ANTIMICROBIAL RESISTANCE IN *ENTEROBACTERIACEAE*

CHARACTERIZATION AND DETECTION

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ISBN: 978-90-9028-809-3

Lay out and cover design: Frits Panday, Rotterdam, the Netherlands
Printed by Optima Grafische Communicatie, Rotterdam, the Netherlands

Printing of this thesis was financially supported by Astellas BV, BD Diagnostics, BioMérieux, Check-Points BV, the Netherlands Society of Medical Microbiology (NVMM) and the Royal Netherlands Society for Microbiology (KNVM)

Antimicrobial resistance in *Enterobacteriaceae*
characterization and detection

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
ter verdediging op woensdag 18 februari 2015
klokke 13.45 uur

door

Sunita Paltansing
geboren te 's Gravenhage
in 1978

PROMOTIECOMMISSIE

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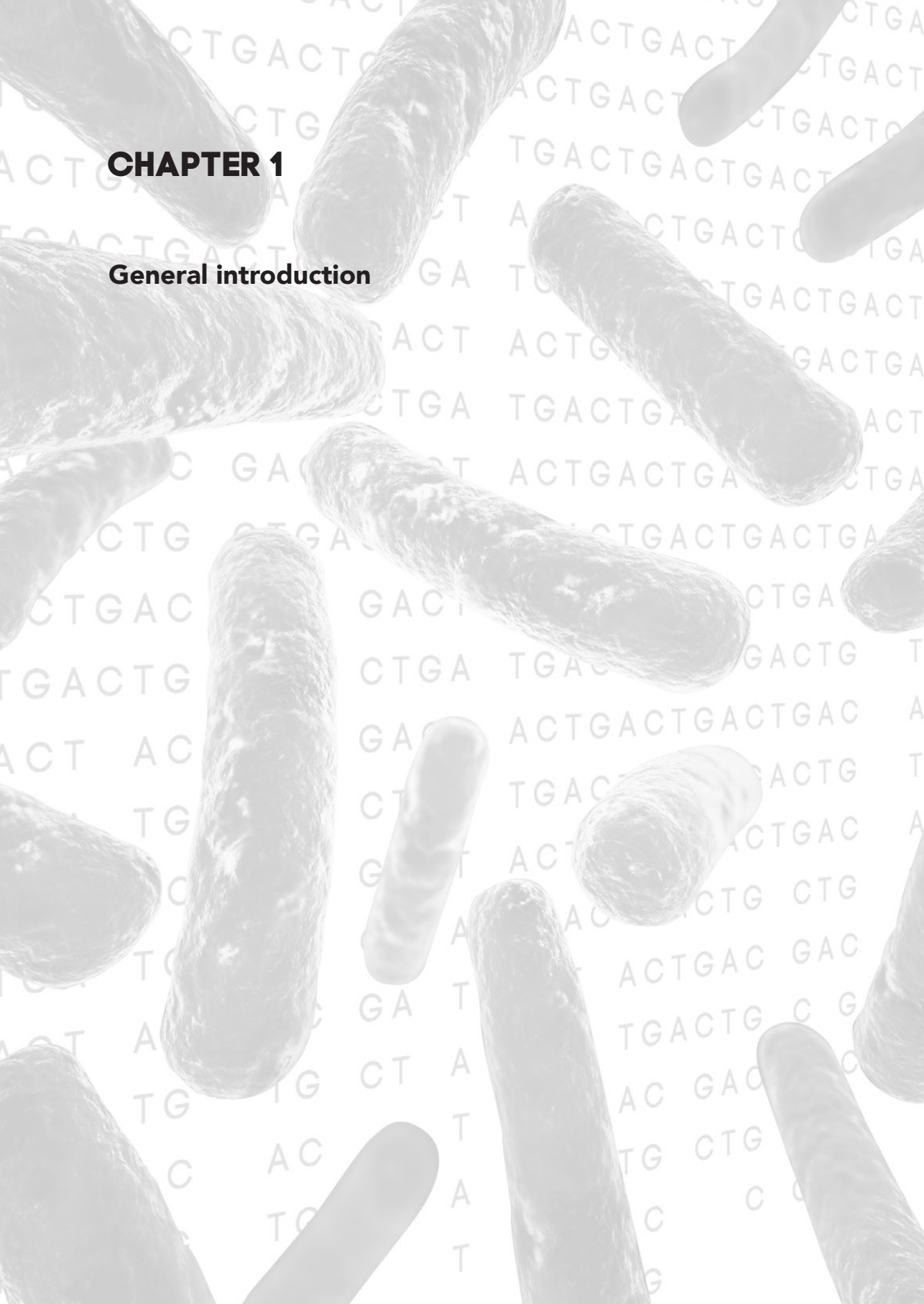
The best and most beautiful things in the world cannot be seen or even touched.
They must be felt within the heart.
Helen Keller (1880-1968)

Contents



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.

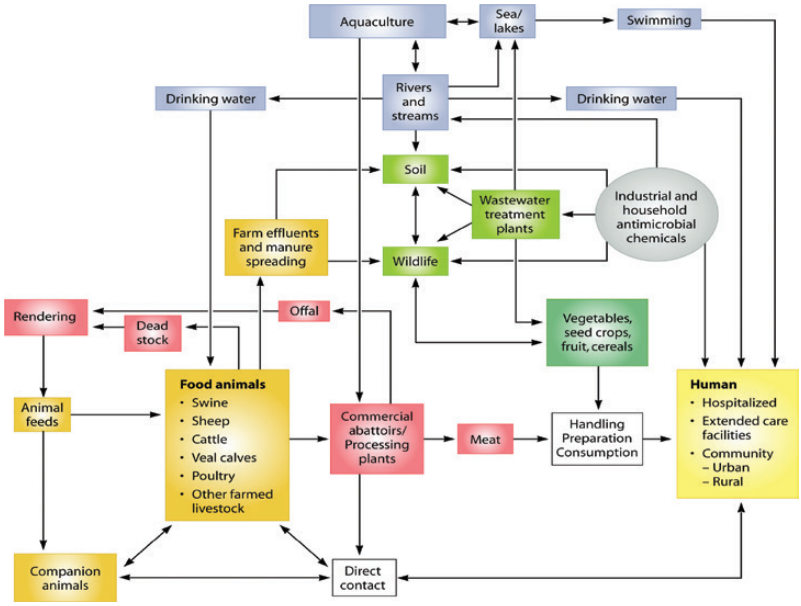


Figure 1. Dissemination of antibiotics and subsequent resistance within agriculture, aquaculture, wastewater treatment and associated environments. (adapted from Davies *et al.*)[34]

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]

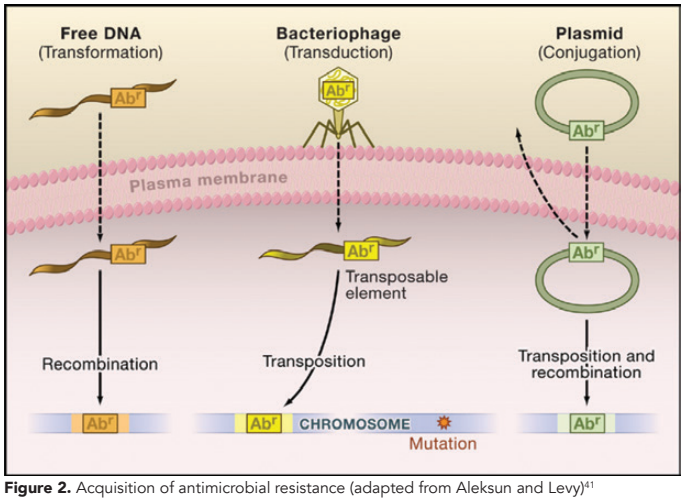


Figure 2. Acquisition of antimicrobial resistance (adapted from Aleksun and Levy)[41]

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).

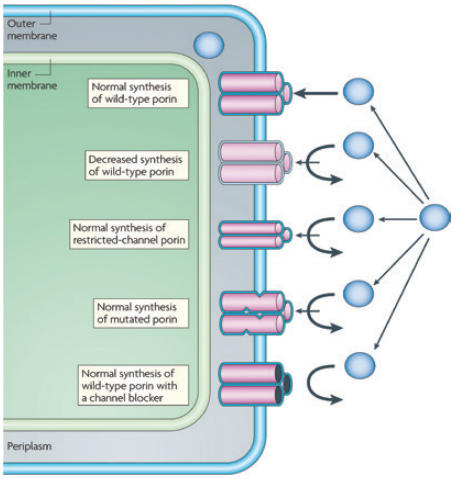


Figure 3. Multidrug resistance mechanisms associated with porin modification. (adapted from Pagès *et. al.*)[65] This figure shows the various resistance mechanisms that are associated with porin modification. The ß-lactam molecules and porin trimers are represented by blue circles and pink cylinders, 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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CHAPTER 2

Molecular characterization of fluoroquinolone and cephalosporin resistance mechanisms in *Enterobacteriaceae*

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Journal of Antimicrobial Chemotherapy 2013 Jan;68(1):40-45

2.1 Abstract

Objectives: To characterize the mechanisms of fluoroquinolone and cephalosporin resistance in *Enterobacteriaceae* from a Dutch teaching hospital in 2008.

Methods: We sequenced *gyrA*, *gyrB*, *parC* and *parE*. The presence of plasmid-encoded genes *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib*, *qepA*, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{CTX-M} and bla_{ampC} was studied by PCR. *Escherichia coli* isolates were further characterized by AFLP and multilocus sequence typing (MLST).

Results: In total, 49 *Escherichia coli*, 16 *Klebsiella pneumoniae* and three *Enterobacter cloacae* isolates were investigated. Mutations in *gyrA* were found in all *E. coli* isolates. Forty-five (92%) *E. coli* isolates carried at least one point mutation in *parC*. Most *E. coli* isolates (59%) also carried mutations in *parE*, of which I529L was the most prevalent. I529L was unequivocally associated with *E. coli* sequence type (ST) 131. This single nucleotide polymorphism (SNP) was later also found in eight out of nine ST131 strains from another collection. Twenty-nine *E. coli* isolates carried extended spectrum ß-lactamase (ESBL) genes, predominantly $bla_{CTX-M-15}$. In *E. coli*, *aac(6')-Ib-cr* was the predominant plasmid mediated resistance mechanism, whereas in *K. pneumoniae qnr* genes were found mostly. In *K. pneumoniae* isolates, *qnr* and *aac(6')-Ib-cr* co-occurred with ESBL genes (n=13; bla_{CTX-M} and bla_{SHV}) and/or bla_{ampC} (n=3; bla_{DHA-1}).

Conclusion: *E. coli* ST131 was the predominant clone, which accumulated a high number of chromosomal mutations. The I529L SNP in *parE* was a signature of most, but not all ST131 strains. In contrast to *E. coli*, fluoroquinolone resistance mechanisms were predominantly plasmid-encoded in *K. pneumoniae*.

2.2 Introduction

Fluoroquinolones are among the most frequently prescribed antimicrobial agents worldwide. Data from the European Antimicrobial Resistance Surveillance Network (EARS-net) show that fluoroquinolone resistance has increased significantly across Europe since 2001, with levels ranging from 7-53% in 2007.[1] Between 2003 and 2007, resistance rates of *Escherichia coli* to ciprofloxacin have risen from 8 % to 14% and for *Klebsiella pneumoniae* from < 1% to 7% in the Leiden University Medical Center (LUMC), a tertiary care hospital in the Netherlands. In 2008, 5% of *E. coli* and 9% of *K. pneumoniae* isolates were extended spectrum ß-lactamase (ESBL) producers.

Quinolone resistance in *E. coli* usually occurs due to stepwise mutations in the quinolone resistance-determining regions (QRDRs) of gyrases (*gyrA* and *gyrB*) and/or topoisomerase IV (*parC* and *parE*), which are located on the bacterial chromosome. Since 1998, plasmid-mediated quinolone resistance (PMQR) determinants have emerged: *qnr (qnrA-D, qnrS)*, *aac(6')-Ib-cr* and *qepA*. *qnr* genes encode for proteins, which protect type II topoisomerases from quinolones. *Aac(6')-Ib-cr* is a variant of aminoglycoside acetyltransferase and is responsible for reduced susceptibility to ciprofloxacin and norfloxacin by N-acetylation of a piperazinyl amine. The *qepA* gene encodes a quinolone efflux protein.[2]

In Europe, many authors have reported the dissemination of PMQR genes in *Enterobacteriaceae,* which are often associated with ESBLs or plasmid-mediated bla_{AmpC} ß-lactamases.[3] However, data regarding the resistance mechanisms of fluoroquinolone resistance in cephalosporin-resistant *Enterobacteriaceae* in the Netherlands are limited.

Therefore, we investigated the resistance mechanisms in a collection of *Enterobacteriaceae* from inpatients and outpatients recovered at the LUMC in 2008. Clonal relatedness among the *E. coli* isolates was characterized by the use of AFLP and multilocus sequence typing (MLST).

2.3 Materials and methods

2.3.1 Bacterial isolates and antimicrobial susceptibility testing

Isolates were screened for reduced susceptibility to second and/or 3rd generation cephalosporins and ciprofloxacin as determined by the VITEK2® system (BioMérieux, Marcy-l'Étoile, France) in our routine laboratory from January to December 2008. MICs for ciprofloxacin and levofloxacin were determined using Etests (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. MICs were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/). Nonrepetitive isolates with resistance to cephalosporins and ciprofloxacin (MIC of ≥ 0.125 mg/L) were included.

2.3.2 Molecular detection of resistance genes

Mutations in *gyrA*, *gyrB*, *parC*, *parE,* and the presence of *qnrA, qnrB, qnrS, aac(6')-Ib, qepA,* and the ß-lactamase genes bla_{TEM}, bla_{SHV}, bla_{OXA}, $bla_{CTX\text{-}M}$ and bla_{AmpC} were investigated by the use of polymerase chain reaction (PCR) and sequence analysis where appropriate.

For the primer design, an alignment of available gene sequences in GenBank® was made using the AlignX program (Vector NTI Advance 11, Invitrogen).

All primer sequences, PCR conditions and annealing temperatures are shown in Table 1. Amplification products were sequenced at the Leiden Genome Technology Center (LGTC®, Leiden, The Netherlands) with the same primer pairs as used for PCR.

Table 1. Primers used in this study

Target	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product size (bp)	Annealing temp (°C)
gyrA	ATGTCGGTCATTGTTGGCCG	GCCATCAGCTCATGRGCRAT	514-530	58
		GCCATCAGTTCATGGGCRAT		
		GCCATCAGTTCGTGGGCGAT		
gyrB	TCTACTGCTTYACCAACAACA	CGTCCGCATCGGTCATGAT	704	58
parC	ATGGCAGAGCGCCTTGCG	GCCGTCRAAGTTTGGCAC	437	58
parE	CGGAAGATATCTGGGATCG	TCTCTTCTTCCGTCAGYGC	703	58
qnrA *qnrA2*	GTTAAACCAGGTCTGCTGAC	TTAGGTCAGCAGCTTCTCCT	480	55
qnrA *qnrA5/6*	GGATTTCTCACGCCAGGATT	ACTCCCAAGGGTTCCAGCA	605	55
qnrB *qnrB1*	GGCTGYCAGTTYTATGATCG	CCAAGMCGYTCCAKGAGC	502	55
qnrB *qnrB3*	CTTAACGCCTTGTAAATCAACG	ATGGCTCTGGCACTCGTTG	584	55
qnrS *qnrS*	ACAATCATACATATCGGCACC	TTAGTCAGGATAAACAACAATAC	646	55
aac-6′-Ib	ATGACTGAGCATGACCTTGC	TTAGGCATCACTGCGTGTTC	518	58
qepA	CGAGTATCGTGATCCGGAC	AAGATGTAGACGCCGAACAT	290	60
bla_{TEM}	TCGGGGAAATGTGCG	TGCTTAATCAGTGAGGACC	972	60
bla_{SHV}	GCCGGTTATTCTTATTTGTCGC	ATGCCGCCGCCAGTCA	1027	60
$bla_{CTX\text{-}M1\ family}$	TCTTCCAGAATAAGGAATCCC	CCGTTTCCGCTATTACAAAC	909	60
$bla_{CTX\text{-}M2\ family}$	ATGATGACTCAGAGCATTCGCCG	TCAGAAACCGTGGGTTACGATTTTC	876	60
$bla_{CTX\text{-}M8\ family}$	AYGATGAGACATCGCGTTAAGCGG	TTAATAACCGTCGGTGACGATTTTC	876	60
$bla_{CTX\text{-}M9\ family}$	ATGGTGACAAAGAGAGTGCAACGG	GATGATTCTCGCCGCTGAAGCC	861	60
$bla_{CTX\text{-}M26\ family}$	ATGATGAGAAAAAGCGTAAGGCGG	TTAATAACCGTCGGTGACAATTCTG	876	60
bla_{MOX}	GCTGCTCAAGGAGCACAGGAT	CACATTGACATAGGTGTGGTGC	520	60
bla_{CIT}	TGGCCAGAACTGACAGGCAAA	TTTCTCCTGAACGTGGCTGGC	462	60
bla_{DHA}	AACTTTCACAGGTGTGCTGGGT	CCGTACGCATACTGGCTTTGC	405	60
bla_{ACC}	AACAGCCTCAGCAGCCGGTTA	TTCGCCGCAATCATCCCTAGC	346	60
bla_{EBC}	TCGGTAAAGCCGATGTTGCGG	CTT CCA CTG CGG CTG CCA GTT	302	60
bla_{FOX}	AACATGGGGTATCAGGGAGATG	CAAAGCGCGTAACCGGATTGG	190	60

PCR was performed in a 50 µL PCR mixture, including 10 µL of template DNA, 0.5 µM concentration of each primer (Biolegio B.V., Nijmegen, the Netherlands) and 25 µL Hotstar Taq Master Mix (Qiagen Benelux B.V., Venlo, the Nederlands). The thermal cycling conditions consisted of an initial incubation of 15 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 20 seconds at different annealing temperatures (listed in Table 1) and 2 minutes at 72°C and a final elongation step of 10 minutes at 72°C. PCR was performed using a MyCycler™ Thermal Cycler (Bio-Rad, Veenendaal, The Netherlands). Products were analyzed by agarose gel electrophoresis.

2.3.3 Molecular typing of E. coli isolates

All *E. coli* isolates were typed comparatively using AFLP, as described previously.[4] Genotypic relatedness was evaluated using BioNumerics software v. 6.1 (Applied Maths, St-Martens-Lathem, Belgium). Isolates linked at 90% or above were considered related and were designated by capital letters A through F. MLST was performed using seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) using the MLST Databases at the Environmental Research Institute, University College Cork website (http://mlst.warwick.ac.uk/mlst/).

2.4 Results and discussion

The primary purpose of the present study was to characterize the resistance mechanisms to fluoroquinolones in enterobacterial isolates of our hospital, since these data from the Netherlands are scarce. A total of 68 nonrepetitive enterobacterial isolates were analyzed: 49 *E. coli*, 16 *K. pneumoniae*, and three *Enterobacter cloacae*. Table 2 shows the results for *E. coli*.

In total, 44 *E. coli* isolates had double mutations in *gyrA*, at codons 83 (S83L) and 87 (S87N, -G, or -H). While these 44 isolates had MICs of ciprofloxacin and levofloxacin well above the I/R breakpoint (> 1 mg/L), four isolates with only one mutation in *gyrA* had MICs below the EUCAST S/I breakpoint of levofloxacin (1 mg/L) but higher than those for wildtype isolates (≤ 0.25 mg/L). Likewise, MICs of ciprofloxacin for these four isolates were below at or just over the S/I breakpoint of ciprofloxacin (0.5 mg/L), but still above wildtype MICs (≤ 0.064 mg/L).

Forty-five (92%) *E. coli* isolates carried at least one point mutation in *parC*, mainly S80I, followed by E84V. No mutations were found in *gyrB*. These findings were in accordance with those of others worldwide.[2]

Thirty-two of the 49 (65%) *E. coli* isolates carried four to five mutations in the QRDR regions of *gyrA*, *parC* and *parE* combined. This number of accumulated mutations is high compared with those reported by others.[5,6] The majority of isolates with a high number of QRDR mutations were *E. coli* ST131.

Table 2. Distribution of fluoroquinolone resistance, amino acid substitutions and ESBL resistance among *E. coli* (n=49) isolates from the LUMC with ST and AFLP type

Isolate no	Patient status[a]	Specimen	ST-type[b]	AFLP type	MIC (mg/L)		*gyrA*	*parC*	*parE*	PMQR	ß-lactamase
					CIP[c]	LVX[d]					
2135	I	abd. drain*	3076	u	0.25	0.5	S83L	WT[e]	WT	-	-
2144	O	abd. drain	48	u	0.25	0.75	S83L	WT	WT	-	*TEM-20*
2466	I	abd. drain	**131**	**D**	0.50	0.75	S83L	WT	S458A, **I529L**	-	-
2027	O	urine	38	u	0.75	0.75	S83L	S57T, S80I	WT	-	*CTX-M-15*
2543	O	urine	58	u	4	6	S83L, D87N	S80I	WT	-	-
1495	O	urine	88	F	6	4	S83L, D87N	E84K	WT	-	-
1508	O	urine	167	u	6	4	S83L, D87N	S80I	WT	-	*CTX-M-1*
2580	O	blood	354	u	6	4	S83L, D87N	S80I	I355T	-	-
2480	O	blood	1011	u	6	8	S83L, D87N	S80I	WT	-	*CTX-M-1*
2717	O	urine	3077	u	8	6	S83L, D87N	S80I	I355T	-	-
2521	I	sputum	167	E	12	8	S83L, D87N	WT	WT	-	-
1965	O	wound	10	u	24	8	S83L, D87N	S80I	WT	-	*CTX-M-1*
2020	O	urine	405	u	>32	6	S83L, D87N	S80I	WT	*aac(6')-Ib-cr*	*CTX-M-15*
2239	O	urine	533	u	>32	12	S83L, D87N	S80I, E84G	WT	-	*SHV-12*
1514	I	urine	624	u	>32	12	D87Y	S80I	WT	-	*CTX-M-1*
1632	O	urine	648	B	>32	12	S83L, D87N	S80I	S458A	*aac(6')-Ib-cr*	*CTX-M-15*
2722	O	urine	617	u	>32	16	S83L, D87N	S80I	S458A	-	*CTX-M-15*
1604	O	urine	88	F	>32	24	S83L, D87N	S80I	S458A	-	-
2207	I	urine	224	u	>32	24	S83L, D87N	E84K	WT	-	-
2413	O	urine	10	u	>32	>32	S83L, D87N	S80I	S458A	-	*CTX-M-1*
1936	I	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	-
2401	I	thor. drain[§]	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	aac(6')-*Ib-cr*	*CTX-M-15*
2506	I	urine	**131**	**D**	>32	>32	S83L, D87G	S80I	S458A, **I529L**	-	*CTX-M-14*
1611	O	urine	**131**	**A**	>32	12	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*
1626	O	urine	**131**	**A**	>32	12	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*
1629	O	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*
1945	O	urine	**131**	**A**	>32	12	S83L, D87N	S80I, E84V	**I529L**	-	*CTX-M-15*
2069	O	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	-	-
2137	O	blood	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	-	*CTX-M-27*
2500	O	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*

Continued

Table 2. Continued

Isolate no	Patient status[a]	Specimen	ST-type[b]	AFLP type	MIC (mg/L)		*gyrA*	*parC*	*parE*	PMQR	ß-lactamase
					CIP[c]	LVX[d]					
2505	O	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*
2517	O	urine	**131**	**A**	>32	16	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	*CTX-M-15*
2574	O	urine	**131**	**A**	>32	>32	S83L, D87N	S80I, E84V	**I529L**	*aac(6')-Ib-cr*	-
2504	O	urine	156	u	>32	>32	S83L, D87N	S80I, E84G	WT	*aac(6')-Ib-cr*	-
2585	I	rectal swab	156	u	>32	>32	S83L, D87N	S80I, E84A	WT	-	*CTX-M-15*
1889	O	urine	167	u	>32	>32	S83L, D87N	S80I	S458A	*aac(6')-Ib-cr*	*CTX-M-15*
1941	O	urine	167	E	>32	>32	S83L, D87N	S80I	WT	-	-
2274	O	urine	167	E	>32	>32	S83L, D87N	S80I	WT	-	-
2218	O	blood	362	u	>32	>32	S83L, D87H	S80I	WT	*aac(6')-Ib-cr*	*CTX-M-55*
2418	I	urine	38	u	>32	>32	S83L, D87N	S80I, E84G	WT	*aac(6')-Ib-cr*	*CTX-M-15*
2525	O	urine	88	u	>32	>32	S83L, D87N	S80R	S458A	-	-
1793	I	rectal swab	453	u	>32	>32	S83L, D87N	S80I	WT	-	*TEM-28*
1502	O	urine	648	B	>32	>32	S83L, D87N	S80I	S458A	*aac(6')-Ib-cr*	-
2230	O	urine	648	B	>32	>32	S83L, D87N	S80I	S458A	-	-
2556	I	urine	648	B	>32	>32	S83L, D87N	S80I	S458A	*aac(6')-Ib-cr*	-
2412	O	urine	1193	u	>32	>32	S83L, D87N	S80I	L416F	-	-
1946	I	urine	393	C	>32	>32	S83L, D87N	S80I	L416F	-	*TEM-52*
2404	I	sputum	393	C	>32	>32	S83L, D87N	S57T, S80I	L416F	*qnrA1*	*CTX-M-9a SHV-12*

[a]Patient status: I = inpatient; O = outpatient
*abd. drain = abdominal drain fluid
$thor. drain = thoracic drain fluid
[b]ST=sequence type as determined with MLST. [c]CIP=ciprofloxacin, [d]LVX=levofloxacin, [e]WT = wildtype, - indicates the absence of a PMQR or extend-spectrum ß-lactamase

An unexplained preponderance of an I529L mutation in *parE*, which is infrequently found by others, prompted us to further characterize these isolates by the use of AFLP and MLST in order to investigate the possible presence of a clone (Figure 1). Results not only showed that ST131 was the most prevalent type among our isolates, but also that the I529L mutation in *parE* was found exclusively in ST131 isolates, irrespective of AFLP type (A and D, Table 2). Thus, I529L was the most prevalent *parE* mutation in our collection. The closely related ST131 isolates of AFLP type A originated from inpatients as well as outpatients.

Only two patients had been admitted to the kidney transplant ward concurrently. Otherwise no epidemiological links between patients could be found. Transfer of a single isolate between the patients with ST131, AFLP type A, therefore seems highly unlikely.

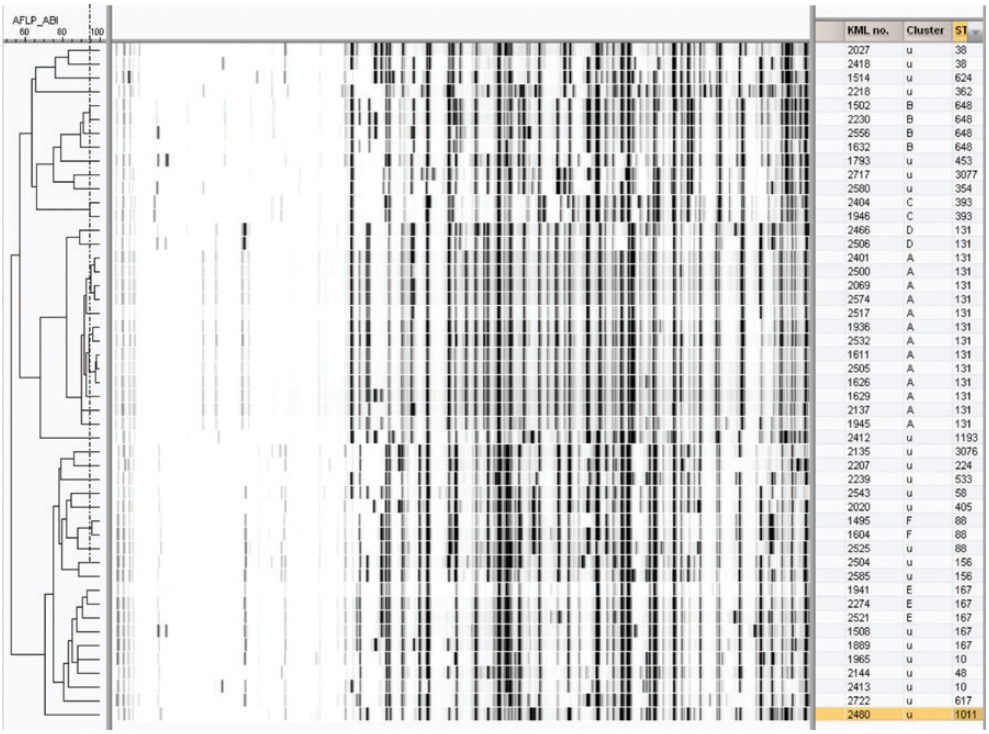

Figure 1. AFLP patterns and dendrogram of 49 cephalosporin- and fluoroquinolone-resistant *E. coli* isolates. Numbers in the horizontal axis indicate percentage similarity as determined by the Pearson correlation coefficient and unweighted pair group method with arithmetic averages. The vertical dotted line marks the isolate delineation level (90%). The KML no. on the right indicates the isolate ID no. The letters indicate the different clusters (A-F) or unrelated isolates (u). The different STs are indicated by numbers.

To investigate whether the I529L mutation was a single nucleotide polymorphism (SNP) of ST131, we sequenced *parE* of nine additional *E. coli* ST131 isolates. These isolates had recently been isolated from residents in the Leiden area after (not before) returning from travel outside Europe. The MICs of ciprofloxacin for these isolates ranged from 0.25 to > 32 mg/L. Eight of these nine isolates showed the I529L SNP, one isolate had a wildtype *parE*. Thus, it can be concluded that the I529L SNP in *parE* can be found in most, but not all ST131 isolates. The contribution of this SNP to resistance to fluoroquinolones is unclear.

The accumulation of resistance mechanisms in *E. coli* ST131 was also manifested by the presence of *aac(6')-Ib-cr* in 10/15 (66%) ST131 isolates, compared to 8/34 (23.5%) non-ST131 isolates. In addition, CTX-M ESBLs, mostly $bla_{\text{CTX-M-15}}$, were found in 11/15 (73%) ST131 isolates, compared to 14/34 (41%) non-ST131 isolates.

Until recently, data on the occurrence of this global ST131 clone in the Netherlands were absent. A study performed from January 2008 to December 2009 in the Rotterdam area, the Netherlands, found ST131 in patients with ESBL-producing *E. coli* isolates from bloodstream infections.[7] Taken these data together with the present study, it may be concluded that ST131 is well established in the Netherlands, at least in this part of the country.

Whereas in *E. coli* chromosomally encoded resistance determinants predominated, in *K. pneumoniae* resistance genes were mostly plasmid-mediated (Table 3). *Qnr* genes were found in 15/16 (94%) *K. pneumoniae* isolates and 5/16 (31%) showed *aac(6')-Ib-cr*. We identified *qnrA* mostly (n=11; 69%); then *qnrB* (n=5; 31%), followed by *qnrS* (n=1; 6%) The presence of PMQR genes in *K. pneumoniae* isolates is frequently associated with combinations of several plasmid encoded ß-lactamase genes, both ESBL and plasmid-mediated AmpC $bla_{\text{DHA-1}}$,[3] which was confirmed by our data.

Table 3. Distribution of fluoroquinolone resistance, amino acid substitutions and extended-spectrum ß-lactam resistance among *E. cloacae* (n=3) and *K. pneumoniae* (n=16) isolates from the LUMC

Species	Isolate no.	Patient status[a]	Specimen	MIC (mg/L)		*gyrA*	*parC*	*parE*	PMQR	ß-lactamase(s)
				CIP	LVX					
E. cloacae	2293	I	urine	0.50	0.75	S83Y	WT	WT	-	-
	2155	I	sputum	0.75	1	S83T D87G	WT	WT	*qnrA1*	*CTX-M-9, SHV-12*
	2503	I	wound	>32	>32	S83Y	WT	WT	-	*CTX-M-1,CTX-M-9*
K. pneumoniae	2288	O	urine	0.38	0.5	WT	WT	WT	-	-
	2437	O	urine	0.38	0.5	WT	WT	WT	*qnrA1, qnrS1*	*CTX-M-3, SHV-71*
	2222	I	bronchoalveolar fluid	0.38	0.75	WT	WT	WT	*qnrA*	*CTX-M-9, SHV-36*
	2261	I	bronchoalveolar fluid	0.38	0.75	WT	WT	WT	*qnrA1*	*CTX-M-14b*
	1867	I	sputum	0.50	1	WT	WT	WT	*qnrA*	*SHV-12*
	2166	I	urine	0.75	1.5	WT	WT	WT	*qnrA*	*SHV-12*
	2255	I	intravenous catheter tip	1.5	3	WT	WT	WT	*qnrA1*	*CTX-M-9, SHV-12*
	2192	I	sputum	2	3	D87Y	WT	WT	*qnrA1*	-
	1884	O	wound	3	0.50	WT	WT	WT	*qnrB1, aac(6')-Ib-cr*	*CTX-M-15*
	1503	I	wound	3	0.75	WT	WT	WT	*qnrB6, aac(6')-Ib-cr*	*CTX-M-3*
	1602	O	urine	3	0.75	WT	WT	WT	*qnrB1, aac(6')-Ib-cr*	*CTX-M-15*
	1788	I	urine	4	0.75	WT	WT	WT	*qnrA -like*	*SHV-12*
	2231	I	urine	16	4	WT	WT	WT	*qnrA, qnrB4*	*DHA-1, SHV-12*
	1811	I	intravenous catheter tip	>32	>32	S83I	S80I	WT	*qnrB4, aac(6')-Ib-cr*	*DHA-1*
	1888	O	urine	>32	6	S83F D87A	S80I	WT	*qnrA, aac(6')-Ib-cr*	*CTX-M-14, SHV-28*
	2195	I	sputum	>32	4	WT	WT	WT	*qnrA, qnrB4*	*DHA-1, SHV-12*

[a]Patient status: I = inpatient; O = outpatient
[b]CIP=ciprofloxacin, [c]LVX=levofloxacin
[d]WT = wildtype; - indicates the absence of a PMQR or extended-spectrum ß-lactamase

We identified *qnrA1* in one out of three *E. cloacae* isolates. It was found in an isolate with a modest reduction in susceptibility, with an MIC of ciprofloxacin of 0.75 mg/L and of levofloxacin of 1 mg/L. This PMQR determinant was described in the Netherlands in *E. cloacae* in 2001,[8] when it caused a large nosocomial outbreak. One of the three *E. cloacae* isolates was highly resistant to both fluoroquinolones (MICs > 32 mg/L), but no resistance mechanisms were found.

Finally, we noted the absence of the *qepA* gene among all isolates studied. This new PMQR determinant is still very rare in Europe, whereas several surveys among *Enterobacteriaceae* of human origin in China have reported a prevalence of 6%.[9]

In conclusion, we found *E. coli* ST131 to be the predominant type among our fluoroquinolone resistant isolates. A high number of resistance mechanisms have accumulated in our ST131 isolates, among which the I529L mutation in *parE* that was unequivocally associated with ST131. In contrast to *E. coli*, fluoroquinolone resistance in *K. pneumoniae* was associated with plasmid-encoded mechanisms much more than with chromosomal mutations.

Funding

This research was supported by the Department of Medical Microbiology of the Leiden University Medical Center, the Netherlands.

Transparency declarations

None to declare

2.5 References

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CHAPTER 3

Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of *Escherichia coli*

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Microbial Drug Resistance 2013 Dec;19(6):469-476

3.1 Abstract

Objectives: Resistance to ciprofloxacin in *Escherichia coli* is increasing parallel to increased use of fluoroquinolones both in the Netherlands and in other European countries. The objective was to investigate the contribution of active efflux and expression of outer membrane proteins in a collection of clinical *E. coli* isolates collected at a clinical microbiology department in a Dutch hospital.

Methods: Forty-seven *E. coli* isolates with a wide range of ciprofloxacin minimum inhibitory concentrations (MICs) and known mutations in the quinolone resistance-determining region (QRDR) were included. A fluorometric determination of bisbenzimide efflux was used with two different efflux pump inhibitors (EPIs). These results were compared to quantitative reverse transcription polymerase chain reaction (qRT-PCR) for the expression levels of *acrA, acrB, tolC, yhiV* and *mdfA* efflux pump genes and the outer membrane proteins *ompF* and *ompX*.

Results: Six isolates (12.7%) showed increased efflux. Although in 35 isolates (76%) overexpression of ≥1 efflux pump genes using qRT-PCR was present. Only the combined overexpression of *acrAB-tolC* and *mdfA* correlated with the phenotypic efflux assay using glucose/Carbonyl Cyanide m-Chloro-Phenylhydrazone (CCCP) with glucose.

Conclusion: Thus, efflux was involved in ciprofloxacin resistance in a limited number of *E. coli* isolates collected at a clinical microbiology department in a Dutch hospital complementing other resistance mechanisms.

3.2 Introduction

The use of fluoroquinolones has increased and correspondingly resistance to these drugs emerged significantly across Europe since 2001, with levels ranging from 7% to 53% in *Escherichia coli* in 2007.[1] Between 2003 and 2007, resistance rates of *E. coli* to ciprofloxacin have risen from 8% to 14% in the Leiden University Medical Center (LUMC), a tertiary care hospital in the Netherlands.

In *E. coli* fluoroquinolone resistance usually occurs due to stepwise mutations in the quinolone resistance determining regions (QRDRs) of the DNA gyrase genes (*gyrA* and *gyrB*) and/or topoisomerase IV genes (*parC* and *parE*) and the presence of plasmid-mediated quinolone resistance determinants (PMQRs). While mutations in the QRDRs are required to achieve a clinical level of resistance, decreased expression of the outer membrane protein (OMP) *ompF* or increased expression of *ompX,* as well as increased efflux pump activity have been shown to contribute to fluoroquinolone resistance. 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. These efflux systems are well characterized and play a major role in *E. coli*.[2] The overexpression of these efflux pump genes is related to antibiotic resistance in clinical isolates.[3,4]

A variety of phenotypic methods have been used to identify active efflux systems in *E. coli*.[5-13] Most of these studies have utilized ethidiumbromide as a substrate. The bisbenzimide dye Hoechst 33342 is also widely used to characterize efflux pump activity in different species, such as *Lactococcus lactis* and *E. coli*.[14,15] This fluorescent marker has several advantages over ethidiumbromide, such as a high quantum yield, and it is less toxic.

A recently described 96-wells plate fluorescence assay allows for a rapid, high throughput evaluation of efflux activity in clinical isolates. Bisbenzimide accumulation and efflux can be studied using bisbenzimide as a fluorescent dye and Carbonyl Cyanide m-Chloro-Phenylhydrazone (CCCP) and Phe-Arg ß-naphtylamide (PAßN) as efflux pump inhibitors (EPIs).[16] The difference in fluorescence between the steady states of bisbenzimide accumulation in the absence and presence of an EPI reveals the contribution of active efflux in a single isolate. However, there is no

uniformity in the interpretation of the results, which makes it difficult to compare the contribution of active efflux between isolates from different studies. Another limitation of phenotypic efflux assays is that they do not provide information about the expression levels of the genes involved in efflux. Most studies that have investigated the contribution of active efflux in *E. coli* isolates use either phenotypic efflux assays[7,8,11,17,18] or quantitative real-time polymerase chain reaction (qRT-PCR).[3,4,19-21] A combined phenotypic and genotypic analysis of efflux pump activity in *E. coli* has been described in a limited number of studies.[6,22,23,24] The purpose of the present study was to investigate the contribution of increased efflux activity and decreased influx to fluoroquinolone resistance in clinical *E. coli* isolates. A fluorometric determination of bisbenzimide efflux, with CCCP and PAßN as EPIs, was used and compared to qRT-PCR for the expression levels of *acrA, acrB, tolC, yhiV* and *mdfA* efflux pump genes and the outer membrane proteins *ompF* and *ompX*.

3.3 Materials and Methods

3.3.1 Bacterial isolates

Forty-seven clinical *E. coli* isolates from a previous study were obtained from different inpatients and outpatients from the Leiden University Medical Center (LUMC), the Netherlands, between January and December 2008. Nonrepetitive isolates with resistance to ciprofloxacin MIC of ≥ 0.125 mg/L were included. All isolates showed ≥ 1 mutation in the quinolone resistance determining regions (QRDRs) of *gyrase A*, *parC* and/or *parE*. The plasmid-mediated quinolone resistance (PMQR) gene *aac(6')-Ib-cr* was present in 17 of the isolates. Isolate no. 2404 contained a *qnrA1* gene. *E. coli* ATCC 25922 and four fully susceptible *E. coli* isolates were included as negative controls.

3.3.2 Susceptibility testing

MICs of ciprofloxacin (CIP) were determined using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guideline. MICs were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/). *E. coli* isolates resistant to at least three different drug classes were defined as multidrug resistant (MDR).

3.3.3 Bisbenzimide accumulation and efflux assays

To determine active efflux, a semi-automated fluorometric method with bisbenzimide as a fluorescence marker was used as previously described. Briefly, cultures were grown in Mueller-Hinton broth to an OD_{600} of 0.6. Bacterial cells were collected by centrifugation at 13,000 rpm and resuspended in PBS (1 mL). The OD of all suspensions was adjusted to 0.3. Aliquots (0.18 mL) were pipetted in a black 96-well microtiter plate (flat-bottomed, Greiner Bio-One, Frickenhausen, Germany). The bacterial cells were added in the format in duplicate: columns 1-10 isolates with PBS; column 11-12 heat-inactivated cells (10 min 90 °C). After adding 45µl bisbenzimide (25 µM), fluorescence was measured in a microtiter plate reader (Mithras LB 940 Berthold Technologies, Bad Wilbad, Germany). The fluorescence was acquired in cycles of 60 seconds at 37°C. Fluorescence during influx was measured using an excitation filter of 350 nm and an emission filter of 460 nm over a 13-min period. The relative fluorescence at each time point was calculated in percentages, using the fluorescence values of the heat-inactivated cells divided by the fluorescence signals obtained in the test isolate. These data were plotted on a graph. The microtiter plate reader was stopped after 13 min to add: (a) PBS (blank), (b) PBS containing glucose as an energy source (final concentration of 0.4%) to measure efflux activity, (c) PBS containing glucose (0.4%) with 172.4 µM PAßN or (d) 38.8 µM CCCP to inhibit efflux. All tests were performed in duplicate. Fluorescence during efflux was measured using an excitation filter of 350 nm and an emission filter of 460 nm. Intrinsic efflux activity was determined using the reference strain *E. coli* ATCC 25922 and four fully susceptible *E. coli* isolates. Efflux activity was expressed as the area between curves (ABC) which was calculated by subtracting the relative fluorescence in the wells containing CCCP/PAßN from that in the wells with glucose at each time point. For these five isolates, the mean and standard deviation (SD) of intrinsic efflux activity was calculated. Efflux was considered to be increased at 1.5x the SD above the mean of intrinsic efflux activity in the susceptible isolates.

3.3.4 RNA extraction and quantitative realtime-PCR (qRT-PCR)

Several colonies from overnight cultures were resuspended in PBS to make suspension of 0.5 McFarland. The suspension was diluted 1:1000 in trypticase soy broth (Beckton Dickinson B.V., Breda, The Netherlands) and incubated at 37°C with shaking (150 rpm). The cultures were grown to a mid-log growth phase, which corresponded to an OD_{600} of 0.3.

Total RNA was extracted using RNeasy kit (Qiagen Benelux B.V, Venlo, the Nederlands) according to the manufacturer's instructions. Genomic DNA was removed by DNase I kit (Invitrogen, Breda, the Netherlands). The concentrations and purity of the resulting RNA samples were measured at 260nm using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

Relative quantification of gene expression was performed by using the OneStep RT-PCR Kit (Qiagen Benelux B.V, Venlo, the Nederlands) with SYBR green (stock 10.000x Sigma-Alderich, Zwijndrecht, the Netherlands) in a CFX96™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands). The genes included in the analysis were *acrA, acrB, tolC, mdfA, yhiV*, *ompF* and *ompX*; glyceraldehyde 3-phosphate dehydrogenase (*gapA*) was used as a reference marker.

For primer design, an alignment of gene sequences in GenBank® was made using the AlignX program (Vector NTI Advance 11, Invitrogen). All primer sequences and annealing temperatures are shown in Table 1.

Table 1. Primer sequences used in this study[a]

Target gene	Primer direction[b]	Sequence (5'- 3')	Annealing temp (°C)	Amplicon length (bp)
gapA	F R	GGCCAGGACATCGTTTCCAA TCGATGATGCCGAAGTTATCGTT	60	100
acrA	F R	CTTAGCCCTAACAGGATGTG TCTTTGAAATTACGCTTCAGG	60	189
acrB	F R	AACGTACRCAGAAAGTGCTC TAATCGCTTCAACTTTGTTTTC	60	183
tolC	F R	GCGCCAGATCACCGGTAAC GTCCTGGCTCAAGCGTGC	60	153
yhiV	F R	CCGTACCGGTGGTTATTCTC ATCGATTTATGCGTCGCTTC	60	193
mdfA	F R	CATTGGCAGCGATCTCCTTT TATAGTCACGACCGAGTTCTTTC	60	102
ompF	F R	GAACTTCGCTGTTCAGTACC CGTACTTCAGACCAGTAGCC	60	209
ompX	F R	ACCTGAAATACCGCTATGAA TCAGTGGTCTGGAATTTACC	60	208

[a]All primers were designed in this study
[b]F, forward; R, reverse

All primers were synthesized by Biolegio B.V., Nijmegen, the Netherlands. The RT-PCR mixture (50 µl) contained 1.2 µl of both forward and reverse gene-specific primers, 2 µl of dNTPs (10mM), 10 µl of 5xQiagen OS RT-PCR buffer, 1.5 µl of 2.5x SYBR green, 1 µg of template RNA, 2 µl of Qiagen OS RT-PCR enzyme mix.
Each sample was placed on a 96-well plate (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) and subjected to one-step reverse transcription at 50°C for 30 min for cDNA synthesis, 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30s and extension at 72°C for 30s. PCR cycling was followed by melting curve analysis of 55-95°C (temperature transition rate of 0.5°C s^{-1}).
In order to assess relative gene expression levels, cycle threshold (CT) values normalized against the housekeeping gene (*gapA*) were calculated and compared with those for *E. coli* ATCC 25922 using the $2^{-\Delta\Delta CT}$ formula. Overexpression was defined as ≥ 1.5 fold increase in the expression of the gene.

3.3.5 Statistical analysis

Data analysis was performed using SPSS (version 20.0, IBM Corp, New York, USA). Student's t-test was performed at a 5% level of significance (one-tailed, unpaired). The strength of a correlation between two variables (effect size) was calculated using the Kendall's tau *b*. This is a statistic method to measure the association between measured quantities.

3.4 Results

3.4.1 Antimicrobial susceptibility testing

The MICs of ciprofloxacin of the 47 clinical *E. coli* isolates varied from 0.5 to 1024 mg/L. Forty-two isolates (89%) were classified as multidrug resistant (Table 2). The MICs of bisbenzimide and the efflux pump inhibitors (EPI): PAßN and CCCP (Sigma-Aldrich, Zwijndrecht, the Netherlands) were determined for all isolates and the quality control strain *E. coli* ATCC 25922 using the agar dilution method in accordance with CLSI guidelines. Based on these results a concentration of 25 µM for bisbenzimide, 38.8 µM for CCCP and 172.4 µM for PAßN were chosen.

Table 2. Overview of antimicrobial susceptibility, mutations, area between the curves and gene expression of efflux pump genes and outer membrane proteins

					Area between the curves		Overexpression of efflux pump genes[g]				
Isolate	**CIP mg/L[a]**	**MDR[b]**	**No of QRDR mutations[c]**	**PMQR[d]**	**CCCP[e]**	**PaßN[f]**	***acrAB-tolC***	***yhiV***	***mdfA***	***ompX* over-expression[h]**	***ompF* down-regulation[i]**
ATCC 25922	0.008	-	ND[j]	ND	2.02	1.60	-	-	-	-	-
Q84	0.016	-	ND	ND	2.33	1.49	-	-	-	-	+
Q82	0.016	-	ND	ND	2.34	1.66	-	-	-	-	+
Q80	0.016	-	ND	ND	2.43	1.31	-	-	-	-	+
Q83	0.016	-	ND	ND	2.51	0.72	-	-	-	-	+
2027	0.5	-	3	-	1.14	0.96	-	+	+	+	+
2144	0.5	+	1	-	2.43	0.71	+	+	+	+	+
2466	1	+	3	-	2.54	1.12	-	-	+	-	+
1508	4	+	3	-	0.16	1.47	-	-	-	+	-
2521	8	+	3	-	1.32	0.59	-	-	-	-	+
2717	8	+	4	-	1.80	1.56	+	+	+	-	+
2543	8	+	3	-	1.83	1.29	-	-	-	-	+
2580	8	+	4	-	1.87	1.23	-	-	+	-	+
2480	8	+	3	-	1.91	0.97	-	-	+	-	+
1495	8	-	3	-	2.31	1.21	-	-	+	+	-
2239	8	+	4	-	2.45	1.73	-	+	+	+	-
1936	16	-	5	+	1.32	1.01	-	-	+	+	+
2020	16	+	3	+	1.69	0.72	-	-	+	+	+
1604	16	+	3	-	1.84	0.72	+	-	+	+	-
1946	16	+	4	-	1.87	0.58	-	-	-	+	+
1965	16	+	3	-	1.95	1.63	-	-	-	-	-
2506	16	+	5	-	2.45	1.13	-	-	-	+	+
1514	16	+	2	-	**2.68**	1.29	-	+	-	+	+
1945	32	+	5	-	1.18	1.07	+	+	+	-	-
2207	32	+	4	-	1.71	0.63	+	+	+	+	+
2574	32	+	5	+	1.84	0.65	-	-	+	-	+
2722	32	+	4	-	1.99	0.64	+	-	+	+	-
2525	32	+	4	-	2.07	**2.80**	-	-	+	-	+
2230	32	-	4	-	2.13	0.23	-	+	+	-	+
2585	32	+	4	-	2.16	0.99	-	-	-	-	+
2069	32	+	5	-	2.24	0.97	+	+	+	+	-
2274	64	+	3	-	0.85	0.64	-	+	+	+	-
1941	64	+	3	-	1.58	1.01	+	-	-	-	-

Continued

Table 2. Continued

Isolate	CIP mg/L[a]	MDR[b]	No of QRDR mutations[c]	PMQR[d]	Area between the curves		Overexpression of efflux pump genes[g]				
					CCCP[e]	PaßN[f]	*acrAB-tolC*	*yhiV*	*mdfA*	*ompX* over-expression[h]	*ompF* down-regulation[i]
2504	64	+	4	+	1.86	0.15	-	-	-	-	-
2412	64	+	4	-	1.93	0.75	+	+	+	+	+
2413	64	+	4	-	2.19	0.79	+	+	+	-	-
1611	64	+	5	+	2.20	1.22	+	-	+	+	+
2404	64	+	5	+	2.34	0.88	+	-	+	-	-
1632	64	+	4	+	2.52	0.94	+	-	+	-	+
2556	64	+	4	+	2.55	0.93	+	+	+	-	+
2500	128	+	5	+	1.31	0.43	-	-	+	-	+
2505	128	+	5	+	1.41	0.44	-	+	+	-	+
2517	128	+	5	+	1.45	0.59	-	-	-	-	+
1626	128	+	5	+	2.20	0.30	+	-	+	+	+
2532	128	+	5	-	2.54	0.93	-	-	-	-	+
1629	128	+	5	+	**3.07**	**1.97**	-	-	+	+	+
2401	128	+	5	+	**3.11**	0.48	+	+	+	-	+
1502	128	+	4	+	**3.11**	0.76	+	-	+	+	+
1793	1024	+	3	-	1.56	0.66	-	-	-	+	+
2218	1024	+	3	+	1.65	0.49	-	+	-	-	-
1889	1024	+	4	+	2.14	1.12	+	-	+	+	+
2418	1024	-	4	+	**3.08**	0.73	-	+	+	-	+

[a]CIP mg/L; MIC of ciprofloxacin as determined with agar dilution
[b]MDR = multidrug resistant: +, if the isolate was resistant to ≥3 different drug classes; -, if the isolate was resistant to <3 different drug classes
[c]QRDR = quinolone resistance determining region. The numbers indicate the number of mutations in the quinolone resistance determining regions of *gyrA*. *parC* and *parE* as determined in a previous study (Paltansing *et al*., 2012)[28]
[d]PMQR = plasmid-mediated quinolone resistance determinant; +. present; -. absent.
[e]and [f]= area between the curves in phenotypic efflux activity in the presence of glucose subtracted from glucose with the addition of the EPIs CCCP or PAßN. The numbers in bold indicate that the ABC was 1.5x SD above the mean for increased phenotypic efflux activity.
[g]as determined by qRT-PCR; +, upregulated gene; -, no upregulation.
[h]as determined by qRT-PCR; +, upregulated gene; -, no upregulation.
[i]as determined by qRT-PCR; +,downregulated gene; -, no downregulation.
[j]ND = not determined

3.4.2 Efflux activity evaluation; accumulation and efflux of bisbenzimide

Table 2 shows the efflux activity, expressed as ABC, for all isolates tested, including the fluoroquinolone-susceptible isolates. The reference strain *E. coli* ATCC 25922 showed inhibition of efflux activity, which was affected by CCCP and less so by PAβN (Figure 1). This was considered as the intrinsic efflux activity of *E. coli* ATCC 25922. The four fully susceptible *E. coli* isolates showed similar results. For the *E. coli* ATCC 25922 and the four susceptible *E. coli* isolates, the ABC between glucose/CCCP with glucose ranged from 2.02-2.51, with a mean of 2.33 (SD: 0.18). For PAβN, the ABC between glucose/PAβN with glucose with ranged from 0.72-1.66, with a mean proportion of 1.36 (SD: 0.38). Efflux was considered to be increased, if the ABC between glucose/CCCP with glucose was ≥ 2.60 or glucose/ PAβN with glucose was ≥ 1.93.

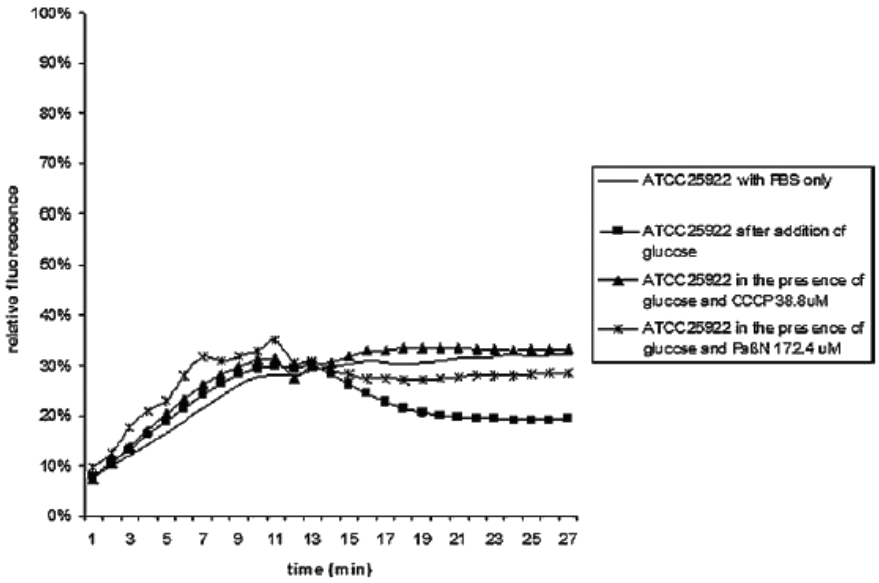

Figure 1. Accumulation (0-13 min) and efflux activity (13-30 min) of bisbenzimide by *E. coli* ATCC 25922 in the presence of glucose and in the presence of the optimum concentration of the EPIs CCCP and PAβN at 37°C.

One of the fluoroquinolone resistant isolates (no 1629) showed inhibition of active efflux in the presence of CCCP as with PAβN (Figure 2). Active efflux in the presence of CCCP only was found in four isolates (9%). In the remaining 33 isolates no increased efflux was found with CCCP. One additional isolate (2525) showed increased efflux with PAβN only.

One isolate showed an ABC of 1.63 (no.1965), which was higher than the intrinsic efflux activity of the reference strain ATCC 25922, but below the level of active efflux. The remaining isolates showed an absence of an inhibitory effect in the experiments with PAβN. There was no association between the increasing MICs values, target alterations or an MDR phenotype and the presence of increased efflux activity.

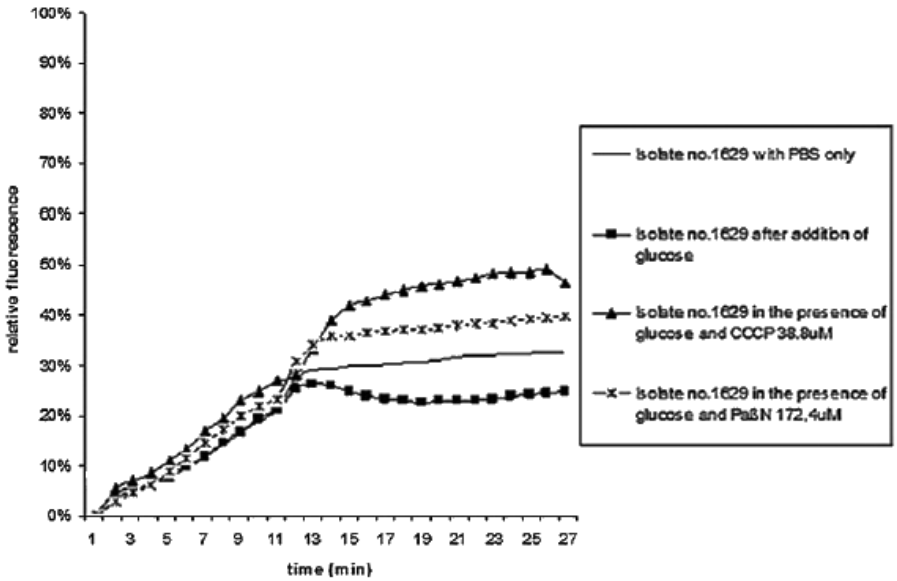

Figure 2. Accumulation (0-13 min) and efflux activity (13-30 min) of bisbenzimide by *E. coli* isolate 1629 in the presence of glucose and in the presence of the optimum concentration of the EPIs CCCP and PABN at 37 °C.

3.4.3 Analysis of quantification of RNA expression by qRT-PCR

Table 2 also shows the expression level of the efflux pump genes for all isolates tested, including the fluoroquinolone-susceptible isolates. Overexpression of ≥1 efflux pump genes was present in 35 isolates (76%). Eighteen isolates showed overexpression of *acrAB-tolC*, 33 isolates showed overexpression of *mdfA* and 17 isolates of *yhiV*. Nine isolates showed expression of three efflux pumps, *acrAB-TolC*, *mdfA* and *yhiV*. Eleven isolates showed no overexpression of any of the efflux pump genes tested. No clear correlation was found between efflux and MICs of ciprofloxacin. Combining (a) the number of QRDR mutations and (b) the presence of plasmid-mediated quinolone resistance determinants with genotypic efflux results did not reveal any correlation. Decreased expression of *ompF* was found in 33 isolates, respectively. Twenty-two isolates showed an upregulation of *ompX*.

3.4.4 Correlation between phenotypic efflux activity and overexpression of efflux pump genes

Nine isolates showed overexpression of all three efflux pump genes investigated (group 1), eight isolates showed *acrAB-tolC* and *mdfA* overexpression (group 2), fifteen isolates showed either *yhiV* or *mdfA* overexpression (group 3) and eleven isolates showed no genotypic overexpression (group 4). A significant correlation between the ABC of glucose/CCCP with glucose and the overexpression of *acrAB-tolC* with the *mdfA* efflux pump gene only (group 2 isolates; p=0.018, Kendall's tau *b*=0.343). The ABC between glucose and PAßN with glucose showed no significant correlations with overexpression with any of the groups tested (p=0.198).

3.5 Discussion

In the present study, the contribution of active efflux and expression of OMPs to fluoroquinolone resistance in a collection of clinical *E. coli* isolates was investigated using a combined phenotypic and genotypic approach.

Efflux pumps are expressed at a basal level in susceptible isolates and can be up-or downregulated in resistant isolates. The current study compared efflux in clinical fluoroquinolone resistant isolates to that in the reference wildtype *E. coli* ATCC

25922 strain with functional efflux genes. In other studies on phenotypic efflux laboratory generated mutant *E. coli* strains have been used to describe active efflux in clinical isolates.[7,22,25] Based on phenotypic tests, six of 47 (12.7%) resistant isolates showed increased efflux activity.

The occurrence of increased efflux seems to be variable in clinical *E. coli* isolates.[6,22,26] Christiansen *et. al*.[6] found an indication of efflux in 76.7% of 60 clinical *E. coli* isolates with an MIC for ciprofloxacin ranging from 0.06-512 mg/L using a modified broth microdilution assay with the use of PAßN and 1-(1-naphtylmethyl) piperazine (NMP) as EPIs. Karczmaraczyk *et. al*.[22] used ethidiumbromide accumulation experiments to determine the activity of the AcrAB-TolC efflux pump. They found increased phenotypic efflux in 8/11 ciprofloxacin resistant *E. coli* isolates from food-producing animals using PAßN. Amabile-Cuevas *et al*.[27] on the other hand using a broth microdilution assay with PAßN found increased efflux in 12% of their clinical isolates, a percentage similar to ours. However, comparison of efflux results in clinical isolates from different studies remains difficult as different phenotypic methods and/or selection criteria have been used to detect phenotypic efflux activity. In the absence of established criteria to define active efflux in clinical isolates, we used the calculation of the ABC.

The fact that most isolates in this collection already showed a high number of accumulated QRDR mutations could also account for the lack of active efflux.[28] A recent publication of Singh *et al*.[29] investigated the contributions and interplay of fluoroquinolone resistance mechanisms at various time points during resistance development in a *E. coli* laboratory standard strain (MG1655) and two isogenic derivatives (Δ*acrAB* and Δ*acrR*). Interestingly, they found that in the initial stages of emerging resistance, efflux pump overexpression occurred before *gyrase* mutations and that once mutations were found, the pump activity had returned to normal levels. The isolates in our collection had accumulated high numbers of QRDR mutations already and may have had their efflux activity already tuned down. However, the six isolates with active efflux were amongst those with a high number of QRDR mutations and high MICs.

Of more than 40 putative transporters in *E. coli*, efflux pumps of the RND family, which are inhibited by PAßN, affect fluoroquinolone MICs when expressed with their own promotors under laboratory growth conditions.[21,30] In both canine/feline and human *E. coli* isolates PAßN has been shown to reverse or reduce the fluoroquinolone MICs.[8,23,31]

In contrast, our results show that PAßN did not have much effect on the extrusion of bisbenzimide, suggesting that active efflux by RND efflux pumps did not contribute to ciprofloxacin resistance in the majority of our clinical isolates.

Using the genotypic approach, relative quantification of efflux gene expression levels was normalized against a fully susceptible reference strain, *E. coli* ATCC 25922, as is customary.[3,4,23] In the present collection, various combinations of gene expression levels were found. This included: (a) alterations in the OMPs and overexpression of efflux pump genes; (b) no OMP alterations but only overexpression of efflux genes (c) only OMPs alterations without overexpression of efflux genes (d) no OMP alterations or overexpression of efflux genes. The results of the OMP alterations showed that outer membrane influx resistance is also involved. Although a significant decrease of bisbenzimide influx was not found. The lack of correlation may be explained by the fact that bisbenzimide uses other routes to enter the bacterial cell.

Convergence between the phenotypic and genotypic method was observed in isolates with overexpression of *acrAB-tolC* and *mdfA* and the ABC of glucose/CCCP with glucose in the phenotypic efflux assay. In *E. coli*, the tripartite *acrAB-tolC* system is considered to be the most important efflux pump.[32] Swick *et al.* investigated fluoroquinolone susceptible and MDR *E. coli* isolates and found that overexpression of *acrAB* correlated with an MDR phenotype.[4] In laboratory-generated mutants, overexpression of the efflux pumps *acrAB* or *mdfA* have been shown to cause three to six-fold increase in fluoroquinolone resistance.[21] Previous studies also reported that the simultaneous overexpression of *acrAB* and *mdfA* results in synergistic increases in resistance to fluoroquinolones.[21,33] Our results show that simultaneous overexpression of these efflux pumps can be detected using the phenotypic assay with glucose and CCCP as inhibitor, which can be used as a screening tool to detect active efflux. In the remaining isolates, no correlation was found between phenotypic detection of active efflux or MICs with mRNA gene expression levels of the efflux genes investigated. Quantification of gene expression levels does not necessarily provide information on the final expression of the gene product and its functionality. Although mRNA expression of multidrug efflux pumps correlated well with protein expression levels using laboratory strains of *Pseudomonas aeruginosa*,[34] clinical isolates also showed discrepancies between mRNA expression and the corresponding protein. Molecular mechanisms causing these discrepancies include the half-lives of specific mRNAs or specific protein[35] and the post-transcriptional control of the protein translation rate.[36]

In conclusion, active efflux was not the predominant resistance mechanism in clinical resistant *E. coli* isolates from our hospital. Living organisms have the capacity to adapt to changing environments. Increased efflux is not the only mechanism involved in the physiological adaptation process. There is a well regulated and coordinated interplay of multiple mechanisms of resistance. The determination of active efflux under defined experimental conditions may contribute to a better understanding of the contribution of active efflux in clinical MDR *E. coli* isolates.

Acknowledgements

We thank Yi Lee and Jouraima Ras for their excellent technical assistance and dr. Wil Goessens and dr. Miguel Viveiros for their helpful discussions.

Disclosure statement

The authors have no competing interests to disclose.

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CHAPTER 4

Increased expression levels of chromosomal AmpC ß-lactamase in clinical *Escherichia coli* isolates and their effect on susceptibility to extended-spectrum cephalosporins

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Microbial Drug Resistance 2014 Sep 4 (epub ahead of print)

4.1 Abstract

Objectives: Forty-nine clinical *Escherichia coli* isolates, both extended-spectrum ß-lactamase (ESBL)-negative and ESBL-positive, were studied to investigate whether increased AmpC expression is a mechanism involved in cefoxitin resistance and if this influences 3rd generation cephalosporin activity.

Methods: Nine of 33 (27.2%) cefoxitin-resistant (minimum inhibitory concentration (MIC) > 8 mg/L) isolates showed hyperproduction of chromosomal AmpC (c-AmpC) based on (i) at least two positive tests using AmpC-inhibitors, (ii) mutations in the promoter/attenuator regions, and (iii) a 6.1 to 163-fold increase in c-*ampC* expression by qRT-PCR.

Results: In ESBL-negative isolates, MICs of ceftazidime and cefotaxime were mostly above wildtype level, but below the S/I breakpoint (EUCAST guideline), except for one isolate with MICs of 4 mg/L. No plasmid-mediated AmpCs were found. Periplasmic extracts of nine c-AmpC hyperproducers were pre-incubated with or without cefuroxime or ceftazidime and analyzed by SDS-PAGE. Cefuroxime and ceftazidime were stable to hydrolysis but acted as inhibitors of the enzyme. None of these isolates showed loss of porins.

Conclusion: Thus, cefoxitin-resistance has low specificity for detecting upregulated c-AmpC production. c-AmpC hyperproducing *E. coli* is mostly still susceptible to 3rd generation cephalosporins but less than wildtype *E. coli*. Surveillance of cefoxitin-resistant *E. coli* to monitor developments in the activity of 3rd generation cephalosporins against c-AmpC hyperproducers is warranted.

4.2 Introduction

Although extended-spectrum ß-lactamases (ESBLs) are a major cause of resistance to cephalosporins in *Escherichia coli*, the involvement of AmpC enzymes is increasingly reported.[1] All isolates of *E. coli* carry a chromosomal *ampC* gene (c-*ampC*). In *Enterobacter*, *Citrobacter*, *Morganella*, and *Serratia*, c-*ampC* expression is usually inducible by ß-lactam antibiotics. Induction of c-*ampC* expression is a complex mechanism involving the regulatory genes *ampR, ampD* and *ampG*.[2] Unlike in the other members of the *Enterobacteriaceae*, c-*ampC* is not inducible in *E. coli* because the *ampR* regulatory gene is lacking. However, expression of c-*ampC* is influenced by mutations in the promoter and attenuator regions, which may result in constitutive hyperproduction of c-AmpC.[3,4] Hyperexpression of c-*ampC* in clinical *E. coli* isolates has been described with varying susceptibility to 3rd-generation cephalosporins.[4-6] Besides c-AmpC hyperproduction, *E. coli* can acquire transferable plasmid-encoded AmpC (p-AmpC) enzymes.[7,8]

In our laboratory, the resistance rate of *E. coli* isolates to ceftazidime corresponds to the ESBL rate (approximately 8% in 2011), but cefuroxime resistance is approximately twice as high. Cefuroxime resistance in ESBL-negative *E. coli* isolates is usually accompanied by cefoxitin resistance, which is suggestive for AmpC production. Instead of cefuroxime, which is our first-line antibiotic, ceftazidime is then the cephalosporin of choice in our hospital.

To investigate whether increased AmpC expression is involved in cefuroxime and cefoxitin resistance in these ESBL-negative isolates and if this influences ceftazidime activity, we investigated a collection of *E. coli* isolates with various combinations of cephalosporin resistance. In addition, all isolates were phenotypically and genotypically characterized for AmpC production. As other mechanisms, such as increased efflux and decreased expression of outer membrane proteins (OMPs), also contribute to cefuroxime resistance in ESBL-negative *E. coli* isolates,[9] the OMPs of different isolates were analyzed.

4.3 Material and methods

4.3.1 Bacterial isolates and antimicrobial susceptibility testing

Between July 2008 and January 2010, nonreplicate clinical isolates of *E. coli* that were either ESBL-producers or showed resistance to at least three different categories of antimicrobial agents (fluoroquinolones, aminoglycosides, ß-lactams, cotrimoxazole) were collected at the Department of Medical Microbiology of the Leiden University Medical Center (LUMC), Netherlands.

From this collection, we selected 22 nonreplicate ESBL-negative *E. coli* isolates that showed resistance to cefoxitin (MIC > 8 mg/L) and cefuroxime. We added a panel of cefoxitin resistant, ESBL-positive isolates (n=4); cefoxitin resistant, cephalosporin susceptible isolates (n=7) and cefoxitin susceptible, ESBL-positive isolates (n=11) to investigate the role of AmpC-production in these subsets of isolates. Five cephalosporin-susceptible isolates were included as controls. Minimum inhibitory concentration (MICs) of cefoxitin, cefuroxime, ceftazidime and cefotaxime were determined using Etests (BioMérieux, France) according to the manufacturer's instructions. MICs were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/). Tests for synergy between cephalosporins and clavulanic acid were performed using combination disk diffusion test for ESBL detection (Rosco Diagnostica A/S, Denmark) according to the Clinical Laboratory Standards Institute's guidelines.[10]

4.3.2 Phenotypic AmpC testing

The AmpC Etest with cefotetan and cefotetan-cloxacillin (BioMérieux, France) was performed according to the manufacturer's instructions. Ratios of the MICs of cefotetan and cefotetan-cloxacillin of ≥ 8 are considered positive for AmpC production. The cefoxitin-boronic acid and cefoxitin-cloxacillin disk tests were performed using paper disks. In brief, 30-µg cefoxitin disks (Becton Dickinson, Germany) were supplemented with 20 µL of phenylboronic acid (stock solution 20 mg/mL) (Sigma-Aldrich, Netherlands) or with 20 µL of cloxacillin (stock 37.5 mg/mL) (Sigma-Aldrich). A test was considered positive for AmpC ß-lactamase production if the inhibition zone around the disk containing cefoxitin with an inhibitor was ≥ 4 mm larger than without the inhibitor.[11]

4.3.3 Real-time quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from cultures grown to mid-log growth phase using the RNeasy kit (Qiagen Benelux B.V, Venlo, Netherlands) according to the manufacturer's instructions. Genomic DNA was removed with the DNase I kit (Invitrogen, Breda, Netherlands). The expression level of the *ampC* gene and the reference gene *gapA* encoding glyceraldehyde 3-phosphate dehydrogenase was assessed by real-time qRT-PCR using the OneStep RT-PCR Kit (Qiagen) with SYBR green (10 000 x stock solution; Sigma-Aldrich). Primer sequences are listed in Table 1.

Table 1. Primer sequences used in this study

Target	Primer direction[a]	Sequence (5'- 3')	Annealing temp (°C)	Amplicon length (bp)	References
gapA	F R	GGCCAGGACATCGTTTCCAA TCGATGATGCCGAAGTTATCGTT	60	100	This study
ampC	F R	CCTCTTGCTCCACATTTGC CCCAGGTAAAGTAATAAGGTTTAC	60	1134	This study
ampC promoter/attenuator region	F R	GATCGTTCTGCCGCTGTG GGGCAGCAAATGTGGAGCAA	60	271	Corvèc *et al.*[b]

[a]F, forward; R, reverse
For primer design, an alignment of gene sequences in GenBank was made using the AlignX program (Vector NTI Advance 11, Invitrogen). The qRT-PCR mixture (50 µl) contained 1.2 µl (0.5 µM) of both forward and reverse primers (Biolegio B.V., Nijmegen, Netherlands), 2 µl of dNTPs (10 mM), 10 µl of 5 x OS RT-PCR buffer (Qiagen), 1.5 µl of 2.5 x SYBR green, 10 µl of template RNA (1:10 diluted), 2 µl of OS RT-PCR enzyme mix (Qiagen). Each sample was placed on a 96-well plate (Bio-Rad Laboratories B.V., Veenendaal, Netherlands) and subjected to one-step reverse transcription at 50°C for 30 min for cDNA synthesis, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR cycling was followed by melting curve analysis of 70-99°C (temperature transition rate of 0.5°C/s).

[b]As described by Corvèc S, Prodhomme A, Giraudeau C, Dauvergne S, Reynaud A, Caroff N. Most *Escherichia coli* strains overproducing chromosomal AmpC ß-lactamase belong to phylogenetic group A. *J Antimicrob Chemother* 2007;**60**: 872-876.

The *ampC* mRNA mean normalized expression was calibrated as fold differences using the mean normalized expression level of the reference *E. coli* strain ATCC 25922 as 1.0 using the delta-delta cycle threshold method as described by Livak *et al.*[12]

4.3.4 Chromosomal ampC gene and promoter/attenuator sequencing

For mutation analysis, the c-*ampC* gene and *ampC* promoter/attenuator region were amplified using primers as described in Table 1. Sequencing was performed at the Leiden Genome Technology Center (LGTC®, Leiden, Netherlands). Sequence analysis was performed using BioNumerics version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium).

4.3.5 Molecular detection of p-ampC ß-lactamase genes

A multiplex PCR was used for the detection of the six p-*ampC* gene families as described by Perez-Perez and Hanson.[8]

4.3.6 Isolation of cell fractions

Cell envelopes were isolated from bacteria grown overnight at 37°C in L-broth, which is composed of 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.002% thymine, pH 7.0.[13] Cells were disrupted by ultrasonication (Branson B-12 Heinemann, Schwäbisch Gmünd, Germany), and sarkosyl (2% end concentration) was added to the lysates to dissolve the inner membrane proteins. After 30 min incubation at room temperature, the insoluble OMPs were collected by centrifugation for 30 min at 16.000 × *g*.

For isolation of whole cell lysates, bacteria exponentially growing in L-broth were converted to spheroplasts as described.[14] Briefly, the cells were pelleted, washed in physiological salt solution and resuspended to 10^{10} cells/mL in 1 mL of 10 mM Tris-HCl (pH 8), 25% sucrose. Subsequently, 10 µL of lysozyme (20 mg/mL) and 2 mL of 1.5 mM EDTA (pH 7.5) were added, and the mixture was incubated on ice for 30 min. Whole cell lysates were then obtained by sonication of the spheroplasts after a freeze-thaw cycle.

To isolate the periplasmic fraction, 5 mM $MgCl_2$ (end concentration) was added to spheroplasts prepared as above. The spheroplasts were then removed by centrifugation for 1 min at 16 000 × *g* and the supernatant was used as periplasmic extract.

4.3.7 SDS-PAGE and Western blotting

Cell fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli *et. al*.[15] with 11% acrylamide, 0.2% SDS and 5 M urea in the running gel unless otherwise indicated. Proteins were either stained in the gels with Coomassie Brilliant Blue or transferred to nitrocellulose membranes by electroblotting.

The blots were incubated with either a polyclonal antiserum raised against the *E. coli* porin PhoE, which cross-reacts with the related porins OmpF and OmpC, [16] or an anti-AmpC antiserum (Aviva Systems Biology, San Diego, USA) and subsequently with alkaline phosphatase-conjugated goat anti-rabbit-IgG antiserum (BioSource International Inc, Camarillo, CA, USA).

The blots were then stained with 0.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate and 0.1 mg/mL nitroblue tetrazolium (Sigma-Aldrich) in 100 mM $NaHCO_3$, 1 mM $MgCl_2$ (pH 9.8) until colour developed.

4.3.8 ß-Lactamase assays

ß-Lactamase activity was determined in whole cell lysates of triplicate cultures using nitrocefin (Calbiochem, Merck KGaA, Darmstadt, Germany) as a chromogenic substrate.[17] Appropriate dilutions of the extracts in 1 mL of 10 mM HEPES, 5 mM $MgCl_2$ (pH 7.2) were incubated at room temperature with 0.05 mM nitrocefin and the initial rate of nitrocefin cleavage was measured by the change in optical density at 486 nm (OD_{486}). A change in OD_{486} corresponds to the degradation of 51 nmol nitrocefin and the measured activity was calculated back to the ß-lactamase activity of 10^8 or 10^9 cells.

To study the degradation of cefuroxime (Hikma Pharmaceuticals, London, United Kingdom) and ceftazidime (Fresenius Kabi, Bad Homburg, Germany), periplasmic extracts were incubated in 1 mL of 10 mM HEPES, 5 mM $MgCl_2$ (pH 7.2) with 50 nmol/mL of the antibiotics, and opening of the ß-lactam ring was measured during 5 min at 275 or 260 nm, respectively, using a Unicam UV1 spectrometer. The degradation of cefazolin (Hikma Pharmaceuticals) measured at 270 nm was used as a control.

Inhibition of ß-lactamase activity by cephalosporins was tested by incubating periplasmic extracts for 1 min with 0.005, 0.05, 0.5, 5 and 50 µM cefuroxime or ceftazidime, prior to the addition of 50 µM nitrocefin and subsequent measurement of nitrocefin hydrolysis.

Preincubation of periplasmic extracts with cefazolin at 5 and 50 µM was used as a control in this assay.

4.3.9 Zymography

For zymography, samples of periplasmic fractions were analyzed by semi-native SDS-PAGE without SDS in the gel[18] and ß-lactamase activity was detected *in situ* using nitrocefin as the substrate as described.[16] The result was photographed immediately.

Table 2. MIC values, phenotypic AmpC test results, mutations detected in the ampC promoter/attenuator region and the fold c-*ampC* expression by qRT-PCR for the 49 clinical *E. coli* isolates included in this study

	MIC mg/L[a]					Phenotypic AmpC test results[b]				
Isolate	**FOX**	**CXM**	**CAZ**	**CTX**	**ESBL**	**AmpC E-test**	**AmpC FOX-BA disk test**	**AmpC FOX-CLX disk test**	**Mutations in *ampC* promoter/attenuator**	***ampC* fold expression**
2483*	**>256**	**64**	**4.0**	**4.0**	-	**+**	**+**	**+**	**-73/-32/-10/+58**	**89.3**
2942*	**24**	**12**	**1.5**	**0.75**	-	**+**	**+**	**+**	**-73/-32/-28**	**20.8**
2529*	**16**	**12**	**1.0**	**0.5**	-	**indet**	**+**	**+**	**-73/-32/-28**	**20.5**
2958*	**16**	**8**	**0.38**	**0.38**	-	**+**	**+**	**+**	**-32**	**56.1**
4478*	**16**	**16**	**0.75**	**0.75**	-	**+**	**+**	**+**	**-73/1bp insertion (-18)**	**25.6**
3430*	**16**	**12**	**1.0**	**0.5**	-	**+**	**+**	-	**-73/-28/1bp insertion(-18)**	**17.3**
2527	48	16	0.5	0.38	-	-	-	-	-76/+22/+26/+27/+32	1.3
3245	32	16	0.38	0.25	-	-	-	+	-88/-82/-18/-1/+58	1.1
3069	32	16	0.5	0.38	-	-	-	-	WT[c]	1.2
3365*	32	12	1.5	0.19	-	indet	-	-	-73/-28	2.6
3831	24	24	0.75	0.25	-	-	-	-	WT	0.5
4408	24	24	0.5	0.25	-	-	-	-	WT	0.4
4112	24	16	0.5	0.25	-	-	-	-	-73/-28	1.2
3559*	24	12	0.5	0.25	-	-	-	+	-88/-82/-18/-1/+58	1.3
2123	24	12	0.5	0.25	-	-	-	-	-73/-28	0.4
4174*	24	8	0.38	0.19	-	-	-	+	WT	1.1
1941	16	12	0.50	0.25	-	-	-	-	-76/+22/+26/+27/+32	0.9
2491	16	12	0.38	0.25	-	-	-	-	-88/-82/-18/-1	2.2
2069	16	12	0.38	0.25	-	-	-	-	-73/-28	2.0
4095	16	8	0.25	0.19	-	-	-	-	WT	0.7
3664	16	8	1	0.19	-	-	-	+	-73/-28	0.9
4479	16	6	1	0.125	-	-	-	+	-88/-82/-18/-1/+58	1.9
4484	12	16	0.50	0.38	-	-	-	-	-88/-82/-18/-1	2.2
4472	12	12	0.75	0.125	-	-	-	-	WT	0.3
4354	12	12	0.50	0.25	-	-	-	-	WT	2.4
3609	12	12	0.38	0.25	-	-	-	-	-73/-28	0.6
3834	12	12	0.38	0.25	-	-	-	-	WT	0.9
2764	12	8	0.50	0.19	-	-	-	-	WT	0.8
3602	12	8	0.25	0.125	-	-	-	-	-88/-82/-18/-1	1.3
3671	8	6	0.38	0.094	-	-	-	-	-73/-28/+17	0.8
3275	6	4	0.25	0.094	-	-	-	-	-73/-28	0.5
3816*	4	3	0.19	0.064	-	-	-	-	-73/-28	0.3
2717	3	4	0.125	0.094	-	-	-	-	-73/-28/+58	2.5
5154*	2	4	0.19	0.094	-	-	-	-	-88/-82/-18/-1/+58	0.6

Continued

Table 2. Continued

Isolate	MIC mg/L[a]				ESBL	Phenotypic AmpC test results[b]			Mutations in *ampC* promoter/attenuator	*ampC* fold expression
	FOX	CXM	CAZ	CTX		AmpC E-test	AmpC FOX-BA disk test	AmpC FOX-CLX disk test		
1495*	**64**	**32**	**6**	**2**	**+**	**+**	**+**	**+**	**-88/-82/-42/-18/-1/+58**	**163.1**
3633*	**24**	**64**	12	**16**	**+**	**+**	**+**	**+**	**-88/-82/-42-/-18/-1/+58**	**69.6**
4197*	**12**	**>256**	32	**>256**	**+**	**+**	**+**	**+**	**-73/-28/+34/+58**	**6.1**
3531*	16	>256	4	16	+	-	-	-	WT	0.9
2026	8	>256	16	>256	+	-	-	-	WT	0.9
3869	4	>256	64	>256	+	-	-	-	WT	2.7
4019	4	>256	12	>256	+	-	-	-	-73/-28	2.3
4433	3	>256	8	>256	+	-	-	-	WT	2.8
3796	3	>256	0.5	>256	+	-	-	-	WT	1.0
1952	3	>256	8	>256	+	-	-	-	-73/-28	1.0
4092	3	6	>256	0.75	+	-	-	-	-76/+22/+26/+27/+32	1.7
4097	2	>256	2	>256	+	-	-	-	-88/-82/-18/-1/+58	1.1
4246	2	>256	2	>256	+	-	-	-	-88/-82/-18/-1/+58	1.0
3847	1.5	>256	4	>256	+	-	-	-	-73/-28	0.9
4196	1.5	>256	0.5	32	+	-	-	-	-88/-82/-18/-1	0.2

[a]MIC in mg/L as determined by Etest, [b]Phenotypic AmpC test results: + , positive; -, negative; indet, indeterminate, [c]WT, wildtype
FOX, cefoxitin; CXM, cefuroxime; CAZ, ceftazidime; CTX,cefotaxime; BA, boronic acid; CLX, cloxacillin
*Isolates that were included for further analysis.
E. coli isolates positive for AmpC activity in ≥ two phenotypic tests + an increased RNA expression level + the presence of mutations in the *ampC* promoter/attenuator mutations associated with c-*ampC* overexpression were considered as c-AmpC hyperproducers and are indicated in bold.

4.4 Results

4.4.1 AmpC production and susceptibility to cephalosporins

The results of phenotypic tests for AmpC, promoter/attenuator sequencing, qRT-PCRs and susceptibility assays of the 49 *E. coli* isolates are presented in Table 2 on the left page and above. For 7/49 *E. coli* isolates, all three phenotypic AmpC tests were positive. For 2/49 *E. coli* isolates, two phenotypic tests were positive. Five isolates showed a positive result only in the AmpC cefoxitin-cloxacillin disk test. The remaining isolates did not show AmpC hyperproduction in any of the phenotypic assays used. Isolates with at least two positive phenotypic tests showed a 6.1 to 163.1-fold increase of expression of the chromosomal *ampC* gene by real-time qRT-PCR (Table 2).

Genetic analysis revealed seven different promoter sequence variants, previously found to be associated with c-AmpC hyperproduction, in the nine isolates with at least two positive phenotypic tests and increased c-*ampC* expression (Table 2). Four isolates (2483, 2942, 2529, and 2958) contained a T to A transversion in the promoter region at position -32, which leads to the optimization of the -35 box from TTG**T**CA to TTG**A**CA. The additional substitution at position -10 in isolate 2483 creates an optimization of the Pribnow box from TA**C**AAT to TA**T**AAT, which explains the even higher expression level of *ampC* in this isolate. An insertion of a single base pair (bp) at position -18 was found in two isolates (4478 and 3430). This insertion increases the spacer region between the wildtype -35 and -10 boxes from 16 to 17 bp, which is the optimal distance between these promoter elements. Two isolates (1495 and 3633) showed substitutions in the promoter region amongst others at positions -42 and -18, which creates an alternate displaced promoter that is identical to the *ampC* promoter sequence of *Shigella*. Only isolate 4197 did not contain a substitution in the promoter region that could explain the increased abundance of the c-*ampC* transcript. In this case, a substitution in the attenuator region at position +34, which is expected to destabilize the stem-loop structure of this element, could account for the increased c-*ampC* expression level.

Twenty-six of the isolates also had several mutations compared with *E. coli* ATCC 25922 (Table 2), but none of these were identified as potentially of influence on the promoter or attenuator function. The promoter/attenuator region in the remaining isolates (n=14) did not show mutations relative to the reference strain. Sequence analysis of the *ampC* coding region did not show any amino-acid changes previously described to lead to extended-spectrum cephalosporinase activity (data not shown). p-AmpC were not detected in any of the 49 isolates.

Thus, nine of the 49 isolates were categorized as c-AmpC hyperproducers on the basis of (i) positive results in at least two phenotypic AmpC tests, (ii) increased c-*ampC* expression in the qRT-PCR experiments, and (iii) mutations in the *ampC* promoter/attenuator regions associated with c-AmpC hyperproduction.

The MICs of cefoxitin, cefuroxime, ceftazidime and cefotaxime are shown in Table 2. Five of six ESBL-negative, c-AmpC hyperproducing isolates showed elevated MICs of ceftazidime, i.e. above the epidemiological cut-off of wildtype (WT) strains of ≤ 0.5 mg/L, whereas only five of the 23 ESBL-negative, cefoxitin-resistant, c-AmpC non-hyperproducers had ceftazidime MICs above the WT cut-off.

MICs of cefotaxime were above WT cut-off of ≤ 0.25 mg/L in all six hyperproducers and in only three of the 23 non-hyperproducers. One ESBL-negative, c-AmpC hyperproducing isolate (2483) had MICs of ceftazidime and cefotaxime of 4 mg/L and thus was intermediately susceptible to ceftazidime and cefotaxime-resistant. MICs of cefuroxime (median: MIC >256 mg/L), ceftazidime (median: MIC 8 mg/L) and cefotaxime (median: MIC >256 mg/L) in the ESBL-positive isolates were higher than MICs of cefuroxime (median: MIC 12 mg/L), ceftazidime (median: MIC 1 mg/L) and cefotaxime (median: MIC 0.25 mg/L) in the ESBL-negative isolates irrespective of c-AmpC production. Four of the 15 ESBL-positive isolates were cefoxitin resistant, three of which were c-AmpC hyperproducers.

4.4.2 Detection of c-AmpC production levels

To verify the hyperproduction of the AmpC enzyme at the protein level, cell extracts of the nine c-AmpC producing isolates were analyzed by zymography. *E. coli* ATCC 25922 (ATCC) and isolates 3816, 5154, 3365, 4174, 3559, and 3531, which did not show increased expression of *ampC* in the qRT-PCR experiments (Table 2), were included as negative controls The zymogram of periplasmic extracts revealed, besides a 28-kDa band present in six of the 15 isolates examined, a very prominent band with the expected apparent molecular weight of ~35 kDa present in all nine isolates with increased *c-ampC* levels (Fig. 1).

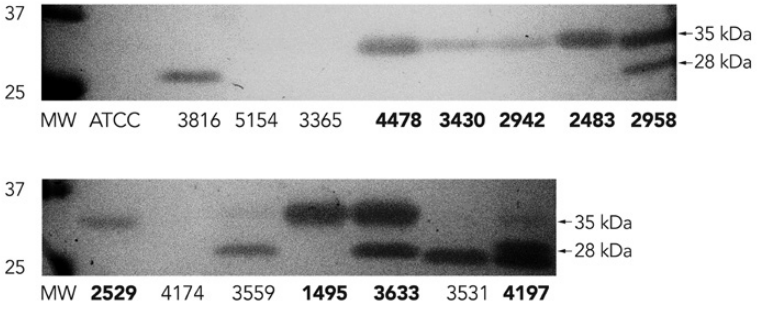

Figure 1. Zymogram analysis of periplasmic extracts revealing expression of the ß-lactamases in clinical *E. coli* isolates with nitrocefin as a ß-lactamase substrate. A predominant band of 35 kDa with ß-lactamase activity was detected in nine isolates with increased c-*ampC* expression levels. A 28-kDa band was found in six isolates. The lane marked MW contains the molecular marker proteins, the molecular mass of which is indicated (in kDa). c-AmpC hyperproducing isolates are indicated in bold, whilst strain ATCC25922 (ATCC) and isolates 3816, 5154, 3365, 4174, 3559, and 3531, which did not show increased expression of c-*ampC* in the qRT-PCR experiments (Table 2) were included as negative controls.

The hypothesis that this protein represents AmpC was further confirmed in Western blotting experiments (Fig. 2), which revealed a reaction of AmpC-specific antibodies with a 35-kDa band in the periplasmic fractions of all but one of these nine isolates; in the deviating isolate (4197), the c-AmpC production levels were apparently too low for detection with the antiserum.

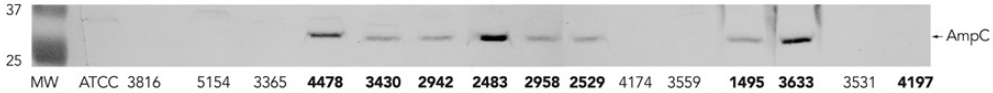

Figure 2. Western blot detection of ß-lactamases. Periplasmic extracts of clinical *E. coli* isolates and reference *E. coli* strain ATCC 25922 (ATCC) were electrophoresed on denaturing SDS-PAGE gels, and the gels were incubated with antisera directed against AmpC. The lane marked MW contains the molecular marker proteins, the molecular mass of which is indicated (in thousands) at the left. c-AmpC hyperproducing isolates are indicated in bold, whilst strain ATCC25922 (ATCC) and isolates 3816, 5154, 3365, 4174, 3559, and 3531, which did not show increased expression of *ampC* in the qRT-PCR experiments (Table 2), were included as negative controls.

4.4.3 Analysis of ß-lactamase activity

To determine whether the increased c-*ampC* expression in the various isolates correlated with increased ß-lactamase activity, the rate of nitrocefin hydrolysis in whole cell lysates of c-AmpC hyperproducing isolates was determined. In comparison to the ß-lactamase activity of *E. coli* ATCC 25922, isolates 2483, 2958, 4478, 2942, 2529 and 3430 demonstrated a 100- to 500-fold higher ß-lactamase activity. In a positive control isolate, i.e. strain EC-8 which produces CTX-M-1, OXA-1 and CMY-2,[16] the rate of nitrocefin hydrolysis was even approximately 10.000 times higher than in *E. coli* ATCC 25922. Nitrocefin hydrolysis rates in lysates of the c-AmpC hyperproducing isolates that also produced ESBL (isolates 1495, 3633 and 4197) was 500- to 1.200-fold higher than in the reference strain. There was no clear correlation between nitrocefin hydrolysis rate and the c-*ampC* expression levels, which is largely due to the contribution of ESBLs and other ß-lactamases in nitrocefin degradation.

Next, we evaluated whether hyperproduced c-AmpC enzyme could hydrolyze the cephalosporins cefuroxime and ceftazidime. For these experiments, periplasmic extracts of isolates 2483 and 4478 were used, because of their high c-*ampC* expression level and the absence of ESBL activity or other ß-lactamases detected by zymography. No hydrolysis of cefuroxime or ceftazidime could be detected (<1 nmol/min/10^9 cells). In control experiments with cefazolin as the substrate, hydrolysis rates of 10.9 and 4.1 nmol/min/10^8 cells were observed in the cell lysates of isolates 2483 and 4478, respectively. In another control experiment using cell lysates of isolate EC-8, hydrolysis rates of 38 and 4.5 nmol/min/10^9 cells for cefuroxime and

ceftazidime, respectively, were detected. Thus, the cephalosporins cefuroxime and ceftazidime are poor substrates for c-AmpC and/or the hyperproduction levels in isolates 2483 and 4478 are not high enough to measure their hydrolysis in whole-cell lysates of these isolates with the assay used.

As c-AmpC-mediated hydrolysis of cefuroxime and ceftazidime was undetectable, we next considered the possibility that these cephalosporins are irreversibly bound by the enzyme thereby acting as enzyme inhibitors. Inhibition of ß-lactamase activity was assessed by preincubating periplasmic extracts of the isolates 2483 and 4478 for 1 min with cefuroxime or ceftazidime at various concentrations and subsequently determining the remaining ß-lactamase activity by measuring the hydrolysis of nitrocefin (Fig. 3; results isolate 4478 not shown). Nitrocefin hydrolysis was inhibited by ~50% after preincubation with cefuroxime at a concentration 10,000-fold lower than that of nitrocefin and was completely inhibited with cefuroxime when used at a 100-fold lower concentration. Ceftazidime appeared a weaker enzyme inhibitor; a 14% reduction in enzyme activity was detected at a concentration 1000-fold lower than that of nitrocefin and 95% reduction was observed at equimolar concentrations. In control experiments, high amounts of the hydrolysable cefazoline only marginally inhibited the hydrolysis of nitrocefin (data not shown).

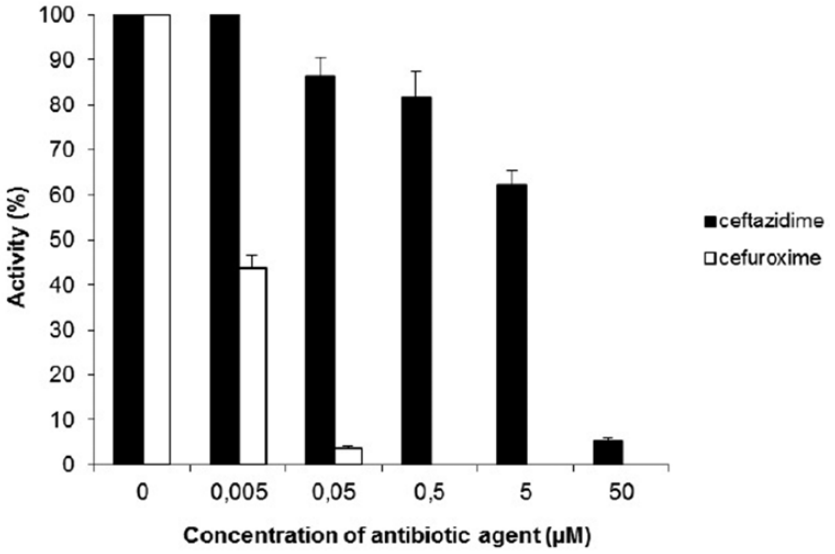

Figure 3. Inhibition of ß-lactamase activity by ceftazidime and cefuroxime. Periplasmic extracts of isolate 2483 were incubated during 1 min with various concentrations of ceftazidime or cefuroxime as indicated on the X-axis. Subsequently, the remaining ß-lactamase activity was determined using nitrocefin as ß-lactamase substrate. ß-Lactamase activity after preincubation without an antibiotic agent is set at 100%. The bars and error bars indicate means and standard deviations of three independent experiments.

The observation that cefuroxime and, to a lesser extent, ceftazidime act as inhibitors of c-AmpC suggests that these antibiotics bind the enzyme to form poorly hydrolysable acyl-enzyme adducts. The formation of such acyl-enzyme complex can be assessed in SDS-PAGE by a reduction in the electrophoretic mobility of the enzyme after binding the substrate.[16] To test this possibility, periplasmic extracts of the isolates 2483 and 4478 were either incubated or not with cefuroxime or ceftazidime and subsequently analyzed by SDS-PAGE and Western blotting with AmpC-specific antiserum. The results showed a slight decrease in the electrophoretic mobility of the ~35 kDa c-AmpC band after preincubation with meropenem, cefuroxime and ceftazidime (Fig.4; results isolate 4478 not shown), consistent with the postulated covalent binding of the substrates to the AmpC protein. This shift was not found in the control experiments using cefazolin (data not shown).

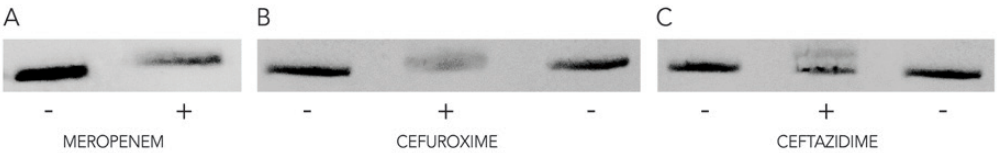

Figure 4. Covalent modification of AmpC with meropenem, cefuroxime and ceftazidime revealed by SDS-PAGE. Periplasmic extract of isolate 2483 was either incubated (+) or not (-) for 20 min at room temperature with 1 mM of the antibiotics and analyzed by SDS-PAGE after boiling of the samples. The gel used for this experiment contained 8% acrylamide and 8 M urea. After blotting, the blot was incubated with anti-AmpC antiserum.

4.4.4 OMP analysis

It is known that reduced permeability of the outer membrane can also contribute to resistance to cephalosporins. The permeability of the outer membrane to ß-lactam antibiotics is largely determined by the presence of a class of abundant, channel-forming OMPs, designated porins. *E. coli* K-12 strains generally produce two porins, OmpF and OmpC, when grown under routine laboratory conditions. To study if the c-AmpC hyperproducing isolates also produced porins, OMPs from various isolates were subjected to SDS-PAGE. The OMP profiles showed abundant bands with similar apparent molecular weights as the porins and OmpA in all isolates examined, except for isolate 3559, which showed only minor bands in this molecular weight range (Fig. 5A and B). Western blot analysis with a porin-specific antiserum confirmed the presence of one to three porins in all isolates and again showed relatively low levels of a porin in isolate 3559 (Fig. 5C).

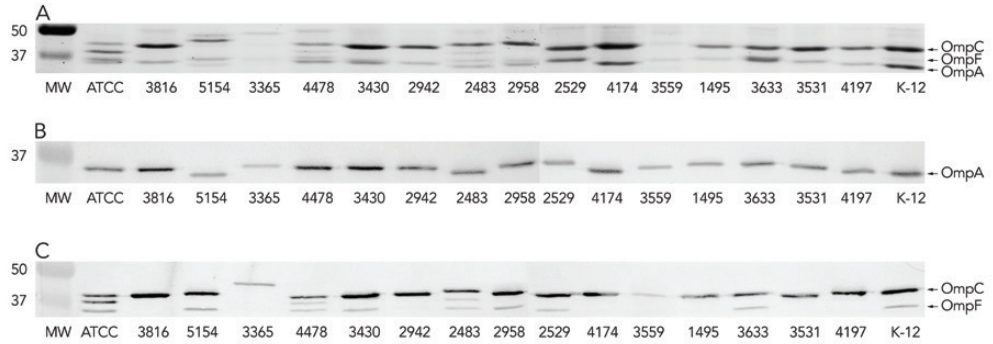

Figure 5. Analysis of the OMP profiles of *E. coli* isolates. OMPs were extracted and separated by SDS-PAGE. The proteins were either stained in the gel (panel A) or blotted and probed with an OmpA-specific monoclonal antibody (panel B) or a porin-specific antiserum (panel C). The lanes marked ATCC and K-12 show the OMP profiles of *E. coli* strain ATCC 25922 and *E. coli* K-12 strain MG1655, respectively, which were which were included for reference. The positions of OmpA,and of the porins OmpC and OmpF in the K-12 reference strain are indicated. Only the relevant part of the gel, containing the major OMPs, is shown. The lane marked MW contains molecular-mass marker proteins and their molecular weights are indicated (in thousands) at the left.

4.5 Discussion

The purpose of the present study was to investigate the contribution of *ampC* expression in cefoxitin-resistant, ESBL-negative isolates and its impact on 3rd generation cephalosporin activity, in a collection of clinical *E. coli* isolates. The phenotypic AmpC tests in nine isolates correlated with the expression level of RNA from the c-*ampC* gene and with mutations in the promoter/attenuator region of the c-*ampC* gene found by others to be associated with c-AmpC hyperproduction.[19-22] Since no plasmid-encoded AmpC was found in any of the isolates, all AmpC activity in our isolates was of chromosomal origin. Thus, c-AmpC hyperproduction was found in nine out of 33 (27.2%) cefoxitin-resistant isolates, i.e. in six of the 29 (20.6%) ESBL-negative isolates and in three of four ESBL-positive isolates.

Since average MICs were higher in c-AmpC hyperproducers than in non-hyperproducers it is concluded that the c-AmpC hyperproduction affects the susceptibility to 3rd generation cephalosporins. In the clinical laboratory, this decrease in inhibitory activity may go largely undetected, because the MICs were in the range between wildtype and the S/I breakpoint of susceptibility, except for one c-AmpC hyperproducing, ESBL-negative isolate which clearly showed loss of susceptibility to the 3rd generation cephalosporins with MICs of 4 mg/L. In the ESBL-positive isolates, the ESBL enzyme probably overrules the effect of c-AmpC hyperproduction on cephalosporins, since the MICs of all three cephalosporins

were significantly higher than in the ESBL-negative isolates and were similar in c-AmpC hyperproducers and non-hyperproducers.

The loss of the expression of porins, which facilitate the transport of ß-lactams into the bacterial cell, are also known to contribute to resistance.[23] Nonetheless, the study of porin expression in clinical isolates is highly complex due to the variety of porins that can be expressed in different strains and the number of regulatory genes and external factors involved. All of the c-AmpC hyperproducing and control isolates studied here in detail still produced at least one porin, and five out of the six AmpC-overproducing, ESBL-negative isolates (no. 2483, 2529, 2958, 4478, and 3430) produced two or even three porins. Therefore, reduced access to the periplasm probably does not play a role in the decreased susceptibility of these strains to cephalosporins.

Compared to other ß-lactamases, c-AmpC ß-lactamases have been reported to show poor hydrolysis rates for ceftazidime and compared to other ß-lactamases,[24] and this was confirmed in the present study. It has been suggested that cephalosporins may be rendered inactive by mere 'trapping' to periplasmic inducible ß-lactamases as speculated in *Enterobacter cloacae* and *Pseudomonas aeruginosa*.[25] In this study, we were able to demonstrate that preincubation of periplasmic extracts from c-AmpC hyperproducing isolates with small amounts of cefuroxime or ceftazidime was sufficient to at least partially inhibit the hydrolysis of nitrocefin in suggesting the formation of a stable cephalosporin-enzyme complex. Additional evidence for the formation of a stable acyl-enzyme complex was obtained by SDS-PAGE and Western blotting, which revealed a decreased electrophoretic mobility of the ß-lactamase after preincubation with cefuroxime or ceftazidime. Antunes *et al*.[26] recently also described covalent trapping as the mechanism of resistance to ceftazidime caused by a deacylation-deficient mutant derivative of the class A TEM-1 ß-lactamase. Similarly, evidence for the mechanism of antibiotic trapping by plasmid-encoded CMY-2 in carbapenem resistance was found in a porin-deficient, carbapenem resistant *E. coli* isolated from a liver transplant patient.[16] Based on our results and supported by data described in literature, the reduced susceptibility to cephalosporins can partially be explained by c-AmpC hyperproduction in the *E. coli* isolates studied. This enzyme, if present in high amounts, is able to 'trap' cephalosporins entering the periplasm into a biologically inactive complex. It is expected that mutant derivatives of such isolates with limited entry of cephalosporins into the periplasm due to the loss of porins will show dramatically decreased susceptibility to cephalosporins.

Such mutants might be selected under continuous selection pressure of cephalosporin treatment.

In the present study, c-AmpC hyperproduction was not predominant among cefoxitin-resistant isolates. As porins were found in the four cefoxitin-resistant non-AmpC producers examined, it is suggestive that efflux may be responsible for the resistance phenotype in these isolates. The contribution of efflux in ß-lactam resistance has been proven in a set of isogenic *E. coli* K-12 strains, in which inactivation of the AcrAB efflux pump often resulted in a significant decrease in MICs of cefoxitin and cefuroxime.[27]

For clinical microbiologists, proper recognition of AmpC-producing *E. coli* is important for clinical management. Administration of 3rd generation cephalosporins has resulted in treatment failure in two patients with c-AmpC hyperproducing isolates.[28] MICs of ceftazidime of 4 mg/L and cefotaxime of 2 mg/L were described in these patients, comparable to the MICs of one c-AmpC hyperproducing isolate (no. 2483) reported in the present study. Recognition of AmpC-production is difficult and no accepted guidelines are available. Resistance to cefoxitin can suggest AmpC-mediated resistance, but because we found it in 27.2% of the cefoxitin-resistant isolates only, it is not a specific marker. Although a comparison of phenotypic tests was not the purpose of this study, AmpC production was most reliably detected using the cefoxitin-boronic acid disk test, which appeared completely consistent with the qRT-PCR results. This test is inexpensive and can be used in all clinical laboratories.

It has been suggested to report *E. coli* with c-AmpC hyperproduction as resistant to 3rd generation cephalosporins irrespective of *in vitro* susceptibility.[29] However, although no longer wild-type strains, the MICs are often still within the susceptibility range and the treatment failures described so far were recognizable by MICs above the S/I breakpoints. In our opinion, the interpretation of 3rd generation cephalosporins should not be changed to 'resistant' on susceptibility reports in order to avoid inappropriate use of other classes of antibiotic agents and the emergence of resistance. We do recommend surveillance to monitor any developments in the resistance to 3rd generation cephalosporins by testing for increased AmpC production in cefoxitin-resistant *E. coli* isolates and by establishing MICs of 3rd generation cephalosporins in c-AmpC hyperproducers.

Funding

This project was in part supported financially by a research grant from the Dutch ZonMW organization.

Disclosure statement

The authors have no competing interests to disclose.

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CHAPTER 5

Extended-spectrum ß-lactamase producing *Enterobacteriaceae* among travelers from the Netherlands

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Emerging Infectious Diseases 2013 Aug;19(8):1206-1213

5.1 Abstract

Objectives: A prospective cohort study was performed among travelers from the Netherlands to investigate the acquisition of carbapenemase-producing *Enterobacteriaceae* (CP-E) and extended-spectrum ß-lactamase producing *Enterobacteriaceae* (ESBL-E) and associated risk factors.

Methods: Questionnaires were administered and rectal swabs were collected and tested before and after return. ESBLs were characterized using a microarray (Checkpoints). Multilocus sequence typing (MLST) was performed on *Escherichia coli* isolates.

Results: Of 370 travelers, 32 (8.6%) were colonized with ESBL-E before travel, 113 (30,5%) acquired an ESBL-E during travel and 26 were still colonized six months after return. No CPE were found. Independent risk factors for ESBL-E acquisition were travel to South and East Asia. MLST showed extensive genetic diversity among *E. coli*. Predominant ESBLs were CTX-M enzymes.

Conclusion: The acquisition rate of ESBL-E in travelers from the Netherlands was high (30.5%). Active surveillance for ESBL-E and CP-E and contact isolation precautions may be recommended at admission to medical facilities for patients, who traveled to Asia during the previous six months.

5.2 Introduction

The effect of international travel on the spread of multidrug resistant *Enterobacteriaceae* (MDR-E) has become more evident in the past few years. Data obtained in prospective studies among returning travelers from Australia, Canada, Sweden and New York during 2007-2010 revealed high carriage rates of extended-spectrum ß-lactamase producing *Enterobacteriaceae* (ESBL-E) varying from 18-25% after foreign travel.[1-4] Two of these studies also reported a pre-travel ESBL-E carriage rate of 7.8%.

The identification of carbapenemase-producing *Enterobacteriaceae* (CP-E) produced another set of challenges. Carbapenemases, such as *Klebsiella pneumoniae* carbapenemases (KPC), New Delhi metallo-ß-lactamase (NDM), OXA-48, VIM and IMP, are plasmid-encoded enzymes, which have emerged worldwide. The rate of acquisition of CP-E after foreign travel is unknown and only reported in case reports, as recently reviewed by Van der Bij and Pitout.[5] In The Netherlands, CP-E were found for the first time in 2010.[6] No data were available on the pre-and post-travel carriage rates among travelers from the Netherlands.

Our objective was to investigate whether Dutch travelers are at risk of acquiring MDR-E (ESBL-E and/or CP-E) by use of a prospective cohort study design. Because detailed microbiological data of the isolates and epidemiological data are crucial for assessing the real public health impacts of these organisms, we also investigated the persistence of intestinal colonization and possible spread to household contacts six months after the travelers returned.

5.3 Materials and Methods

5.3.1 Study design

A prospective cohort study was conducted at the travel clinic at the Leiden University Medical Center (LUMC) and at the Hollands Midden Municipal Health Services (MHS) in Leiden, The Netherlands. During March-September 2011, all adults who made an appointment for travel advice and had the intention to travel to areas outside Europe, North America and Australia were invited to participate in the study.

Travelers < 18 years of age and those who traveled > 3 months were excluded.Only one person of a couple or travel group was included. Participants were asked to

complete an electronic questionnaire and to deliver a rectal swab immediately before and immediately after travel. Questionnaires were used to collect demographic data, previous medical history and travel information. Travelers who acquired a MDR-E after foreign travel were asked to fill out a third questionnaire and deliver a third rectal swab six months after return.

If travelers were positive for MDR-E six months after return, their household contacts were also requested to submit a rectal swab and questionnaire. Household contacts were defined as persons who shared the same household with a participant on a regular basis. MDR-E positive participants were asked to deliver a fourth rectal swab at the same time. The study was approved by the Leiden University Medical Center medical ethics committee.

5.3.2 Bacterial isolates

Rectal swabs were collected with the Stuart Agar Gel Medium Transport Swabs (Copan Diagnostics, Corona, CA). The swabs were inoculated in trypticase soy broth (TSB) supplemented with cefotaxime 0.25 mg/L and vancomycine 8 mg/L (MP products, Groningen, The Netherlands) and incubated for 24 hours at 37°C. After overnight incubation the TSB broths were subcultured on chromogenic ESBL screening agar (ESBL-ID, BioMérieux) and sheep blood agar as a growth control. All Gram-negative rods growing on the ESBL-ID were identified by MaldiTof-MS with BioTyper software (version 3.0; Bruker Daltonics, Germany) and antimicrobial susceptibility testing was perform using the VITEK2® system (BioMérieux, Marcy-l'Étoile, France). All isolates underwent ESBL confirmatory disk testing by disk diffusion for ceftazidime and cefotaxime or cefepime (in cefoxitin resistant isolates) with and without clavulanic acid as recommended by the Clinical Laboratory Standards Institute's guidelines.

MICs for meropenem and ertapenem were determined using Etests (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. MICs were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/).

5.3.4 Molecular characterization of ß-lactamases

Molecular characterization of the ß-lactamase genes in ESBL-E was performed by using Check-MDR CT103 microarray, version 1.1 (Checkpoints B.V., Wageningen, The Netherlands). The principals of the microarray system and interpretation software have been described previously.[7] Concisely, the system combines ligation-

mediated amplification with the detection of amplified products on a microarray to detect the various carbapenemase genes: (OXA-48, NDM-1, IMP, VIM and KPC), CTX-M groups (CTX-M group 1, 2, 9 or combined 8/25) and the most prevalent ESBL-associated single nucleotide polymorphisms (SNPs) in TEM and SHV-variants. Furthermore, the six plasmid-mediated AmpC ß-lactamases can be identified (www.lahey.org/studies).

5.3.5 Molecular typing of Escherichia coli isolates

Multilocus sequence typing (MLST) was performed on all *E. coli* isolates using seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) to determine the corresponding sequence type (ST) and to designate the sequence type complex (STC) by using the MLST Databases at the Environmental Research Institute, University College Cork website (http://mlst.warwick.ac.uk/mlst/).

5.3.6 Data analysis

A logistic regression model was used to determine risk factors for the acquisition of ESBL-E/CP-E after foreign travel for a total of 338 participants. Associations between acquiring an ESBL-E/CP-E post-travel and different variables are calculated as odds ratios and p-values. Participants who were positive for ESBL-E/CP-E before travel were analyzed separately. Database processing and statistical analyses (univariate and multivariate analysis) were performed by using the SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). MLST analysis was performed using BioNumerics software v.6.6 (Applied Maths, St-Martens-Lathem, Belgium).

5.4 Results

5.4.1 Study population and travel characteristics

In total, 521 participants were invited to participate in the study. 370 travelers completed two questionnaires and sent in two rectal swabs and were included in the analysis (Figure 1). The median age of the study population was 33 years (range 19-82) and 234 (63.2%) were women. The median length of stay abroad was 21 days (range 6-90 days). The most common reason for travel was vacation (n=277). Of the 370 participants, 113 (30.5%), whose pretravel swabs were negative, acquired MDR-E during foreign travel. Of these 113 participants, 19 (16.8%) still

carried MDR-E six months after return. In 32 of the 370 participants (8.6%), MDR-E was identified before travel. Twenty (62.5%) of these 32 participants returned with MDR-E, 7 of whom were still colonized after six months (35%). No MDR-E was found before or after travel in 225 participants (60.8%).

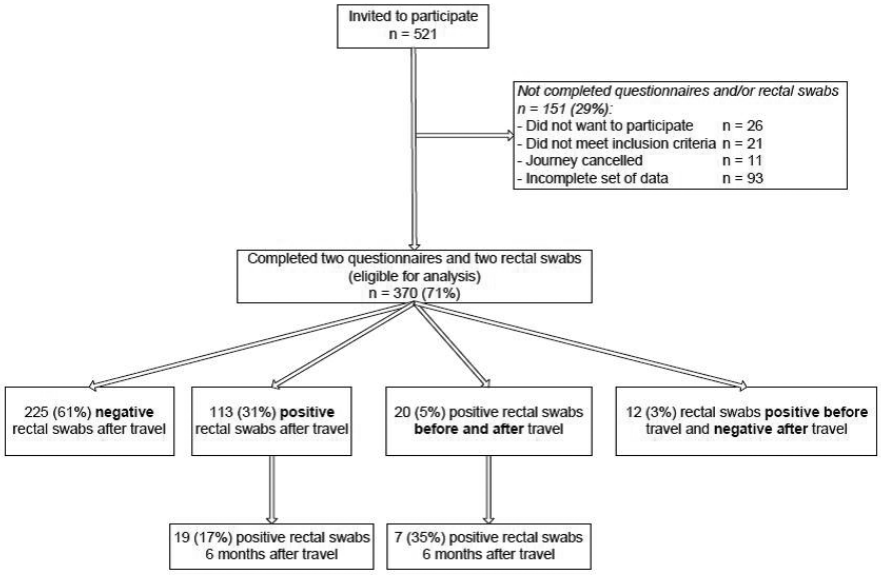

Figure 1. Flowchart of participants in the study

5.4.2 Travel-associated risk factors for ESBL-acquisition in returning travelers

For the analysis of travel-associated risk factors, data of 338 returning travelers with negative pretravel swabs were used (Table 1). In total, 65 countries were visited; these are subdivided in 10 subcontinents. The most common destinations were Indonesia (n=62), Thailand (n=30), Malaysia (n=27), Cambodia (n=21), China (n=39), Kenya (n=30), Tanzania (n=24), Surinam (n=20) and South Africa (n=19).

The highest ESBL-E acquisition rates were identified among travelers who visited countries in Asia: 73% in South Asia and 67% in East Asia. Univariate and multivariate analysis showed that the travel destinations South and East Asia were significant risk factors for the acquisition of ESBL-E in returning travelers ($p<0.001$). Participants traveling to Asia (all subcontinents) were more likely to return with an ESBL-E after a self-arranged trip (odds ratio 1.7; $p=0.07$) and stay in hostels/lodges (odds ratio 1.9; $p= 0.08$), although this was not statistically significant. There were no other risk factors for the acquisition of ESBL-E after foreign travel.

Table 1. Person and travel characteristics and risk factors for ESBL-E acquisition in a cohort of 338 travelers from the Netherlands*

Variable	No.(%) negative pre- and post- travel N=225 (%)	No. (%) positive post-travel only, N=113	Univariate analysis OR (95% CI)	p-value †	Multivariate analysis OR (95% CI)	p-value
Gender, female	144 (64)	69 (61.1)	0.88 (0.55-1.41)	0.60		
Age						
18-25 years	54 (24.0)	28 (24.8)	1.0			
26-33 years	66 (29.3)	24 (21.2)	0.39 (0.17-0.92)	0.03		
34-51 years	56 (24.9)	28 (24.8)	0.67 (0.28-1.61)	0.37		
≥ 52 years	43 (21.8)	33 (29.2)	1.46 (0.60-3.54)	0.41		
Vegetarian	13 (5.8)	6 (5.3)	0.91 (0.34-2.47)	0.86		
Health care worker	59 (26.2)	27 (23.9)	0.88 (0.52-1.49)	0.64		
Daily contact with farm animals	8 (3.6)	4 (3.5)	1.0 (0.29-3.38)	0.99		
Visit to identified risk areas during previous 12 months			1.01 (0.64-1.61)	0.96		
None	138 (61.3)	69 (61.1)				
Africa	26 (11.6)	15 (13.3)				
Asia	21 (9.3)	12 (10.6)				
India	5 (2.2)	4 (3.5)				
Middle East	26 (11.6)	13 (11.5)				
Central America and Carribean Region	17 (7.6)	7 (6.2)				
South America	6 (2.7)	5 (4.4)				
Medical problem ‡						
None	161 (71.6)	84 (74.3)				
Inflammatory bowel disease	2 (0.9)	1 (0.9)				
Chronic diarrhea	3 (1.3)	0				
Chronic constipation	3 (1.3)	1 (0.9)				
Irritable bowel syndrome	17 (3.1)	7 (6.2)				
Diabetes mellitus	3 (1.3)	1 (0.9)				
Gastroesophageal reflux	12 (5.3)	4 (3.5)				
Recurrent UTIs	4 (1.8)	1 (0.9)				
Autoimmune disease	7 (3.1)	2 (1.8)				
Abdominal pain with unknown origin	5 (2.2)	2 (1.8)				
Gallbladder problems	4 (1.7)	1 (0.9)				
Transplantation	1 (0.4)	0				
Coeliac disease	0	2 (1.8)				
Other	30 (13.3)	18 (15.9)				
Antibiotic use during 12 months before travel	47 (20.9)	17 (15.1)	0.85 (0.56-1.29)	0.45		
Hospitalization during 12 months before travel						
<3 months prior	5 (2.2)	3 (2.7)				
3-6 months prior	2 (0.9)	1 (0.9)				
6-9 months prior	1 (0.4)	1 (0.9)				
9-12 months prior	2 (0.9)	2 (1.8)				
Travel destination, by subcontinents¶						
Southeast Asia	73 (32.4)	37 (32.7)	1.01 (0.63-1.64)	0.96		
East Asia	11 (4.9)	22 (19.5)	4.70 (2.19-10.1)	<0.001	3.95 (1.78-8.73)	0.001
South Asia	7 (3.1)	18 (15.9)	5.90 (2.39-14.60)	<0.001	5.09 (2-12.92)	0.001
Central Asia	2 (0.9)	1 (0.9)	1.0 (0.089-1.11)	0.99		
Middle East	13 (5.8)	2 (1.8)	0.29 (0.07-1.33)	0.11	0.28 (0.06-1.30)	0.103
North Africa	6 (2.7)	4 (3.5)				
Central Africa	39 (17.3)	17 (15)				
Southern Africa	23 (10.2)	3 (2.7)	0.24 (0.07-0.82)	0.02	0.24 (0.07-0.85)	0.027
Central America and the Caribbean	21 (9.3)	7 (6.2)	0.64 (0.26-1.56)	0.33		
South America	30 (13.3)	2 (1.8)	0.12 (0.027-0.50)	0.004	0.14 (0.03-0.59)	0.008

Continued

Table 1. Continued

Variable	No.(%) negative pre- and post- travel N=225 (%)	No. (%) positive post-travel only, N=113	Univariate analysis OR (95% CI)	p-value †	Multivariate analysis OR (95% CI)	p-value
Median duration of stay in days (range)	21 (6-90)	22 (6-89)	0.99 (0.976-1.004)	0.17	1.0 (0.97-1.0)	0.22
Type of travel						
Self-arranged travel	95 (42.2)	52 (46)	1.17 (0.74-1.84)	0.51		
Backpacking	51 (22.7)	25 (22.1)	0.97 (0.56-1.67)	0.91		
Organized group travel	62 (27.6)	27 (23.9)	0.83 (0.49-1.39)	0.47		
Cruise	1 (0.4)	0				
Other	16 (7.1)	9 (8)				
Own (holiday) home	16 (7.1)	3 (2.7)				
Other	3 (1.3)	3 (2.7)				
Reason for travel						
Vacation	166 (73.8)	83 (73.5)				
Visit family/friends	8 (3.6)	8 (7.1)				
Business	15 (6.7)	9 (8.0)				
Study	18 (8)	7 (6.2)				
Volunteer work	10 (4.4)	5 (4.4)				
Travel group composition						
Alone	25 (11.1)	14 (12.4)	1.13 (0.56-2.27)	0.73		
With 1 partner	102 (45.3)	44 (38.9)	0.77 (0.49-1.22)	0.26		
More partners	44 (19.6)	30 (26.5)				
Group travel	54 (24.0)	25 (22.1)	1.23 (0.78-1.93)	0.37		
Accomodation during travel						
Luxury hotels	78 (34.7)	34 (30.1)	0.81 (0.50-1.32)	0.40		
Hostels	50 (22.2)	30 (26.5)	1.27 (0.75-2.13)	0.38		
Budget hotels	49 (21.8)	27 (23.9)	1.13 (0.66-1.93)	0.66		
Own holiday home	16 (7.1)	3 (2.7)				
Camping	10 (4.4)	6 (5.3)				
With family/friends	8 (3.6)	5 (4.4)				
Locals	7 (3.1)	3 (2.7)				
Boat	4 (1.8)	2 (1.8)				
Other	3 (1.3)	3 (2.7)				
Diarrhea during travel	83 (36.9)	45 (39.8)	1.13 (0.71-1.80)	0.60		
Companion travelers with diarrhea	115 (51.1)	61 (54)	1.1 (0.71-1.77)	0.62		
Antibiotic use during travel	10 (4.4)	9 (8.0)	1.86 (0.73-4.72)	0.19	1.98 (0.72-5.47)	0.16

*Data are presented as no. (%), unless stated otherwise. Blank cells indicate no data available for value. OR, odds ratio; UTI, urinary tract infection.
†Variables with $p < 0.2$ in the univariate analysis were included in the multivariate logistic regression model.
‡Participants could report >1 medical problem.
§ Travel destinations visited by the travelers who completed the study were divided in 10 subcontinents (n = no. of travelers per destination. One participant could have visited >1 country): Southeast Asia: Cambodia (n = 21), Philippines (n = 1), Indonesia (n = 62), Laos (n = 9), Malaysia, (n = 27), Singapore (n = 9), Thailand (n = 30) and Vietnam (n = 17); East Asia: People's Republic of China (n = 39), Japan (n = 1), Mongolia (n = 4) and Taiwan (n = 1); South Asia: Bangladesh (n = 1), India, (n = 20) Maldives (n = 2), Nepal (n = 8) and Sri Lanka (n = 5); Central Asia: Kazakhstan,(n = 2), Kyrgyzstan (n = 2) Uzbekistan (n = 2) and Turkmenistan (n = 1); Middle East: Iran (n = 1), Jordan (n = 1), Turkey (n = 14) Emirates(n = 3); North Africa: Egypt (n = 10) and Morocco (n = 5); Central Africa: Benin (n = 1), Cameroon (n = 1), Congo (n = 7), Gambia (n = 2), Ghana (n = 1), Kenya (n = 30), Liberia (n = 1), Rwanda (n = 1), Sierra Leone (n = 1), Tanzania (n = 24) and Uganda (n = 9); Southern Africa: Angola (n = 1), Botswana (n = 5), Lesotho (n = 2), Madagascar (n = 3), Malawi (n = 5), Mauritius (n = 1), Mozambique (n = 2), Namibia (n = 7) South Africa (n = 19), Swaziland (n = 6), Zambia (n = 6)and Zimbabwe. (n = 1); Central America and the Caribbean: Belize (n = 2), Bonaire (n = 1), Costa Rica (n = 9), Cuba (n = 5), Curacao (n = 1), Dominican Republic (n = 4), Grenada (n = 1), Guatemala (n = 4), Honduras (n = 2), Mexico (n = 9), Nicaragua (n = 5) and Panama (n = 3); South-America: Argentina (n = 3), Bolivia (n = 2), Brazil (n = 5), Chile (n = 2), Ecuador (n = 3), Guyana (n = 3, Peru (n = 3), Surinam (n = 20), Trinidad and Tobago (n = 2) and Venezuela (n = 1).

The incidence proportions of ESBL-E after foreign travel are listed in Table 2.

Table 2. Incidence proportions and incidence rates for ESBL-E in 338 Dutch travelers

Destination	No of travelers	No of travelers with ESBL-E (%)	Incidence proportion % (SE)	Person days (all travelers)	Mean duration of travel (all travelers)	ESBL incidence rate per 100 pdt* (SE†)
Southeast Asia	110	37 (34)	34 (4.5)	2980	27	1.24 (0.20)
East Asia	33	22 (67)	67 (8.3)	776	24	2.83 (0.60)
South Asia	25	18 (72)	72 (9.2)	599	24	3.01 (0.70)
Central Asia	3	1 (30)	33 (33.3)	94	31	1.06 (1.06)
North Africa	10	4 (40)	40 (16.3)	112	11.2	3.57 (1.76) ‡
Middle Africa	56	17 (30)	30 (6.2)	1637	29	1.04 (0.25)
Southern Africa	25	3 (12)	12 (6.6)	631	25	0.48 (0.27)
Middle East	15	2 (13)	13 (9.1)	222	14.8	0.90 (0.64)
Central America and the Caribbean	28	7 (25)	25 (8.3)	544	19	1.29 (0.48)
South America	32	2 (6)	6 (4.4)	922	29	0.22 (0.15)
All travelers	338	113 (33)	33 (2.6)	8536	25	1.32 (0.12)

* Pdt: person days of travel;
† SE standard error.
‡ The ESBL incidence rate per 100 person days of travel is represented by 4 ESBL-E carrying returning travelers from North Africa. Three of them had traveled for 7 days and one traveler had a 25-day stay abroad, which explains the high standard error.

5.4.3 Microbiological results and molecular characterization

A total of 133 participants were colonized with an MDR-E after travel. This group consisted of 113 travelers, who had initially negative pretravel swabs. In addition, 20 participants who had positive pretravel swabs also returned colonized with MDR-E. The ESBL-E of these 133 post-travel swabs consisted of 146 *E. coli*, 10 *K. pneumoniae* and 2 *Enterobacter cloacae* isolates.

No CP-E were found among the pre-and post-travel isolates. Molecular characterization of the post-travel isolates demonstrated that CTX-M group 1 ESBL (n=110) predominated (CTX-M-1 like, n=4; CTX-M-3 like, n=1; CTX-M-15 like, n=85; CTX-M-32 like, n=20), followed by CTX-M group 9 ESBL (n=42), CTX-M group 2 (n=2) and CTX-M group8/25 (n=1). One *E. coli* isolate carried an SHV-ESBL (238S+240K). In addition some isolates coproduced plasmid-mediated AmpC ß-lactamase, ACT/MIR (n=1) or CMY-2 (n=2).

Thirty-four ESBL-E were isolated from pretravel rectal swabs from 32 participants. Twenty-nine (85.3%) of them were positive for *E. coli,* 4 *Klebsiella pneumoniae* (11.8%) and 1 for *Citrobacter freundii* (2.9%). The CTX-M group 1 ESBL (n=22) predominated (CTX-M-1 like, n=4; CTX-M-15 like, n=16; CTX-M-32 like, n=2); the remaining ESBL isolates belonged to CTX-M group 9 ESBL (n=8) and CTX-M group 2 (n=1).

Two *E. coli* isolates carried an SHV-ESBL (238S+240K).

Co-resistance to other classes of antimicrobial drugs was common in pre- and post-travel isolates; 67% displayed resistance to cotrimoxazole, 36% to ciprofloxacin, 37% to tobramycin, 35% to gentamicin and 29% to nitrofurantoin. All isolates were susceptible to colistin and carbapenems.

5.4.4 MLST of ESBL-producing E. coli isolates

MLST of 146 *E. coli* isolates from the post-travel swabs identified 86 different sequence types (ST); 31 new STs were found. The most prevalent STs were: ST38 (12%; n=17), ST10 (7%; n=10) and ST131 (4%; n=9). The distribution of the CTX-M groups and types and STs is displayed in Figure 2. There was no association between ST and ESBL-type, nor were STs associated with specific travel destinations. Pretravel isolates showed a similar diversity of STs, of which three were ST131.

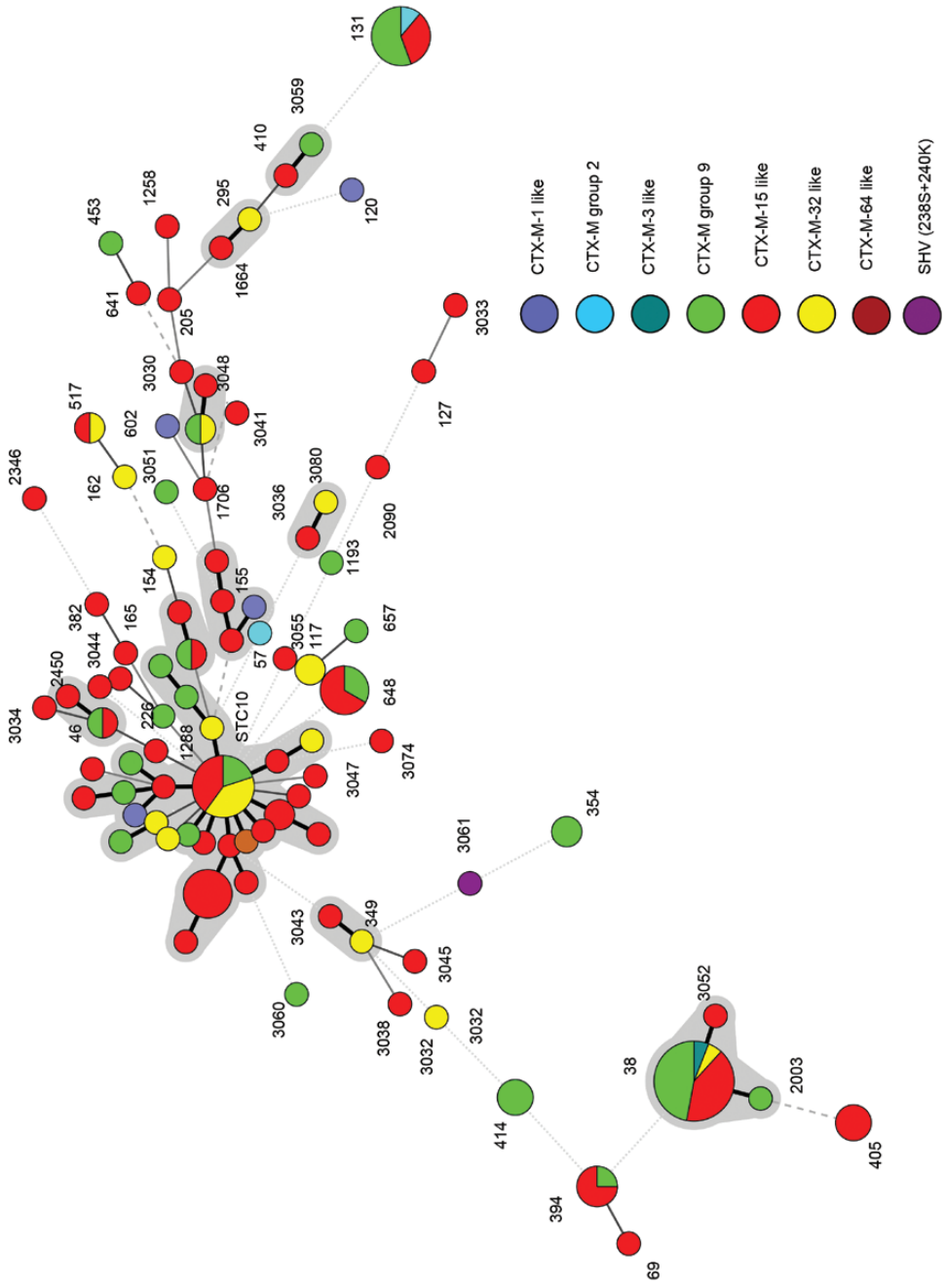

Figure 2. Multilocus sequence typing of *Escherichia coli* (n=146) from the post-travel isolates of 133 travelers from the Netherlands. The numbers indicate the most prevalent sequence types (STs). Gray shadow indicates that >1 ST belongs to the same complex. The following sequences belong to STC10: ST4,10, 34, 43, 44, 48, 167, 193, 215, 218, 227, and 617. Thick connecting lines indicate single-locus variants; thin connecting lines indicate variants with 2–3 loci differences; dashed connecting lines indicate variants with 4 loci differences; dotted connecting lines indicate 5–7 loci differences.

5.4.5 Prolonged carriage and household contacts

Of the 133 participants whose samples were positive for ESBL-E after return, 127 (95.4%) completed the follow-up survey and provided samples after six months. ESBL-E was isolated from 26 samples (20.4%) (Table 3). None of these participants reported the use of antibiotics, or were hospitalised during the previous six months; none were healthcare workers and none reported contact with farm animals. Seven participants reported diarrhea.

Of 113 participants, who had initially negative pretravel swabs and positive swabs immediately after return, 19 (16.8%) were still colonized after six months. Of these, 7 participants had samples that were positive for *E. coli* with the same ST six months after return. Nine participants carried an *E. coli* with a different ST six months after return. Three were positive for a different species six months after return. Eleven household contacts of 4 MDR-E positive participants agreed to cooperate and submitted a rectal swab. ESBL-producing *E. coli* was isolated from 2 (18.1%) household contacts, each from different households. The first household contact carried a different ESBL-producing *E. coli* than the associated traveler before and after travel. Both isolates carried a CTX-M group 9 enzyme. The second household contact was positive for SHV-ESBL producing *E. coli* ST2599. The associated traveler's sample were positive for *E. coli* ST617 and ST38 immediately after travel, *K. pneumoniae* six months after return and a CTX-M-15 like *E. coli* ST3363 in the fourth rectal swab.

Of 20 participants whose samples were positive before and after return, 7 participants (35.0%) were still colonized six months after return. Of these 7 participants, 5 carried a similar strain: 2 carried a CTX-M- group 9-producing *E. coli* with an identical ST as before travel, 2 carried a similar ST but with a different CTX-M group enzyme as before travel and one participant carried a CTX-M group 1 producing *K. pneumoniae* during the study period. Two participants returned with an *E. coli* with a different ST. No household contacts participated in this subgroup of travelers.

Table 3. Microbiological and molecular characteristics of rectal swabs samples collected from travelers from the Netherlands immediately pre-and post-travel and 6 months after return*

				Immediate post-travel samples								
	Pretravel swab			Isolate 1			Isolate 2			Post-travel sample 6 months after return†		
ID	Species	CTX-M group	ST	Species	CTX-M group	ST	Species	CTX-M group	ST	Species	CTX-M group	ST
25	Neg	NA	NA	*E. coli*	9	131	Neg	NA	NA	*E. coli*	9	131
45	Neg	NA	NA	*E. coli*	1	405	*E. coli*	9	38	*E. coli*	1	405
56	Neg	NA	NA	*E. coli*	1	3036	*E. coli*	1	517	*E. coli*	1	3267
60	Neg	NA	NA	*E. coli*	1	648	*K. p.*	1	ND	*E. coli*	1	648
61	Neg	NA	NA	*E. coli*	1	648	*E. coli*	9	227	*E. coli*	1	131
62	Neg	NA	NA	*E. coli*	9	3037	Neg	NA	NA	*E. coli*	9	501
80	Neg	NA	NA	*E. coli*	1	131	Neg	NA	NA	*E. coli*	9	1177
86	Neg	NA	NA	*E. coli*	1	93	*E. coli*	1	2090	*E. cloacae*	9	ND
137	Neg	NA	NA	*E. coli*	1	155	*E. coli*	1	617	*E. coli*	9	131
204	Neg	NA	NA	*E. coli*	1	38	Neg	NA	NA	*K. p.*	1	ND
211	Neg	NA	NA	*E. coli*	1	3044	Neg	NA	NA	*K. p.*	1	ND
222	Neg	NA	NA	*E. coli*	9	2003	Neg	NA	NA	*E. coli*	9	2003
238	Neg	NA	NA	*E. coli*	9	414	Neg	NA	NA	*E. coli*	9	10
251	Neg	NA	NA	*E. coli*	1	34	Neg	NA	NA	*E. coli*	1	450
309	Neg	NA	NA	*E. coli*	1	3045	Neg	NA	NA	*E. coli*	1	3045
373	Neg	NA	NA	*E. coli*	1	38	Neg	NA	NA	*E. coli*	1	3266
387	Neg	NA	NA	*E. coli*	1	131	Neg	NA	NA	*E. coli*	1	131
454	Neg	NA	NA	*E. coli*	9	10	Neg	NA	NA	*E. coli*	9	10
474	Neg	NA	NA	*E. coli*	1	154	Neg	NA	NA	*E. coli*	1	131
12	*E. coli*	9	38	*E. coli*	1	3074	Neg	NA	NA	*E. coli*	1	38
105	*E. coli*	1	191	*E. coli*	1	120	*E. coli*	1	38	*E. coli*	1	120
255	*E. coli*	9	131	*E. coli*	1	617	Neg	NA	NA	*E. coli*	9	131
269	*K. p.*	1	ND	*K. p.*	1	ND	Neg	NA	NA	*K. p.*	1	ND
283	*E. coli*	9	131	*E. coli*	1	46	Neg	NA	NA	*E. coli*	9	131
505	*E. coli*	1	1163	*E. coli*	1	69	Neg	NA	NA	*E. coli*	9	3268
512	*E. coli*	9	657	*E. coli*	9	657	Neg	NA	NA	*E. coli*	1	657

*ID, participant identification number; CTX-M, extended-spectrum ß-lactamase enzyme; ST, sequence type; Neg, no species were isolated from sample; *E., Escherichia; K.p., Klebsiella pneumoniae;* NA, not applicable; ND, no sequence type data available.
†None of the participants with a positive rectal swab sample after 6 months reported antimicrobial drug use during the 6 months after return.

5.5 Discussion

The results of this study show a high ESBL-E carriage rate of 30.5% among healthy participating travelers from the Netherlands after return. This finding is worrisome, because this ESBL-E carriage rate is higher compared with those in recent studies that identified international travel as an independent risk factor for ESBL-E colonization.[1-4] It is striking that none of the potential travel-associated risk factors investigated in this study, other than traveling to South and East Asia, were found to contribute to this high ESBL-E carriage rate. Additional risk factors were not revealed by including the univariate analysis the 13 participants who had a positive pretravel swab, and acquired an ESBL-producing *E. coli* during travel with a different ST than before travel. Gastroenteritis during travel was found to be associated with the risk of ESBL-E acquisition in Swedish travelers.[3] That association was not found in this study, which may reflect less fecal-oral contamination while traveling. Baaten *et. al*. reported that diseases transmitted by the fecal-oral route among travelers to non-industrialized countries have declined because of improved hygiene standards at the destination as measured by the human developmental index, sanitation index and the water source index.[8] The sanitation index (SI) levels, which represent the proportion of the population that has access to sanitation, were the lowest for Sub-Saharan Africa and the Indian subcontinent. On the basis of these indices, we would expect the incidence of ESBL-E acquisition to be similar among travelers in countries in Asia and Africa. Nonetheless, participating travelers to Asia had the highest post-travel colonization rates. Travelers to Asia most likely differ in their eating habits compared to travelers to African countries, since the former are more likely to eat in individual establishments outside of hotels or from street vendors. Thus, the high incidence rate found in returning travelers from Asia in this study may result from the increased risk for foodborne exposure.

No CP-E were found despite the fact that countries were visited where CP-E are prevalent in hospitals and in the environment.[5] Other known risk areas besides India, for the acquisition of CP-E, such as the USA, Greece, Italy and the Balkan region were not included in this study, because these travelers do not visit the Travel Clinic of the LUMC. Many citizens from the Netherlands have relatives in North-African countries or Turkey whom they visit frequently. OXA-48 producing bacteria are endemic to these countries.[9] These travelers do not consult travel clinics and may well return carrying OXA-48 producing isolates unnoticed.

Peirano *et. al.*[2] reported that the prevalence of ST131, an uropathogenic *E. coli*, notorious for its worldwide expansion and spread of CTX-M-15, was similar among travelers and non-travelers from the Calgary region. The most prevalent ESBL among the travelers participating in this study was the CTX-M-15 like enzyme. However, this enzyme was found in a plethora of different sequences types of *E. coli*. Participants in the Leiden area not only showed a great heterogeneity of sequence types, but also harboured different CTX-M-types after travel and six months after return. The majority of the *E. coli* strains identified in the participants in this study were of sequence types that clustered around ST10 and belonged to sequence type complex 10 (STC10). STC10 strains essentially belong to the non-virulent, commensal phylogenetic group A.[10] In a recent study based in France, isolates belonging to STC10 were found to be the most prevalent among fecal samples of healthy carriers of nalidixic acid resistant (but ESBL-negative) *E. coli*.[11] It is also the most prevalent STC in the MLST database. Data from this study show that transmissible genetic elements containing resistance genes are exchanged with naïve *E. coli* strains of the human intestinal microbiota during foreign travel combined with foodborne exposure.

Although 26 participants had positive results for ESBL-E six months after travel, they were not all positive for the same enterobacterial strain that was identified immediately after travel. In 8 travelers colonized with *E. coli,* an ESBL of the same CTX-M group was found in the immediate post-travel sample as after six months, but *E. coli* with a different ST was detected. In 11 travelers, the strain persisted during the study period. It is possible that more strain types were present in the rectal samples where colony morphology of different strains was not discriminative. However, it is also possible that the transfer of ESBL genes between strains within a host is a frequent occurrence. Or, the acquisition of a new ESBL-E occurs at the expense of the resident strain.

Interhousehold transmission of ESBL-E has been demonstrated in the community setting.[12,13] Clonally related strains could be found for 66% of the isolates from infected community patients and their corresponding household contacts.[13] Because of the limited data on household contacts in the present study, the transmission dynamics of ESBL-E in households after foreign travel remain to be discovered.

The high pretravel ESBL-E carriage rate among our study participants (8.6%) was an unexpected finding. Two recent studies on the ESBL-E carriage rate in the community have been conducted in the Amsterdam area. In the first study, 10.1% of

the fecal samples from outpatients with gastrointestinal discomfort being assessed by their general practitioners yielded ESBL-E, predominantly CTX-M-15 producing *E. coli*.[14] In a second study, investigating the prevalence of ESBL-E carriage in the general community, a carriage rate of 8.5% was found (E.A. Reuland, unpublished data). Although no data on travel history were given, the investigators pointed out that foreign travel might be responsible for at least part of ESBL-E carriage rates among outpatients from the Netherlands. This finding is supported by data from our study: 50% of participants who had a positive pretravel swab had traveled during the previous 12 months. This high percentage of carriers identified in this study before travel points towards ongoing importation of ESBL-E. Other potential reservoirs for ESBL-E are poultry and other retail meat, which have been found to be contaminated with ESBL-producing *E. coli* strains harboring the genes on identical plasmids as found in human isolates.[15, 16]

International travel is growing and the number of intercontinental flights has increased during the past decade. The findings in this study support the role of international travel on the ESBL-E acquisition and carriage rates among travelers from the Netherlands, especially to South and East Asia. The high pre- and post-travel carriage rates among persons traveling from the Netherlands indicate that the consequences of increased foreign travel are already manifest in this country. The lack of apparent travel-associated risk factors, the spread of CTX-M enzymes through a highly diverse population of *E. coli,* the association of ESBL-production with multidrug resistance, and the possible role of other sources make containing the spread difficult. These factors also complicate the implementation of other strategies, such as pretravel advice, and imply that all travelers to Asia should be considered for carriage of ESBL-E. Although CP-E were not found in this study, CP-E have been introduced into the Netherlands by returning travelers,[6,17-19] and introduction by asymptomatic travelers to the Netherlands from countries where CP-E are endemic, may largely go unnoticed. There is no reason to assume that, after CP-E are introduced, their spread will be less dynamic than of ESBL-E. This inference has serious implications for implementation of screening methods and effective infection control strategies. On the basis of the results of this study, we recommend active surveillance of CP-E and ESBL-E and at least temporary contact isolation precautions for patients being admitted to hospitals after travel to Asia during the previous six months.

Acknowledgements

We thank the technicians from the Department of Medical Microbiology for the cultures performed in this study and Check-Points B.V. for technical support.

This study was partly funded by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID SG Research Grant)

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CHAPTER 6

Characterization of multidrug resistance, clonal type and virulence of *Escherichia coli* by the use of bench-top sequencing

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Submitted for publication

6.1 Abstract

Objectives: Whole genome sequencing (WGS) technology is a high throughput method to rapidly identify all genes of interest in clinical multidrug resistant *Escherichia coli.*

Methods: Ten well characterized clinical isolates of *E. coli* were selected for WGS by the use of Ion Torrent to determine the presence of antimicrobial resistance mechanisms for ß-lactam antibiotics, fluoroquinolones, aminoglycosides and cotrimoxazole. In addition, Ion Torrent sequencing was used to assess the multilocus sequence type (MLST) and the presence of 13 virulence genes. Results were compared with conventional PCR and sequence data for 18 antimicrobial resistance genes.

Results: For 17 antimicrobial resistance genes WGS results were consistent with conventional PCR and sequencing data. Only bla_{SHV} genes were difficult to recognize, due to a low coverage rate. In addition, this technique identified 13 virulence genes and determined the ST type correctly in all isolates.

Conclusion: It is concluded that Ion Torrent sequencing of clinical isolates is an excellent approach to rapidly identify antimicrobial resistance genes, to detect virulence genes and to determine the sequence type in one single run.

6.2 Introduction

Multidrug resistant *Escherichia coli* increases in incidence across Europe.[1] As such, accurate and rapid antimicrobial susceptibility testing in multidrug resistant isolates delivers direct clinical benefit. Besides determining species and antimicrobial susceptibility testing, other characteristics, such as virulence genes provide valuable information on the emergence of virulent and epidemic strains. For that, a single test which provides all characteristics is indispensable. Whole genome sequencing (WGS) technology provides rapid complete genome sequencing and thereby potentially full characterization of the genetic profile of a bacterial strain. During recent years there has been a dramatic reduction in cost and an increase in the quality of WGS, making this technology economically feasible as a routine tool, not only for scientific research[2,3] but also for clinical diagnostics[4-11] and surveillance. The introduction of affordable and fast bench-top next-generation sequencer machines like the Ion Torrent Personal Genome Machine® (PGM) makes this technology feasible as a routine tool for clinical diagnostics. The Ion Torrent PGM® has successfully been applied in niche areas, such as the detection of drug resistance in *Mycobacterium tuberculosis* strains,[12] genomic typing of *Neisseria meningitidis*[13] and in a retrospective outbreak investigation of extended spectrum ß-lactamase (ESBL)-producing *E. coli*.[14] WGS for detection and characterization of microorganisms directly from clinical samples has been published recently.[6]

However, there is little knowledge regarding the concordance between Ion Torrent sequencing data and conventional PCR and/or phenotypic testing in clinical multidrug resistant *E. coli* isolates, typing results, and the presence of virulence genes in an endemic setting. The purpose of the present study was to assess the performance of the Ion Torrent PGM® for the combined detection of antimicrobial resistance in clinical *E. coli* isolates, i.e. the resistance genes for ß-lactam antibiotics, including carbapenems, fluoroquinolones, aminoglycosides and cotrimoxazole, housekeeping and virulence genes.

6.3 Materials and Methods

6.3.1 Bacterial strains and antimicrobial susceptibility testing

Ten clinical *E. coli* isolates with various sequence types were selected as representative of multidrug resistant phenotypes. Of these isolates, phenotypic antimicrobial susceptibility (AST) results, as well as conventional PCR and sequencing data on selected antimicrobial resistance genes and multilocus sequence typing (MSLT) data from a previous study were available[15] to assess the concordance with Ion Torrent sequencing data. No conventional PCR data were available for genes encoding resistance to cotrimoxazole and aminoglycosides, nor for the presence of virulence genes.

All isolates were identified by MaldiTof-MS with BioTyper software (version 3.0; BrukerDaltonics, Germany) and antimicrobial susceptibility testing was performed using the VITEK2® system (BioMérieux, Marcy-l'Étoile, France). ESBL production was investigated by the combination disk test with ceftazidime and cefotaxime or cefepime (in cefoxitin resistant isolates) with and without clavulanic acid as recommended by the Clinical Laboratory Standards Institute's guidelines. MICs for meropenem were determined using Etests (BioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. MICs were interpreted using EUCAST criteria (http://www.eucast.org/clinical_breakpoints/).

Screening for *gyrA*, *gyrB*, *parC*, *parE*, *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib*, *qepA*, the ß-lactamase genes bla_{TEM}, bla_{SHV}, bla_{OXA}, and bla_{CTX-M} was performed as described previously.[15] Phenotypic screening and confirmation of carbapenemases was performed using the Modified Hodge test and multiplex PCR of the carbapenemase genes $bla_{KPC,}$ $bla_{NDM,}$ $bla_{OXA-48,}$ bla_{VIM} and bla_{IMP}.[16]

6.3.2 DNA isolation

E. coli isolates were incubated on sheep blood agar (BioMérieux, Marcy-l'Étoile, France) overnight at 37°C. DNA isolation was performed using the DNAeasy Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The purity of the DNA was measured with a NanoDrop 1000 apparatus (NanoDrop products), and the quantity was estimated by a fluorescence-based method using a Qubit double-stranded DNA BR assay kit and a Qubit fluorometer (Life Technologies, Germany) according to the manufacturer's instructions.

6.3.3 Emulsion PCR and sequencing on the Ion Torrent

The genomic DNA samples were processed into Ion Torrent sequencing libraries using the Ion Xpress™ Plus gDNA Fragment Library Preparation method (Life Technologies; 4471269, Germany) using the guidelines provided by the manufacturer. Libraries were generated using 1 µg of the genomic DNA. Each sample was assigned a unique sequence barcode during this step. The final library fragment size was set to 300bp using a standard agarose gel size selection. Template preparation by ePCR and Ion sphere enrichment procedures were performed using the OneTouch system and Enrichment system, respectively using standard protocols (Ion OneTouch™ 200 Template Kit v2; 4478316). Samples were sequenced on Ion 318-chips (Life Technologies; 4469496), using the standard single end 200 bp read lengths [Ion PGM™ Sequencing 200 Kit v2; 4482006]. On each Ion Torrent 318- chip, two barcoded samples were sequenced. Primary analysis of the sequencing data to generate FASTQ files was done using Torrent Suite version 2.2.

6.3.4 Data analysis

The analysis of the sequenced reads has two major components: one is focused on the identification of genes by means of coverage and variant calling; the other one is focused on the typing of variants to determine the sequence type. First, all reads are mapped to the reference genome (AC 000091.1) using the TMAP aligner with default settings. Then the unaligned reads are extracted from the resulting BAM file using SAMTools, which are subsequently converted back to FASTQ files using an in-house script (Figure 1 on the next page).

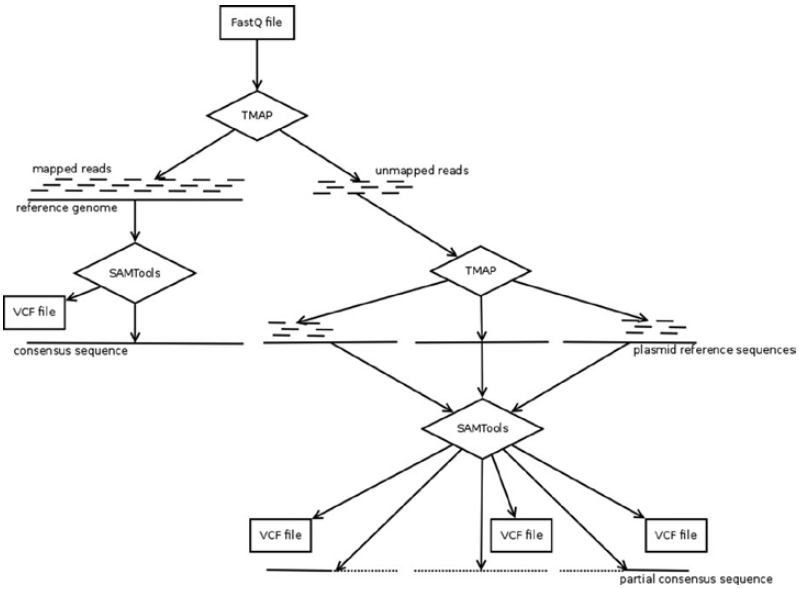

Figure 1. The short reads are mapped to the genomic reference sequence using TMAP. Unmapped reads are mapped in parallel to candidate plasmid reference sequences. For reference sequences on genome and plasmids variants are called and a consensus sequence is generated.

For more information about the used tools and their versions, see Table 1. All in-house scripts and the pipeline are available online.[17] All calculations were done on a cluster with 360Us running the Open Grid Scheduler.

Table 1. Tools and version number used for the analysis

Name	Version	Description
TMAP	0.0.19	Ion Torrent Aligner
SAMTools	0.1.19	Alignment manipulation tools
Piletools	0.21.dev	Mpileup manipulation tools.[a]
Wiggelen	0.2.0	Wiggle track manipulation tools.[b]
Unmap	None	Convert BAM to FASTQ.[c]
Fastools	0.8.0	FASTA/FASTQ manipulation tools.[d]

[a]https://pypi.python.org/pypi/piletools
[b]https://pypi.python.org/pypi/wiggelen
[c]https://git.lumc.nl/lgtc-bioinformatics/ngs-misc
[d]https://pypi.python.org/pypi/fastools

6.3.5 Gene identification

The reads from all sequenced genomes were aligned with the sequences of all 674 genes of interest (https://git.lumc.nl/j.f.j.laros/paltansing-ecoli/blob/master/analysis/genes.csv for the list of genes). Two metrics were used in order to identify these genes. First, the fraction of non-N content was assessed in order to calculate the horizontal coverage of the gene. Then, the number of variants compared to the reference sequence was used to distinguish between different gene variants. A coverage rate of 100% without variants was chosen to define the presence of a gene.

6.3.6 MLST

Consensus sequence construction of the Ion Torrent sequencing data of the seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*) was performed and converted to FASTA with Fastools. The FASTA sequences were entered into the MLST database of the Environmental Research Institute, University College Cork website (http://mlst.warwick.ac.uk/mlst/) to determine the sequence type.

6.3.7 Virulence genes

The presence/absence of thirteen virulence genes coding for fimbriae: *fimA, fimI, fimC* and *fimD*; iron-hydroxamate transporter units: *fhuB* and *fhuD*; flagellin: *fliC*; lipopolysaccharide *rfaY*, *rfaK*, *fep*E and *fepC*; genes coding for invasion: *aslA*, *csgA* and *csgB* was investigated.

6.4 Results

6.4.1 Performance data

The turnaround time from DNA isolation to sequence reads was about 24 hours. Sequencing yielded 2.29×10^6 reads on average per strain with a mean read length of 235 nucleotides. Of these reads, 80% mapped to the reference genome, giving an average coverage of 92x.

6.4.2 Sequencing of cultured isolates

WGS led to the same species identification of the 10 isolates conventionally identified as *E. coli* (16S rRNA gene).

Table 2. Phenotypical susceptibility and resistance genes detected in clinical *E. coli* isolates using Ion Torrent sequencing

	ß-lactams							Fluoroquinolones		Aminoglycosides			
	AMP	CAZ	CTX	ESBL[a]		MER		CIP		GEN/TOB		CTR	
Isolate	**P**	**P**	**P[a]**	**P[a]**	***bla* gene(s)**	**P**	**G[b]**	**P**	**G**	**P**	**G**	**P**	**G**
1508	R	R	R	+	**TEM-1** **CTX-M-1**	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I**	R	*aadA5*	R	*sul2* *dfrA17*
1632	R	R	R	+	**TEM-1** **OXA-1** **CTX-M-15**	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I** ***parE*:S458A** ***aac(6')-Ib-cr***	R	*aac-3-II* *aac-6-Ib* *aacA4*	R	*sul1* *dfrA17*
1941	R -	S	S	-	-	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I**	R	*aadA1*	R	*sul2* *dfrA1*
2218	R	R	R	+	**OXA-1** **CTX-M-55**	S	-	R	***gyrA*:S83L, D87H** ***parC*:S80I** ***aac(6')-Ib-cr***	R	*aacA4* *aac-6-Ib*	R	*sul1* *dfrA12*
2404	R -	R	R	+	**CTX-M-9a** SHV-12*	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I** ***parE*:L416F** ***qnrA1***	I	*ant-3-9a/b* *aadA*	R	*sul1* *dfrA16*
2480	R	R	R	+	**TEM-1** **CTX-M-1**	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I**	R	*ant-3-9a/b* *aadA*	R	*sul1* *dfrA-12*
2500	R	R	R	+	**TEM-1** **OXA-1** **CTX-M-15**	S	-	R	***gyrA*:S83L, D87N** ***parC*:S80I, E84V** ***parE*:I529L** ***aac(6')-Ib-cr***	I	*aac-6-Ib* *aacA4* *aadA5*	R	*sul1* *dfrA17*
2506	R	R	R	+	**TEM-1** **CTX-M14**	S	-	R	***gyrA*:S83L, D87G** ***parC*:S80I** ***parE*:S458A,** ***I529L***	R	*aadA5*	R	*sul2* *dfrA17*
4300	R	R	R	Indet	**OXA-1** SHV-12*	I	-	R	***gyrA*:S83L, D87N** ***parC*:S80I** ***parE*:S458A**	R	*aadA1*	R	*sul1*
6059	R	R	R	+	**TEM-1** **OXA-1** **CTX-M-15** SHV-12*	R	-	R	***gyrA*:S83L, D87N** ***parC*:E84V** ***aac(6')-Ib-cr***	R	*aac-3-II* *aacA4* *aac-6-Ib* *aadA5*	R	*sul1* *dfrA17*

AMP, ampicillin; CAZ, ceftazidime; CTX, cefotaxime; ESBL,extended-spectrum-ß-lactamase; MER, meropenem; CIP, ciprofloxacin; GEN, gentamicin; TOB, tobramycin; CTR, cotrimoxazole; P, phenotype;G, genotype;S, sensitive; I, intermediate, R, resistant
[a]ESBL confirmatory disk testing+, positive; -, negative; [b]-, no genotype found; indet, indeterminate. *bla*, ß-lactamase
Bold face indicates that conventional PCR and sequencing as well as Ion Torrent sequencing data were concordant in these isolates.
*indicates that conventional PCR and sequencing as well as Ion Torrent sequencing data were not concordant in these isolates.
Italicized text indicates genes identified with Ion Torrent sequencing.

An overview of the phenotypic AST and the resistance genes of individual isolates as detected by the Ion Torrent is shown in Table 2 on the left page.

Ion Torrent sequencing data of these isolates showed 100% concordance with conventional PCR and sequencing data for the ß-lactamases: bla_{TEM}, bla_{OXA} and bla_{CTX-M} types. For the bla_{CTX-M} type of ESBLs, conventional and Ion Torrent sequencing results were concordant in all ESBL positive isolates (n=8). WGS data correlated with previously identified single nucleotide polymorphisms in the quinolone resistance determining regions of *gyrA*, *parC* and *parE*, associated with resistance to ciprofloxacin in all cases. In addition, the presence of plasmid-mediated quinolone resistance genes *qnr* and *aac(6')-Ib-cr* were consistent with the results as found with conventional PCR and sequencing, as previously described.[15] No *qepA* or *oxqAB* loci were found by either method.

Three isolates contained bla_{SHV-12} by conventional PCR and amplicon sequencing. In one isolate (2404), the bla_{SHV} gene was found with Ion Torrent sequencing. However, it was not able to discriminate between the exact bla_{SHV}-type. In the remaining two isolates, 4300 and 6059, the presence of bla_{SHV-12} could not be detected with a 100% coverage rate. The coverage rate for bla_{SHV-12} in these two isolates was only 97.2% and 97.7%, respectively.

The MICs of meropenem for the isolates 4300 and 6059 were 1.5 mg/L and 4 mg/L, respectively. No carbapenemase production was detected among these two isolates with the Modified Hodge test. Two multiplex real-time PCR for detection of KPC, NDM, OXA-48, VIM and IMP- type carbapenemases were negative in both isolates. This is in agreement with Ion Torrent sequencing data, in which carbapenemase genes were not detected in either genome.

No conventional PCR was performed for genes encoding resistance to cotrimoxazole and aminoglycosides. We sought whether representative genes were present, as described in the literature. Aminoglycoside resistance was typically associated with the presence of *aac*-like and *aadA*-like genes. Other aminoglycoside resistance loci included *ant-3* in two isolates. No *aph*-like enzymes were detected.

Cotrimoxazole resistance was associated with the presence of *sul* and *dfrA* genes in nine isolates; no *dfrA* variant was found in isolate 4300, which was resistant to cotrimoxazole.

6.4.3 Typing data

Table 3 summarizes the MLST results. For all ten isolates, conventional typing results were concordant with the Ion Torrent sequence data generated.

Table 3. Results from the 10 *E. coli* isolates showing alleles and ST-type

Isolate	*adk*	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	ST
1508	10	11	4	8	8	13	2	167
1632	92	4	87	96	70	58	2	648
1941	10	11	4	8	8	13	2	167
2218	62	100	17	31	5	5	4	362
2404	18	106	17	6	5	5	4	393
2480	6	4	159	44	112	1	17	1011
2500	53	40	47	13	36	28	29	131
2506	53	40	47	13	36	28	29	131
4300	6	4	324	1	20	12	7	3583
6059	35	37	29	25	4	5	73	405

ST, sequence type

6.4.4 Virulence genes

Thirteen virulence genes coding for fimbriae: *fimA, fimI, fimC* and *fimD*; iron-hydroxamate transporter units: *fhuB* and *fhuD*; flagellin: *fliC*; lipopolysaccharide *rfaY*, *rfaK*, *fep*E and *fepC*; genes coding for invasion: *aslA*, *csgA* and *csgB* were identified (Table 4). Compared to the other sequence types, ST131 strains isolated from urine samples, both harbored most of the virulence characteristics investigated, i.e. 12 out of 13. In two *E. coli* ST167, also from urine samples, only 8 out of 13 virulence genes were found. None of the genes coding for fimbriae (*fimA, fimI, fimC, fimD*) and no *rfaK* encoding a lipopolysaccharide were found in these strains. Although phenotypic AST results for *E. coli* ST648 (isolate 1632 from urine) were similar to the *E. coli* ST 131 strains, only six virulence genes were found. The remaining sequence types did not show any relation to the number of virulence genes.

Table 4. Virulence genes present in 10 *E. coli* isolates as found with Ion Torrent sequencing

			Virulence genes												
Isolate	**ST**	**specimen**	***fimA***	***fimI***	***fimC***	***fimD***	***fhuB***	***fhuD***	***fliC***	***rfaY***	***rfaK***	***fepE***	***aslA***	***csgA***	***csgB***
2500	131	urine	+	+	+	+	+	+	-	+	+	+	+	+	+
2506	131	urine	+	+	+	+	+	+	-	+	+	+	+	+	+
6059	405	blood	+	+	+	+	+	+	+	-	-	+	+	+	+
2218	362	blood	+	+	+	+	+	+	+	-	-	+	+	+	+
2404	393	sputum	+	+	+	+	+	+	+	-	-	+	+	+	+
2480	1011	blood	+	+	+	+	+	+	-	-	-	+	+	+	+
4300	3583	blood	+	+	+	+	+	+	+	-	-	+	-	+	+
1508	167	urine	-	-	-	-	+	+	+	+	-	+	+	+	+
1941	167	urine	-	-	-	-	+	+	+	+	-	+	+	+	+
1632	648	urine	-	-	-	-	+	+	+	-	-	+	+	+	-

+, gene present;-, gene absent
ST, sequence type

6.5 Discussion

In the present study, we conducted a pilot project to determine if WGS technology allows for an efficient extended characterization of routine isolates, beyond what is customary in a routine diagnostic setting. Using WGS on multidrug resistant *E. coli* isolates, we were able to obtain species on the basis of the 16S rRNA gene, multilocus sequence type, as well as the identification of antimicrobial resistance and virulence genes. In a recent study, prediction of antimicrobial susceptibility was performed using a study database of >100 known resistant loci, including plasmid-mediated and chromosomal resistance genes on 74 *E. coli* and 69 *Klebsiella pneumoniae* isolates, that were sequenced using the Illumina HiSeq 2000 platform.[18] High sensitivity and specificity was obtained compared with phenotypic methods in routine use.

One disadvantage of the Illumina HiSeq is that it has a long run time of 12 days at high costs. The Ion Torrent PGM® has a turnaround time of 24 hours from DNA isolation to sequence reads. It is clear that for most uses in routine clinical microbiology a fast, cheap and compact bench-top machines, is preferable to the large, expensive, high-capacity machines like the Illumina HiSeq.

This report describes the application of the 318-chip for the Ion PGM platform, which supports for up to 200 bp read lengths. Long reads add to a higher coverage rate of the genome, which is important to detect single nucleotide polymorphisms

(SNPs) at crucial sites. In this study, all SNPs in the seven housekeeping genes were accurately detected. With regard to SHV-type detection, no reliable discrimination of the *bla*$_{SHV}$-type could be made. Accurate SNP calling is important to distinguish the exact *bla*$_{SHV}$-type, because this family of ß-lactamases comprises both non-ESBL and ESBL variants. In this study, isolates with *bla*$_{SHV-12}$ showed a low coverage rate, most likely due to poor mapping to the reference. This problem may be overcome in future with the generation of longer reads (>200 bp) or alternative methods for mapping based assembly as performed in this study.

Although clinical decision making based on WGS data was not the purpose of the present study, missing the *bla*$_{SHV}$ genes would not have affected treatment or infection control measures in two cases, since *bla*$_{CTX-M}$ already accounted for ESBL-activity. The remaining case (no. 4300) would be of concern if no phenotypic AST was conducted, as the presence of *bla*$_{SHV}$ with extended-spectrum activity could not be detected and no plasmid-mediated *bla*$_{AmpC}$ or carbapenemases were identified. As no carbapenemases were found in our isolates, it is likely that porin loss combined with ESBL-production is responsible for reduced susceptibility to meropenem,[19, 20] which needs further investigation. The WGS approach to investigate the resistance mechanisms porin expression and efflux pump activity have to be systematically investigated in association with phenotypic results. In addition, alternative sequence-based methods in the field of bacterial transcriptomics such as RNA-Seq needs to be explored.[21]

The WGS approach has created the additional opportunity to identify virulence genes, which provides information on the virulence potential of a specific isolate. Routine typing, surveillance and outbreak detection of verotoxigenic *E. coli* is already applicable and may also be used for typing and surveillance of other pathogens.[22] Several research studies using WGS have revealed that *E. coli* ST131 possess a variable complement of genes encoding established virulence factors in urinary tract infections.[23-25]

Broadly disseminated international clones such as ST131, ST405 and ST648 have emerged among *E. coli* that produce CTX-M ß-lactamases as important causes of community-onset urinary tract and bloodstream infections. These STs were found to have more virulence factors present compared to other ST38 and non-STs.[26] We were able to show that most of the virulence factors investigated were present in ST131 (12 out of 13) and ST405 (11 out of 13) compared to ST648 (6 out of 13). The precise role of these virulence factors remains to be elucidated; however

the combination of antimicrobial resistance and the presence of certain virulence factors might be important for their spread. Identifying virulence genes with the WGS approach is still at an early stage, but with the increase usage of this method providing new understanding of virulence factors in different STs. In addition to resistance prediction, the identification of virulence genes combined with patient characteristics may potentially predict the virulence potential of an isolate in the human host, i.e. the probability of recurrent infection and progression of an uncomplicated infection to bacteraemia.

Sequence based diagnostics will become a part of the clinical microbiology in the next era. The huge potential of WGS lies in its completeness: once sequenced the genetic information results in full characterization of clinical isolates. Therefore, WGS is a promising alternative to current diagnostic and reference tests. The data of this retrospective, proof-of-principle experiment is promising for the future. Further work is required to assess the performance and cost-effectiveness of antimicrobial resistance prediction from WGS data in a routine diagnostic setting. The advances provided by WGS, combined with clinical outcome data, should greatly enhance our understanding of the genetic basis of antimicrobial resistance, molecular epidemiology and virulence traits.

Acknowledgements

We thank Professor dr. A.C.M. Kroes for supporting this work and for his critical discussions. The authors declare no conflicts of interests.

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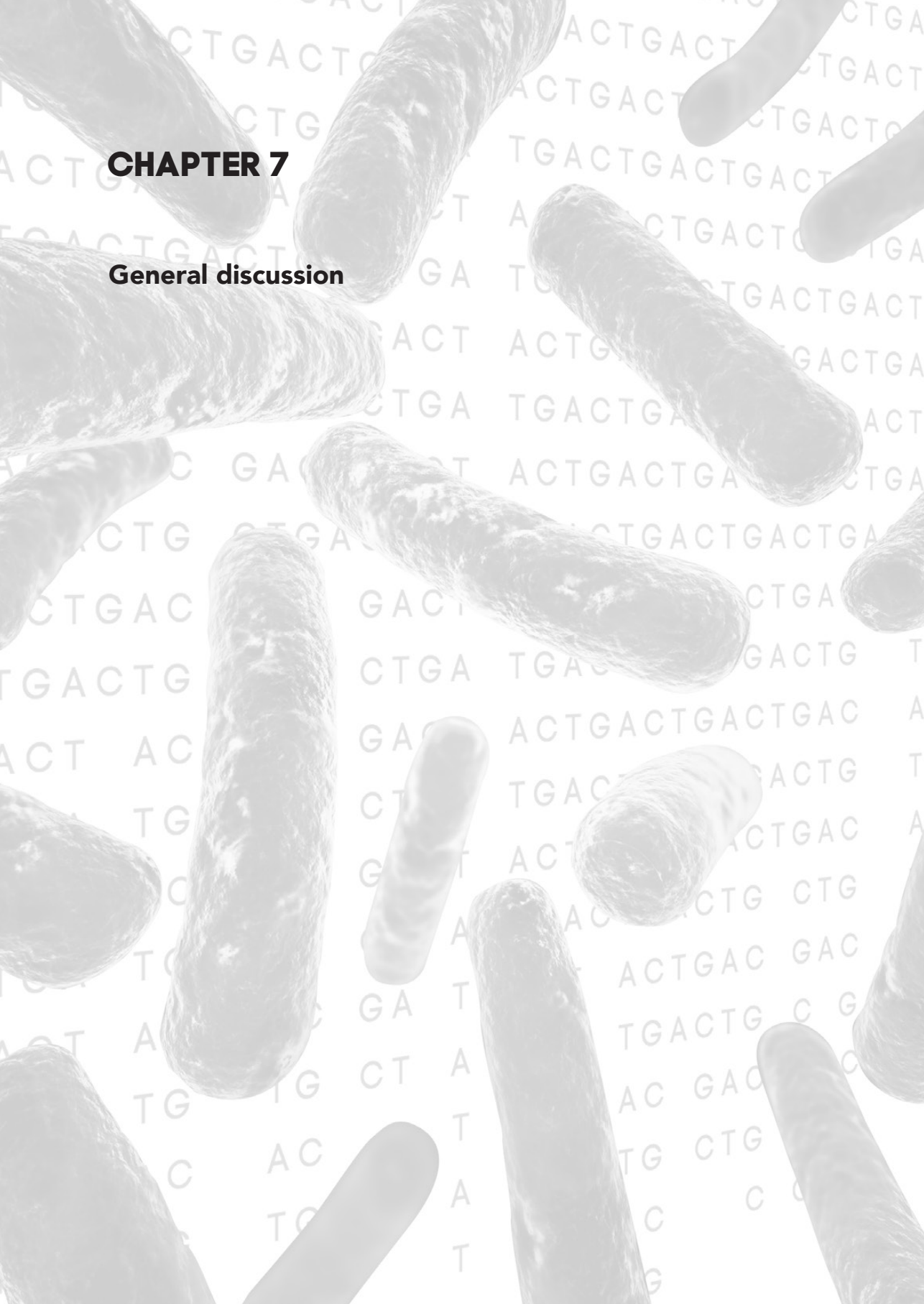

CHAPTER 7

General discussion

7.1 The continuing problem of antimicrobial resistance

Antimicrobial resistance among *Enterobacteriaceae* has progressively increased and has accelerated within the past 15 years. The spread of resistance is unlikely to stop and may compromise the value of established antimicrobial agents. The increase in resistance is mainly due to the spread of clones carrying genes encoding for extended-spectrum ß-lactamases or carbapenemases.[1-3] A report from the European Center for Disease Prevention and Control published in 2009 estimated that approximately 400,000 infections in Europe were associated with resistant strains, resulting in 25,000 extra deaths and 2.5 million extra hospital days.[4] The Dutch surveillance report NETHMAP 2013 indicates that 6% of the clinical *Klebsiella pneumoniae* and 8% of the *Escherichia coli* isolates recovered from unselected hospital departments are highly resistant microorganisms.[5] Increasing resistance rates to 3rd generation cephalosporins are reported from 3.5% in 2008 to 5.7% in 2012 for *E. coli* and from 4.0% to 7.5% for *K. pneumoniae* respectively. At present, cefuroxime and gentamicin are still used as empirical therapy in case of sepsis in the Leiden University Medical Center (LUMC). Given the rise of multidrug resistance and the mechanisms involved in *Enterobacteriaceae* in our hospital setting, it remains to be seen whether this will continue to be the appropriate choice. Despite the recognized need for new antimicrobials for clinical use, the reality is that only two new classes of antibiotics, oxazolidinone and lipopeptide, active against Gram-positive bacteria have been brought to market in the past 14 years.[6] The Pew Charitable Trusts is a large independent non-profit, non-governmental organization founded in 1948, that has assessed antibiotics currently in clinical development, which is available online and updated regularly (http://www.pewhealth.org/other-resource/antibiotics-currently-in-clinical-development-85899541594).However, given the inevitability that some in development will fail to obtain final approval, it is clear that there are too few drugs in development to meet current and anticipated patient needs.

The aim of the research described in this thesis was to obtain more insight into the four principal mechanisms of antimicrobial resistance in multidrug resistant *Enterobacteriaceae*, recovered at the LUMC, the Netherlands. This discussion will try to define some useful future strategies, based upon the results of this thesis and the other recent insights that will first be briefly summarized.

7.2 Necessity of rapid molecular detection

Antibiotic resistance genes were present among clinical isolates at very low levels prior to the introduction of antibiotics, and it is largely the selective pressure of antibiotic use, not only in humans but also in pets and livestock, which has caused the rise. The increasing mobility of people across the globe has further contributed to the spread of antimicrobial resistance. The rise in antimicrobial resistance relates not only to the number of individuals infected or colonized with antimicrobial resistant *Enterobacteriaceae*, but also to the diversity of underlying resistance mechanisms, which will continue to increase in the years ahead. In multidrug resistant strains of today, it is common that all four of principal mechanisms play a role in resistance, with strains having multiple determinants affecting susceptibility to each antimicrobial agent.

As shown in all chapters of this thesis, molecular characterization of antimicrobial resistance mechanisms illustrates the heterogeneity of antimicrobial resistance mechanisms involved in multidrug resistant *Enterobacteriaceae*. Besides important transferable extended-spectrum ß-lactamases, CTX-M, SHV, TEM, OXA, plasmid mediated AmpC ß-lactamases, carbapenemases, other enzymes such as the plasmid-mediated quinolone resistance Qnr and AAC-6'-Ib-cr should be tested for *Enterobacteriaceae*. As described in chapter 2, 3, and 4 molecular characterization revealed that chromosomally-encoded resistance traits such as mutations in the GyrA, ParC, ParE, chromosomal efflux activity, c-AmpC with its promoter/attenuator region are of importance as well. Although multiplex PCR and sequence analysis, qRT-PCR and microarrays are well established methods, as used in various chapters of this thesis, their use is insufficient to investigate the numerous genes involved. In the current era of multidrug resistance, molecular tests need to be rapid and highly multiplexed and multifaceted in a single assay. Therefore, improved diagnostic approaches for the rapid and adequate detection of antimicrobial resistance, epidemiological markers and virulence characteristics are essential. Besides early and sensitive detection of resistance mechanisms by non-phenotypic tests, preventive measures and active surveillance will become more important in the near future.

7.3 Implications for future strategies

7.3.1 Potential for whole genome sequencing

Microbiological laboratories play a central role in the combat against antimicrobial resistance trends by rapid and proper recognition of multidrug resistant *Enterobacteriaceae*. The use of phenotypic tests, PCR-based techniques and microarrays has all demonstrated their utility to detect resistance. To obtain a better understanding of the current molecular epidemiology and characteristics of multidrug resistant *Enterobacteriaceae* more information is required, e.g. the type of antimicrobial resistance mechanism, presence or absence of resistance genes and specific mutations conferring resistance.

With the emergence of the new tools of whole genome sequencing (WGS), it allows to sequence full bacterial genomes in an efficient way. WGS enables an integrated approach to study the complete genome of a bacterial isolate, which can be determined within hours, providing identification to the species, its susceptibility to antibiotics, its virulence characteristics and epidemiological markers, as described in chapter 6. This technique has become fast, making WGS compatible with the routine microbiology workflow.[7,8] A sample preparation method for rapid single colony WGS of different bacterial species has been validated for clinical use.[9] It is therefore to be considered likely that applications of WGS will play an important role in the approach to antimicrobial resistance and the following paragraphs will discuss some relevant topics for future research, related to this development.

7.3.2 Understanding and predicting antimicrobial resistance

First attempts to predict antimicrobial susceptibilities using WGS data in different species, such as *Staphylococcus aureus*,[10] *Mycobacterium tuberculosis* [11] as well as for *E. coli* and *K. pneumoniae* [12] have been reported recently with promising results. Despite rapid advances in this field and major advantages of this technique, WGS for the detection of antimicrobial resistance is still not yet widely adopted by clinical microbiology laboratories. The WGS approach in the absence of any phenotypic support for the functionality of a resistance gene or mutation could lead to major errors (reported as resistant and actually susceptible) and preclude the use of potentially useful therapies. Conversely, the absence of an identifiable gene or mutation sequence does not guarantee susceptibility. To be used confidentially in clinical practice, reliable genotypic prediction of the antimicrobial resistance

phenotype has to be demonstrated to the same standards as any new phenotypic method, using large diverse sets of unrelated isolates. Comprehensively validated genotypic prediction of antimicrobial resistance, ready for implementation in clinical practice will require multiple large studies.

In fact, it is likely that phenotypic methods will continue to be used, at least for the near future, to screen microbial isolates for unrecognized resistance patterns, and, thus, their mechanisms of resistance, before gene-level inquiry is pursued. However, the need for routine phenotyping will diminish as laboratories increasingly use WGS data, combined with phenotypic data, to elucidate the contribution of all underlying genetics in resistance. Further investigation is required to determine to what extent it will be possible to rely on genotype alone. The need for phenotypic verification of the genotype might be partly circumvented by WGS transcriptomics using RNA-Seq, thus, providing direct evidence of functional resistance rather than using gene identification.[13]

Although WGS is not suitable for resistance prediction in clinical practice yet, sequencing the whole genome provides full characterization of a bacterial strain, providing all genetic information on species identification, sequence type and virulence determination in a single test. WGS as a single platform for extracting all the information required is an attractive alternative in the routine diagnostic setting.

7.3.3 Routine typing and virulence determination

In addition to resistance prediction, completely sequenced genomes can provide for epidemiological typing and detection of virulence genes. Analysis of sequence typing and virulence factors in clinically relevant isolates has already been found useful in outbreak investigations.[14,15] Several studies using WGS have revealed that *E. coli* ST131 possess a variable complement of genes encoding established virulence factors in urinary tract infections.[16,17]

For useful application in clinical microbiology, it is necessary to conduct the relevant WGS analysis in realtime. Routine typing, surveillance and outbreak detection of verotoxigenic *E. coli* is already applicable and may also be used for typing and surveillance of other pathogens.[18] The expanded use of WGS in clinical microbiology has the potential to produce the equivalent of a HapMap for microbes (http://hapmap.ncbi.nlm.nih.gov/). When combined with data generated by the human HapMap, the risk of infection by specific strains or clones carrying characterized virulence factors in individuals of a particular haplotype could begin

to be assessed. Even more promising would be simultaneous genotyping of host and bacterial isolate, to define critical host factors in response to infection and progression of disease and treatment tailored to the patient's host characteristics.

7.3.4 Data management and reporting

Although WGS provides detailed information that will, in theory, enable routine diagnostic microbiology solely on the basis of the bacterial genomes, it is a formidable challenge to define and extract the appropriate information from the large amount of sequence data that is generated.[19,20] Thus, to facilitate the use of WGS data for routine diagnostics, typing and surveillance, it is important that the sequence data can be automatically and quickly converted to clinically relevant information that can be easily interpreted without bioinformatics skills. At present, there are several databases for the investigation of antimicrobial resistance genes, including the Antibiotic Resistance Genes Database,[21] Resfinder for acquired antimicrobial resistance genes[22] and Antibiotic Resistance Cassette Database (RAC).[23] For bacterial typing, different comprehensible web-tools for WGS analysis are available, such as the Bacterial Isolate Genome Sequence Database (BIGSDB),[24-26] MLST predictor,[27] and snp-Tree.[28] VirulenceFinder, for detection of *E. coli* virulence genes has been added recently.[29] While most of these databases are new and well updated, they have diverse sources. Reference data should be stored and maintained in one centralized, expandable and up-to-date database, e.g. to include novel resistance determinants.

Predictions about the resistance and susceptibility need to be accurate for proper patient management. One solution is the creation of an international, online database, divided into two parts. The first part is a depository into which accredited diagnostic laboratories routinely deposit WGS data, combined with clinical and epidemiological data, from across the world. These data could provide for an expert panel to discuss and propose new markers for antimicrobial drug resistance or to link genotype directly to disease outcome. Harmonization of these genotypic markers could be modelled in an international up-to-date diagnostic WGS database for routine clinical microbiology laboratories with approved markers for automated WGS data interpretation.

7.3.5 Screening for gastrointestinal colonization

Early detection of multidrug resistant *Enterobacteriaceae* (MDR-E) carriage could allow timely implementation of infection control measures and the appropriate selection of antimicrobials. On-admission surveillance for ESBL-E has been associated with a reduced incidence of MDR-E infections during hospitalization.[30] In 2013, a guideline for the detection of highly resistant microorganisms (HRMO) has been implemented nationally.[31] Patients are placed in contact isolation and screened for HRMO-carriage, if patients are transferred from a foreign hospital, or if they come from any ward with an ongoing outbreak.[31]

Travel to geographic areas with high prevalence of antimicrobial resistant bacteria has been shown to be a risk factor for the acquisition of MDR-E.[32] Prospective cohort studies among travelers returning from Australia, Canada, Sweden and New York revealed high carriage rates of varying from 18-25% after foreign travel [33-36] and showed that travel to India or the Indian subcontinent was the highest risk factor. The study presented in chapter 5 also highlighted international travel as a significant risk factor for the acquisition of MDR-E. The high pre- and post-travel carriage rate of 8.6% and 30.5% of MDR-E respectively as found in chapter 5 has recently been confirmed in another group of 122 returning healthy travelers from the Netherlands.[37] The high rates of ESBL fecal colonization as found in chapter 5 highlights the importance of a targeted screening strategy for Dutch hospitals. The present results implicate that it is necessary to perform active surveillance of MDR-E in patients on admission, who returned from Asia during the previous 6 months, which are not considered to be high risk carriers yet.

Colonization of healthy subjects with antimicrobial resistant *Enterobacteriaceae* could contribute to the amplification of resistance both in the community and nosocomial settings.[38-40] Metagenomic analysis of the fecal microbiome of 162 individuals from Denmark, Spain and China were compared to environmental and agricultural metagenomic data sets. The presence of resistance genes was significantly higher in the gut microbiome, reaching an average of 0.266% of the total number of gut genes. Individuals from China showed the highest diversity of resistance genes, whereas individuals from Denmark showed the lowest levels of diversity and abundance.[41] Another study on the gut microbiome also found a higher abundance of resistance determinants in the microbiota of individuals from Spain, Italy and France compared with individuals from Denmark, the United States and

Japan[42] WGS directly on rectal swabs may allow realtime generation of locally and nationally relevant epidemiological data, allowing strains to be tracked, their relative importance evaluated and control efforts to be meaningfully targeted. Furthermore, the higher level of discrimination will permit the detection and monitoring of newly emerging strains. Linked with clinical surveillance data, this could provide as early warning system.

7.4 Culture-free sequencing

WGS technology is developing at incredible speed and has already transformed the research landscape in microbiology. In the next five years, future developments of workflow and pipelines will facilitate research on antimicrobial resistance mechanisms, genes, virulence markers and epidemiological profiling in a diagnostic setting.[43-45] Full bacterial genomes can already be obtained from a single DNA molecule using the PacBio long-read, single cell molecule, real-time sequencing technology.[46-48] This technique has recently been used to investigate the resistome of cow manure, in which novel and diverse antimicrobial resistance determinants conferring resistance to chloramphenicol, kanamycin, tetracycline, or ß-lactam antibiotics were found.[49]

If we assume that WGS will become increasingly affordable, rapid, and simple to use and that technologies and databases will evolve to the extent that WGS will be highly reproducible and reliable, application in clinical microbiology directly on clinical samples seems a matter of time. Current and realistic future applications of WGS in routine clinical microbiological laboratories are illustrated in Figure 1.

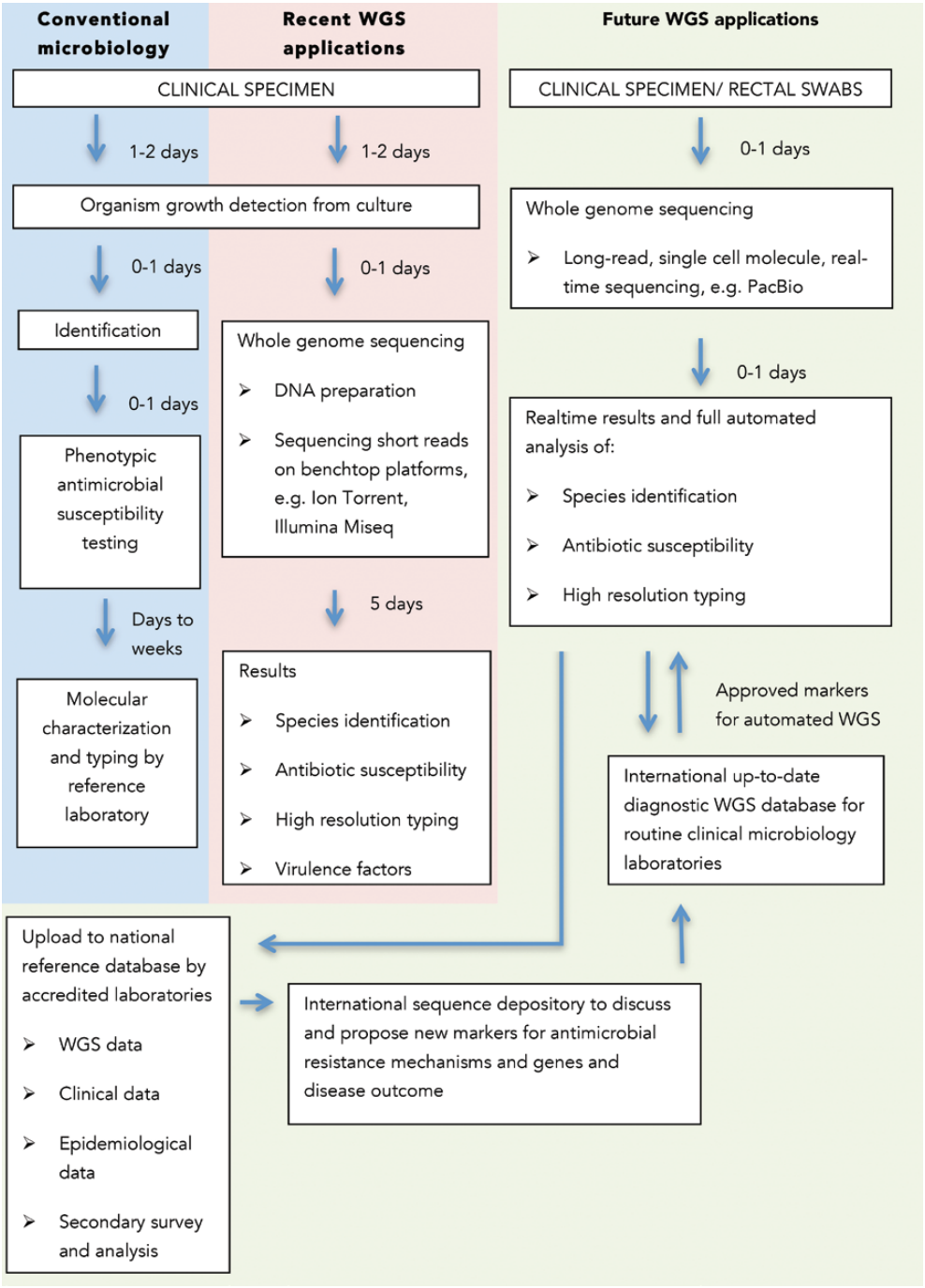

Figure 1. A comparison of the current and future methods in routine clinical microbiology

The eventual adaptation of WGS in every clinical laboratory will provide all information: identification to the species, its susceptibility to antibiotics, its virulence characteristics and epidemiological markers. Because results are obtained rapidly, WGS may ultimately replace most if not all current phenotypical microbiological methods. If WGS would fulfill such expectations, this will revolutionize diagnostics and clinical practice. Empirical antibiotic treatment will be reduced in time to a minimum or tailored therapy may even be started immediately. Furthermore, monitoring and surveillance of antimicrobial resistance could be performed in real-time.

Another promising approach is sequence-based metagenomics, which involves extracting and random sequencing of DNA directly from a variety of environment (e.g. soil, agriculture, livestock, river/sea/lake, aquaculture and the human gut) including DNA from uncultured bacteria.[50-53] The application of metagenomics will not only facilitate future identification of novel resistance mechanisms, but will be used for the prediction of antibiotic resistance and distinct host features, for example in the human microbiome.[54]

To be fully useful, WGS data will have to be shared on local, national and international level; integrated post-genomic approaches to store epidemiological and genomic data are under development.[55, 56] In this respect we could and should learn from micro-organisms who survive thanks to their ability and practice to exchange resistance information rapidly. *Enterobacteriaceae* readily exchange information at a high speed and so should we.

7.5 References

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Nederlandse samenvatting

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Inleiding

In de gezondheidszorg wordt de behandeling van bacteriële infecties met antibiotica in toenemende mate bemoeilijkt door het voorkomen van bacteriën, die resistent zijn tegen meerdere soorten antibiotica. De zogenaamde eerste-, tweede- en soms ook derdelijns antibiotica zijn steeds vaker onbruikbaar als gevolg hiervan. Het in de afgelopen jaren sterk toegenomen gebruik van antimicrobiële middelen, zowel binnen als buiten het ziekenhuis, ligt zonder twijfel ten grondslag aan deze ontwikkelingen. Antibioticagebruik, en dan vooral suboptimaal, langdurig of herhaald, is de belangrijkste factor die leidt tot selectie en verspreiding van resistente bacteriestammen. Maar ook het gebruik ervan in de veeteelt kan via vleesconsumptie, direct contact met dieren of besmetting van het milieu leiden tot ongewenste verspreiding van resistente bacteriën. Tot slot kan reizen naar het buitenland leiden tot import van resistente bacteriën.

Het is duidelijk dat de ziektelast van resistentie in Europa toeneemt. Een schatting is dat dit neerkomt op zo'n 25.000 extra sterfgevallen per jaar en 2,5 miljoen extra ziekenhuisligdagen. Wanneer multiresistente bacteriën infecties veroorzaken, is de gebruikelijke behandeling met antibiotica soms onwerkzaam en treedt dus een vertraging op in de behandeling. Daarnaast vergt het geïsoleerd verplegen van deze patiënten extra inspanningen en is belastend voor zowel het ziekenhuispersoneel als voor de patiënt. Verder nemen ook de kosten voor verpleging en verzorging toe. Ook bij patiënten, die buiten het ziekenhuis een infectie oplopen en niet een medische voorgeschiedenis hebben waarin van antibioticumgebruik sprake is, wordt een multiresistente bacterie steeds vaker gevonden als oorzakelijk agens.

Bij een ernstige infectie wordt microbiologische diagnostiek ingezet om de oorzakelijke bacterie te determineren en tevens de gevoeligheid voor antibiotica te bepalen. Er zijn verschillende methoden om deze gevoeligheid te meten. Veelal wordt antibioticaresistentie bepaald door middel van geautomatiseerde apparatuur. Dit gebeurt op basis van optische meting van bacteriële groei in reeksen met verschillende antibioticaconcentraties. Zowel de kweek- als de geautomatiseerde methode levert een uitslag die verdeeld kan worden in gevoelig (S), intermediair

gevoelig (I) of resistent (R). Dit geeft echter geen informatie over de achterliggende resistentiemechanismen, die hieraan ten grondslag liggen. Ook duurt dit proces enkele dagen totdat de definitieve uitslag bekend is.

Resistentiemechanismen

Resistentie tegen antibiotica bij bacteriën kan een gevolg zijn van verschillende resistentiemechanismen. De bacteriële resistentiemechanismen zijn:

1. Productie van een enzym dat het antibioticum inactiveert.
2. Veranderingen in het aangrijpingspunt, waarop het antibioticum werkzaam is.
3. Een verhoogde activiteit van efflux pompen, waardoor het antibioticum naar buiten wordt gepompt.
4. Barrières om de bacterie binnen te dringen door een verandering in permeabiliteit van de celmembraan, bijvoorbeeld door verlies van buitenmembraaneiwitten op de bacteriële celwand, zodat een antibioticum niet meer wordt opgenomen.

Een bacterie kan zowel een intrinsieke als een verworven resistentie hebben tegen een antibioticum. Intrinsieke resistentie is een stabiele genetische eigenschap die gecodeerd is in het bacteriële chromosomale DNA. Intrinsieke antibiotica-resistentiegenen kunnen permanent actief zijn of geïnduceerd worden door de blootstelling aan een specifiek antibioticum. Een bacterie kan ook resistent zijn door een intrinsieke eigenschap zoals het ontbreken van een bindingsplaats voor een specifieke antibioticum. Bacteriën kunnen ook resistentie verwerven door een mutatie in het bacteriële chromosomale DNA. In de meeste gevallen van verworven resistentie hebben bacteriën verschillende stappen nodig, waarbij elke stap op zich slechts een lichte verandering in gevoeligheid geeft. Bestaande resistentiemechanismen kunnen ook actiever worden door bijkomende mutaties. Behalve door mutaties kunnen bacteriën resistentie ontwikkelen door het verwerven van nieuw genetisch materiaal. Sommige bacteriën wisselen chromosomaal DNA uit in een proces dat *transformatie* heet. Het nieuw ingebrachte DNA kan recombineren met vergelijkbare sequenties in het chromosomale DNA van de ontvangende bacterie. Transformatie zorgt voor genetische variatie die zich vooral verspreid van de 'moeder-' naar de 'dochtercel' *(verticaal)*. Daarnaast kunnen bacteriën mobiele genetische elementen bevatten: plasmiden, (conjugatieve) transposonen en integronen. Genetische informatie die aanwezig is

in deze elementen kan worden overgedragen tussen verschillende stammen van één bacteriesoort en tussen verschillende bacteriesoorten. Dit wordt *horizontale genoverdracht* genoemd. De mobiele genetische elementen vergroten de mogelijkheid tot overdracht van resistentiegenen en resistentieontwikkeling.

Voor een effectief antibioticumbeleid is meer inzicht nodig in de epidemiologie van de onderliggende antimicrobiële resistentiegenen van multiresistente bacteriën, de verspreiding van deze resistentiegenen en de factoren die bijdragen aan de selectie en de transmissie van multiresistente bacteriën. Het identificeren en karakteriseren van resistentiegenen van een groot aantal isolaten is echter zeer arbeidsintensief. De nieuwe generatie sequencing-technologieën, de zogenaamde 'next-generation sequencing' (NGS) technieken, zijn in staat grotere stukken DNA tegelijkertijd te sequencen en dus geschikter voor het ontrafelen van hele genomen. Hierdoor kan binnen korte tijd een enorme hoeveelheid DNA-sequentie worden geproduceerd.
In dit proefschrift zijn de resistentiemechanismen bij *Enterobacteriaceae* bestudeerd. *Enterobacteriaceae* zijn normale darmbewoners, die op andere plaatsen in het lichaam milde tot levensbedreigende infecties kunnen veroorzaken. *Enterobacteriaceae* zijn Gram-negatieve staven en de meest voorkomende zijn: *Escherichia coli, Klebsiella* en *Enterobacter.* Minder vaak worden *Proteus, Serratia, Citrobacter* en *Morganella* gevonden. Voor alle genoemde species geldt, dat ze over diverse resistentiemechanismen tegelijk kunnen beschikken en zich in toenemende mate manifesteren als multiresistente bacteriën.

De snel toenemende resistentie tegen antibiotica in deze groep van bacteriën is voor een belangrijk deel toe te schrijven aan de productie van extended-spectrum-ß-lactamases (ESBLs) en AmpC ß-lactamases. Deze enzymen zijn in staat de meest voorgeschreven antibiotica, cefalosporines, tegen infecties met *Enterobacteriaceae* onwerkzaam te maken. Een ander veelgebruikt antibioticum is ciprofloxacine, welke behoort tot de klasse van de fluoroquinolonen. Ciprofloxacine is een breedspectrum antibioticum dat zowel tegen Gram-positieve als Gram-negatieve bacteriën werkzaam is. Het blokkeert de DNA replicatie door te binden aan DNA gyrase, waardoor tweezijdige breuken in het DNA ontstaan. Selectie voor bacteriën met puntmutaties in de genen die coderen voor DNA gyrase en topoisomerase IV *(gyrA, gyrB, parC* and *parE)* zijn lang veronderstelt als belangrijkste oorzaak voor resistentie tegen ciprofloxacine. Naast puntmutaties op het bacteriële chromosoom

zijn ook plasmid-geassocieerde mechanismen, zoals de genen *qnr* en *aac-(6')-Ib-cr*. Ook kan een verhoogde activiteit van effluxpompen en/of verlies van porines op de bacteriële celwand bijdragen aan resistentie tegen fluoroquinolonen.

Hoofdstuk 2

In hoofdstuk 2 is de moleculaire karakterisering van antimicrobiële resistentie genen in 68 multiresistente *Enterobacteriaceae*, afkomstig van verschillende LUMC-patiënten in het jaar 2008 beschreven. Met behulp van moleculaire technieken (PCR) zijn de chromosomale genen *gyrase A, gyrase B, parC* en *parE* in kaart gebracht. Daarnaast is onderzocht of deze bacteriën de plasmidaal-gecodeerde genen *qnrA, qnrB, qnrS, aac(6')-Ib-cr* en *qepA* bevatten. Ook werd de aanwezigheid van ESBLs onderzocht. Tenslotte is clonale verwantschap van de *Escherichia coli* isolaten onderzocht.

In totaal werden 49 *E. coli*, 16 *Klebsiella pneumoniae* and 3 *Enterobacter cloacae* isolaten onderzocht. In alle *E. coli* isolaten werd een mutatie in het *gyrase A* gen gevonden. In 45 (92%) van de *E. coli* isolaten werd tenminste 1 mutatie in het *parC* gen gevonden. In 59% van de *E. coli* isolaten waren ook mutaties in het *parE* gen aanwezig. De meest voorkomende mutatie in het *parE* gen was I529L, welke was geassocieerd met *E. coli* met sequentie type 131 (ST131).

Bovendien werd in 29 *E. coli* isolaten een ESBL gen gevonden; de meest voorkomende was $bla_{\text{CTX-M-15.}}$ In de onderzochte *Klebsiella* isolaten werden voornamelijk de plasmidaal gecodeerde *qnr*, en *aac(6')-Ib-cr* genen samen met ESBL genen (n=13; $bla_{\text{CTX-M}}$ and bla_{SHV}) en/of bla_{ampC} (n=3; $bla_{\text{DHA-1}}$) aangetoond. *E. coli* isolaten hebben voornamelijk chromosomale mutaties, die leiden tot resistentie tegen fluoroquinolonen. De I529L mutatie in het *parE* gen komt in de meeste, maar niet in alle ST131 isolaten voor. In tegenstelling tot de *E. coli* isolaten komen plasmidale resistentie genen vaker voor bij de onderzochte *K. pneumoniae* isolaten.

Hoofdstuk 3

Het onderzoek in hoofdstuk 3 beschrijft een studie naar de verhoogde activiteit van effluxpompen in 47 *E. coli* stammen, met een verminderde gevoeligheid voor ciprofloxacine en cefalosporines, afkomstig van verschillende LUMC-patiënten in het jaar 2008. Het doel van deze studie was te onderzoeken of verhoogde efflux pompactiviteit bijdraagt aan resistentie tegen fluoroquinolonen in *E. coli* isolaten. De activiteit van effluxpompen is bestudeerd met een geoptimaliseerde fluorometrische methode met behulp van bisbenzimide als fluorescentiemarker. De expressie van effluxpomp genen zoals *acrA, acrB, tolC, yhiV* en *mdfA* zijn onderzocht met behulp van kwantitatieve real-time reverse transcriptie-PCR (qRT-PCR). Ook zijn de expressieniveau's van de buitenmembraan-eiwitten *ompF* en *ompX* bepaald.
Met behulp van de RT-PCR methode kon in 35 isolaten overexpressie van messenger RNA van ≥1 efflux pomp aangetoond worden. Met behulp van de fluorometrische methode werd in 6 isolaten een verhoogde efflux pomp activiteit aangetoond. Alleen de gecombineerde overexpressie van *acrAB-TolC* en *mdfA* correleerde met de resultaten van de fenotypische fluorometrische methode. De bijdrage van actieve effluxpomp activiteit aan de resistentie tegen fluoroquinolonen was beperkt.

Hoofdstuk 4

In een tweede onderzoek naar cefalosporine resistentie, beschreven in hoofdstuk 4, is in 49 klinische *E. coli* isolaten onderzocht of een verhoogde expressie van het AmpC enzym de oorzaak is van resistentie tegen cefoxitin en of dit van invloed is op de activiteit van 3e generatie cefalosporinen.
Negen van 33 (27,2%) cefoxitin-resistente isolaten (minimaal remmende concentratie (MIC) > 8 mg/L) toonde hyperproductie van chromosomaal AmpC (c-AmpC) op basis van (i) ten minste twee positieve fenotypische testen met AmpC-remmers, (ii) mutaties in de promoter/attenuator gebieden en (iii) een 6,1 tot 163-voudige toename van c-AmpC expressie door kwantitatieve RT-PCR. In de ESBL-negatieve isolaten waren de MICs van ceftazidim en cefotaxim meestal boven wildtype niveau, maar onder het S/I breekpunt (EUCAST richtlijn), met uitzondering van één isolaat met MICs van 4 mg/L. Er werden geen plasmidaal gecodeerde AmpC

genen aangetoond. Periplasmatische extracten (uit de ruimte tussen de binnenste celmembraan en buitenste membraan) van 9 c-AmpC hyperproducerende isolaten werden vooraf geïncubeerd met of zonder cefuroxim of ceftazidim en met behulp van SDS-PAGE geanalyseerd. Cefuroxim en ceftazidim waren bestand tegen hydrolyse maar fungeerden als remmers van het enzym. Geen van deze isolaten toonde verlies van buitenmembraaneiwitten.
Uit deze studie is gebleken dat resistentie tegen cefoxitin niet specifiek is voor c-AmpC hyperproducerende stammen. c-AmpC hyperproducerende *E. coli* isolaten zijn meestal nog steeds gevoelig voor de 3e generatie cefalosporinen, maar wel minder gevoelig dan wildtype *E. coli*. Monitoring van cefoxitin-resistente *E. coli* op de ontwikkelingen in de resistentie tegen de 3e generatie cefalosporines wordt aangeraden.

Hoofdstuk 5

Nederlanders reizen steeds meer naar verre bestemmingen waar relatief veel resistente bacteriën voorkomen. In deze prospectieve cohortstudie is onderzocht in hoeverre resistente darmbacteriën worden opgelopen tijdens deze verre reizen, hoe lang men deze bacteriën bij zich blijft houden en of deze 'import-bacteriën' zich ook verspreiden onder niet-reizende huisgenoten. Het gaat hierbij om dragerschap van carbapenemase producerende *Enterobacteriaceae* (CP-E) en extended-spectrum-ß-lactamase producerende *Enterobacteriaceae* (ESBL-E) en de geassocieerde risicofactoren. Er werden bij 370 Nederlandse reizigers van de Travel Clinic van het LUMC en de GGD Hollands Midden vragenlijsten en rectumuitstrijken afgenomen vóór en na terugkeer van een buitenlandse reis. De ESBL-enzymen werden gekarakteriseerd met behulp van een micro-array (Check-Points BV). Bovendien zijn alle *E. coli* isolaten getypeerd. Van 370 reizigers waren 32 (8,6%) gekoloniseerd met ESBL-E voor de reis, 113 (31%) verwierf een ESBL-E en 26 reizigers waren zes maanden na terugkeer nog gekoloniseerd. Er werden geen CP-E gevonden in deze studie. Onafhankelijke risicofactoren voor ESBL-E dragerschap waren reizen naar Zuid- en Oost-Azië. Typering van de *E. coli* isolaten toonde uitgebreide genetische diversiteit. CTX-M enzymen waren het overheersende ESBL-type, dat werd gevonden. Het percentage ESBL-E dragerschap na een buitenlandse reis in Nederlandse reizigers was hoog (31%). Actieve surveillance van ESBL-E en CP-E

en minimaal contactisolatie voorzorgsmaatregelen worden aanbevolen bij opname van patiënten, die in de afgelopen 6 maanden naar Azië hebben gereisd.

Hoofdstuk 6

De nieuwe generatie sequencing-technologieën, de zogenaamde 'next-generation sequencing' (NGS) technieken, zijn in staat grotere stukken DNA tegelijkertijd te sequencen en dus geschikter voor het ontrafelen van hele genomen. Hierdoor kan binnen korte tijd een enorme hoeveelheid DNA-sequentie worden geproduceerd. In hoofdstuk 6 zijn 10 goed gekarakteriseerde klinische *E. coli* isolaten geselecteerd voor sequentie analyse met behulp van de Ion Torrent bench-top sequencer.
In deze isolaten is de aanwezigheid van antimicrobiële resistentie mechanismen voor ß-lactam antibiotica, fluoroquinolonen, aminoglycosiden en co-trimoxazol onderzocht. Bovendien werd Ion Torrent sequentie analyse gebruikt om de isolaten te typeren en de aanwezigheid van 13 virulentiegenen te bepalen. Resultaten werden vergeleken met conventionele PCR en sequenties voor 18 antimicrobiële resistentiegenen. Voor 17 antimicrobiële resistentiegenen waren de NGS resultaten vergelijkbaar met conventionele PCR en sequentieanalyse. Alleen de SHV genen waren moeilijk te identificeren vanwege een lage dekkingsgraad van dit gen. Daarnaast is met behulp van NGS de aanwezigheid van 13 virulentiegenen geïdentificeerd. Tenslotte kon met deze techniek ook in alle gevallen het correcte sequentietype worden vastgesteld. NGS met behulp van de Ion Torrent sequencer van klinische isolaten maakt het mogelijk om het hele genoom van een bacterie te onderzoeken in één test.

Conclusie

Zoals weergegeven in alle hoofdstukken van dit proefschrift toont moleculaire karakterisering van antimicrobiële resistentie mechanismen de heterogeniteit aan van antimicrobiële resistentiemechanismen in multiresistente *Enterobacteriaceae*. Naast de belangrijke overdraagbare ESBL-enzymen, CTX-M, SHV, TEM, plasmidaal-gemedieerde AmpC ß-lactamasen en carbapenemases zouden multiresistente isolaten in elk geval ook onderzocht moeten worden op de aanwezigheid van

andere resistentie genen, zoals de plasmidaal-gemedieerde resistentie genen *qnr* en *aac-6'-Ib-cr*.
Zoals beschreven in hoofdstuk 2, 3, en 4 is met moleculaire karakterisering gebleken dat chromosomaal gecodeerde resistentie, zoals de mutaties in GyrA, ParC, ParE, chromosomale efflux activiteit, c-AmpC met zijn promoter/attenuator regio ook een belangrijke rol spelen. Hoewel verschillende technieken als multiplex PCR en sequentieanalyse, qRT-PCR en microarrays zijn gebruikt in verschillende hoofdstukken van dit proefschrift, is het gebruik hiervan niet toereikend om alle betrokken genen te kunnen onderzoeken. In het huidige tijdperk van multiresistente bacteriën zijn moleculaire technieken nodig, die het hele genoom kunnen onderzoeken in één test.

De toepassing van NGS in een klinisch microbiologisch laboratorium zal alle informatie verstrekken: de identificatie van de species, de gevoeligheid voor antibiotica, de virulentie kenmerken en typering en tenslotte ook de hoeveelheid van de aanwezige species. Mogelijk kan NGS uiteindelijk de meeste, zo niet alle huidige fenotypische microbiologische methoden vervangen. Als NGS aan deze verwachtingen kan voldoen, zal dit een revolutie betekenen in de microbiologische diagnostiek en de klinische praktijk. Naast het gebruik van snelle en gevoelige detectie van resistentiemechanismen door niet-fenotypische testen, zal actieve realtime monitoring en surveillance belangrijker gaan worden.

Dankwoord

Dankwoord

Het meest gelezen onderdeel van een proefschrift en is ook een van de weinige hoofdstukken dat niet eindeloos is gecorrigeerd door supervisoren, co-auteurs en peer-reviewers. Dit is helemaal van mezelf!
Hoewel alleen mijn naam als enige op de voorkant van dit proefschrift staat, had ik dit niet kunnen schrijven zonder hulp van vele anderen. Alle collega's van de afdeling Medische Microbiologie en Infectieziekten wil ik daarom bedanken voor de prettige samenwerking, werksfeer en collegialiteit.
Verder wil ik alle medeauteurs bedanken voor hun bijdrage aan dit proefschrift. Daarnaast wil ik een aantal mensen in het bijzonder noemen.

Sandra Bernards, mijn co-promotor, voor de zeer betrokken begeleiding in het onderzoek, de leerzame discussies en de onvoorwaardelijke steun tijdens het corrigeren van vele manuscripten. Ik heb er veel van geleerd.

Louis Kroes, mijn promotor, voor de nuchtere kijk op de zaak, het einddoel helder krijgen en de ruimte die jij me gaf om onderzoek te doen. Vooral je ondersteuning tijdens de afronding van het proefschrift in combinatie met mijn nieuwe baan heb ik erg gewaardeerd.

Margriet Kraakman: ik heb het geluk gehad dat ik werd bijgestaan door een geweldige analist. Geen enkel experiment was voor jou teveel. Mede dankzij jouw praktische werk is dit proefschrift een feit.

Tijdens mijn onderzoek heb ik zes stagiaires mogen begeleiden. De studenten, Anne-Marie van Welzen, Jouraima Ras, Ivo Kors, Yi Li, Anouk Tengeler en Romy Mesman bedankt voor jullie enthousiasme om iets te willen leren en jullie bijdrage aan dit onderzoek.

Els Wessels; na mijn stage parasitologie in India werd jij in maart 2008 mijn nieuwe collega en waren we lange tijd kamergenoten! Dank je voor de prettige samenwerking, gezellige gesprekken en het ontwerpen en bestellen van primers en probes en eerste hulp bij moleculaire problemen. Samen met Eric Claas zijn jullie een geweldig moleculair team. Eric bedankt voor het bijbrengen van de Brabantse taal.

Alle (oud) arts-assistenten van de afdeling Medische Microbiologie bedankt voor het delen van veel gezelligheid, gezamenlijke uitjes, lief en (promotie-)leed. Martha, wij begonnen 6 maanden na elkaar met de opleiding tot arts-microbioloog. Veel deden we dan ook samen, waarbij we elkaar nooit in de weg zaten. Een echte symbiose! Onze samenwerking heeft veel voor me betekend. In november 2012 had ik de eer om naast je te staan bij jouw promotie. Bedankt dat jij nu mijn paranimf wilt zijn.

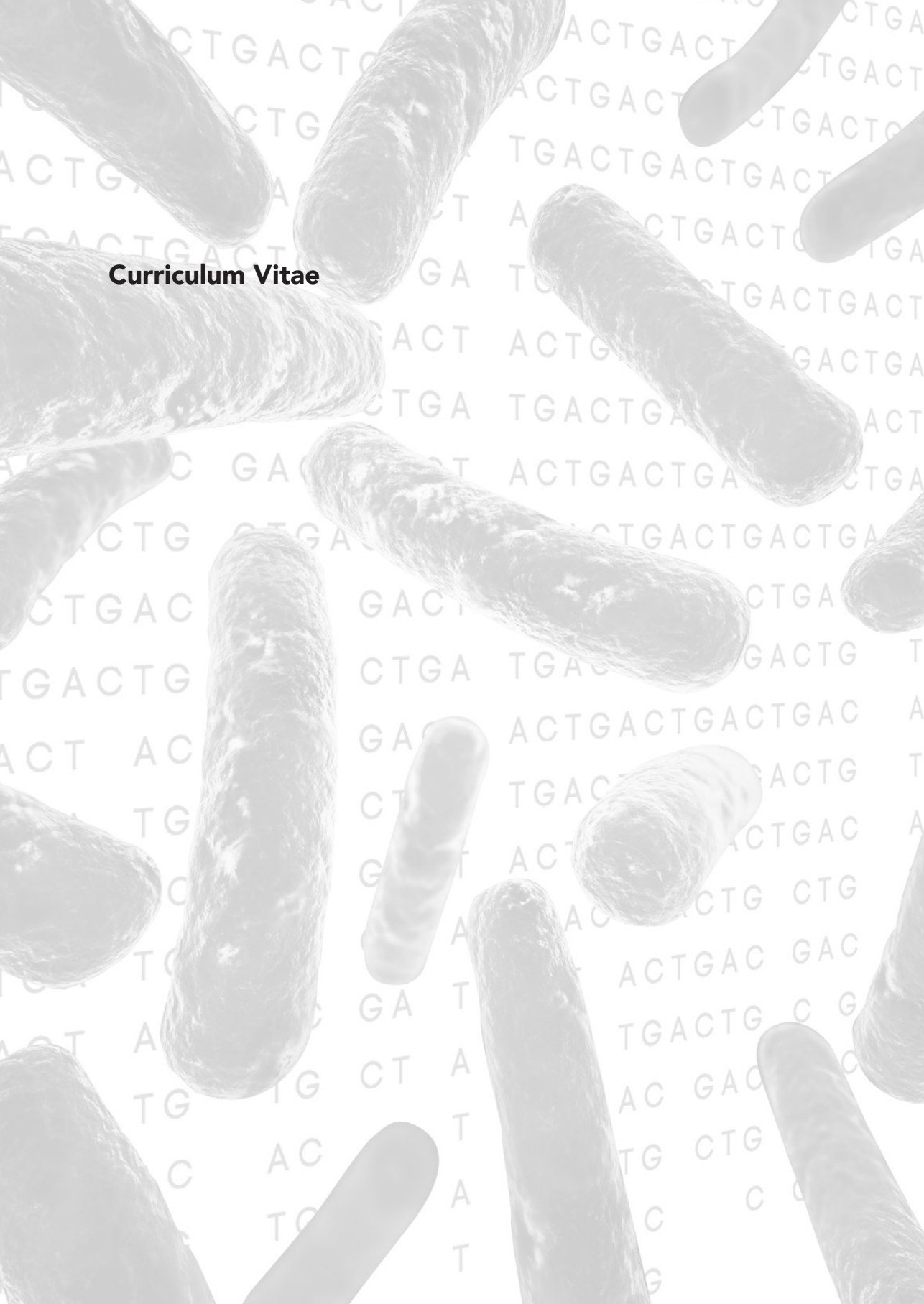
Mijn ouders, zusje Savita en zwager Jaswant, voor het vertrouwen dat jullie altijd in mij gehad hebben. Bedankt voor de vele dagen, dat jullie hebben opgepast om mij de ruimte te geven om te doen wat voor mij belangrijk is.
Mijn schoonfamilie wil ik bedanken voor alle belangstelling en gezelligheid. Met de goede verzorging van de inwendige mens is het samenzijn steeds weer een feest.

Een goede werksfeer is belangrijk, maar vriendschap buiten het vak mogelijk nog meer. Dus daarom wil ik ook mijn vrienden en vriendinnen bedanken voor alle ondersteuning en meeleven, gezellige etentjes en ontspannende gesprekken.

Elise, Bernard en Norbert: bedankt voor al jullie steun en vertrouwen tijdens de afronding van mijn proefschrift.

Lieve Vinod, het boekje ligt er, eindelijk! Jouw rustige kijk ('geef mij maar jouw lijstje,…. dan gooi ik het weg'…), goede adviezen, onvoorwaardelijke liefde en steun waren onmisbaar. Promoveren in combinatie met kleine kinderen en een nieuwe baan was geen gemakkelijke klus. De laatste loodjes waren zwaar, maar jij hebt ervoor gezorgd dat ik alle ruimte had om het proefschrift af te kunnen maken.

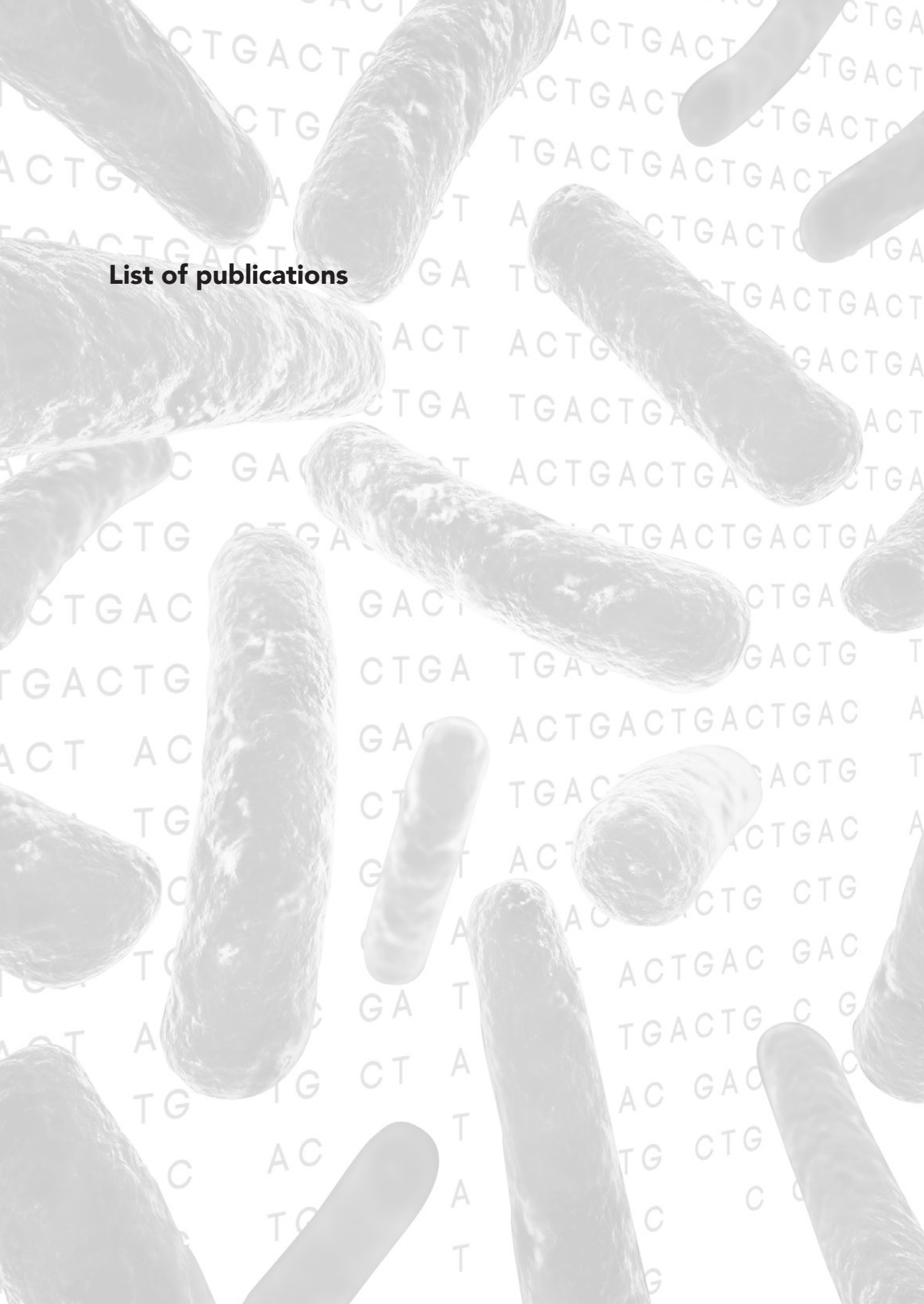
Er is zoveel meer dan alleen opleiding, werk en onderzoek. Onze dochters Jiya (2009) en Jahnavi (2013) maken het leven waard om geleefd te worden.
Mama houdt van jullie, ik heb jullie lief, mijn hele leven.



Curriculum Vitae

Curriculum Vitae

Sunita Paltansing werd op 18 februari 1978 geboren in Den Haag. Ze is de dochter van Randjieth Paltansing en Satwatie Mohabir. Haar ouders zijn afkomstig uit Suriname en wonen sinds 1974 in Nederland.
Ze behaalde haar VWO-eindexamen met acht vakken in 1996 aan het Stedelijk College in Zoetermeer. Vervolgens studeerde zij Biomedische Wetenschappen aan de Universiteit Leiden. In 1999 startte zij daarnaast met de studie Geneeskunde. Het doctoraalexamen voor beide studies behaalde zij in 2002 en het artsexamen in 2004. In het Leids Universitair Medisch Centrum werkte zij 6 maanden als arts-niet in opleiding op de afdeling Klinische Genetica, waarna zij in maart 2005 begon aan haar opleiding tot arts-microbioloog op de afdeling Medische Microbiologie (opleider prof. dr. A.C.M. Kroes). In 2008 begon zij aan het promotie-onderzoek bij de afdeling Medische Microbiologie onder leiding van dr. A.T. Bernards, waarvan de resultaten in dit proefschrift beschreven worden. In september 2010 werd zij geregistreerd als arts-microbioloog, waarna zij nog tot 1 oktober 2013 werkzaam bleef op de afdeling in het LUMC. Sindsdien is zij werkzaam als arts-microbioloog in het IJsselland Ziekenhuis in Capelle aan den IJssel.



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List of publications

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