins. Molecular detection of resistance genes is performed using PCR and sequence analysis of the specific targets for fluoroquinolone and ß-lactam resistance. Clonal relatedness among the *E. coli* isolates is characterized by the use of AFLP and multilocus sequence typing.

Chapter 3 will go into further detail with the investigation of the contribution of increased efflux activity and decreased influx to fluoroquinolone resistance in clinical *E. coli* isolates. Therefore a modified high-throughput phenotypic efflux assay and quantitative reverse transcription PCR for the expression levels of specific targets is used.

As described in **Chapter 4**, AmpC-mediated resistance is another clinically relevant and emerging resistance mechanism in *Enterobacteriaceae*. This study investigates whether increased AmpC expression is a mechanism involved in cefoxitin resistance in clinical cefoxitin-resistant *E. coli* isolates and if this influences 3rd generation cephalosporin activity. Therefore phenotypic AmpC assays, conventional and multiplex PCR and sequence analysis where appropriate, quantitative reverse transcription PCR and in depth analysis are all used.

Chapter 5 concerns a prospective cohort study among travelers from the Netherlands to investigate the acquisition and duration of rectal carriage of the predominant resistance mechanism in *Enterobacteriaceae*, i.e. the presence of ß-lactamases: ESBLs, plasmid-mediated AmpCs and carbapenemases, after foreign travel. Rapid molecular characterization of all *Enterobacteriaceae* is performed using Check-MDR CT103, a commercially available microarray, and MLST is performed on all *E. coli* isolates. In addition, potential travel-associated risk factors are investigated.

In **Chapter 6** the performance of the next-generation sequencing bench top, the Ion Torrent Personal Genome Machine® is used for the combined detection of antimicrobial resistance, housekeeping, and virulence genes in clinical *E. coli* isolates.

Finally in **Chapter 7**, implications of the findings as well as the role of innovative applications in the rapid detection of antimicrobial resistance mechanisms for clinical microbiology in the next era are discussed, with special emphasis on the possible role of whole genome sequencing in future clinical microbiology.

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