Broad-spectrum β-lactamase in *Enterobacteriaceae*: detection, prevalence, and source tracking

Guido Maarten Voets

Printing of the this thesis was financially supported by Infection & Immunity Center Utrecht

Cover Design: Richard van der Linden - turaziel.deviantart.com/gallery/

Print: Gildeprint drukerijen, Enschede, The Netherlands

ISBN: 978-90-393-6062-0

Broad-spectrum β-lactamase in *Enterobacteriaceae*: detection, prevalence, and source tracking

Broad-spectrum β-lactamase in *Enterobacteriaceae*: detectie, prevalentie en het zoeken naar een bron

(met een samenvatting in het Nederlands)

Proefschrift

ter verkijging van de graad van doctor aan de Universiteit utrecht op gezag van de rector magnificus, prof. dr. G. J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 12 december 2013 des middags om 12.45 uur.

door

Guido Maarten Voets

geboren op 20 oktober 1982 te Leiden

promotor: Prof. Dr. M. J. M. Bonten

co-promotoren: Dr. A. C. Fluit

Dr. J. W. T. Cohen Stuart

Voor iedereen met ADHD
Voor mijn ouders
Voor God

Table of Contents

CHAPTER I

GENERAL INTRODUCTION

Introduction

Throughout time the name of an age is usually given by a future generation. There have already been many suggestions for our current age. These names tend to focus on one of the achievements of humanity, e.g., the information age. One suggestion is closely tied to our healthcare system, i.e., the age of antibiotics. Studies in the past decades have shown, however, that the achievement of antibiotics might become obsolete in the near future. One might call the near future the age of antibiotic resistance.

Before antibiotics infectious diseases were deadly diseases with high mortality rates. With the introduction of antibiotics the danger of infectious diseases seemed a thing of the past. In reaction to the wide-spread use of antibiotics bacteria developed or acquired resistance mechanisms. In fact, resistance to all types of currently available antibiotics has been described. Vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Mycobacterium tuberculosis* are well-known examples. In the last two decades a rise in the prevalence of multi-resistant *Enterobacteriaceae*, has been demonstrated. Especially the emergence of isolates carrying Extended-Spectrum ß-Lactamases (ESBLs) and carbapenemases has contributed to a new impulse into the field of antibiotic resistance research. β-Lactam-resistant *Enterobacteriaceae* are the subject of this thesis and questions regarding detection, prevalence, and a potential source for humans will be addressed.

Enterobacteriaceae: pathogenicity

The *Enterobacteriaceae* is one of the most well-known families of the small gram-negative rods. Currently, the family consists of 52 genera and 280 species. [1] Clinical important genera include, but are not limited to, *Enterobacter*, *Escherichia*, *Citrobacter*, *Klebsiella*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia*. *Enterobacteriaceae* are intimately linked to the colonization of the gastrointestinal tract of several vertebrae and many pathologic processes ranging from gastrointestinal syndromes to wound infections and from respiratory to urinary tract infections. The most common disease caused by members of the *Enterobacteriaceae*, second only to *Vibrio cholerae*, is gastroenteritis and related infections (e.g., enteritis or dysentery). The causative strains of these diarrheal diseases differ from their commensal counterparts, but nonetheless belong to the same genera or species, e.g., *Escherichia* spp. [2] Such diseases may be considered just an inconvenience in the developed world, but its

estimated that more than 2 million people succumbed to diarrheal diseases in 2001 making it the third-ranking cause of deaths by infectious diseases worldwide. [3]

Escherichia coli are present in the intestinal tract of domesticated animals, bird and humans. It is estimated that at least 10^7 CFU *E. coli* are present in 1 gram of human feces. [4, 5] Under normal circumstances this population is considered as commensals, however, age, illness or environmental changes can alter this. *E. coli* are, next to diarrhea, associated with urinary tract infections, respiratory tract infections, sepsis, primary peritonitis, and meningitis. Especially the mortality rate of meningitis in neonates is reported to be as high as 17 to 38%. Of the survivors 58% develop long-term or permanent complications. [6]

Klebsiella spp. can be isolated from a wide variety of mammals, reptiles, birds, insects and environmental sources. [7-11] *Klebsiella* infections are associated with urinary tract infections, pneumonia, bloodstream infections, septicemia, and wound infections. The mortality from these *Klebsiella* infections can be as high as 50% in case of *Klebsiella* induced pneumonia. [12]

Niches occupied by *Enterobacter* spp. are difficult to identify because most studies regarding the environmental distribution of *Enterobacter* predate the current taxonomy. In general *Enterobacter* species can be found in avian, reptiles, and mammals, dating as far back as 12,000 years, as well as in numerous vegetables. Clinical conditions associated with *Enterobacter* spp. are complicated urinary tract infections, bacteremia, endocarditis, meningitis, pneumonia, and wound infections. [10, 13-16]

Besides nosocomial- and community acquired infections, *Enterobacteriaceae* infections that originate from a different host have been reported. Infamous examples of outbreaks were reported in 2011, when over four thousand people got infected with an enterohermorrhagic *E. coli*, and in 2012, when over one thousand people got infected with *Salmonella* due to the consumption of salmon. [17, 18]

Many classes of antibiotics have been useful to treat infections with *Enterobacteriaceae* including tetracyclines, sulfonamides/trimethoprim, fluoroquinolones, aminoglycosides and ß-lactam antibiotics such ampicillin or amoxicillin, cephalosporins, and carbapenems. In the Netherlands in particular third-generation cephalosporins are used as part of the empiric treatment of severe infections that may be caused by *Enterobacteriaceae*.

Beta-lactam antibiotics

In 1928 Sir Alexander Flemming discovered the first classical antibiotic. He coined the term penicillin for the antimicrobial substance released by the fungus *Penicillium rubens* under stress conditions. [19] He showed that the drug was very effective against gram-positive isolates. Later Baron Florey showed the *in vivo* bactericidal action of penicillin in 1939. Strikingly, the first mentioning of the antimicrobial capabilities of the *Penicillium* fungi was already described in 1875 by John Tydall, to whom Fleming refers. Penicillin was the first member of a large group of antibiotics sharing a ß-lactam ring in their chemical structure. However, by 1957 over 80% of the hospitals reported penicillin-resistant *Staphylococcus aureus*, as well as penicillin-resistant *Streptococcus pneumoniae* and *Neisseria gonorrhea*. [20-23] The development of antibiotics and the subsequent rise of resistance became the continuous cycle for next few decades.

The members of the β-lactam family are divided based on their structural differences into four groups: penicillins, monobactams, cephalosporins, and carbepenems (Figure 1).

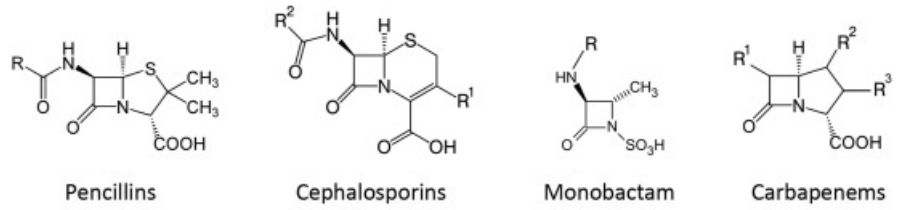


Figure 1: General molecular structures of β-lactam antibiotics.

These antibiotics inhibit the synthesis of the peptidoglycan layer of the bacterial cell wall by binding to the penicillin-binding proteins (PBPs). In fact PBPs were discovered by the binding of penicillin to the protein rather than by discovering their biological function. PBPs differ per species but share conserved regions. A PBP is a transpeptidase that facilitates the last transpeptidation step of the synthesis of the peptidoglycan layer. PBPs vary in their affinity for binding different types of β-lactam antibiotics and the number of different PBPs varies among bacteria. [24] The binding of β-lactam antibiotics to PBPs is facilitated by their structural similarity with D-alanyl-D-alanine. During normal synthesis of the peptidoglycan layer PBPs use D-alanyl-D-alanine to crosslink the N-acetyl-muramic subunits of the immature peptidoglycan layer. However, β-lactam antibiotics can bind irreversibly by acylation to a serine residue of the active site of a susceptible PBP due to their characteristic β-lactam ring. This irreversible binding inhibits the PBPs and prevents the final cross-linking of the immature peptidoglycan layer and therefore cell wall synthesis. [25]

The inhibition of the PBPs by β-lactam antibiotics also causes a build-up of peptidoglycan precursors in the periplasmic space. This enhances the antimicrobial activity of β-lactam antibiotics, as peptidoglycan precursors are part of a signal to reorganize the bacterial cell wall that triggers the activation of autolytic cell wall hydrolases. [25]

Thus, the bactericidal activity of β-lactam antibiotics is two-fold: decreasing the production of new peptidoglycan and indirect triggering of degradation of the existing peptidoglycan by autolytic hydrolysis. These two modes of action lead to the loss of structural integrity resulting ultimately in cell lysis. [25]

The penicillins is arguable the most famous group of the β-lactam antibiotics, due to its historical significance. Besides normal penicillins, e.g., benzylpenicillin and phenoxymethylpenicillin, pencillinase-resistant penicillins were developed, e.g., oxacilline and cloxacilline. Penicillins can differ in half-life, affinity, and effectiveness against certain organisms. [26]

Cephalosporins derive their name from fungi belonging to the genus *Cephalosporium*, which later was renamed to *Acremonium*. Cephalosporins tend to be grouped into "generations", which can loosely be coupled to their antimicrobial activity. First generation cephalosporins are predominately active against gram-positive bacteria. Consecutive generations have an increased activity against gram-negative bacteria in comparison to their preceding generation, albeit this is often accompanied by a reduced activity against gram-positive bacteria. The so-called fourth generation cephalosporins are an exception to this rule and have a broad spectrum of activity against both gram-positive as well as gram-negative bacteria. Cephamycins are a subgroup of the second-generation cephalosporins, which, e.g., include cefoxitin and cefmetazole. Cephamycins are efficacious antibiotics against anaerobic microbes, unlike most cephalosporins. Third-generation and fourth-generation cephalosporins with an oxyimino side chain like cefotaxime and ceftazidime are known as extended-spectrum cephalosporins, coinciding with their effectiveness against many bacteria.

The last group of antibiotics that belong to the β-lactam antibiotics are the carbapenems. Members of this group have several advantages in comparison to the other β-lactam antibiotics. Firstly, they have the broadest antimicrobial spectrum in comparison to the other β-lactam antibiotics. They are active against both gram-positive as well as gram-negative bacteria. Secondly, the most common β-lactamases, the main mechanism of β-lactam resistance in bacteria, are unable to hydrolyze carbapenems. Thirdly, agents of the carbapenems group are the only β-lactam antibiotics capable of inhibiting L,D-transpeptidases, a transpeptidase that makes use of cysteine as its active residue. [27]

Monobactams differ from other β-lactam antibiotics by the lack of the second ring bound to the β-lactam ring itself. The monobactam aztreonam has a high affinity

for PBPs of gram-negative bacteria, but is largely ineffective against gram-positive bacteria and anaerobes.

Due to the rise in resistance against (third-generation) cephalsoporins, however, the need to use the last group of β-lactam antibiotics that was developed, the carbapenems, becomes ever more frequent. In 2003, as measured by sales, more than half of all commercially available antibiotics in use were β-lactam compounds. [28]

Table 1: Overview of β-lactam antibiotics naming scheme.

Type	Group	Sub-group	Examples
Penicillins	Narrow spectrum	Penicillinase sensitive	Benzylpenicillin Clometocillin Penamecillin Phenoxymethylpenicillin
		Penicillinase resistant	Cloxacillin Oxacillin Meticillin Nafcillin
	Extended spectrum		Amoxicillin Ampicillin Piperacillin Ticarcillin
Cephalosporins	1st generation		Cefazolin Cefapirin Cefatrizine Cefazaflur
	2nd generation		Cefotiam Cefbuperazone Cefuroxime
		Cephamycins	Cefoxitin Cefotetan Cefmetazole
	3rd generation	ESBLs	Ceftriaxone Ceftazidime Cefodizime Cefotaxime Cefpimizole Ceftiofur
	4th generation	ESBLs	Cefepime Cefozopran Cefpirome Cefquinome
Carbapenems			Biapenem Ertapenem Imipenem Meropenem
Monobactam			Aztreonam Tigemonam Carumonam Nocardicin A

Enterobacteriaceae: **resistance and prevalence**

Unfortunately, antibiotic resistance for all antibiotics useful for treatment of infections with *Enterobacteriaceae*, including third-genereation cephalosporins, has been documented in numerous studies. Best data are available for invasive *E. coli* and *Klebsiella pneumonia* in Europe which were collected by the European Centre for Disease Prevention and Control (ECDC) (Figure 2 and 3). [29]

In general resistance rates for *K. pneumonia* are higher than for *E. coli*, but the resistance rates between different antibiotics for each species show considerable differences. For both species the prevalence of third-generation cephalosporin-resistant isolates is higher than for aminoglycoside-resistant isolates. For both species carbapenem-resistant isolates are still rarely encountered in most countries, however, carbapenem-resistant *K. pneumonia* are reported in Greece, Italy, and Cyprus in alarming numbers. In general, countries in the south and east of Europe report more resistant isolates than countries in the north and west of Europe. Especially multi-antibiotic resistance is a problem. In 2011 3.7% of all European invasive *E. coli* isolates was resistant to third-generation cephalosporins and two other groups of antibiotics, e.g., aminopencillins, aminoglycosides and fluorquinolones. Individual countries varied greatly in the percentage of isolates resistant to antibiotics from 3 groups (0.8% in Iceland to 18.2% in Cyprus).

In 2011 in the Netherlands the prevalence for third-generation cephalosporin-resistant *E. coli* and *K. pneumoniae* was 5.7% (CI 95% 5-6) and 8.1% (CI 95% 6-10), respectively. [29] In 2009/2010 3% of the *E. coli* causing urinary tract infections was resistant to three or more classes of antibiotics. [30]

The relative low prevalence of third-generation cephalosporin-resistant isolates in western and northern Europe in comparison to eastern and southern Europe may be attributed to sparser application of antibiotics in the clinical setting, as well as better detection methods and possible containment policies. Regardless of prevalence, an increasing trend in the case of antimicrobial non-susceptible and resistant isolates was noted in all countries, including the Netherlands (Table 2).

The increasing prevalence of antimicrobial non-susceptible and resistant isolates is not limited to Europe. Reports from across the world demonstrate an increase in prevalence of drug-resistant *Enterobacteriaceae*. [31-36] Drug-resistant *Enterobacteriaceae* have also been discovered in farm animals and companion animals. [37-40] Bacteria found in these animal compartments have been shown to be zoönotic or evidence points to possible zoönosis. [18, 41-44]

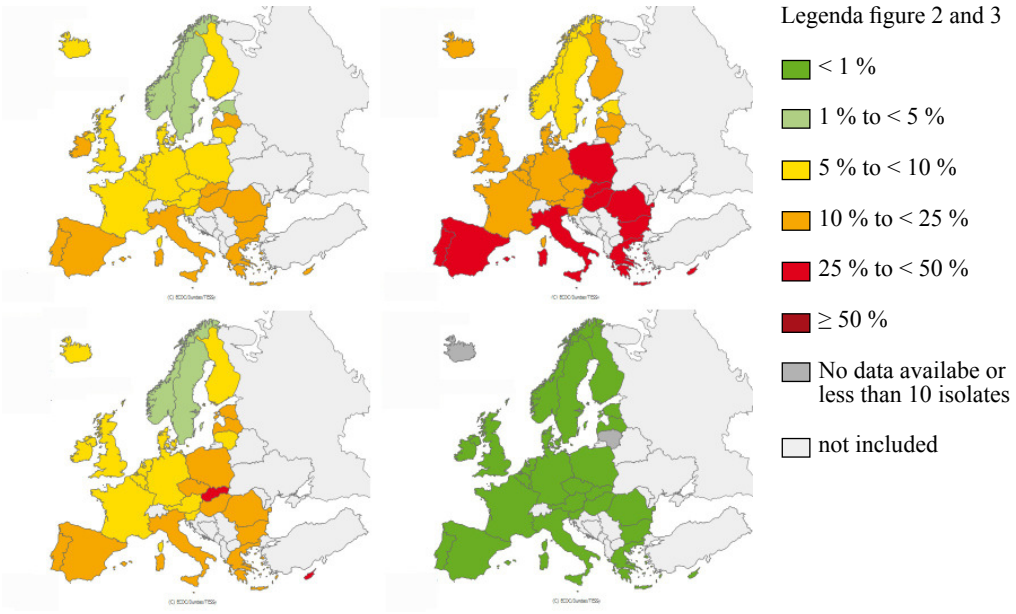


Figure 2: Antibiotic-resistant *E. coli* in 2011 in Europe. Left top: prevalence of aminoglycoside resistance. Right top prevalence of fluoroquinolone resistance. Left bottom: prevalence of third-generation cephalosporin resistance. Right bottom: prevalence of carbapenem resistance. [29]

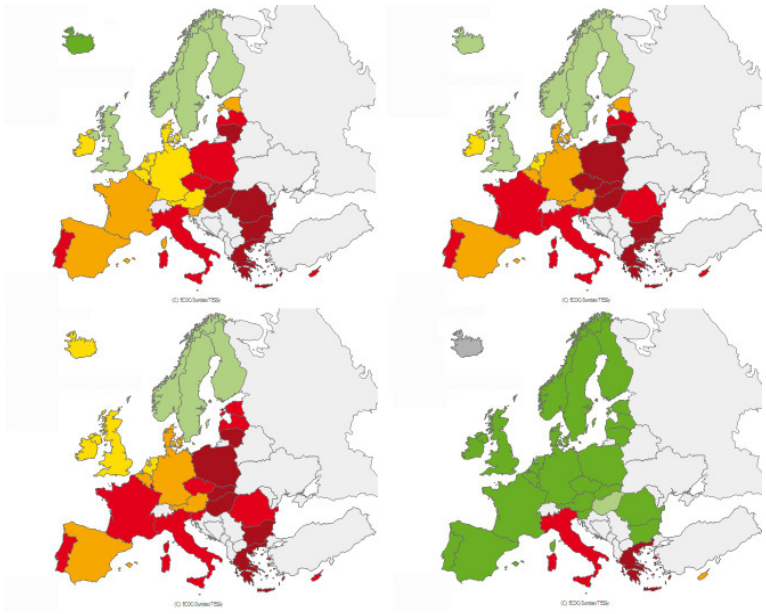


Figure 3: Antibiotic-resistant *K. pneumonia* in 2011 in Europe. Left top: prevalence of aminoglycoside resistance. Right top: prevalence of fluoroquinolone resistance. Left bottom: prevalence of third-generation cephalosporin resistance. Right bottom: prevalence of carbapenem resistance. [29]

Table 2: Annual percentage (%) of antimicrobial resistant isolates from the Netherlands from 2003 to 2011. – means no information was gathered in this year. Adapted from Antimicrobial resistance surveillance report. [29]

Pathogens by antimicrobial classes	2003	2004	2005	2006	2007	2008	2009	2010	2011
Escherichia coli									
Aminopenicilins	45	43	48	47	49	48	45	48	49
Aminoglycosides	3	3	4	3	5	6	4	7	8
Fluoroquinolones	7	7	10	11	13	14	11	14	14
Third-gen. cephalosporins	1	1	2	3	4	5	4	5	6
Carbapenems	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Klebsiella pneumoniae									
Aminoglycosides	-	-	5	4	5	7	3	7	8
Fluoroquinolones	-	-	6	4	4	7	4	7	7
Third-gen. cephalosporins	-	-	4	4	7	8	6	7	8
Carbapenems	-	-	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Pseudomonas aeruginosa									
Piperacillin (± tazobactam)	-	-	4	2	2	6	3	4	6
Ceftazidime	-	-	5	5	4	6	4	3	5
Carbapenems	-	-	5	2	2	6	3	3	3
Aminoglycosides	-	-	7	4	3	4	1	2	5
Fluoroquinolones	-	-	9	9	5	8	7	4	7

β-lactam resistance mechanisms in *Enterobacteriaceae*

As described before, an alarming number of *Enterobacteriaceae* have developed and/or acquired several mechanisms to counter β-lactam antibiotics. To understand these mechanisms, it is important to know that, as gram-negative bacteria, *Enterobacteriaceae* have two lipid bilayer membranes separated by the periplasmic space. Within the periplasmic space the peptidoglycan layer is located. The peptidoglycan layer adds to the structure and rigidity of the bacterial cell and protects against osmotic shock (Figure 4). The assembly of the peptidoglycan layer is the target of β-lactam antibiotics. In order to reach its target a β-lactam antibiotic must pass the outer lipid bilayer.

β-lactam antibiotics cannot pass the lipid bilayer. For this they require the presence of porins that allow their passage. Normally, porins serve as a passive form of uptake of, e.g., nutrients which cannot pass the lipid bilayer. Porins are vital to the survival of *Enterobacteriaceae*. *Enterobacteriaceae* have several types of porins at their disposal. In order to limit the concentration of β-lactam antibiotics they can down regulate the expression of porins mostly used by β-lactam antibiotics, e.g., OmpF or OmpC (*E. coli*) and OmpK35 or OmpK36 in (*K. pneumoniae*). At the same time it may up-regulate restricted-channel porins in order to compensate for the loss in uptake of vital compounds. [45, 46] Alternatively, porin expression may remain normal,

but the porin may be altered to restrict the uptake of β-lactam antibiotics. This can be achieved by mutation of the porin or the addition of channel blocker, e.g., deoxycholic acid. [47, 48] Another mechanism is not to restrict the inflow of β-lactam antibiotics, but to increase the outflow by up regulating the expression of efflux pumps that can actively transport the β-lactam antibiotic across the lipid bilayer into the extra cellular compartment, e.g., by MexB and MexY. [49]

Another mechanism to develop resistance is the mutation of the target of β-lactam antibiotics, the PBPs. This mechanism is rarely detected in *Enterobacteriaceae*, but it is more common among other gram-negative bacteria. These mutations that lead to a decreased affinity of the PBP for a third-generation cephalosporin can lead to a reduction of cross-linking. [49-51]

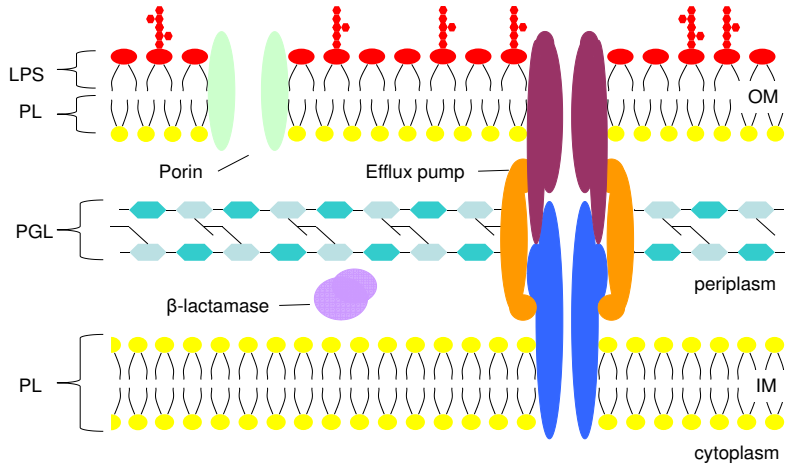


Figure 4: Membrane structure of gram-negative bacteria. The inner membrane (IM) is comprised out of a phospholipid (PL) bilayer. Proteins, e.g., an efflux pump, are incorporated into the IM as well. The periplasm is an aqueous compartment of the membrane in between the IM and the outer membrane (OM). Both the peptidoglycan layer (PGL) as well as many soluble proteins, e.g., β-lactamases, can be found in the periplasm. The OM has phospholipids on the inner leaflet and lipopolysaccharides (LPS) in the outer leaflet. The OM, just like the IM, has proteins, e.g., porins, incorporated into its structure. Proteins that are also covalently attached to the PGL anchor the OM.

The main mechanism of resistance against ß-lactam antibiotics in *Enterobacteriaceae*, however, is the production of ß-lactamases that hydrolyze the β-lactam ring. This mechanism is also the most common mechanism found in third-generation cephalosporin-resistant *Enterobacteriaceae*. A few species are capable of hyper-producing, provided certain mutations occur, their chromosomal β-lactamase (*Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa*) conferring resistance to extended-spectrum β-lactam antibiotics. If a hyper-producer has an additional resistance mechanism, as described above, it may provide resistance to carbapenems or cefepime. Most members of the *Enterobacteriaceae*, however, do not derepress or hyper-produce their chromosomal

β-lactamase. They acquire a β-lactamase due to horizontal transfer mainly in the form of a plasmid that harbors a β-lactamase-encoding gene. β-Lactamases can differ greatly on basis of their amino acid sequence, mechanism of hydrolysis, substrate preference, and resistance to β-lactamase inhibitors. Therefore two schemes have been developed to classify β-lactamase(s) (families).

The functional classification, also known as the Jacoby-Bush classification, is based on the substrate and inhibitor profiles that attempts to group enzymes in a manner that correlates to a phenotype. [52] However, as the presence of several mechanisms influence the phenotype of an isolate it is rather difficult to relate a particular phenotype to a β-lactamase group defined by this scheme.

The other classification, the Ambler classification, is a molecular classification system based on the amino acid sequence. [53] Despite the fact that this provides less information about their substrate profile, due to possible variance of hydrolysis profiles within a family of β-lactamases, this scheme is the more widely used. The Ambler classification divides the β-lactamases that use a serine residue for β-lactam ring hydrolysis into class A, C, and D. The metalloenzymes, which use zinc ions for their substrate hydrolysis, are classified as Class B. Class A β-lactamases are in general sensitive to inhibition by clavulanate, though for class A carbapenemases boronic acid is used. Ambler class B β-lactamases are in general sensitive to inhibition by ethylene-diamine-tetra-acetic acid (EDTA) which chelates the zinc ions. Ambler C class β-lactamases are in general insensitive to the above mentioned inhibitors. The effect of these inhibitors on Ambler D class β-lactamases is varied.

Within in every Ambler class a number of families are grouped of which the most common will be outlined below. The largest family, with more than 200 members, is formed by the TEM-ß-lactamases. [54] Its first discovered member, TEM-1, is a contestant for the most commonly encountered β-lactamase in *Enterobacteriaceae*. TEM-1 is, however, a narrow-spectrum β-lactamase only capable of conferring resistance to penicillins. It is ineffective against (extended-spectrum) cephalosporins and carbapenems. Amino acid substitutions, however, that alter the active site to allow β-lactam antibiotics with larger side-chains to enter the active site result in changes in substrate profile. Several members of the TEM family are ESBLs, e.g., TEM-3, TEM-12, and TEM-52, capable of hydrolyzing extended spectrum cephalosporins. [55]

The SHV family is also an important member of the Ambler class A with more than 150 members. [54] Like the TEM family most members of the SHV family are narrow spectrum β-lactamases, but with the proper amino acid substitutions are ESBLs, e.g., SHV-2, SHV-5, and SHV-12. [55]

The CTX-M family is also part of Ambler class A. It was named for its greater activity against cefotaxime in comparison to, e.g., ceftazidime, and the location of its discovery, München. The CTX-M family originates from the chromosome of

Kluyvera species. They only share 40% amino acid sequence with the TEM- and SHV-family ß-lactamases. Since their discovery in 1983, CTX-M has become the dominant ESBL family found in studies. Especially CTX-M-9, CTX-M-14, and CTX-M-15 are widespread in Europe. [56]

Other plasmid-mediated ESBL families belonging to the Ambler class A include, among others, the PER, VEB, and GES family. They are less frequently found than the TEM, SHV, or CTX-M family. The GES family even contains a few members that are classified as carbapenemases. However, the most (in)famous carbapenemase family within the Ambler A class is undoubtedly the KPC family. The KPC family has caused outbreaks in the USA, Israel, and Greece. [57-59] It is considered the most widespread carbapenemase. [60] Less notable Ambler A class carbapenemase families, due to a lower spread, are the families known as IMI, SME, and NMC.

The Ambler B class includes all metallo-β-lactamases. All families in this Ambler group have at least several members that are classified as carbapenemases. Most notable are the IMP, VIM, and NDM family. IMP and VIM spread across the world in the 1990's and 2000's and are both capable of hydrolyzing every β-lactam antibiotic with the exception of monobactams. The NDM family was discovered in 2009 and spread quickly across the globe probably due to tourism, specifically medical tourism. [61, 62]

Table 4: Overview of β-lactamase naming scheme.

Ambler Class		Family	Example Member
A	Narrow spectrum	TEM	TEM-1
		SHV	SHV-1
	ESBL	TEM	TEM-12
		SHV	SHV-12
		CTX-M	CTX-M-9
	Carbapenemase	GES	GES-5
		IMI	IMI-1
		KPC	KPC-1
B	Carbapenemase	IMP	IMP-1
		NDM	NDM-1
		VIM	VIM-1
C	AmpC	ACC	ACC-1
		CMY	CMY-1
		DHA	DHA-1
	Carbapenemase	pYMG	pYMG-1
D	Narrow spectrum	OXA	OXA-1
			OXA-2
	Broad spectrum	OXA	OXA-16
			OXA-18
	Carbapenemase	OXA	OXA-48
			OXA-54

The Ambler class C includes all AmpC type β-lactamases. They tend to be able to hydrolyze all cephalosporins but especially cephamycins (e.g., cefotoxin), though the hydrolysis rates can differ significantly among themselves and with ESBLs of the Ambler A class. These β-lactamases can either be encoded on the chromosome in which case they can be either inducible (e.g., in *E. cloacae* and *C. freundii*) or constitutively (*E. coli*) expressed. In order to become resistant, however, *E. coli* must become a hyperproducer, requiring mutations in the promoter of the AmpC gene. AmpC β-lactamases, however, can also be carried on plasmids. To date only one class C carbapenemase family has been detected, namely pYMG-1. [63]

The Ambler class D consists mainly of the OXA β-lactmases. The plasmid-mediated OXA family is named for its high hydrolytic activity against oxacillin and cloxacillin. It is a highly diverse family sharing as little as 20% amino acid sequence homology with one another. It is therefore not striking that the individual OXA β-lactamases can, e.g., be narrow-spectrum β-lactamases, resemble an ESBL-phenotype, or even be classified as a carbapenemase.

Detection Methods

In a clinical environment it is standard diagnostic practice to determine the antibiogram of isolates causing severe infections by automated systems like the Vitek-2. Phenotypic tests are often used to determine the resistance mechanism. For this purpose several detection methods have been developed to provide clinically relevant information. They, however, can be labor intensive, time consuming, and give inconclusive results. Detection methods include, but are not limited to, Etests, double-disk synergy tests, the combination disk method, and the modified Hodge test.

An example of an automated system is the Vitek-2 (BioMérieux, Marcy l'Etiole, France). It simultaneously assesses the antibacterial activity of several β-lactams antibiotics, both with and without the presence of an inhibitor. According to literature the test has a high specificity with ESBLs, but performs poorer with induced AmpC isolates. [64-66] Other automated systems exist as well, most notably the Phoenix, however, it is considered the lesser of the two. [67]

Etests are generally used to determine minimal inhibitory concentrations of antibiotics. Etests are also designed for specific resistance mechanisms, i.e. ESBLs or metallo-β-lactamases, allowing for an indication of the type of mechanism causing the resistance. EBSL Etests, specifically, are designed to quantify the synergy of an extended-spectrum cephalosporin and clavulanate regarding a particular isolate. However, the appearance of a so-called phantom zone or deformation of the inhibition ellipse is also considered a positive result (Figure 5). However, these latter two possible positive results can be difficult to determine, which may lead to false-

positive or false-negative results. MIC values may also lie beyond the range of the strips rendering the test inconclusive.[68, 69]

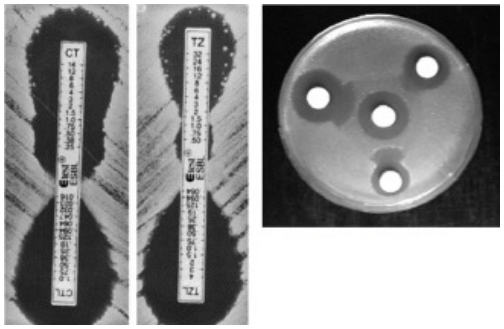


Figure 5: On the left Etests (adjusted from Stürenburg [70]) showing, next to a regular zone, a phantom zone. On the right a DDST which shows phantom zones resulting from synergy between two disks.

The double-disk synergy test, or DDST, was developed to differentiate between isolates resistant to cephalosporins by overproductions of AmpC's or those that produce ESBLs. In this test an isolate is plated on agar which contains one or more disks with a β-lactam antibiotic and one disk with amoxicillin-clavulanate positioned at a precise distance from another. The test is considered positive when a decreased susceptibility to a particular ESBL is present and if the inhibition zone is enhanced between the two disks, showing a synergy between the two compounds (Figure 5). The tests can be inconclusive though, if for instance a decreased susceptibility to β-lactam antibiotics is present, but no clear synergy is present. Results of the test can also differ on basis of the distance of two disks. [71, 72]

In a variant of the DDST, the combination disk method the inhibition zone around a disk of a β-lactam antibiotic is compared to an inhibition zone around a disk of the same β-lactam antibiotic plus clavulanate. The test is considered positive if a difference of 5 mm or 50% is detected. Though the test is easy to perform it requires accurate measurements regarding the zone.[73]

The modified Hodge test designed for the detection of carbapenemase production. It makes use of a carbapenem disk in the center and an inoculum of a sensitive isolate. Inocula of controls and an unknown isolate are made linear to the carbapenem disk. If the sensitive isolate can grow towards the carbapenem along the line of the unknown isolate the test is considered positive. However, it suffers from relatively large number of false-positive results. [74]

Isolate Typing

When a hospital encounters an increased number of bacterial isolates from the same species that are resistant to a particular antibiotic, it warrants investigation if an outbreak is occurring, especially when the isolates are detected in the same ward or in short period of time. For this purpose several bacterial isolate typing methods

have been developed. These methods can be divided in phenotypic and genotypic methods, but in general nowadays only genotypic methods are used.

Genotypic typing methods make use of the bacterial DNA to determine if isolates may be related or not. Genotypic methods include, but are not limited to, multi-locus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and DiversiLab. Genotypic methods have as an advantage that they usually give more detailed information in comparison to phenotypic methods, but tend to be more expensive.

For MLST several species specific house-keeping genes that are not under evolutionary pressure are sequenced. On basis of the DNA sequence a number is allocated to an allele. The combination of allele types is assigned an unique MLST number. The advantage of this technique is that it allows for the comparison of isolates from across the world, especially using electronic databases which store centrally all the different allele and MLST types that have been reported. [75] However, MLST has a relatively low discriminatory power as it sequences only a limited number of house-keeping genes. Whole genome sequencing that is increasingly used to study transmission routes will alleviate the lack of discriminatory power, but the technique is still too costly and not sufficiently rapid for implementation in routine diagnostics. [76]

Other genotypic techniques, known as finger-printing techniques, also make use of DNA, but they do not provide information on what is encoded in these sequences. PFGE, which has been widely used and still is regularly used, falls in this category. PFGE makes use of restriction endonucleases to cut a limited number (10-20) of specific sequences in the genome. The resulting DNA fragments are separated by size which generates for each isolate a pattern or 'finger-print'. Other finger-printing methods make use of conserved repeat sequences which can vary in length and number per isolate. DiversiLab (BioMérieux) is a commercial automated system that makes use of a repetitive-sequence-based PCR method. However, finger-print techniques suffer from the fact that small differences in execution result in differences in the patterns which can make analysis difficult, nor does the sharing of pattern between two isolates one technique ensure that the two isolates will not differ from another in another location in the genome. [77]

Plasmids: Transfer and Typing

As mentioned before, the most common mechanism for *Enterobacteriaceae* to become resistant to third-generation cephalosporins is to acquire an external resistance gene. This resistance gene is acquired through horizontal gene transfer, i.e. the transmission of DNA between two organisms of distinctive lineages. This can be achieved by different mechanisms: transduction, transformation, and conjugation. During transduction a virus-like particle, a bacteriophage, can transfer DNA from

one bacterium to another. During transformation bacteria take up external DNA released by dead cells. However, conjugation is considered the most important mode of transfer for *Enterobacteriaceae*. During conjugation a connection between two cells is realized and single-stranded DNA is transferred from one cell to another. This requires a specialized set of proteins that are encoded on many mobile genetic elements, e.g., plasmids that encode *tra* genes. After conjugation both the donor as well as the acceptor has at least one copy of the plasmid that was transferred. The products of *tra* genes are however not plasmid specific. This means that the products of conjugative plasmids may mobilize other genetic elements, e.g., non-conjugative plasmids and transposons.

Plasmids are circular double-stranded DNA molecules that can vary in size from a few thousand to hundreds of thousands base pairs. The number of plasmids and type of plasmids, harbored by an isolate can vary greatly. It is however impossible for an isolate to possess two plasmids that share the same type of origin of replication. This phenomenon is known as incompatibility and plasmids with the same origin of replication belong to the same incompatibility group. Regardless of their type plasmids replicate independently from the chromosome and generally do not encode house keeping functions for the host, but rather maintenance mechanisms of the plasmid, i.e. so called addictions systems, and genes that can benefit the host in certain environments, e.g., antibiotic resistance or virulence genes.

So, an increase in antibiotic resistance may not only be caused by the classic spread of a single resistant organism, but also by the spread of an antibiotic resistance encoding mobile element that is transferred between two organisms that come in contact. In literature several outbreaks involving horizontal gene transfer have been described. [e.g. 78, 79]

To compare plasmids several techniques have been developed. Using these methods it is possible to compare plasmids based on their origin of replication, conserved regions, and size. Plasmid based replicon typing (PBRT) makes use of the fact that plasmids of different incompatibility groups have different origins of replication. Primers unique to these sites on the plasmid are used in a polymerase chain reaction (PCR). On the basis of the presence of an amplification product a plasmid can be assigned to an incompatibility group. However, this method does not relate any genetic information present on the plasmid. [80] Specifically, for the incompatibility group known as IncI1, because of its wide-spread prevalence, a plasmid-based multi-locus sequence typing (pMLST) was developed to improve discriminatory power. Conserved genes on these plasmids are sequenced. As with the ‘isolate’ multi-locus sequence typing, based on the DNA sequence a number is associated with the allele type found. The combination of several allele types provides a pMLST type. [81]

The size of a plasmid can be determined using a variant of classical PFGE. Though this provides additional information in comparison to two previous described

techniques, other methods such as sequencing or specific PCR are required to detect specific genes, e.g., *bla*-genes that encode β-lactamases.

Thesis Objectives

The rise in prevalence of antibiotic-resistant *Enterobacteriaceae*, especially third-generation cephalosporin-resistant *Enterobacteriaceae*, is a major threat to public health. Despite the knowledge of the rise in prevalence, knowledge about the underlying genetic mechanisms responsible for this resistance within the Netherlands remains scarce.

This thesis aims to provide a deeper insight into the genetic background of the prevalent mechanisms of third-generation cephalosporin resistance in the Netherlands. However, the detection of third-generation cephalosporin resistance is not straightforward. Therefore techniques to detect ESBLs were evaluated and developed.

In chapter 2 the performance of the commercially available Brilliance™ CRE Agar (Oxoid, Basingstoke, United Kingdom) to selectively isolate and detect carbapenemase-producing *Enterobacteriaceae* was evaluated.

In chapter 3 the ability of the commercially available Check-MDR CT102 DNA microarray (CheckPoints, Wageningen, The Netherlands) used to detect the most common carbapenemases as well as the most common ß-lactamase groups with ESBLs was evaluated. The assay uses ligation-mediated amplification probes. Amplification products can hybridize to specific locations on a chip and are subsequently visualized. This allows for a fast indication of the β-lactamase involved.

In chapter 4 a set of new multiplex PCRs is described that enables the amplification of most members of the 16 most common β-lactamase families using a single amplification protocol.

In chapter 5 was investigated whether the DiversiLab (BioMérieux) bacterial typing system is suitable to compare *E. coli* and *Klebsiella* spp. isolates analysed at different centers. This would allow interlaboratory comparison of multi-antibiotic resistant isolates. The typing system is an automated repetitive-sequence-based PCR technique that creates several amplification products which are run on a commercial gel, which in turn is read by a BioAnalyzer. Dedicated software analyzes the intensity of peaks and computes the relatedness of isolates.

In chapter 6 the distribution of β-lactamases conferring resistance to third-generation cephalosporins among clinical *Enterobacteriaceae* in the Netherlands was determined.

In the study described in chapter 6 several extended-spectrum β-lactamases that have been rarely reported in human derived isolates were found. In a different study it was suggested that these ESBLs may be derived from poultry.

In chapter 7 the possibility for poultry as a source of AmpC β-lactamases in human isolates was investigated. Therefore, the human- and poultry-derived isolates and their plasmids sharing these AmpC ß-lactamases were further characterized.

The aim of chapter 8 was to investigate the ability of poultry-associated *E. coli* to survive in the human gut microbiome using an *in vitro* model system that mimics the healthy human microbiome. Furthermore, the ability of these isolates to transfer third-generation cephalosporin resistance to *Enterobacteriaceae* in the human gut microbiome was investigated.

References

1. Euzéby JP. List of Prokaryotic names with Standing in Nomenclature. www.bacterio.cict.fr (19-05 2013, date last accessed).

2. Janda JM, Abbott SL. In: Press A, ed. *The Enterobacteria*. Washington, D.C., 2006; p. 17-9.

3. Smolinski MS, Hamburg MA, Lederberg J. *Microbial Threats to Health: Emergence, Detection, and Response*. Washingtion, D.C.: The National Academies, 2003; p. 23-51.

4. Vidotto MC, Muller EE, de Freitas JC et al. Virulence factors of avian *Escherichia coli*. *Avian Dis* 1990; **34**: 531-538.

5. Bettelheim KA. The genus *Escherichia*. *The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*: Springer-Verslag, New York, N.Y., 1991; 2696-736.

6. Johnson JR, Oswald E, O'Bryan TT et al. Phylogenetic distribution of virulence-associated genes among *Escherichia coli* isolates associated with neonatal bacterial meningitis in the Netherlands. *J Infect Dis* 2002; **185**: 774-84.

7. Silvanose CD, Bailey TA, Naldo JL et al. Bacterial flora of the conjunctiva and nasal cavity in normal and diseased captive bustards. *Avian Dis* 2001; **45**: 447-51.

8. Blaylock RS. Normal oral bacterial flora from some southern African snakes. *Onderstepoort J Vet Res* 2001; **68**: 175-82.

9. Buenviaje GN, Ladds PW, Melville L et al. Disease-husbandry associations in farmed crocodiles in Queensland and the Northern Territory. *Aust Vet J* 1994; **71**: 165-73.

10. Gordon DM, FitzGibbon F. The distribution of enteric bacteria from Australian mammals: host and geographical effects. *Microbiology* 1999; **145**: 2663-71.

11. Kuzina LV, Peloquin JJ, Vacek DC et al. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (*Diptera: Tephritidae*). *Curr Microbiol* 2001; **42**: 290-4.

12. Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 1998; **11**: 589-603.

13. Bangaert RL, Ward ACS, H. SE et al. A survey of aerobic bacteria in the feces of captive raptors. *Avian Dis* 1988; **32**: 743-8.

14. Osterblad M, Pensala O, Peterzens M et al. Antimicrobial susceptibility of *Enterobacteriaceae* isolated from vegetables. *J Antimicrob Chemother* 1999; **43**: 503-9.

15. Jorge MT, Ribeiro LA, da Silva ML et al. Microbiological studies of abscesses complicating Bothrops snakebite in humans: a prospective study. *Toxicon* 1994; **32**: 743-8.

16. Rhodes AN, Urbance JW, Youga H et al. Identification of bacterial isolates obtained from intestinal contents associated with 12,000-year-old mastodon remains. *Appl Environ Microbiol* 1998; **64**: 651-8.

17. Altmann M, Spode A, Altmann D et al. Timeliness of surveillance during outbreak of Shiga Toxin-producing *Escherichia coli* infection, Germany, 2011. *Emerg Infect Dis* 2011; **17**: 1906-9.

18. Friesema IH, de Jong AE, Fitz James IA et al. Outbreak of *Salmonella Thompson* in the Netherlands since July 2012. *Euro Surveill* 2012; **17**: 20303.

19. Fleming A. Classics in infectious diseases: on the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae* by Alexander Fleming. *Brit J Exp Pathol* 1929; **10**: 226-36.

20. Finland M, Frank PF, Wilcox C. In vitro susceptibility of pathogenic Staphylococci to seven antibiotics. *Am J Clin Pathol* 1950; **20**: 325-34.

21. Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998; **339**: 520-32.

22. Whitney CG, Farley MM, Hadler J et al. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States. *N Engl J Med* 2000; **343**: 1917-24.

23. Eickhoff TC, Finland M. Changing susceptibility of meningococci to antimicrobial agents. *N Engl J Med* 1965; **272**: 395-8.

24. Bayles KW. The bactericidal action of penicillin: new clues to an unsolved mystery. *Trends Microbiol* 2000; **8**: 274-8.

25. Fisher JF, Meroueh SO, Mobashery S. Bacterial resistance to beta-lactam antibiotics: compelling opportunism, compelling opportunity. *Chem Rev* 2005; **105**: 395-424.

26. Garrod LP. The relative antibacterial activity of four penicillins. *Br Med J* 1960; **2**: 1695-6.

27. Mainardi JL, Villet R, Bugg TD et al. Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. *FEMS Microbiol Rev* 2008; **32**: 386-408.

28. Elander RP. Industrial production of beta-lactam antibiotics. *Appl Microbiol Biotechnol* 2003; **61**: 385-92.

29. ECDC. Antimicrobial resistance surveillance report. http://ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2011.pdf (19-05 2013, date last accessed).

30. RIVM. Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands. http://www.swab.nl/swab/cms3.nsf/uploads/6F9140D61805A468C12577530037D22B/$FILE/Nethmap_2010_def.pdf (19-05 2013, date last accessed).

31. Vento TJ, Cole DW, Mende K et al. Multidrug-resistant gram-negative bacteria colonization of healthy US military personnel in the US and Afghanistan. *BMC Infect Dis* 2013; **13**: 68.

32. Simner PJ, Zhanel GG, Pitout J et al. Prevalence and characterization of extended-spectrum beta-lactamase- and AmpC beta-lactamase-producing *Escherichia coli*: results of the CANWARD 2007-2009 study. *Diagn Microbiol Infect Dis* 2011; **69**: 326-34.

33. Nakano R, Nakano A, Abe M et al. Regional outbreak of CTX-M-2 beta-lactamase-producing *Proteus mirabilis* in Japan. *J Med Microbiol* 2012; **61**: 1727-35.

34. Gales AC, Castanheira M, Jones RN et al. Antimicrobial resistance among Gram-negative bacilli isolated from Latin America: results from SENTRY Antimicrobial Surveillance Program (Latin America, 2008-2010). *Diagn Microbiol Infect Dis* 2012; **73**: 354-60.

35. Brink A, Coetzee J, Clay C et al. The spread of carbapenem-resistant *Enterobacteriaceae* in South Africa: risk factors for acquisition and prevention. *S Afr Med J* 2012; **102**: 599-601.

36. Tan R, Liu J, Li M et al. Epidemiology and antimicrobial resistance among commonly encountered bacteria associated with infections and colonization in intensive care units in a university-affiliated hospital in Shanghai. *J Microbiol Immunol Infect* 2013: Epub, ahead of print.

37. Dierikx C, van Essen-Zandbergen A, Veldman K et al. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet Microbiol* 2010; **145**: 273-8.

38. Geser N, Stephan R, Hachler H. Occurrence and characteristics of extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae* in food producing animals, minced meat and raw milk. *BMC Vet Res* 2012; **8**: 21.

39. Dierikx CM, van Duijkeren E, Schoormans AH et al. Occurrence and characteristics of extended-spectrum-beta-lactamase- and AmpC-producing clinical isolates derived from companion animals and horses. *J Antimicrob Chemother* 2012; **67**: 1368-74.

40. Ewers C, Grobbel M, Stamm I et al. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. *J Antimicrob Chemother* 2010; **65**: 651-60.

41. Ewers C, Bethe A, Semmler T et al. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. *Clin Microbiol Infect* 2012; **18**: 646-55.

42. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect* 2011; **17**: 873-80.

43. Meyer E, Gastmeier P, Kola A et al. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum beta-lactamase-producing *Escherichia coli*. *Infection* 2012; **40**: 685-7.

44. Liebana E, Carattoli A, Coque TM et al. Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. *Clin Infect Dis* 2013; **56**: 1030-7.

45. Guillard T, Duval V, Moret H et al. Rapid detection of aac(6')-Ib-cr quinolone resistance gene by pyrosequencing. *J Clin Microbiol* 2010; **48**: 286-9.

46. Hu WS, Chen HW, Zhang RY et al. The expression levels of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxAR and BaeSR two-component systems, play important roles in the ceftriaxone resistance of *Salmonella enterica* serovar Typhimurium. *Antimicrob Agents Chemother* 2011; **55**: 3829-37.

47. Lauman B, Pagel M, Delcour AH. Altered pore properties and kinetic changes in mutants of the *Vibrio cholerae* porin OmpU. *Mol Membr Biol* 2008; **25**: 498-505.

48. Bredin J, Saint N, Mallea M et al. Alteration of pore properties of *Escherichia coli* OmpF induced by mutation of key residues in anti-loop 3 region. *Biochem J* 2002; **363**: 521-8.

49. Moya B, Beceiro A, Cabot G et al. Pan-beta-lactam resistance development in *Pseudomonas aeruginosa* clinical strains: molecular mechanisms, penicillin-binding protein profiles, and binding affinities. *Antimicrob Agents Chemother* 2012; **56**: 4771-8.

50. Garcia-Cobos S, Arroyo M, Campos J et al. Novel mechanisms of resistance to beta-lactam antibiotics in *Haemophilus parainfluenzae*: beta-lactamase-negative ampicillin resistance and inhibitor-resistant TEM beta-lactamases. *J Antimicrob Chemother* 2013; **68**: 1054-9.

51. Leski TA, Tomasz A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. *J Bacteriol* 2005; **187**: 1815-24.

52. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. *Antimicrob Agents Chemother* 2010; **54**: 969-76.

53. Ambler RP. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci* 1980; **289**: 321-31.

54. Lahey. ß-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes. www.lahey.org/studies/webt.asp (21-05 2013, date last accessed).

55. Livermore DM. beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev* 1995; **8**: 557-84.

56. Chen Y, Delmas J, Sirot J et al. Atomic resolution structures of CTX-M beta-lactamases: extended spectrum activities from increased mobility and decreased stability. *J Mol Biol* 2005; **348**: 349-62.

57. Bradford PA, Bratu S, Urban C et al. Emergence of carbapenem-resistant *Klebsiella* species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 beta-lactamases in New York City. *Clin Infect Dis* 2004; **39**: 55-60.

58. Leavitt A, Navon-Venezia S, Chmelnitsky I et al. Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. *Antimicrob Agents Chemother* 2007; **51**: 3026-9.

59. Pournaras S, Protonotariou E, Voulgari E et al. Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece. *J Antimicrob Chemother* 2009; **64**: 348-52.

60. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* 2009; **9**: 228-36.

61. Kumarasamy KK, Toleman MA, Walsh TR et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis* 2010; **10**: 597-602.

62. Yong D, Toleman MA, Giske CG et al. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother* 2009; **53**: 5046-54.

63. Kim JY, Jung HI, An YJ et al. Structural basis for the extended substrate spectrum of CMY-10, a plasmid-encoded class C beta-lactamase. *Mol Microbiol* 2006; **60**: 907-16.

64. Schwaber MJ, Navon-Venezia S, Chmelnitsky I et al. Utility of the VITEK 2 Advanced Expert System for identification of extended-spectrum beta-lactamase production in *Enterobacter* spp. *J Clin Microbiol* 2006; **44**: 241-3.

65. Stuart JC, Diederen B, Al Naiemi N et al. Method for phenotypic detection of extended-spectrum beta-lactamases in enterobacter species in the routine clinical setting. *J Clin Microbiol* 2011; **49**: 2711-3.

66. Sanders CC, Peyret M, Moland ES et al. Ability of the VITEK 2 advanced expert system To identify beta-lactam phenotypes in isolates of *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *J Clin Microbiol* 2000; **38**: 570-4.

67. Gherardi G, Angeletti S, Panitti M et al. Comparative evaluation of the Vitek-2 Compact and Phoenix systems for rapid identification and antibiotic susceptibility testing directly from blood cultures of Gram-negative and Gram-positive isolates. *Diagn Microbiol Infect Dis* 2012; **72**: 20-31.

68. Cormican MG, Marshall SA, Jones RN. Detection of extended-spectrum beta-lactamase (ESBL)-producing strains by the Etest ESBL screen. *J Clin Microbiol* 1996; **34**: 1880-4.

69. Leverstein-van Hall MA, Fluit AC, Paauw A et al. Evaluation of the Etest ESBL and the BD Phoenix, VITEK 1, and VITEK 2 automated instruments for detection of extended-spectrum beta-lactamases in multiresistant *Escherichia coli* and *Klebsiella* spp. *J Clin Microbiol* 2002; **40**: 3703-11.

70. Sturenburg E, Mack D. Extended-spectrum beta-lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. *J Infect* 2003; **47**: 273-95.

71. Jarlier V, Nicolas MH, Fournier G et al. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988; **10**: 867-78.

72. Drieux L, Brossier F, Sougakoff W et al. Phenotypic detection of extended-spectrum beta-lactamase production in *Enterobacteriaceae*: review and bench guide. *Clin Microbiol Infect* 2008; **14 Suppl 1**: 90-103.

73. Linscott AJ, Brown WJ. Evaluation of four commercially available extended-spectrum beta-lactamase phenotypic confirmation tests. *J Clin Microbiol* 2005; **43**: 1081-5.

74. Pasteran F, Mendez T, Guerriero L et al. Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae*. *J Clin Microbiol* 2009; **47**: 1631-9.

75. Wirth T, Falush D, Lan R et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol Microbiol* 2006; **60**: 1136-51.

76. Nubel U, Nachtnebel M, Falkenhorst G et al. MRSA transmission on a neonatal intensive care unit: epidemiological and genome-based phylogenetic analyses. *PLoS One* 2013; **8**: e54898.

77. Goering RV. Pulsed field gel electrophoresis: a review of application and interpretation in the molecular epidemiology of infectious disease. *Infect Genet Evol* 2010; **10**: 866-75.

78. Borgia S, Lastovetska O, Richardson D et al. Outbreak of carbapenem-resistant *Enterobacteriaceae* containing $bla_{\text{NDM-1}}$, Ontario, Canada. *Clin Infect Dis* 2012; **55**: e109-17.

79. Mathers AJ, Cox HL, Kitchel B et al. Molecular dissection of an outbreak of carbapenem-resistant *Enterobacteriaceae* reveals Intergenus KPC carbapenemase transmission through a promiscuous plasmid. *mBio* 2011; **2**: e00204-11.

80. Carattoli A, Bertini A, Villa L et al. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* 2005; **63**: 219-28.

81. Garcia-Fernandez A, Chiaretto G, Bertini A et al. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. *J Antimicrob Chemother* 2008; **61**: 1229-33.

Chapter II

Evaluation of the Oxoid Brilliance™ CRE Agar for detection of carbapenemase-producing *Enterobacteriaceae*

James Cohen Stuart (1), Guido Voets (1), Wouter Rottier (1), Sebastiaan Voskuil (1), Jelle Scharringa (1), Karin Van Dijk, Ad C. Fluit (1), Maurine Leverstein - Van Hall (1,2)

1. Department of Medical Microbiology, University Medical Centre Utrecht, The Netherlands

2. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

European Journal of Clinical Microbiology & Infectious Diseases; 2013; Epub

Abstract

Introduction: Adequate detection of carbapenemase producing *Enterobacteriaceae* (CPE) is essential for adequate antibiotic therapy, and for infection control purposes, especially in an outbreak setting. Selective agars play an important role in the detection of CPE.

Methods: The Oxoid Brilliance™ CRE Agar (Thermo Fisher Scientific) was evaluated for detection of carbapenemase producing *Enterobacteriaceae* using 255 non-repetitive *Enterobacteriaceae* isolates, including 95 CPE (36 KPC, 4 KPC plus VIM, 4 NDM, 6 GIM, 20 VIM, and 25 OXA-48 producing isolates).

Results: The sensitivity of the CRE agar for detection of CPE was 94% (89/95), but differed per carbapenemase gene (100% for KPC, NDM and GIM, 90% for VIM and 84% for OXA-48 producing isolates). The specificity of the CRE agar was 71%, due to growth of AmpC and/or ESBL producing isolates.

Conclusion: The CRE agar is a sensitivity tool for detection of KPC and metallo-carbapenemase producing *Enterobacteriaceae*, although detection of OXA-48 producers is less optimal. The relative low specificity requires confirmation of carbapenemase production for isolates recovered from the CRE agar.

Introduction

The emergence and spread of carbapenemase producing-*Enterobacteriaceae* (CPE) poses a considerable threat to clinical patient care and public health. CPE are characterised by their resistance to virtually all beta-lactam antibiotics, as well as to fluoroquinolones, aminoglycosides and co-trimoxazole. Invasive infections with these strains are associated with high rates of morbidity and mortality (2,3). Swift and accurate detection of CPE is essential for infection control purposes, especially in an outbreak setting, and may influence choice of antibiotic therapy. Selective agars play an essential role when screening clinical specimens to detect CPE.

The aims of this study were threefold: 1. To determine the test characteristics of the Oxoid *Brilliance*™ CRE Agar (Thermo Fisher Scientific, United Kingdom)(CRE Agar) for detection of CPE. 2. To determine the capacity of the CRE agar to detect carbapenem non-susceptible *Enterobacteriaceae*. 3. To investigate the species-specificity of the colony colours.

Methods

The test collection included 255 well characterized non-repeat *Enterobacteriaceae* isolates, including 95 carbapenemase-positive and 160 carbapenemase-negative isolates. PCR and sequencing of beta-lactamases was used as the reference test (10).

The 95 carbapenemase-positive isolates consisted of 73 *Klebsiella pneumoniae*, 10 *Escherichia coli*, 8 *Enterobacter* spp., 2 *Proteus mirabilis*, and 2 *Serratia marcescens* isolates producing the following carbapenemases: 36 KPC-2/3, 4 KPC plus VIM, 4 NDM-1, 6 GIM, 20 VIM, 25 OXA-48 (Table 1). The 160 carbapenemase-negative control isolates (Table 2) included 87 *E. coli*, 42 *Enterobacter* spp, 16 *K. pneumoniae*, 8 *Klebsiella oxytoca*, 4 *P. mirabilis*, one *Citrobacter freundii*, one *P. stuartii*, and one *S. marcescens,* of which 78 produced an ESBL (47 CTX-M, 14 TEM, 10 SHV, 7 other ESBL genes or combinations), 22 isolates an ESBL plus derepressed chromosomal AmpC (15 CTX-M, 4 SHV and 3 other ESBL genes or combinations), 11 isolates a plasmid AmpC, 26 isolates a derepressed chromosomal AmpC, 4 isolates a plasmid AmpC plus ESBL, 8 isolates were K1 hyperproducing *K. oxytoca*, and 11 isolates were beta-lactamase negative.

The isolates (one per patient) originated from the following sources: 72 isolates were collected at a Dutch beta-lactamase reference centre (University Medical Centre Utrecht), 29 were from Greece, 20 from New York, 118 from Dutch ESBL surveillance studies (7,9), 6 from Germany, and 10 isolates were ATCC or NCTC reference strains.

As described previously (4), the selective plates were inoculated with 10 µL of a 0.5 McFarland suspension, corresponding to approximately 10^6 colony forming units (CFU). Plates were incubated under aerobic conditions and read after 24 and 48 h.

The ertapenem and meropenem MICs were determined using broth micro-dilution (Merlin, Germany). Imipenem MICs were determined using Etest (bioMérieux, France). Ertapenem and meropenem MICs of 21 OXA-48 producing isolates were determined using Etest. Using EUCAST breakpoints (www.eucast.org), 86 (91%), 83 (87%), and 82 (86%) of the carbapenemase producing isolates were non-susceptible to ertapenem, meropenem and imipenem, respectively, and all except one (99%) of the carbapenemase producers were non-susceptible to at least one carbapenem (Table 1). Of the carbapenemase negative isolates, 52 (33%), 17 (11%), and 18 (11%) were non-susceptible to ertapenem, meropenem and imipenem, respectively, and 56 (35%) were non-susceptible to one or more of the carbapenems.

Results

The sensitivity for detection of CPE after both 24 and 48 hours was 89/95 (94%) (Table 1), and differed per carbapenemase gene (100% for KPC, GIM, and NDM, 90% for VIM, and 84% for OXA-48). Compared to the detection of other carbapenemase genes, the sensitivity for detection of OXA-48-positive CPE was significantly lower (97% versus 84%, p=0.039). However, the sensitivity of the CRE agar tot detect OXA-48 producers depended on the co-production of an ESBL. All isolates co-producing OXA-48 plus ESBL were able to grow on the CRE agar, whereas only one of the 5 isolates producing OXA-48 without an ESBL were detected (p<0.001, Table 1).

The specificity for detection of CPE after 24 and 48 hours was only 71% and 63%, respectively (Table 2). The relative low specificity was due to growth of AmpC and/or ESBL producing isolates with increased carbapenem MICs. Extending the incubation beyond 24 h is therefore not indicated.

The majority of the 46 carbapenemase-negative isolates growing on the CRE agar were *E. cloacae* (24 (52%)) with a derepressed AmpC gene, and *E. coli* and *K. pneumoniae* with ESBL genes (CTX-M, SHV) or (plasmidal or chromosomal) AmpC genes (Table 2). The sensitivity and specificity of the CRE agar to detect *Enterobacteriaceae* isolates non-susceptible to at least one carbapenem were 88% (123/140) and 103/115 (90%), respectively.

The sensitivity to detect carbapenem I/R isolates (EUCAST) depended on the presence of a carbapenemase gene. CPE isolates were detected with a sensitivity of 88/94 (94%) (Table 1), versus a sensitivity of only 76% (35/46) for isolates non-susceptible to at least one carbapenem due to other beta-lactamases (p=0.004). The 11 carbapenemase-negative isolates with increased carbapenem MICs that failed to grow on the agar were 8 chromosomal AmpC producing *E. cloacae* isolates, and 3 ESBL producing isolates (one *K. pneumoniae*, *one E. coli*, and one *P. mirabilis*) (Table 2).

The colony colours of all *K. pneumoniae*, *Enterobacter* spp., *P. mirabilis* and *S. marcesens* isolates were blue, and differentiating between those species based on the colour was not possible. *E. coli* colonies were brownish.

Discussion

This study shows that the CRE agar is a sensitive tool for detection of KPC and metallo-carbapenemase producing *Enterobacteriaceae*. However, the detection of OXA-48 producing *Enterobacteriaceae* is significantly lower (84%), in line with the results of a previous study (11). The sensitivity to detect CPE was comparable to the reported 91% sensitivity of the ChromID carba plate (Biomerieux, France)(11). The

sensitivity to detect KPC, VIM and NDM producing isolates was comparable with the 85%-100% sensitivity of the CHROMagar KPC plate (Chromagar Microbiology, France) (1,6,8), but detection of OXA-48 producing isolates cannot be compared because such isolates were not included in the evaluations of the CHROMagar KPC plate. In contrast to our findings, a detection sensitivity of 100% for OXA-48 producing isolates was recently reported using a novel screening medium (5).

The specificity of the CRE agar was relatively low (71%), due to growth of ESBL and/or AmpC producing isolates. This finding is consistent with previous studies showing that AmpC and/or ESBL producing isolates with decreased permeability may have increased carbapenem MICs, with ertapenem most affected (12). Although the manufacturer claims that the CRE agar contains a modified carbapenem (www.oxoid.com), the selective antibiotic(s) used in the CRE agar have not been disclosed, and the reason for the low specificity therefore remains unknown.

This study has several limitations. First, the findings have to be confirmed in a study evaluating the CRE agar with clinical specimens in the routine setting of a laboratory for clinical microbiology. Second, no IMP producing *Enterobacteriaceae* were included in this evaluation. Finally, the stability of the medium has not been tested in this study.

In conclusion, the CRE agar is a sensitivity tool for detection of KPC and metallo-carbapenemase producing *Enterobacteriaceae*, although detection of OXA-48 producers is less optimal. The relative low specificity requires confirmation of carbapenemase production for isolates recovered from the CRE agar.

Acknowledgements

The Oxoid Brilliance™ CRE Agar plates used for this study were kindly provided by Thermo Fisher Scientific, United Kingdom.

We wish to thank Vivi Miriagou, Laboratory of Bacteriology, Hellenic Pasteur Institute, Athens, Greece, and Collin MacKenzie, Colin R. MacKenzie, Institute of Medical Microbiology and Hospital Hygiene, University Hospital, Heinrich-Heine-University, Düsseldorf, Germany, for providing carbapenemase producing isolates.

Part of this data has been presented at the 22nd ECCMID, 31 March - 3 April, London, UK

Reference List

1. **Adler, A., S. Navon-Venezia, J. Moran-Gilad, E. Marcos, D. Schwartz, and Y. Carmeli**. 2011. Laboratory and clinical evaluation of screening agar plates for detection of carbapenem-resistant *Enterobacteriaceae* from surveillance rectal swabs. J.Clin.Microbiol. **49**:2239-2242.

2. **Bilavsky, E., M. J. Schwaber, and Y. Carmeli**. 2010. How to stem the tide of carbapenemase-producing *Enterobacteriaceae*?: proactive versus reactive strategies. Curr.Opin.Infect.Dis. **23**:327-331.

3. **Gupta, N., B. M. Limbago, J. B. Patel, and A. J. Kallen**. 2011. Carbapenem-resistant *Enterobacteriaceae*: epidemiology and prevention. Clin.Infect.Dis. **53**:60-67.

4. **Huang, T. D., P. Bogaerts, C. Berhin, A. Guisset, and Y. Glupczynski**. 2010. Evaluation of Brilliance ESBL agar, a novel chromogenic medium for detection of extended-spectrum-beta- lactamase-producing *Enterobacteriaceae*. J.Clin. Microbiol. **48**:2091-2096.

5. **Nordmann, P., D. Girlich, and L. Poirel**. 2012. Detection of carbapenemase producers in *Enterobacteriaceae* using a novel screening medium. J.Clin. Microbiol. **50**:2761-2766.

6. **Panagea, T., I. Galani, M. Souli, P. Adamou, A. Antoniadou, and H. Giamarellou**. 2011. Evaluation of CHROMagar KPC for the detection of carbapenemase-producing *Enterobacteriaceae* in rectal surveillance cultures. Int.J.Antimicrob.Agents **37**:124-128.

7. **Platteel, T. N., J. W. Cohen Stuart, A. J. de Neeling, G. M. Voets, J. Scharringa, S. N. van de, A. C. Fluit, M. J. Bonten, and M. A. Leverstein-van Hall**. 2011. Multi-centre evaluation of a phenotypic extended spectrum beta-lactamase detection guideline in the routine setting. Clin.Microbiol.Infect. **19**:70-76.

8. **Samra, Z., J. Bahar, L. Madar-Shapiro, N. Aziz, S. Israel, and J. Bishara**. 2008. Evaluation of CHROMagar KPC for rapid detection of carbapenem-resistant *Enterobacteriaceae*. J.Clin.Microbiol. **46**:3110-3111.

9. **Cohen Stuart, J., B. Diederen, N. N. Al, A. Fluit, N. Arents, S. Thijsen, B. Vlaminckx, J. W. Mouton, and H. M. Leverstein-van**. 2011. Method for phenotypic detection of extended-spectrum beta-lactamases in *Enterobacter* species in the routine clinical setting. J.Clin.Microbiol. **49**:2711-2713.

10. **Voets, G. M., A. C. Fluit, J. Scharringa, J. Cohen Stuart, and M. A. Leverstein-van Hall**. 2011. A set of multiplex PCRs for genotypic detection of extended-spectrum beta-lactamases, carbapenemases, plasmid-mediated AmpC beta-lactamases and OXA beta-lactamases. Int.J.Antimicrob.Agents **37**:356-359.

11. **Wilkinson, K. M., T. G. Winstanley, C. Lanyon, S. P. Cummings, M. W. Raza, and J. D. Perry**. 2012. Comparison of four chromogenic culture media for carbapenemase-producing *Enterobacteriaceae*. J.Clin.Microbiol. **50**:3102-3104.

12. **Woodford, N., J. W. Dallow, R. L. Hill, M. F. Palepou, R. Pike, M. E. Ward, M. Warner, and D. M. Livermore**. 2007. Ertapenem resistance among *Klebsiella* and *Enterobacter* submitted in the UK to a reference laboratory. Int.J.Antimicrob.Agents **29**:456-459.

Table 1: Carbapenem minimum inhibitory concentrations (MICs), European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretation, and growth characteristics on Oxoid Brilliance™ CRE Agar (Thermo Fisher Scientific, United Kingdom) (CRE agar) of 95 carbapenemase-positive *Enterobacteriaceae* (CPE) isolates

Carbapenemase-positive isolates growing on CRE agar	**Species**	**N**	**Ertapenem MIC (mg/L)**	**Meropenem MIC (mg/L)**	**Imipenem MIC (mg/L)**
KPC	*E. coli*	4	4 - >8 (R)	4 - 16 (I/R)	4 - >32 (I/R)
	K. pneumoniae	32	>8 (R)	>32 (R)	4 - >32 (R)
KPC + VIM-1	*K. pneumoniae*	4	>8 (R)	>32 (R)	>32 (R)
NDM-1	*K. pneumoniae*	2	8 - >8 (R)	16 - >32 (I/R)	>32 (R)
	K. pneumoniae	1	2 (R)	8 (I)	1.5 (S)
	E. coli	1	8 (R)	32 (R)	>32 (R)
GIM-1	*E. cloacae*	1	>8 (R)	32(R)	>32 (R)
	E. cloacae	2	>8 (R)	8 (I)	2 (S)
	S. marcesens	2	4 - >32 (R)	8 - >32 (I/R)	6 - >32 (I/R)
	E. coli	1	2 (R)	8 (I)	1.5 (S)
VIM-1	*K. pneumoniae**	1	0.125 (S)	1 (S)	2 (S)
	K. pneumoniae	3	0.125-0.5 (S)	8-16 (I/R)	32 - >32 (R)
	K. pneumoniae	9	2 - >8 (R)	8 - >32 (I/R)	12 - >32 (R)
	E. coli	1	0.25 (S)	2 (S)	6 (I)
	E. cloacae	1	0.5 (S)	8 (I)	24 (R)
	E. cloacae	1	2 (R)	2 (S)	8 (I)
	E. cloacae	1	1 (I)	16 (R)	>32 (R)
	P. mirabilis	1	≤0.0625 (S)	0.5 (S)	>32 (R)
OXA-48 with ESBL	*K. pneumoniae*	3	0.75 - 4 (I/R)	0.5 - 1 (S)	1 - 2 (S)
	K. pneumoniae	1	4 (R)	1 (S)	3 (I)
	K. pneumoniae	9	>8 (R)	6 - >32 (I/R)	3 - >32 (I/R)
	K. pneumoniae	4	3 - >32 (R)	4 - >32 (I/R)	1 - 2 (S)
	E. coli	1	4 (R)	1.5 (S)	1 (S)
	E. coli	1	6 (R)	8 (I)	6 (I)
	E. cloacae	1	>32 (R)	4 (I)	4 (I)
OXA-48 without ESBL	*K. pneumoniae*	1	2 (R)	1 (S)	3 (I)
Total growing on CRE agar		89			
Carbapenemase-positive isolates not growing on CRE agar	**Species**	**N**	**Ertapenem MIC (mg/L)**	**Meropenem MIC (mg/L)**	**Imipenem MIC (mg/L)**
VIM-1	*E. coli*	1	0.25 (S)	2 (S)	6 (I)
	P. mirabilis	1	≤0.0625 (S)	0.5 (S)	>32 (R)

OXA-48 without ESBL	*K. pneumoniae*	1	4 (R)	32 (R)	24 (R)
	K. pneumoniae	1	3 (R)	8 (I)	6 (I)
	K. pneumoniae	1	12 (R)	4 (I)	12 (R)
	K. pneumoniae	1	16 (R)	6 (I)	16 (R)
Total not growing on CRE agar		6			

* carbapenemase producing isolate susceptible to all 3 carbapenems according to EUCAST breakpoints.

Table 2: Carbapenem MICs, EUCAST interpretation, and growth characteristics on CRE agar of 160 carbapenemase-negative *Enterobacteriaceae* isolates

Carbapenemase-negative isolates growing on CRE agar	**Species**	**Beta-lactamase**	**N**	**Ertapenem MIC (mg/L)**	**Meropenem MIC (mg/L)**	**Imipenem MIC (mg/L)**
ESBL	*E. coli*	CTX-M-1	2	0.125 (S)	≤0.25 (S)	0.19 (S)
	E. coli	CTX-M-15	5	≤0.0625 - 0.5 (S)	≤0.25 (S)	0.19 (S)
	E. coli	CTX-M-15	2	1 (I)	≤0.25 (S)	0.25 (S)
	E. coli	CTX-M-15	1	>8 (R)	4 (I)	3 (I)
	K. pneumoniae	GES-6 + SHV-5	1	>8 (R)	>32 (R)	>32 (R)
	K. pneumoniae	CTX-M-15	1	>8 (R)	16 (R)	4 (I)
	K. pneumoniae	CTX-M-15	1	8 (I)	2 (S)	0.5 (S)
	K. pneumoniae	CTX-M-15	3	>8 (R)	4 - 16 (I/R)	1.5 - 2 (S)
	K. pneumoniae	SHV-12	1	4 (R)	8 (I)	1.5 (S)
Derepressed AmpC* plus ESBL	*E. cloacae*	CTX-M-9	1	0.5 (S)	0.5 (S)	>32 (R)
	E. cloacae	CTX-M-9	7	1 - 2 (I/R)	≤0.25 - 0.5 (S)	0.19 - 0.5 (S)
	E. cloacae	SHV-12	1	0.5 (S)	≤0.25 (S)	0.38 (S)
	E. cloacae	SHV-12	2	2 (R)	≤0.25 - 0.5 (S)	0.38 (S)
	E. cloacae	SHV-12	1	>8 (R)	32 (R)	8 (R)
	E. cloacae	CTX-M-15	1	>8 (R)	16 (R)	4 (I)
	E. cloacae	CTX-M-15	1	>8 (R)	32 (R)	2 (S)
	E. cloacae	CTX-M-3	1	0.5 (S)	≤0.25 (S)	0.38 (S)
	E. cloacae	SHV-2 + GES-6	1	0.5 (S)	0.5 (S)	0.5 (S)
	E. cloacae	CTX-M-9+ SHV-12	1	8 (R)	1 (S)	0.75 (S)
	E. cloacae	CTX-M-9 + SHV-12	1	>8 (R)	16 (R)	8 (I)

Plasmid AmpC	*E.coli*	CMY-2	1	>8 (R)	32 (R)	>32 (R)
Derepressed AmpC*	*E. coli*	CMY-2	1	≤0.0625 (S)	≤0.25 (S)	16 (R)
	E. cloacae	AmpC	4	1 - 2(I/R)	≤0.25 (S)	0.25 - 0.38 (S)
	E. cloacae	AmpC	1	2 (R)	≤0.25 (S)	3 (I)
	E. cloacae	AmpC	1	>8 (R)	8 (I)	>32 (R)
ESBL plus pAmpC**	*K. pneumoniae*	CMY-2 + SHV-5	1	0.25 (S)	≤0.25 (S)	0.25 (S)
	K. pneumoniae	MIR-3 + SHV-4	1	1 (I)	≤0.25 (S)	0.5 (S)
	E. coli	MIR-3 + CTX-15	1	≤0.0625 (S)	≤0.25 (S)	12 (R)
Total with growth			46			

Carbapenemase-negative isolates not growing on CRE agar	**Species**	**Beta-lactamase**	**N**	**Ertapenem MIC (mg/L)**	**Meropenem MIC (mg/L)**	**Imipenem MIC (mg/L)**
ESBL	Diverse	Diverse ESBL/AmpC	58	≤0.0625 - 0.5 (S)	≤0.25 (S)	0.125 - 1 (S)
	K. pneumoniae†	SHV-2	1	4 (R)	1 (S)	0.5 (S)
	P. mirabilis†	CTX-M-2	1	≤0.0625 (S)	≤0.25 (S)	4 (I)
	E. coli†	SHV-5	1	0.25 (S)	≤0.25 (S)	12 (R)
Derepressed AmpC* plus ESBL	*E. cloacae*	CTX-M-9	3	0.125 - 0.5 (S)	≤0.25 - 0.5 (S)	0.25 - 0.75 (S)
	C. freundii	SHV-2	1	0.125 (S)	≤0.25 (S)	0.75 (S)
Plasmid AmpC	*E. coli*	Div.	9	≤0.0625 - 0.125 (S)	≤0.25 (S)	0.19 - 0.38 (S)
Derepressed AmpC*	*E. cloacae*	AmpC	6	≤0.0625 - 0.5 (S)	≤0.25 - 0.5 (S)	0.25 - 1.5 (S)
	E. coli	AmpC	6	≤0.0625	≤0.25	0.125 - 25 (S)
	E. cloacae†	AmpC	4	1 - 4 (I/R)	≤0.25 - 2 (S)	0.25 - 2 (S)
	E. cloacae†	AmpC	4	>8 (I/R)	16 - 32 (R)	32 - >32 (R)
ESBL plus pAmpC**	*K. pneumoniae*	CMY-36 + SHV-5	1	0.25 (S)	≤0.25 (S)	0.19 (S)
K1, non-ESBL TEM/ SHV, or no beta-lactamase	Diverse	Diverse	19	≤0.0625 - 0.5 (S)	≤0.25 (S)	0.19 - 0.75(S)
Total without growth			114			

* inferred from a cefoxitin MIC ≥ 16mg/L, combined with a cefotaxime and ceftazidime MIC >32 and absence of synergy between clavulanic acid and cefotaxime and ceftazidime.

** plasmid AmpC

† isolates non-susceptible to at least one carbapenem not growing on the CRE agar

Chapter III

Detection of carbapenemase-producing *Enterobacteriaceae* with a commerical DNA microarray

Stuart JC [1], Voets G, Scharringa J, Fluit AC, Scharringa J, Leverstein - Van Hall MA

Department of Medical Microbiology, University Medical Centre Utrecht, The Netherlands

James Cohen Stuart [1], Guido Voets [1], Jelle Scharringa [1], Ad C. Fluit [1] and Maurine Leverstein - Van Hall [1,2]

1. Department of Medical Microbiology, University Medical Centre Utrecht, The Netherlands

2. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

Journal of Medical Microbiology; 2012; 61; p. 809-812

Abstract

The Check-MDR CT102 DNA microarray enables detection of the most prevalent carbapenemases (NDM, VIM, KPC, OXA-48 and IMP) and extended-spectrum b-lactamase (ESBL) gene families (SHV, TEM and CTX-M). The test performance of this microarray was evaluated with 95 *Enterobacteriaceae* isolates suspected of being carbapenemase producers, i.e. with meropenem MICs ≥0.5 mg l^{-1}. The collection of isolates contained 70 carbapenemase producing isolates, including 37 bla_{KPC}-, 20 bla_{VIM}-, five bla_{OXA-48}-, four bla_{KPC}/bla_{VIM}- and four bla_{NDM}-positive isolates; and 25 carbapenemase-gene-negative isolates. ESBLs were produced by 51 of the isolates. PCR and sequencing of b-lactamase genes was used as reference test. For detection of carbapenemases, the sensitivity of the microarray was 97% (68/70), with 100% specificity. The two negative isolates tested positive when the microarray test was repeated; these isolates were an OXA-48- and a KPC-producing isolate. For ESBL detection, the sensitivity was 100% (51/51) and the specificity was 98% (43/44), although 20% of the SHV-12 ESBLs were categorized as SHV-2-like ESBLs. In conclusion, the CDT102 microarray is a rapid and accurate tool for the detection of carbapenemase and ESBL genes, although the array seems less suitable for epidemiology of ESBL genes.

Introduction

Carbapenemase-producing *Enterobacteriaceae* are an emerging problem worldwide. Rapid and accurate detection of carbapenemase-producing strains is pivotal for adequate antibiotic therapy and infection control, especially in an outbreak setting. Phenotypic detection of carbapenemases in *Enterobacteriaceae* has several disadvantages. First, the two most frequently used confirmation tests, the modified Hodge test and the carbapenemase inhibition test, require overnight incubation and do not provide information on the carbapenemase gene. Second, the modified Hodge test is difficult to interpret and has limited specificity because extended-spectrum b-lactamase (ESBL)- and/or AmpC producing isolates with decreased permeability may give false positive results (Pasteran et al., 2009, 2010), and a low sensitivity has been reported for detection of NDM producing isolates (Girlich et al., 2012). Third, although the carbapenemase inhibition tests with boronic acid derivatives/cloxacillin and DPA/EDTA are sensitive and specific for detection of Ambler class A and B carbapenemases (Giske et al., 2011; Tsakris et al., 2010), respectively, the available data are mainly from VIM- and KPC producing isolates. Fourth, the carbapenemase inhibition tests cannot differentiate between ESBL- and AmpC producing isolates with decreased permeability and OXA-48 carbapenemase, which is encoded by an emerging carbapenemase gene and has recently been implicated in numerous outbreaks in several regions in the world (Cuzon et al., 2011; Pitart et al., 2011; Goren et al., 2011; O'Brien et al., 2011).

Because of the limitations of the phenotypic carbapenemase confirmation tests, genotypic detection of carbapenemase genes is the gold standard, although this only detects a prespecified set of known carbapenemase genes. The Check-MDR CT102 microarray (Check points Health BV) has been designed for detection of genes encoding NDM, KPC, VIM, IMP and OXA-48 (Naas et al., 2011; Woodford et al., 2011), which are currently the most prevalent carbapenemases (Cohen Stuart & Leverstein-Van Hall, 2010). In addition, this microarray enables detection of the most clinically relevant ESBL Abbreviations: ESBL, extended-spectrum b-lactamase. gene families, i.e. bla_{CTX-M}, bla_{SHV} and bla_{TEM}. goal of this study was to determine the test characteristics of this microarray for the detection of carbapenemase genes.

Methods

The ability of the Check-MDR CT102 microarray to detect carbapenemase genes was evaluated in 95 well-characterized nonduplicate *Enterobacteriaceae* isolates, which had a meropenem MIC of ≥0.5 mg l^{-1}, and, as such, were suspected of being carbapenemase producers (Cohen Stuart & Leverstein-Van Hall, 2010). Of these isolates, 45 were from a b-lactamase reference centre (University Medical Centre, Utrecht, The Netherlands), collected in 2010 for detection of carbapenemase genes, 24 were from Greece, 20 were from New York, and six were ATCC or NTCC reference strains. The collection contained 65 *Klebsiella pneumoniae*, nine *Escherichia coli* and two *Proteus mirabilis* isolates as well as 19 *Enterobacter* isolates (15 *E. cloacae*, four *E. aerogenes* and one *E. cancerogenus*). Of the 95 isolates, 70 were carbapenemase-positive (37 KPC-2 or KPC-3, 20 VIM-1, five OXA-48, four NDM-1, four VIM-1 plus KPC-2) and 25 were carbapenemase-negative (17 ESBL and eight AmpC, one of which produced plasmid-borne CMY-2 and seven of which were chromosomal AmpC-hyper producing *E. cloacae*). Of all the isolates (carbapenemase-positive and -negative), 51 (54%) harboured an ESBL gene (19 bla_{CTX-M}, 30 bla_{SHV} and two bla_{CTX-M} plus bla_{SHV}). As reference test for the presence of b-lactamase genes, PCR and sequencing were performed as described previously (Voets et al., 2011), using the Ultraclean Microbial DNA Isolation kit (Mo Bio Laboratories). The principles of the microarray system and interpretation software have been described previously (Cohen Stuart et al., 2010). Concisely, the system combines ligation-mediated amplification with detection of amplified products on a microarray to detect the various carbapenemase genes (bla_{OXA-48}, bla_{NDM}, bla_{IMP}, bla_{VIM} and bla_{KPC}), bla_{CTX-M} groups (bla_{CTX-M} groups 1, 2 and 9, or combined 8/25), and the most prevalent ESBL-associated single nucleotide polymorphisms (SNPs) in bla_{TEM} and bla_{SHV} variants. The assay cannot provide a sequence or Lahey number (www.lahey.org/studies/) of the bla_{TEM} and bla_{SHV} genes (e.g. bla_{TEM-6} or bla_{SHV-2}), but reports which group they belong to (Cohen Stuart et al., 2010). The microarray is designed to analyse three isolates at a time, which makes it costly to analyse a single isolate. The microarray assays were

performed according to the manufacturer's instructions using software version 20110215T170816R29 and including the use of two separate rooms (one room for DNA isolation and ligation, and one for amplification, hybridization and detection). The time to result of the microarray system was 8 h (3 h for DNA isolation and 5 h for ligation, amplification and detection). To mimic a routine clinical setting, the test characteristics were based on the first test result. When there were discrepancies between the microarray result of the carbapenemase gene detection and the PCR and sequencing result, the array was repeated.

Results

In all except two of the 70 carbapenemase-producing isolates, the microarray detected the correct carbapenemase gene, corresponding to a sensitivity of (68/70) 97% (Table 1). One OXA-48 producer and one KPC producer were interpreted as carbapenemase-negative by the microarray interpretation software, although visual inspection of the microarray showed a weak signal on the OXA-48 and KPC spot, respectively. The two false-negative isolates tested positive when the microarray was repeated. There were no carbapenemase false-positive results (specificity 100 %). The micro-array detected all ESBL genes (sensitivity of

100 %). However, one ESBL-negative (OXA-48-producing) *K. pneumoniae* isolate was reported as $bla_{\text{CTX-M-1}}$-positive, corresponding with a specificity and ESBL detection rate of 43/44 (98%). In addition, 6 of the 30 SHV-12-producing isolates (20%) were reported as ESBL $bla_{\text{SHV-2}}$-positive instead of belonging to the $bla_{\text{SHV-4}}$ group, because only the G238S substitution of the enzyme was detected in the bla_{SHV} gene by the microarray, and not the E240K substitution. All microarray results were obtained within one working day.

Table 1. Comparison between PCR/sequencing results and microarray results

PCR/sequencing results *(n)*	Number of concordant microarray results (%)	Number of discordant microarray results (%)	Microarray results of discrepant isolates
Carbapenemases (70)			
KPC (37)	36/37 (100 %) *	1/37 (3 %)	1 isolate *bla*$_{KPC}$-negative*
NDM (4)	4/4 (100 %)	0/4 (0 %)	NA
VIM (20)	20/20 (100 %)	0/20 (0 %)	NA
OXA-48 (5)	4/5 (100 %) *	1/5 (20 %) *	1 isolate *bla*$_{OXA-48}$-negative*
VIM plus KPC (4)	4/4 (100 %)	0/4 (0 %)	NA
Carbapenemas-negative (25)	31/31 (100 %)	0/31 (0 %)	NA
ESBLs (51)			
CTX-M (19)	19/19 (100 %)	0/19 (0 %)	NA
SHV (30)	24/30 (80 %)	6/30 (20 %)	6 isolates SHV-2 group (G238S) instead of SHV-12 (SHV-4 group G238S, E40K)
CTX-M plus SHV (2)	2/2 (100 %)	0/2 (0 %)	NA
ESBL-negative (44)	43/44 (98 %)	1/44 (2 %)	1 isolate CTX-M-1 group

NA, Not applicable. *The microarray detected both carbapenemase genes in the repeated microarray test.

Discussion

This study shows the Check-MDR CT102 microarray has a high sensitivity and specificity for detection of carbapenemases. This is in line with two previous reports evaluating this microarray. One study, involving 144 carbapenemase-producing isolates, found sensitivity and specificity values of 100% for detection of bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-48} carbapenemase genes, whereas values of 85% and 100 %, respectively, were found for bla_{KPC} (Naas et al., 2011). Another evaluation of the microarray with 41 carbapenemase producers, reported sensitivity and specificity values of 100% (Woodford et al., 2011). However, the present study is the first to exclusively use isolates suspected of carbapenemase production because of a meropenem MIC of ≥0.5 mg l^{-1}, i.e. a set of isolates that would require confirmation of carbapenemase production in the clinical setting. The sensitivity and specificity values for ESBL detection were also high. However, the microarray did not detect

the SHV E240K substitution in 20% of SHV-12-producing isolates, making this assay less suitable for epidemiological purposes involving SHV ESBLs. Although the latter finding was not mentioned in the two previous evaluations of this microarray (Naas et al., 2011; Woodford et al., 2011), it has been reported before (Platteel et al., 2011) in an evaluation of another microarray from the same manufacturer (Check KPC ESBL). A limitation of this evaluation is the fact that the capacity to detect TEM ESBLs or IMP carbapenemases has not been tested. Another limitation is that unknown carbapenemase genes may not have been detected by both the microarray and the reference method, i.e. PCR and sequencing. We conclude that this microarray assay is a practical and rapid tool for detection of genes encoding NDM, OXA-48, VIM and KPC carbapenemases in a routine clinical setting. Finally, because the microarray is designed to analyse three isolates at a time, it is costly to analyse a single isolate.

Acknowledgements

We wish to thank Vivi Miriagou, Department of Bacteriology, Hellenic Pasteur Institute, Athens, Greece, for providing the strains, and Sebastiaan Voskuil for providing technical support.

References

Cohen Stuart, J. & Leverstein-Van Hall, M. A. on behalf of the Dutch Working Party on the Detection of Highly Resistant Microorganisms (2010). Guideline for phenotypic screening and confirmation of carbapenemases in *Enterobacteriaceae*. *Int J Antimicrob Agents* **36**, 205–210.

Cohen Stuart, J., Dierikx, C., Al Naiemi, N., Karczmarek, A., Van Hoek, A. H., Vos, P., Fluit, A. C., Scharringa, J., Duim, B. & other authors (2010). Rapid detection of TEM, SHV and CTX-M extended-spectrum β-lactamases in *Enterobacteriaceae* using ligation-mediated amplification with microarray analysis. *J Antimicrob Chemother* **65**, 1377–1381.

Cuzon, G., Ouanich, J., Gondret, R., Naas, T. & Nordmann, P. (2011). Outbreak of OXA-48-positive carbapenem-resistant *Klebsiella pneumoniae* isolates in France. *Antimicrob Agents Chemother* **55**, 2420–2423.

Girlich, D., Poirel, L. & Nordmann, P. (2012). Value of the modified Hodge test for detection of emerging carbapenemases in *Enterobacteriaceae*. *J Clin Microbiol* **50**, 477–479.

Giske, C. G., Gezelius, L., Samuelsen, Ø., Warner, M., Sundsfjord, A. & Woodford, N. (2011). A sensitive and specific phenotypic assay for detection of metallo-b-lactamases and KPC in Klebsiella pneumoniae with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. *Clin Microbiol Infect* **17**, 552–556.

Goren, M. G., Chmelnitsky, I., Carmeli, Y. & Navon-Venezia, S. (2011). Plasmid-encoded OXA-48 carbapenemase in *Escherichia coli* from Israel. *J Antimicrob Chemother* **66**, 672–673.

Naas, T., Cuzon, G., Bogaerts, P., Glupczynski, Y. & Nordmann, P. (2011). Evaluation of a DNA microarray (Check-MDR CT102) for rapid detection of TEM, SHV, and CTX-M extended-spectrum blactamases and of KPC, OXA-48, VIM, IMP, and NDM-1 carbapenemases. *J Clin Microbiol* **49**, 1608–1613.

O'Brien, D. J., Wrenn, C., Roche, C., Rose, L., Fenelon, C., Flynn, A., Murphy, V., FitzGerald, S. F., Fenelon, L. E. & other authors (2011). First isolation and outbreak of OXA-48-producing *Klebsiella pneumoniae* in an Irish hospital, March to June 2011. *Euro Surveill* **16**, pii:19921.

Pasteran, F., Mendez, T., Guerriero, L., Rapoport, M. & Corso, A. (2009). Sensitive screening tests for suspected class A carbapenemase production in species of

Enterobacteriaceae. *J Clin Microbiol* **47**, 1631–1639.

Pasteran, F., Mendez, T., Rapoport, M., Guerriero, L. & Corso, A. (2010). Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class A carbapenemase in species of *Enterobacteriaceae* by incorporating boronic acid. *J Clin Microbiol* **48**, 1323–1332.

Pitart, C., Sole´ , M., Roca, I., Fa` brega, A., Vila, J. & Marco, F. (2011). First outbreak of a plasmid-mediated carbapenem-hydrolyzing OXA-48 β-lactamase in *Klebsiella pneumoniae* in Spain. *Antimicrob Agents Chemother* **55**, 4398–4401.

Platteel, T. N., Cohen Stuart, J. W., Voets, G. M., Scharringa, J., van de Sande, N., Fluit, A. C., Leverstein-Van Hall, M. A. & ESBL national surveillance working group (2011). Evaluation of a commercial microarray as a confirmation test for the presence of extendedspectrum β-lactamases in isolates from the routine clinical setting. *Clin Microbiol Infect* **17**, 1435–1438.

Tsakris, A., Poulou, A., Pournaras, S., Voulgari, E., Vrioni, G., Themeli-Digalaki, K., Petropoulou, D. & Sofianou, D. (2010). A simple phenotypic method for the differentiation of metallo-blactamases and class A KPC carbapenemases in *Enterobacteriaceae* clinical isolates. *J Antimicrob Chemother* **65**, 1664–1671.

Voets, G. M., Fluit, A. C., Scharringa, J., Cohen Stuart, J. & Leverstein-van Hall, M. A. (2011). A set of multiplex PCRs for genotypic detection of extended-spectrum b-lactamases, carbapenemases, plasmid-mediated AmpC b-lactamases and OXA b-lactamases. *Int J Antimicrob Agents* **37**, 356–359.

Woodford, N., Warner, M., Pike, R. & Zhang, J. (2011). Evaluation of a commercial microarray to detect carbapenemase-producing *Enterobacteriaceae*. *J Antimicrob Chemother* **66**, 2887–2888.

Chapter IV

A set of multiplex PCRs for genotypic detection of extended-spectrum β-lactamases, carbapenemases, plasmid-mediated AmpC, β-lactamases, and OXA β-lactamases.

Guido M. Voets 1, Ad C. Fluit [1], Jelle Scharringa [1], James Cohen Stuart [1], Maurin A. Leverstein-van Hall [1, 2]

[1] Department of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands

[2] National Institute for Public Health and the Environment, Bilthoven, The Netherlands

International Journal of Anitmicrobial Agents; 2011; 37; p. 356-359

Abstract

Worldwide, resistance of Gram-negative micro-organisms to third-generation cephalosporins and carbapenems due to β-lactamases is an increasing problem. Although the CTX-M, TEM and SHV extended-spectrum β-lactamases are most widely disseminated, other β-lactamase families have recently emerged as well, such as plasmid-mediated AmpC β-lactamases and carbapenemases. Here, we describe a new set of multiplex PCRs with one amplification protocol enabling detection of twenty-five prevalent β-lactamase families, including ESBLs, carbapenemases, plasmid-mediated AmpC β-lactamases, and OXA β-lactamases.

Introduction

The increasing prevalence of Gram-negative bacteria producing β-lactamases, which hydrolyze third-generation cephalosporins or carbapenems, is a world-wide problem. The most prevalent β-lactamases are extended-spectrum β-lactamases (ESBLs) of the CTX-M, TEM and SHV families [1]. However, the prevalence of Gram-negative bacteria that are resistant to third-generation cephalosporins due to increased expression of chromosomal β-lactamases, like SME and IMI-1, or the acquisition of plasmid-mediated β-lactamases such as IMI-2, NDM-1, KPC, IMP, OXA, PER, and VEB as well as AmpC β-lactamases is increasing [2-6].

Several multiplex PCRs for the detection of β-lactamase families have been published [7-9]. Especially, the set of multiplex PCRs by Dallenne et al. detects a broad range of epidemiological important β-lactamase families [7]. However, this set does not detect the SME, IMI, NDM, NMC-A, GIM, SIM, and SPM ß-lactamase families, as well as several OXA-groups within the OXA family.

The aim of this study was to develop a set of multiplex PCRs for the detection of the majority of genes encoding clinically important β-lactamases causing third-generation cephalosporin or carbapenem resistance using a single amplification protocol.

To achieve this aim we developed seven multiplex PCRs for the detection of plasmid-mediated AmpC ß-lactamases (ACC, ACT, DHA, CMY, FOX, LAT, MIR, MOX), metallo-carbapenemases (GIM, NDM, SIM, SPM), serine carbapenemases (IMI SME, NMC-A) and OXA β-lactamases (OXA groups 23, 24, 48, 1, 2, 51, 4, and 58) using SuperTaq polymerase (HT Biotechnology Ltd., Cambridge, UK) (Table 1). For detection of genes encoding CTX-M, TEM, SHV, GES, VEB, PER, KPC, VIM, IMP ß-lactamase families, four multiplex PCRs of Dallene et al. (multiplex I, II, IV and VI) [7] were evaluated using the same amplification protocol as used for our PCRs, including the SuperTaq polymerase instead of the AmpliTaq polymerase (Sigma Aldrich, St Quentin Fallavier, France) originally used by the authors.

Material and Methods

Primers were designed using the DNASTAR Lasergene 8 program (Madison, USA). The optimisation of the amplification conditions of each PCR, both the newly presented PCRs in this paper as well as the complementary PCRs of Dallenne et al. [7], resulted in the following PCR protocol. DNA was isolated using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The DNA concentrations of the samples were equalised to approximately 50 ng/µl and 1 µl was added to each multiplex PCR in a 25 µl reaction mixture, containing 1x PCR buffer, 50 µM of each deoxynucleotide triphospate, 1 U SuperTaq, and a variable concentration of primers and $MgCl_2$ (Table 1). Amplification was carried out as follows: initial denaturation at 94°C for 1 min; 30 cycles of 30 sec at 94°C, 40 sec 60°C, 1 min 72°C, and a final elongation step at 72°C for 1 min. Amplification products were visualized after running at 100 V for 1 h on a 1% agarose gel containing gel red (Biotum, Hayward, USA). A 1 kb DNA ladder (Invitrogen, Breda, The Netherlands) was used as a size marker. DNA sequencing was performed by Baseclear (Leiden, The Netherlands). *In silico* PCR simulations were performed using nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and MegAlign (DNAstar Lasergene 8).

Results and discussion

Amplification products of the expected sizes were obtained with all control strains, confirming the group specificity of the primers (Figure 1). In addition, the expected amplification products were also obtained with the adapted complementary PCRs described by Dallenne et al. [7] (data not shown). To determine the specificity of the primers for allotypes for which no positive control strain was available *in silico* PCR simulation was performed. All allotypes to which the primers anneal, allowing for 1 mismatch except for the 3'-nucleotide and allotypes with more mismatches, but in the same target gene-group were identified (Table 1). *In silico* PCR simulation was also performed to check for any unwanted cross-reaction with members of the *Enterobacteriaceae*, *Acinetobacter baumanni*, and *Pseudomonas aeruginosa*. The 3'-end of the SPM-GBM-R primer anneals with 21 of 27 bp to the ACC deaminase gene of a *Pseudomonas* spp. (GenBank ACC. No. EU520401) and the 3'-end O58-GD3M-R anneals with 17 of 22 bp to the biotin sulfoxide reductase gene of *E. coli* (GenBank ACC. No. CP001846) and also with 17 of 22 bps to the molybdopterin guanine dinucleotide-containing S/N-oxide reductase gene of *E. coli* (GenBank ACC. No. CP001637). The cross-reaction of SPM-GBM-R primer was not a problem with the *P. aeruginosa* positive control. The cross-reactions of O58-GD3M-R may result in poorer resolution of the MGD3 multiplex PCR when used in combination with an *E. coli*.

It should be noted that the MGC2 multiplex PCR yielded a 160 bp non-specific

amplification product (Figure 1 B, lane 1 and 2). DNA sequencing showed that this product was identical to a part of a CMY-2-group gene in the control strain (lane 1 and 2). In 6 isolates that were expected to remain negative another 160 bp non-specific amplification product was noted. DNA sequencing showed that this product was part of an ACT-like AmpC (GenBank ACC. No. EF078894.1) (data not shown). The CMY-2 group gene amplification resulted from annealing of the ACT-GCM2-R primer and CY2-GC2M-F primer to genes of the CMY-2-group despite several mismatches. The ACT-like AmpC gene amplification resulted from the annealing of the ACT-GCM2 primers to the ACT-like AmpC gene. However, these amplification products were not considered to be a problem for the interpretation of the multiplex PCR because it they are smaller than the expected specific products. Furthermore, the amplification products were derived from a CMY-2-group or ACT-like gene, which are both targets of the MGC2 multiplex PCR. In conclusion, we describe a set of multiplex PCRs that, when used in combination with the multiplexes I, II, IV, and VI of Dallene et al. [7], can detect a wide range of β-lactamases using the same amplification conditions. This enables the detection of the majority of clinically important β-lactamases causing third generation cephalosporin or carbapenem resistance.

Acknowledgements

The authors wish to thank Patrice Nordmann for the kindly providing isolates Nord-03 and Nord-04, Kyungwon Lee for isolate PSA097, and Ana Gales for isolate PSA098.

References

1. Gniadkowski M. Evolution of extended-spectrum beta-lactamases by mutation. Clin Microbiol Infect 2008;14 Suppl 1:11-32.

2. Nordmann P, Poirel L. Emerging carbapenemases in Gram-negative aerobes. Clin Microbiol Infect 2002;8(6):321-31.

3. Naas T, Poirel L, Nordmann P. Minor extended-spectrum beta-lactamases. Clin Microbiol Infect 2008;14 Suppl 1:42-52.

4. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev 2009;22:161-82

5. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-beta-lactamase gene, *bla(NDM-1)*, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 2009;53:5046-54.

6. Walther-Rasmussen J, Hoiby N. Class A carbapenemases. J Antimicrob Chemother 2007;60:470-82.

7. Dallenne C, Da Costa A, Decre D, Favier C, Arlet G. Development of a set of Multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. J Antimicrob Chemother 2010;65:490-5.

8. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 2006;27:351-3.

9. Mendes RE, Kiyota KA, Monteiro J, Castanheira M, Andrade SS, Gales AC, et al. Rapid detection and identification of metallo-beta-lactamase-encoding genes by multiplex real-time PCR assay and melt curve analysis. J Clin Microbiol 2007;45:544-7.

10. Carrer A, Poirel L, Pitout JD, Church D, Nordmann P. Occurrence of an SME-2-producing *Serratia marcescens* isolate in Canada. Int J Antimicrob Agents 2008;31:181-2.

11. Aubron C, Poirel L, Ash RJ, Nordmann P. Carbapenemase-producing *Enterobacteriaceae*, U.S. rivers. Emerg Infect Dis 2005;11:260-4.

12. Lee K, Yum JH, Yong D, Lee HM, Kim HD, Docquier JD, et al. Novel acquired metallo-beta-lactamase gene, bla(SIM-1), in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob Agents Chemother 2005;49:4485-91.

13. Cohen Stuart J, Dierikx C, Al Naiemi N, Karczmarek A, Van Hoek AH, Vos P, et al. Rapid detection of TEM, SHV and CTX-M extended-spectrum beta-lactamases in *Enterobacteriaceae* using ligation-mediated amplification with microarray analysis. J Antimicrob Chemother 2010;65:1377-81.

14. Perez-Perez FJ, Hanson ND. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol 2002;40:2153-62.

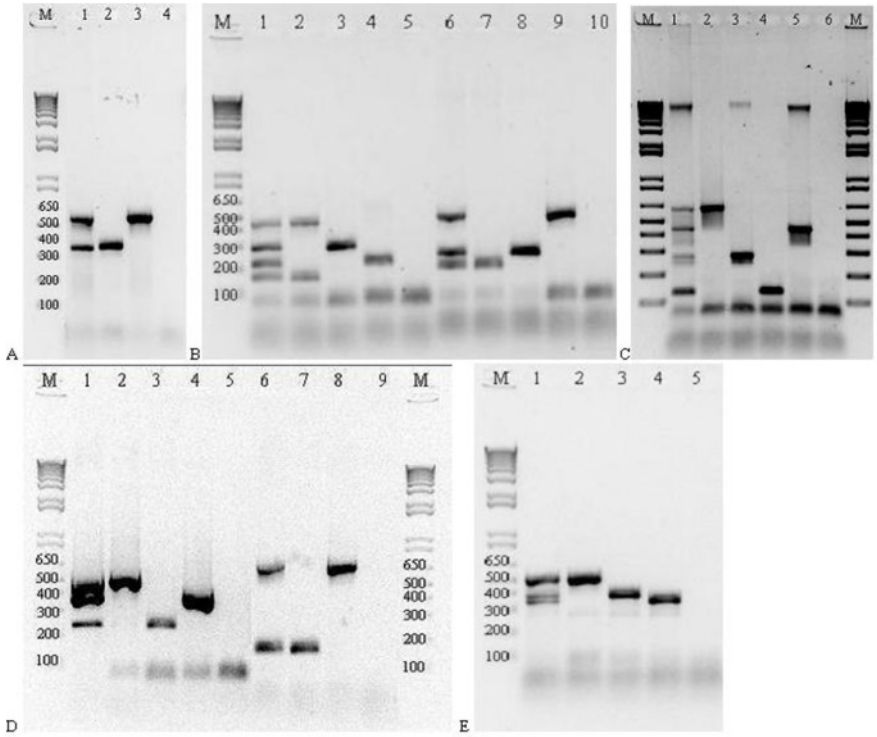


Figure 1: Multiplex PCRs. All multiplex PCR products were separated in a 1% agarose gel. (A) Multiplex PCR for the detection of the carbapenemase families SME, IMI and NMC-A. Lanes: M. molecular size marker (in bp); 1. Nord-03 + Nord-04; 2: Nord-03; 3: Nord-04; 4: negative control. (B) Lanes 1-5: multiplex PCR for CIT-, EBC-, and FOX-groups of plasmid-mediated AmpC β-lactamases; lanes 6-10 multiplex PCR for OXA-1, -2, and -51-groups. Lanes: M: molecular size marker (in bp); 1: CP040 + CP038 + CP093; 2: CP040; 3: CP038; 4. CP093; 5: negative control; 6: CP116 + CP082 + NCTC-13420; 7: CP116; 8: CP082; 9: NCTC-13420; 10: negative control. (C) Multiplex PCR for the detection of the metallo-carbapenemase families SIM, GIM, SPM and NDM. Lanes: M. molecular size marker (in bp); 1. PSA096 + PSA097 + PSA098 + NCTC-13443; 2: PA097; 3: PSA098; 4: NCTC-13443; 5: PSA096; 6: negative control. (D) Lanes 1-5 multiplex PCR for OXA-23, -24, -48-groups; lanes 6-9 multiplex PCR for OXA-10 and OXA-58 groups. Lanes: M: molecular size marker (in bp); 1: CP081 + NCTC-13303 + NCTC-13442; 2: CP081; 3: NCTC-13303; 4: NCTC-13442; 5: negative control; 6; CP089 + NCTC-13305; 7: CP089; 8: NCTC-13305; 9: negative control; M: molecular size marker (in bp). (E) Multiplex PCR for CMY-1-, MOX-, DHA-, and ACC-groups of plasmid-mediated AmpC β-lactamases. Lanes: M: molecular size marker (in bp); 1: CP043 + CP039 + CP044; 2: CP043; 3: CP039; 4: CP044; 5: negative control.

Table 1: Group–specific primers used in the multiplex PCRs. All allotypes without parentheses have no or 1 mismatch with the primers, except for the 3'-nucleotide, designed for this target group. NCTC = National Collection of Type Cultures

Multiplex	β-lactamase(s) targeted	Primer Name	Sequence (5' - 3')	Positive Control	Amplicon size (bp)	Primer conc. (pmol/µl)	$MgCl_2$ conc. (mM)
MGA	SME-1 to SME-3	SME-GAM-F	GAG GAA GAC TTT GAT GGG AGG AT	Nord-03 [11]	334	0.8	1.5
		SME-GAM-R	TCC CCT CAG GAC CGC CAA G			0.7	
	IMI-1 to IMI-3, NMC-A	IMI-GS-F	GGT GTC TAC GCT TTA GAC ACT GGC TC	Nord-04 [12]	536	0.5	
		IMI-GS-R	GCA CGA ATA CGC GCT GCA CCG G			0.6	
MGB	GIM-1	GIM-GBM-F	CGT TGC CAG CTT TAG CTC AGG	PSA098 [this study]	279	0.3	2.5
		GIM-GBM-R	GCA ACT TGA TAC CAG CAG TGC G			0.3	
	SIM-1	SIM-GBM-F	TTG CGG AAG AAG CCC AGC CAG	PSA097 [13]	613	0.3	
		SIM-GBM-R	GCG TCT CCG ATT TCA CTG TGG C			0.3	
	NDM-1	NDM-GBM-F	CCC GGC CAC ACC AGT GAC A	NCTC- 13443	129	0.7	
		NDM-GBM-R *	GTA GTG CTC AGT GTC GGC AT			0.6	
	SPM-1	SPM-GBM-F	GGG TGG CTA AGA CTA TGA AGC C	PSA096 [9]	447	1.2	
		SPM-GBM-R	GCC GCC GAG CTG AAT CGG			1.4	
MGC 1	CMY-1, CMY-8 to CMY-11, CMY-19, MOX-1 to MOX-4 & [MOX-5 to MOX-7]	CY1-GC1M-F	GCT GCT CAA GGA GCA CAG GAT CCC G	CP043, CP113 [14]	522	0.5	1.0
		CY1-GC1M-R	GGC ACA TTG ACA TAG GTG TGG TGC ATG			0.5	
	DHA-1 to DHA-3, DHA-7	DHA-GC1M-F	CTT TCA CAG GTG TGC TGG GTG CG	CP039 [14]	403	0.5	
		DHA-GC1M-R	CCG TAC GCA TAC TGG CTT TGC GC			0.6	
	ACC-1 to ACC-4	ACC-GC1M-F	TCC AGC CGC TGA TGC AGA AGA AT	CP044 [14]	365	1.1	
		ACC-GC1M-R	CCA YGC TTT TAG ATA AGC CAT CAG CTG			1.0	
MGC 2	CMY-2-group †, LAT-1, and LAT-4 **	CY2-GC2M-F	ACT GGC CAG AAC TGA CAG GCA AA	CP040, CP122, CP 168 [14]	466	1.7	1.0
		CY2-GC2M-R	GTT TTC TCC TGA ACG TGG CTG GC			1.7	

	ACT-1 to ACT-3, MIR-1 to MIR-4, MIR-8 and [ACT-5] ***	ACT-GC2M-F	TCG GTA AAG CCG ATG TTG CGG	CP038 [14]	302	0.6	
		ACT-GC2M-R	CTT CCA CTG CGG CTG CCA GTT			0.6	
	FOX-1 to FOX-8	FOX-GC2M-F	CAT GGG GTA TCA GGG AGA TGC C	CP093 [14]	218	0.1	
		FOX-GC2M-R	GCC GCT GCT CGC CCA TCG			0.1	
MGD 1	OXA-23, -27, -49, -73, -133, -146	O23-GDM-F	CCT GAT CGG ATT GGA GAA CCA G	NCTC-13421, CP081 [14]	516	0.6	2.0
		O23-GDM-R	GAT GCC GGC ATT TCT GAC CG			0.7	
	OXA-24 (= -33, -40), -25, -26, -72, -139, -160 & [OXA-143, -182]	O24-GDM-F	GGT CGA TAA TTT TTG GTT AGT TGG CCC	NCTC-13302 , NCTC-13303	237	0.3	
		O24-GDM-R	CCA TTA GCT TGC TCC ACC CAA CCA G			0.3	
	OXA-48, -54, & [OXA-181]	O48-GDM-F	CCA AGC ATT TTT ACC CGC ATC KAC C	NCTC-13442	389	1.1	
		O48-GDM-R	GYT TGA CCA TAC GCT GRC TGC G			1.2	
MGD 2	OXA-1 (=30), -31, -47	O1-GD2M-F	CAA CGG ATT AAC AGA AGC ATG GCT CG	CP116 [14]	198	0.1	2.5
		O1-GD2M-R	GCT GTR AAT CCT GCA CCA GTT TTC CC			0.1	
	OXA-2, -3, -15, -21, -32, -34, -36, -46, -53, -141, -144, -161 & [OXA-118, -119]	O2-GD2M-F	GAC CAA GAT TTG CGA TCA GCA ATG CG	CP082, CP087 [14]	256	0.8	
		O2-GD2M-R	CYT TGA CCA AGC GCT GAT GTT CYA CC			0.8	
	OXA-51 group ††	O51-GD2M-F	GAC CGA GTA TGT ACC TGC TTC GAC C	NCTC-13420	497	1.6	
		O51-GD2M-R	GAG GCT GAA CAA CCC ATC CAG TTA ACC			1.5	
MGD 3	OXA-10 group ††† and [OXA-5]	O10-GD3M-F	CGC CAG AGA AGT TGG CGA AGT AAG	CP089 [14]	138	1.1	2.0
		O10-GD3M-R	GAA ACT CCA CTT GAT TAA CTG CGG			1.1	
	OXA-58, -96, -97, -164	O58-GD3M-F	GTG CTG AGC ATA GTA TGA GTC GAG C	NCTC-3305	630	0.5	
		O58-GD3M-R	GGT CTA CAG CCA TTC CCC AGC C			0.6	

* = primer sequence from David Livermore, Health Protection Agency, UK (personal correspondence) ; ** = a.k.a. the EBC group [15] ; *** = a.k.a. the CIT group[15] ; † = CMY-2, -4 to -7, -12 to -16, -18, -21, -23, -24, -27 to -36, -38 to -41, -43 to -45, -47 to -49, -53 to -55, and -59 ; †† = OXA-51, -64 to -71, -75 to -80, -82 to -84, -86 to -95, -98 to -100, -106 to -113, -115 to -117, -128, -130 to -132, -138, -144, -148 to -150, -172 to -180 ; ††† = OXA-4, -7., -10, -11, -13, -14, -16, -17, -19, -28, -56, -74, -101, -129, -142, -145, -147

Chapter V

International Multicenter Evaluation of the DiversiLab Bacterial Typing System for *Escherichia coli* and *Klebsiella* SPP.

Guido M. Voets[a], Maurine A. Leverstein-van Hall[a,b], Susanne Kolbe-Busch[c], Adri van der Zande[d], Deirdre Church[e], Martin Kaase[f], Andrea Grisold[g], Mathew Upton[h], Elaine Cloutman-Green[i], Rafael Cantón[j], Alexander W. Friedrich[k], Ad C. Fluit[a], The DiversiLab Study Group

[a] Department of Medical Microbiology, University Medical Center Utrecht Utrecht, the Netherlands

[b] Bronovo Hospital, the Hague and Diaconessenhuis, Leiden. the Netherlands

[c] Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Düsseldorf, Germany

[d] Laboratorium Microbiologie Twente Achterhoek, Enschede, the Netherlands

[e] Department of Pathology & Laboratory Medicine and Medicine, Calgary, Canada

[f] Department of Medical Microbiology, Bochum, Germany

[g] *Institute of Hygiene, Microbiology and Environmental Medicine, Graz,* Austria

[h] University of Manchester School of Medicine, Manchester, United Kingdom

[i] Great Ormond Street Hospital for Children, London, United Kingdom

[j] Servicio de Microbiología, Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain

[k] Department of Medical Microbiology, University of Groningen, Groningen, the Netherlands

Revised version accepted for publication in Journal of Clinical Microbiology

Abstract

Internationally successful multi-drug resistant clones are globally increasing in prevalence, which makes the ability to identify these clones urgent. However, adequate easy-to-perform and reproducible typing methods are lacking. We investigated whether DiversiLab (DL), an automated repetitive-sequence-based PCR bacterial typing system (bioMérieux), is suitable to compare isolates analyzed at different geographic centers. A total of 39 *Escherichia coli* and 39 *Klebsiella* spp. isolates previously typed by the coordinating center were analyzed. Pulsed-field gel electrophoresis (PFGE) confirmed the presence of one cluster of six isolates, three clusters of three isolates, and three clusters of two isolates for each set of isolates. DL was performed in 11 centers in six different countries using the same protocol. A total of 425 *E. coli* and 422 *Klebsiella* spp. DL profiles were obtained. DL showed a lower discriminatory power than PFGE. Local DL data showed a low concordance as indicated by the adjusted Rand's and adjusted Wallace's coefficients (*E. coli*: range 0.132-0.740 and 0.070-1.0; *Klebsiella* spp.: 0.091-0.864 and 0.056-1.0, respectively). Central analysis showed significantly improved concordance (*E. coli*: 0.473-1.0 and 0.290-1.0; *Klebsiella* spp. 0.513-0.965 and 0.425-1.0). Misclassification of profiles for individual isolates was mainly due to inconsistent amplification, which is most likely due to variations in the quality and amount of the isolated DNA used for amplification. Despite local variations, DL may have the potential to track clonal outbreaks in an international setting, provided there is strict adherence to standardized, reproducible, DNA isolation methods and analysis protocols, all supported by a central database for profile comparison.

Introduction

The prevalence of internationally successful multidrug-resistant clones, e.g., *Klebsiella pneumoniae* ST258 and *Escherichia coli* ST131, is increasing globally (1-4). The spread of these high-risk clones is aided by an increase in international travel, medical treatment abroad, and repatriated patients (5, 6). The ability to identify these epidemic clones is of importance to understand the epidemiology of these isolates and may alert hospitals of the emergence of epidemic strains. This requires a reliable typing method capable of identifying epidemic clones that can be performed at different centers together with an internationally accessible database for comparisons (7). Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) have been used for this purpose. The main drawback of PFGE, however, is poor reproducibility due to technical variation and the time consuming nature of the method, whereas MLST lacks sufficient discriminatory power. Multiple-variable number of tandem repeat analysis (MLVA) and amplified fragment length polymorphism analysis (AFLP) are also typing methods with databases, but these methods are not widespread and also suffer from technical

limitations (8-10).

The DiversiLab (DL) bacterial typing system (bioMérieux, Marcy l'Etoile, France) that allows to obtain results within a day may offer an alternative, although it is based on repetitive-sequence-based PCR (rep-PCR) which also suffers from poor reproducibility (11, 12). By standardisation of its procedures (PCR and analysis of the amplification products) and the use of a commercial microfluidics system DL improved its reproducibility and has the potential for multicenter comparisons of typing data, thereby possibly facilitating identification of international clones. However, comparisons between different centers have not yet been performed. The aim of this study was to evaluate the interlaboratory reproducibility of DL for *E. coli* and *Klebsiella* spp. in an international, multicentre setting. Eleven centers in six countries typed 39 *E. coli* and 39 *Klebsiella* spp. isolates, which were previously characterized by PFGE and represent either outbreaks or unique isolates.

Materials and Methods

Isolates and centers. In total, 39 *E. coli* isolates and 39 *Klebsiella* spp. (34 *K. pneumoniae* and 5 *Klebsiella oxytoca*) that had been typed previously by PFGE were selected from the collection of the Hospital Hygiene Department of the University Medical Center Utrecht and a study into the population distribution of ß-lactamases conferring resistance to third-generation cephalosporins in human clinical isolates in the Netherlands (13). Repeated PFGE typing confirmed in both groups one cluster of six isolates, three clusters of three isolates each, three clusters of two isolates, and 18 unique isolates. Isolates were initially identified by standard microbiology methods and later confirmed by MALDITOF MS (Bruker Daltonics, Germany).

The isolates, from a single plate, were shipped on M40 Transystem Amies Agar Gel transport swabs (Copan Italia SpA, Brescia, Italia) to the 11 participating centers in 6 countries across Europe (Austria, England, Germany, Spain, The Netherlands) and Canada.

Typing. All centers used the same protocol. DNA was isolated using an UltraClean® Microbial DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer's instructions with two changes for the *Klebsiella* spp. isolates. It was recommended to all centers to use a 10 µl loop of bacteria and 900 µl of the MD3 solution based on previous experience (8). Nanodrop™, a highly sensitive spectrophotometer, or equivalent was used to quantify the DNA. The minimal required concentration was 25 ng/µl. The DNA was required to have an OD 260/280 ratio >1.7 and OD 260/230 ratio >1.3. Each center performed the PCR with AmpliTaq (Invitrogen, Breda, The Netherlands) and the kits specified by the manufacturer for *E. coli* (kit no. 270613) and *Klebsiella* (kit no. 270615). PCR products were analyzed

using standard chips (no. 270670). Each center uploaded the chip results to its own bioMérieux DiversiLab website for local analysis.

Data analysis and statistical methods. Analysis of the profiles was performed at two levels. First, at the level of the individual laboratory and secondly all profiles were examined by staff at the coordinating center. All centers received the same protocol for analysis of the data at the first level. DL results to be used for comparisons were recommended to lack automatic warnings and have peak intensities of at least 100 or above for at least one peak. Analysis was performed using Pearson correlation in the dedicated DL software of the manufacturer (version 3.4). Isolates with a similarity <95% were considered different and isolates with a similarity >98% were considered indistinguishable. All isolates with a similarity >95% and <98% were judged manually using the pattern overlay of the analysis tool in the software.

Statistical analysis (adjusted Rand's and adjusted Wallace's coefficients) was performed using the online tool of the Instituto de Medcina Molecular of the University of Lisbon (http://darwin.phyloviz.net/ComparingPartitions/index.php). The discriminatory power was estimated by Simpson's index of diversity (14). Ninety-five percent confidence intervals (CI95) for discriminatory indices were calculated according to the method. Non-overlapping confidence intervals were regarded as representing statistically significant differences in discriminatory power (15).

Isolates designated as non-typeable were included in the statistical analysis as unique values. All non-processed, non-viable, and isolates that could not be amplified were removed from the statistical analysis. PFGE data were not communicated to the local centers.

Central analysis was performed by the chief investigators (GV, AF). The data were also transferred to a dedicated website created by bioMérieux with the same DL analysis software as used by the individual centers for the analysis of the combined data. The data were judged manually using the pattern overlay of the analysis tool in the software. Statistical analysis was performed as described above for local analysis.

Results

Typing data of 425 of 429 *E. coli* and 422 of 429 *Klebsiella* spp. were available for analysis. Three of the *E. coli* samples were not processed (<1%) and one showed no amplification in one of the centers (<1%). Similarly, seven of the *Klebsiella* spp. samples (1%) were not processed (<1%). The analyses performed by the individual centers were confirmed by the central laboratory.

The local analyses showed little consensus (Figure 1A), which is confirmed by the adjusted Rand's and adjusted Wallace's coefficients (Tables S1 and S2). Nevertheless, the local analyses of the *E. coli* DL data agreed in 96% of the cases for half of the unique isolates according to PFGE (Figure 1A; bottom nine isolates). A similar level of consensus was obtained for the cluster of isolates 31E and 32E and the cluster consisting of isolates 14E, 24E and 25E. However, there was no consensus in the assignment for the isolates in the three other clusters. The lack of consensus was confirmed by the adjusted Rand's and adjusted Wallace's coefficients (Tables S1 and S2). Both statistical analyses showed poor overall values (range 0.132-0.740 and 0.070-1.0, respectively). An exception for the adjusted Wallace's coefficients was the comparison with center 3, which was due to the fact that analysis here resulted in one big cluster that encompassed the smaller clusters identified by other centers.

For *E. coli* DL analysis from local centers showed less discriminatory power compared to PFGE. Examples of this are the DL clusters with isolates 31E and 32E, 18E and 37E, and 14E, 24E, and 25E (Figure 1A).

The central analysis of *E. coli* DL also resulted in less discriminatory power in comparison to the PFGE analysis, which is reflected by lower Simpson's index of diversity (Table S3). The Simpson's index of diversity was 0.964 (95% CI 0.935-0.992) for PFGE and ranged from 0.709 (95% CI 0.550-.867) to 0.864 (95% CI 0.767-0.961). However, it showed improved consensus between the data from the different centers (Figure 1B and Tables S4 and S5) compared to local analysis. This was most apparent in the cluster consisting of 22E and 39E, the cluster of 18E and 37E, and the cluster of 34E up to and including 11E in Figure 1B, which improved significantly in concordance. This is reflected in the adjusted Rand's and adjusted Wallace's coefficients (Tables S4 and S5). The adjusted Rand's coefficients have a mean value of 0.8 (range 0.473-1.0). The average is mainly lowered by results obtained by center 11. The directional adjusted Wallace's coefficient also reflects the improved concordance with a mean value of 0.83 and for 6 of the 11 centers the mean was above 0.9 (range 0.290-1.0).

The *Klebsiella* spp. were not separated into different species as most of them were *K. pneumoniae* (n=34) and only a few *K. oxytoca* (n=5) were included and these were unique isoaltes based on PFGE. The assignments of *Klebsiella* spp. agreed in 97% of the cases for two thirds of the unique isolates according to PFGE (Figure 2A; last 12 isolates). Concordance of isolates belonging to a cluster was larger than found for *E. coli*, though more outliers were present. This is reflected in overall higher and more consistent values of the adjusted Rand's and adjusted Wallace's coefficients (Table S6 and S7), although both statistical analyses show poor overall values (range 0.091-0.864 and 0.056-1.0, respectively). An exception to this finding for the adjusted Wallace's coefficients is center 9, where analysis identified one big cluster, which encompassed the smaller clusters that other centers identified.

In comparison to the PFGE analysis for *Klebsiella* spp. DL also had less discriminatory power in the local analysis (Figure 2A and Tables S6 and S7). Examples of this are the isolates 03K and the isolates 35K and 37K.

A marked improvement in the degree of concordance between the centers was obtained by central analysis and the discriminatory power remained acceptable (Figure 2B and Table S3). Most notably, the formation of the clusters containing the isolates 23K, 09K, and 36K, the cluster of 02K and 33K, the cluster of 26K and 31K, the cluster of 37K and 35K and the cluster consisting of 21K and 38K improved in concordance significantly. This is reflected in the statistical analysis (Table S8 and S9); the adjusted Rand's coefficients have a mean value of 0.71 (range 0.513-0.965). The values of the adjusted Wallace's coefficient also increased in comparison to the local analyses to a mean value of 0.73 (range 0.425-1.0). It has to be noted that although different clusters could be assigned to the isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K, the patterns of these clusters were closely related and that these clusters and unique isolates may be considered a clonal complex (Figure S1).

Discussion

We performed an international multicenter evaluation of the DiversiLab bacterial typing system for *E. coli* and *Klebsiella* spp. In order to assess whether DL is suitable to compare isolates analyzed at different centers. Some local studies (8, 16-18) showed that DL performed well for several species, e.g., *Klebsiella* spp., and to a lesser extent, for some others, e.g., *E. coli*. Moreover, it was shown to identify some of circulating high risk clones such as ST131 *E. coli* harbouring CTX-M-15 extended spectrum β-lactamasae and clonal complex 147 from *K. pneumoniae* expressing carbapenemases (19-20).

In our study, 11 centers from six different countries typed 39 *E. coli* and 39 *Klebsiella* spp. isolates that were selected based on PFGE results. The main findings of the study were: 1) DL had a lower discriminatory power for these sets of isolates than PFGE; 2) the clustering obtained by the different centers was only partly concordant; 3) central analysis improved the clustering to an acceptable level. In comparison to PFGE typing, DL had less discriminatory power, creating larger clusters and clustering of isolates that using PFGE are considered unique (Figure 1-4). This has also been demonstrated by other studies (21, 22). Isolates considered to be different by DL are also considered to be different by using PFGE.

A number of factors might have contributed to the less than optimal concordance between the centers. The main factors are incorrect clustering by Pearson correlation and misclassification of isolates due to variation in the amplification products between isolates. In some cases the amplification signals were faint or

even completely absent causing incorrect clustering by Pearson correlation. In the central analysis, which completely relied on interpretation of the overlays, a more consistent clustering was obtained (see Figures 1B and 2B). This was further aided by the fact that, for each isolate, at least 10 replicates were available. This allowed for assessing and mitigation of continuous minor changes in the patterns of the isolates when examining individual clusters. Sometimes, replicates for the same isolates did not cluster next to each other but across the whole cluster (Figure S1). These data indicate that the reproducibility of one or more of the steps before analysis is insufficient. Several issues can be responsible for this lack of reproducibility: 1) variation between persons performing the assays; 2) amplification may be inconsistent; 3) inconsistent quality and/or amount of DNA obtained during its isolation. However, this also indicates the importance of a central database to compare isolates. Moreover, person-to-person variation, variation in thermocycler performance and the quality and amount of DNA isolated are known from the experience at the central laboratory to lead to variation in the results (unpublished data). This study lacked an experimental design to measure person-to-person variation within a center. Regardless, it is difficult to control for person-to-person variation, particularly within a multicenter setting.

Inconsistent DNA amplification is most likely not due to the PCR kits used as these are quality checked for consistent performance and the same batches were used in all centers included in this study. However, the thermocycler used may be a source of variation, especially since during the amplification process in a rep-PCR based system and sometimes less than optimal interactions between primers and target occur and slight differences in initial conditions such as small variations in primer concentrations can have a major impact on the results. Although it is possible at a local level to assign a single machine to the DL assays, this is not feasible in a multicenter setting. One of the most important parameters in amplification is the quality and amount of DNA added. The study protocol required a minimum amount and quality of the DNA (25 ng/µl and an OD 260/280 ratio >1.7 and OD 260/230 ratio >1.3). This, however, still allows for variations between centers. In addition, the possible use of different quantification platforms may contribute to variation as different platforms may yield different results (unpublished observations). Moreover, only 2 µl of DNA solution is added and small absolute variations lead to a large relative variation in volume. Furthermore, amplification products of different lengths are generated with one amplification protocol leading to competition for amplification. The variation in DNA quality and amount may be addressed by a more robust protocol. The use of automatic DNA extraction can provide a useful contribution in better controlling DNA amount and quality. It should be noted though, that the use of different types of automatic extraction machines still might contribute to problems with reproducibility of amplification.

The final two factors that contributed to the differences in the assignment of isolates

to different clusters are errors in administration and/or exchange of isolates and/or results and failure to adhere to protocol. These two factors were most notable with isolates included for analysis that generated poor signals or warning signals, even if a good result of a retest was present.

Despite the ability of the amplification protocol and the DL system to type every isolate in most centers, some centers reported isolates that were non-typeable according to the given criteria. Some centers retested isolates because the quality of the initial data was unacceptable. All centers performed at least a small number of retests, whereas a few required a considerable number of retests, though the number of retests varied greatly, e.g., one center retested three isolates once and another center retested 90% (70 of 78) of the isolates and repeated one isolate seven times. This also indicates that adequate training is required. No pattern could be discerned with particular isolates being retested more often than others among the centers.

We conclude that DiversiLab may have the potential to track clonal outbreaks in an international setting, at least for *E. coli* and *Klebsiella* spp., although with lower discriminatory power than PFGE. However, this will require more reproducible DNA amplification and isolation methods, strict adherence to protocols and an international database to allow comparison of isolates. In addition, reference isolates should be used with every chip to inform on the quality of each amplification.

Acknowledgements

We would like to thank the members of the DiversiLab Study Group, namely, Ana Fernández-Olmos (Servicio de Microbiología, Hospital Universitario Ramón y Cajal and Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain), Ashley McEwan (Medical Microbiology, Central Manchester Foundation Trust, Manchester, UK), Roland Schulze-Röbbecke (Institut für Medizinische Mikrobiologie und Krankenhaushygiene, Düsseldorf, Germany), Artur J. sabat (Department of Medical Microbiology, University of Groningen, Groningen, the Netherlands), Jelle Scharringa (Department of Medical Microbiology, University Medical Center Utrecht Utrecht, The Netherlands), James Cohen Stuart (Department of Medical Microbiology, University Medical Center Utrecht Utrecht, The Netherlands).

We thank Viviane Monnot of bioMérieux for her support and efforts in this study. The study was partially funded by bioMérieux. Elaine Cloutman-Green was funded by the National Institute of Health Research in the UK.

References

1. **Pournaras S, Protonotariou E, Voulgari E, Kristo I, Dimitroulia E, Vitti D, Tsalidou M, Maniatis AN, Tsakris A, Sofianou D.** 2009. Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece. J. Antimicrob. Chemother. **64:**348-352.

2. **Navon-Venezia S, Leavitt A, Schwaber MJ, Rasheed JK, Srinivasan A, Patel JB, Carmeli Y.** 2009. First report on a hyperepidemic clone of KPC-3-producing *Klebsiella pneumoniae* in Israel genetically related to a strain causing outbreaks in the United States. Antimicrob. Agents Chemother. **53:**818-820.

3. **Giakkoupi P, Papagiannitsis CC, Miriagou V, Pappa O, Polemis M, Tryfinopoulou K, Tzouvelekis LS, Vatopoulos AC.** 2011. An update of the evolving epidemic of blaKPC-2-carrying *Klebsiella pneumoniae* in Greece (2009-10). J. Antimicrob. Chemother. **66:**1510-1513.

4. **Lavigne JP, Vergunst AC, Goret L, Sotto A, Combescure C, Blanco J, O'Callaghan D, Nicolas-Chanoine MH.** 2012. Virulence potential and genomic mapping of the worldwide clone Escherichia coli ST131. PLoS One **7:**e34294.

5. **Tangden T, Cars O, Melhus A, Lowdin E.** 2010. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. Antimicrob. Agents Chemother. **54:**3564-3568.

6. **Rogers BA, Aminzadeh Z, Hayashi Y, Paterson DL.** 2011. Country-to-country transfer of patients and the risk of multi-resistant bacterial infection. Clin. Infect. Dis. **53:**49-56.

7. **Sabat AJ, Budimir A, Nashev D, Sa-Leao R, van Dijl J, Laurent F, Grundmann H, Friedrich AW.** 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. Euro Surveill. **18:**20380.

8. **Fluit AC, Terlingen AM, Andriessen L, Ikawaty R, van Mansfeld R, Top J, Cohen Stuart JW, Leverstein-van Hall MA, Boel CH.** 2010. Evaluation of the DiversiLab system for detection of hospital outbreaks of infections by different bacterial species. J. Clin. Microbiol. **48:**3979-3989.

9. **Tenover FC, Arbeit R, Archer G, Biddle J, Byrne S, Goering R, Hancock G, Hebert GA, Hill B, Hollis R, R, Jarvis WR, Kresiwirth B, Eisner W, Maslow J, McDougal LK, Miller JM, Mulligan M, Pfaller MA.** 1994. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. J. Clin. Microbiol. **32:**407-415.

10. **van Belkum A, Tassios PT, Dijkshoorn L, Haeggman S, Cookson B, Fry NK, Fussing V, Green J, Feil E, Gerner-Smidt P, Brisse S, Struelens M.** 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clin. Microbiol. Infect. **13 Suppl 3:**1-46.

11. **Versalovic J, Koeuth T, Lupski JR.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823-6831.

12. **Versalovic J, Lupski JR.** 2002. Molecular detection and genotyping of pathogens: more accurate and rapid answers. Trends Microbiol. **10:**S15-21.

13. **Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, Bonten MJ, Hall MA.** 2012. Population distribution of Beta-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands. PLoS One **7:**e52102.

14. **Hunter PR, Gaston MA.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:** 2465-2466.

15. **Grundmann H, Hori S, Tanner G.** 2011. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. J. Clin. Microbiol. **39:** 4190-4192.

16. **Bourdon N, Lemire A, Fines-Guyon M, Auzou M, Perichon B, Courvalin P, Cattoir V, Leclercq R.** 2011. Comparison of four methods, including semi-automated rep-PCR, for the typing of vancomycin-resistant *Enterococcus faecium*. J. Microbiol. Methods **84:**74-80.

17. **Brolund A, Haeggman S, Edquist PJ, Gezelius L, Olsson-Liljequist B, Wisell KT, Giske CG.** 2010. The DiversiLab system versus pulsed-field gel electrophoresis: characterisation of extended spectrum beta-lactamase producing *Escherichia coli* and Klebsiella pneumoniae. J. Microbiol. Methods **83:**224-230.

18. **Te Witt R, Kanhai V, van Leeuwen WB.** 2009. Comparison of the DiversiLab system, Pulsed-Field Gel Electrophoresis and Multi-Locus Sequence Typing for the characterization of epidemic reference MRSA strains. J. Microbiol. Methods 77:130-133.Table S1: Adjusted Rand coefficients of *E. coli* local analyses

19. **Pitout JD, Campbell L, Church DL, Wang PW, Guttman DS, Gregson DB.** 2009. Using a commercial DiversiLab semiautomated repetitive sequence-based PCR typing technique for identification of *Escherichia coli* clone ST131 producing CTX-M-15. J. Clin. Microbiol. **47:**1212-1215.

20. **Hasan CM, Turlej-Rogacka A, Vatopoulos AC, Giakkoupi P, Maatallah M, Giske CG.** 2013. Dissemination of bla in Greece at the peak of the epidemic of 2005-2006: clonal expansion of *Klebsiella pneumoniae* clonal complex 147. Clin. Microbiol. Infect. doi: 10.1111/1469-0691.12187.

21. **Lau SH, Cheesborough J, Kaufmann ME, Woodford N, Dodgson AR, Dodgson KJ, Bolton EJ, Fox AJ, Upton M.** 2010. Rapid identification of uropathogenic Escherichia coli of the O25:H4-ST131 clonal lineage using the DiversiLab repetitive sequence-based PCR system. Clin Microbiol Infect. **16**:232-237.

22. **Shutt CK, Pounder JI, Page SR, Schaecher BJ, Woods GL.** 2005. Clinical evaluation of the DiversiLab microbial typing system using repetitive-sequence-based PCR for characterization of *Staphylococcus aureus* strains. J. Clin. Microbiol. **43:**1187-1192.

23. **Tenover FC, Gay EA, Frye S, Eells SJ, Healy M, McGowan JE, Jr.** 2009. Comparison of typing results obtained for methicillin-resistant *Staphylococcus aureus* isolates with the DiversiLab system and pulsed-field gel electrophoresis. J. Clin. Microbiol. **47:**2452-2457.

Legends to the figures

FIG 1 Panel A. Comparison of the local clustering of *E. coli* and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same color. Panel B. Comparison of the central clustering of *E. coli* and the clustering according to PFGE. Isolates belonging to one cluster according to central analysis or PFGE are indicated by the same color.

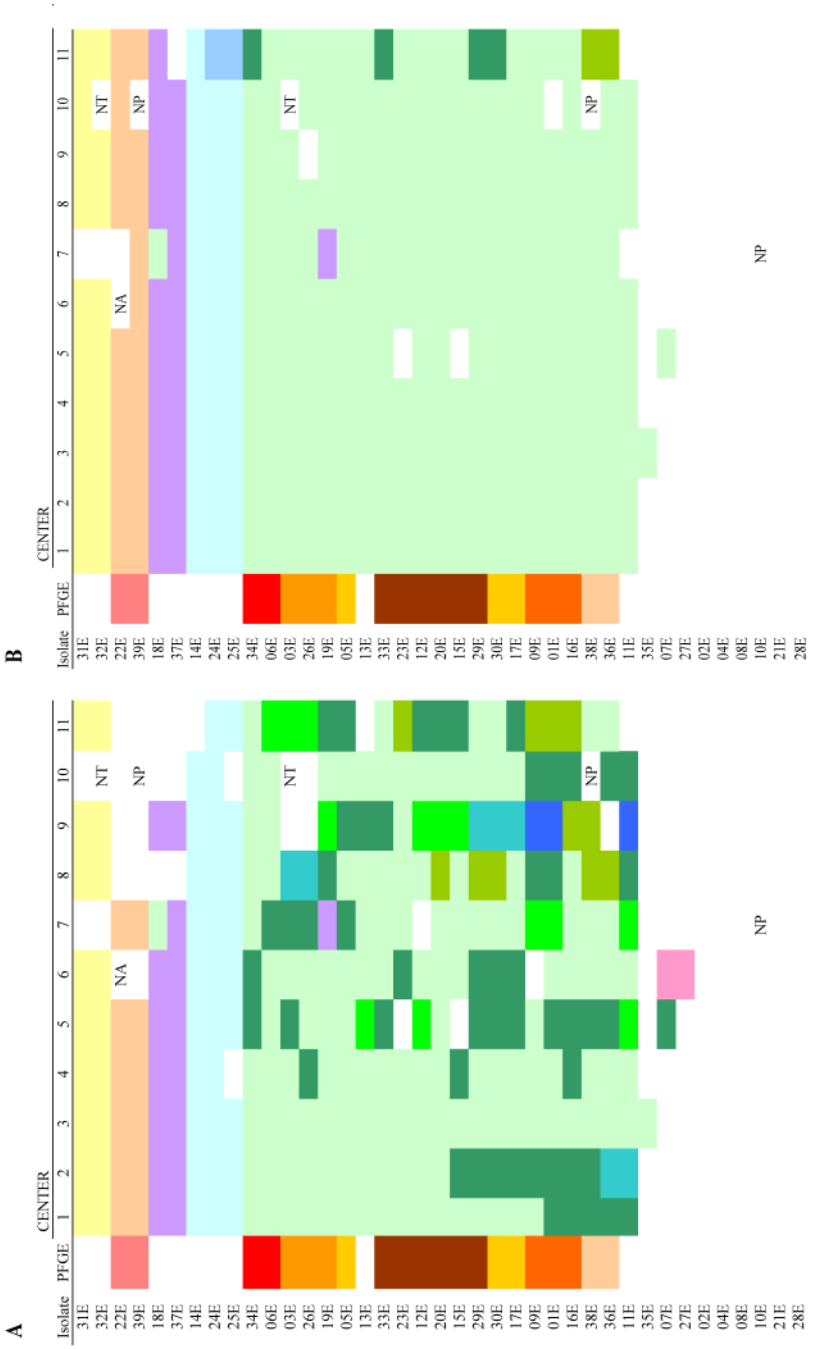
Isolates left blank were considered unique isolates according to central analysis. CTR: Center. NT: Non-typeable. NP: Not processed by the center. NA = No amplification.

FIG 2 Panel A. Comparison of the local clustering of *Klebsiella* spp. and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same color. Panel B. Comparison of the central clustering of *Klebsiella* spp. and the clustering according to PFGE. Isolates belonging to one cluster according to central analysis or PFGE are indicated by the same color.

Isolates left blank were considered unique isolates according to central analysis. CTR: Center. NT: Non-typeable. NP: Not processed by the center. NA = No amplification.

SUPPLEMENTARY FIG 1 Clonal complex of isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K according to the central analysis. Three clusters were detected: cluster A, B, and C. The colors of the clusters correspond to the clusters and unique isolates in Figure 4. Isolates a, b, and c are 11K isolates who do not group with the other 11K isolates (key 79-86). The pattern for isolate d, is most likely due to a poor DNA sample.

Figure 1



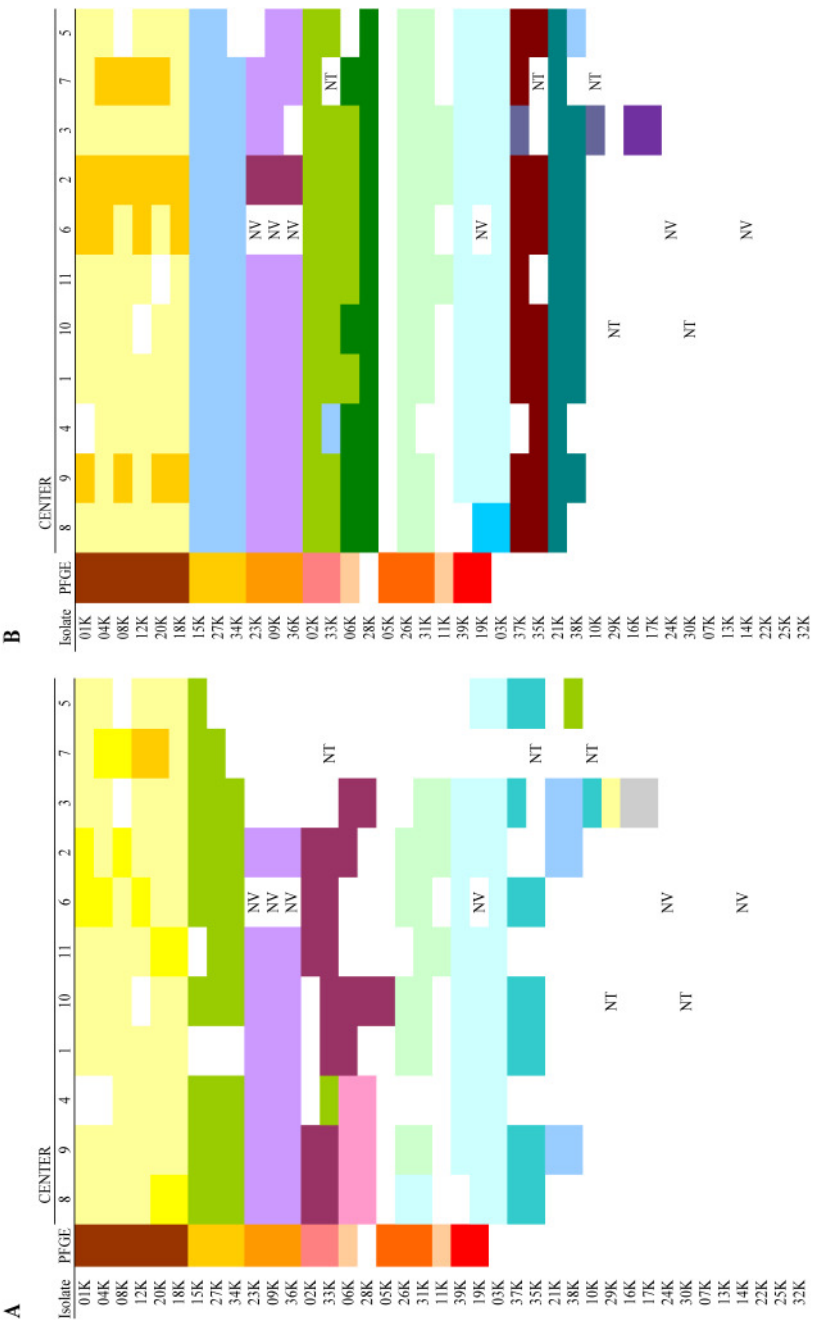

A
B
Isolate
PFGE
CENTER
8
9
4
1
10
11
6
2
3
7
5
01K
04K
08K
12K
20K
18K
15K
27K
34K
23K
09K
36K
02K
33K
06K
28K
05K
26K
31K
11K
39K
19K
03K
37K
35K
21K
38K
10K
29K
16K
17K
24K
30K
07K
13K
14K
22K
25K
32K
NV
NT

Figure 2

Supplementary Figure 1

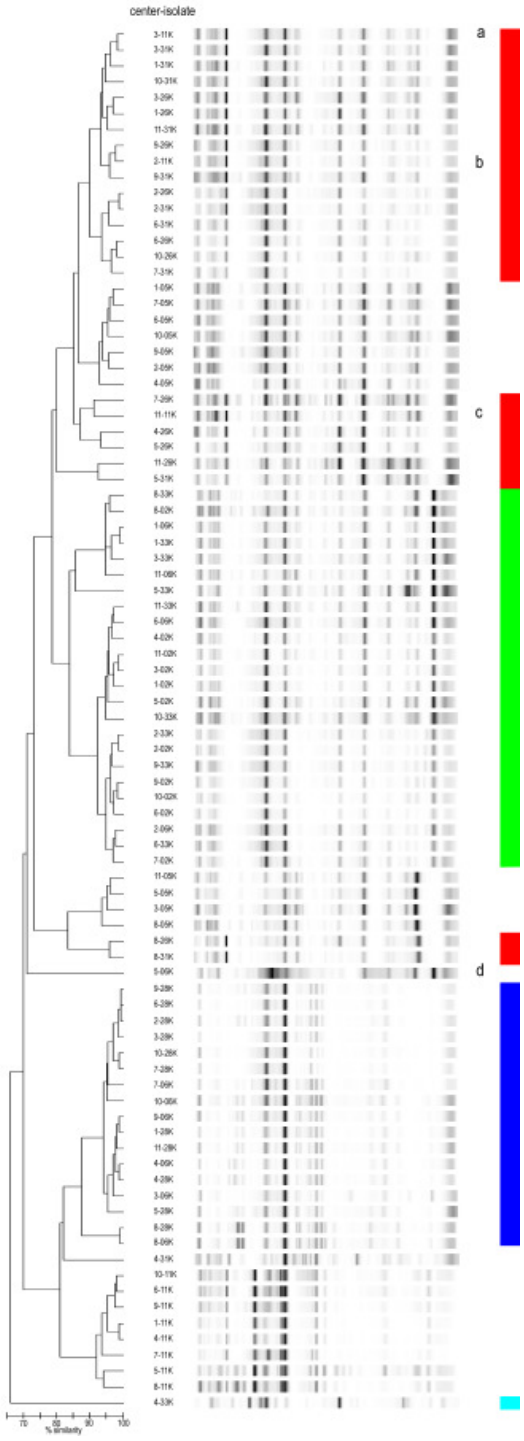


Table S1: Adjusted Rand coefficients of *E. coli* local analyses

	CTR. 1	CTR. 2	CTR. 3	CTR. 4	CTR. 5	CTR. 6	CTR. 7	CTR. 8	CTR. 9	CTR. 10	CTR. 11
CTR. 1											
CTR. 2	0.606										
CTR. 3	0.647	0.454									
CTR. 4	0.612	0.441	0.739								
CTR. 5	0.306	0.339	0.329	0.351							
CTR. 6	0.446	0.409	0.576	0.485	0.297						
CTR. 7	0.335	0.306	0.359	0.315	0.328	0.273					
CTR. 8	0.392	0.357	0.344	0.332	0.183	0.3	0.432				
CTR. 9	0.233	0.297	0.133	0.148	0.152	0.232	0.18	0.3			
CTR. 10	0.73	0.484	0.489	0.56	0.172	0.361	0.423	0.516	0.301		
CTR. 11	0.265	0.232	0.222	0.231	0.317	0.202	0.305	0.288	0.278	0.31	

Table S2: Adjusted Wallace coefficients of *E. coli* local analyses

	CTR. 1	CTR. 2	CTR. 3	CTR. 4	CTR. 5	CTR. 6	CTR. 7	CTR. 8	CTR. 9	CTR. 10	CTR. 11
CTR. 1		0.489 (0.296-0.683)	1.000 (1.000-1.000)	0.681 (0.394-0.968)	0.235 (0.086-0.383)	0.418 (0.207-0.630)	0.272 (0.050-0.495)	0.282 (0.070-0.494)	0.134 (0.046-0.222)	0.595 (0.282-0.908)	0.167 (0.044-0.291)
CTR. 2	0.796 (0.702-0.890)		1.000 (1.000-1.000)	0.66 (0.398-0.922)	0.316 (0.122-0.509)	0.498 (0.251-0.745)	0.310 (0.124-0.495)	0.305 (0.087-0.523)	0.185 (0.074-0.296)	0.507 (0.229-0.785)	0.165 (0.037-0.293)
CTR. 3	0.478 (0.228-0.728)	0.294 (0.146-0.442)		0.586 (0.294-0.877)	0.207 (0.052-0.361)	0.406 (0.157-0.655)	0.233 (0.023-0.443)	0.208 (0.062-0.354)	0.071 (0.014-0.128)	0.324 (0.092-0.555)	0.125 (0.035-0.214)
CTR. 4	0.556 (0.292-0.820)	0.331 (0.156-0.507)	1.000 (1.000-1.000)		0.251 (0.081-0.422)	0.415 (0.176-0.654)	0.238 (0.009-0.466)	0.225 (0.087-0.362)	0.083 (0.014-0.152)	0.439 (0.178-0.700)	0.14 (0.024-0.256)
CTR. 5	0.442 (0.262-0.621)	0.366 (0.148-0.584)	0.814 (0.520-1.000)	0.58 (0.256-0.905)		0.396 (0.193-0.599)	0.359 (0.067-0.652)	0.166 (0.011-0.321)	0.097 (0.000-0.207)	0.202 (0.000-0.419)	0.236 (0.022-0.450)
CTR. 6	0.478 (0.280-0.676)	0.347 (0.134-0.560)	0.988 (0.975-1.000)	0.583 (0.296-0.871)	0.238 (0.100-0.376)		0.234 (0.054-0.415)	0.225 (0.060-0.390)	0.136 (0.036-0.235)	0.325 (0.106-0.545)	0.131 (0.010-0.252)
CTR. 7	0.434 (0.146-0.722)	0.302 (0.128-0.476)	0.787 (0.464-1.000)	0.465 (0.123-0.807)	0.302 (0.012-0.592)	0.329 (0.141-0.516)		0.364 (0.195-0.533)	0.111 (0.021-0.201)	0.455 (0.143-0.766)	0.215 (0.032-0.398)
CTR. 8	0.647 (0.378-0.917)	0.431 (0.208-0.654)	1.000 (1.000-1.000)	0.633 (0.353-0.913)	0.203 (0.028-0.378)	0.451 (0.217-0.685)	0.529 (0.265-0.794)		0.201 (0.068-0.335)	0.646 (0.371-0.921)	0.23 (0.062-0.398)
CTR. 9	0.898 (0.802-0.994)	0.763 (0.587-0.938)	1.000 (1.000-1.000)	0.681 (0.478-0.885)	0.347 (0.166-0.528)	0.799 (0.664-0.933)	0.473 (0.279-0.666)	0.588 (0.425-0.751)		0.799 (0.681-0.918)	0.383 (0.195-0.570)
CTR. 10	0.944 (0.878-1.000)	0.462 (0.238-0.686)	1.000 (1.000-1.000)	0.772 (0.483-1.000)	0.149 (0.000-0.313)	0.404 (0.219-0.589)	0.396 (0.087-0.706)	0.43 (0.161-0.699)	0.185 (0.077-0.294)		0.228 (0.042-0.414)
CTR. 11	0.642 (0.471-0.812)	0.389 (0.177-0.601)	1.000 (1.000-1.000)	0.658 (0.424-0.891)	0.481 (0.314-0.648)	0.44 (0.205-0.676)	0.523 (0.360-0.686)	0.384 (0.148-0.620)	0.219 (0.016-0.421)	0.615 (0.419-0.811)	

Table S3: Simpson's index of diversity of *E. coli* and *Klebsiella* sp. central analysis

Center	Species	SID	CI
1	*E. coli*	0.709	(0.550-0.867)
2		0.709	(0.550-0.867)
3		0.680	(0.515-0.846)
4		0.709	(0.550-0.867)
5		0.735	(0.584-0.887)
6		0.710	(0.550-0.869)
7		0.738	(0.585-0.891)
8		0.709	(0.550-0.867)
9		0.735	(0.584-0.887)
10		0.787	(0.651-0.922)
11		0.864	(0.767-0.961)
PFGE		0.964	(0.935-0.992)
1	*Klebsiella* sp.	0.960	(0.932-0.987)
2		0.957	(0.929-0.984)
3		0.958	(0.931-0.985)
4		0.969	(0.944-0.994)
5		0.973	(0.951-0.995)
6		0.957	(0.929-0.985)
7		0.976	(0.958-0.993)
8		0.965	(0.937-0.993)
9		0.972	(0.955-0.988)
10		0.968	(0.946-0.989)
11		0.965	(0.942-0.987)
PFGE		0.964	(0.935-0.992)

Table S4: Adjusted Rand coefficients of *E. coli* central analysis

	CTR. 1	CTR. 2	CTR. 3	CTR. 4	CTR. 5	CTR. 6	CTR. 7	CTR. 8	CTR. 9	CTR. 10	CTR. 11
CTR. 1											
CTR. 2	1.000										
CTR. 3	0.933	0.933									
CTR. 4	1.000	1.000	0.933								
CTR. 5	0.805	0.805	0.743	0.805							
CTR. 6	1.000	1.000	0.932	1.000	0.8						
CTR. 7	0.787	0.787	0.724	0.787	0.603	0.785					
CTR. 8	1.000	1.000	0.933	1.000	0.805	1.000	0.787				
CTR. 9	0.933	0.933	0.867	0.933	0.743	0.931	0.723	0.933			
CTR. 10	0.854	0.854	0.786	0.854	0.66	0.851	0.649	0.854	0.788		
CTR. 11	0.554	0.554	0.503	0.554	0.405	0.547	0.486	0.554	0.503	0.474	

Table S5: Adjusted Wallace coefficients of *E. coli* central analysis

	CTR. 1	CTR. 2	CTR. 3	CTR. 4	CTR. 5	CTR. 6	CTR. 7	CTR. 8	CTR. 9	CTR. 10	CTR. 11
CTR. 1		1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.755 (0.463-1.000)	1.000 (1.000-1.000)	0.731 (0.435-1.000)	1.000 (1.000-1.000)	0.874 (0.651-1.000)	0.745 (0.453-1.000)	0.384 (0.132-0.635)
CTR. 2	1.000 (1.000-1.000)		1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.755 (0.463-1.000)	1.000 (1.000-1.000)	0.731 (0.435-1.000)	1.000 (1.000-1.000)	0.874 (0.651-1.000)	0.745 (0.453-1.000)	0.384 (0.132-0.635)
CTR. 3	0.875 (0.652-1.000)	0.875 (0.652-1.000)		0.875 (0.652-1.000)	0.656 (0.337-0.974)	0.872 (0.644-1.000)	0.633 (0.309-0.957)	0.875 (0.652-1.000)	0.765 (0.483-1.000)	0.647 (0.329-0.965)	0.336 (0.091-0.581)
CTR. 4	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)		0.755 (0.463-1.000)	1.000 (1.000-1.000)	0.731 (0.435-1.000)	1.000 (1.000-1.000)	0.874 (0.651-1.000)	0.745 (0.453-1.000)	0.384 (0.132-0.635)
CTR. 5	0.863 (0.622-1.000)	0.863 (0.622-1.000)	0.857 (0.607-1.000)	0.863 (0.622-1.000)		0.86 (0.613-1.000)	0.598 (0.254-0.943)	0.863 (0.622-1.000)	0.743 (0.441-1.000)	0.613 (0.275-0.951)	0.291 (0.052-0.530)
CTR. 6	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.749 (0.451-1.000)		0.731 (0.427-1.000)	1.000 (1.000-1.000)	0.871 (0.643-1.000)	0.74 (0.443-1.000)	0.376 (0.122-0.630)
CTR. 7	0.851 (0.602-1.000)	0.851 (0.602-1.000)	0.844 (0.584-1.000)	0.851 (0.602-1.000)	0.607 (0.258-0.956)	0.848 (0.593-1.000)		0.851 (0.602-1.000)	0.728 (0.417-1.000)	0.604 (0.258-0.950)	0.35 (0.096-0.604)
CTR. 8	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.755 (0.463-1.000)	1.000 (1.000-1.000)	0.731 (0.435-1.000)		0.874 (0.651-1.000)	0.745 (0.453-1.000)	0.384 (0.132-0.635)
CTR. 9	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.743 (0.441-1.000)	1.000 (1.000-1.000)	0.718 (0.411-1.000)	1.000 (1.000-1.000)		0.732 (0.429-1.000)	0.362 (0.112-0.612)
CTR. 10	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.714 (0.386-1.000)	1.000 (1.000-1.000)	0.7 (0.365-1.000)	1.000 (1.000-1.000)	0.853 (0.598-1.000)		0.372 (0.103-0.640)
CTR. 11	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	0.663 (0.300-1.000)	1.000 (1.000-1.000)	0.795 (0.499-1.000)	1.000 (1.000-1.000)	0.825 (0.534-1.000)	0.656 (0.299-1.000)	

Table S6: Adjusted Rand coefficients of *Klebsiella* spp. local analyses

	CTR. 8	CTR. 9	CTR. 4	CTR. 1	CTR. 10	CTR. 11	CTR. 6	CTR. 2	CTR. 3	CTR. 7	CTR. 5
CTR. 8											
CTR. 9	0.696										
CTR. 4	0.473	0.656									
CTR. 1	0.551	0.863	0.545								
CTR. 10	0.557	0.777	0.552	0.736							
CTR. 11	0.676	0.657	0.503	0.641	0.498						
CTR. 6	0.681	0.693	0.506	0.549	0.558	0.574					
CTR. 2	0.519	0.721	0.559	0.626	0.586	0.605	0.469				
CTR. 3	0.354	0.641	0.427	0.503	0.472	0.413	0.382	0.557			
CTR. 7	0.146	0.235	0.166	0.207	0.186	0.092	0.124	0.140	0.192		
CTR. 5	0.328	0.561	0.234	0.640	0.385	0.332	0.333	0.375	0.554	0.229	

Table S7: Adjusted Wallace coefficients of *Klebsiella* sp. local analyses

	CTR. 8	CTR. 9	CTR. 4	CTR. 1	CTR. 10	CTR. 11	CTR. 6	CTR. 2	CTR. 3	CTR. 7	CTR. 5
CTR. 8		0.811 (0.695-0.927)	0.440 (0.252-0.628)	0.577 (0.424-0.731)	0.623 (0.427-0.818)	0.582 (0.416-0.747)	0.616 (0.370-0.861)	0.531 (0.355-0.706)	0.388 (0.167-0.608)	0.086 (0.000-0.300)	0.260 (0.030-0.489)
CTR. 9	0.609 (0.411-0.808)		0.540 (0.301-0.779)	0.786 (0.701-0.871)	0.749 (0.513-0.986)	0.507 (0.300-0.713)	0.530 (0.354-0.706)	0.644 (0.454-0.835)	0.607 (0.361-0.853)	0.133 (0.000-0.314)	0.403 (0.144-0.663)
CTR. 4	0.512 (0.271-0.752)	0.836 (0.661-1.000)		0.619 (0.492-0.747)	0.672 (0.426-0.918)	0.462 (0.260-0.664)	0.487 (0.150-0.825)	0.620 (0.369-0.870)	0.509 (0.248-0.770)	0.100 (0.000-0.340)	0.196 (0.000-0.447)
CTR. 1	0.528 (0.288-0.768)	0.957 (0.913-1.000)	0.487 (0.201-0.773)		0.784 (0.505-1.000)	0.532 (0.300-0.764)	0.460 (0.245-0.676)	0.613 (0.387-0.839)	0.525 (0.228-0.822)	0.120 (0.000-0.315)	0.491 (0.192-0.791)
CTR. 10	0.504 (0.321-0.687)	0.807 (0.704-0.910)	0.468 (0.266-0.670)	0.694 (0.574-0.814)		0.394 (0.215-0.573)	0.441 (0.250-0.632)	0.541 (0.369-0.714)	0.463 (0.238-0.687)	0.106 (0.000-0.297)	0.284 (0.055-0.513)
CTR. 11	0.807 (0.669-0.944)	0.935 (0.870-1.000)	0.551 (0.357-0.745)	0.806 (0.694-0.918)	0.676 (0.450-0.902)		0.627 (0.316-0.938)	0.742 (0.584-0.900)	0.547 (0.306-0.787)	0.057 (0.000-0.312)	0.300 (0.020-0.580)
CTR. 6	0.762 (0.593-0.931)	1.000 (1.000-1.000)	0.526 (0.338-0.714)	0.681 (0.528-0.834)	0.759 (0.588-0.931)	0.529 (0.292-0.766)		0.602 (0.375-0.829)	0.597 (0.391-0.803)	0.070 (0.000-0.348)	0.370 (0.131-0.609)
CTR. 2	0.507 (0.319-0.695)	0.819 (0.698-0.940)	0.509 (0.323-0.696)	0.641 (0.493-0.788)	0.639 (0.446-0.832)	0.511 (0.324-0.698)	0.430 (0.209-0.652)		0.594 (0.453-0.736)	0.082 (0.000-0.293)	0.292 (0.114-0.470)
CTR. 3	0.326 (0.112-0.540)	0.680 (0.412-0.947)	0.368 (0.150-0.587)	0.483 (0.204-0.762)	0.481 (0.211-0.751)	0.332 (0.119-0.544)	0.317 (0.096-0.537)	0.524 (0.258-0.789)		0.111 (0.000-0.309)	0.413 (0.128-0.697)
CTR. 7	0.485 (0.120-0.849)	1.000 (1.000-1.000)	0.487 (0.124-0.850)	0.742 (0.483-1.000)	0.741 (0.481-1.000)	0.233 (0.000-0.676)	0.231 (0.000-0.675)	0.484 (0.119-0.849)	0.741 (0.482-1.000)		0.491 (0.131-0.851)
CTR. 5	0.445 (0.156-0.734)	0.920 (0.840-1.000)	0.289 (0.000-0.652)	0.921 (0.841-1.000)	0.601 (0.214-0.988)	0.371 (0.074-0.668)	0.402 (0.091-0.713)	0.524 (0.131-0.917)	0.841 (0.728-0.953)	0.149 (0.000-0.436)	

Table S8: Adjusted Rand coefficients of *Klebsiella* sp. central analysis

	CTR. 8	CTR. 9	CTR. 4	CTR. 1	CTR. 10	CTR. 11	CTR. 6	CTR. 2	CTR. 3	CTR. 7	CTR. 5
CTR. 8											
CTR. 9	0.758										
CTR. 4	0.726	0.625									
CTR. 1	0.889	0.777	0.707								
CTR. 10	0.834	0.885	0.67	0.846							
CTR. 11	0.721	0.671	0.599	0.852	0.71						
CTR. 6	0.654	0.656	0.439	0.802	0.655	0.782					
CTR. 2	0.857	0.746	0.679	0.966	0.815	0.892	0.763				
CTR. 3	0.763	0.642	0.616	0.88	0.717	0.836	0.699	0.917			
CTR. 7	0.719	0.71	0.774	0.699	0.706	0.626	0.52	0.67	0.6		
CTR. 5	0.686	0.574	0.497	0.711	0.625	0.552	0.635	0.682	0.615	0.514	

Table S9: Adjusted Wallace coefficients of *Klebsiella* sp. central analysis

	CTR. 8	CTR. 9	CTR. 4	CTR. 1	CTR. 10	CTR. 11	CTR. 6	CTR. 2	CTR. 3	CTR. 7	CTR. 5
CTR. 8		0.683 (0.479-0.888)	0.682 (0.417-0.948)	0.96 (0.920-1.000)	0.801 (0.545-1.000)	0.721 (0.458-0.984)	0.577 (0.330-0.824)	0.96 (0.920-1.000)	0.839 (0.745-0.934)	0.606 (0.395-0.817)	0.605 (0.326-0.884)
CTR. 9	0.852 (0.747-0.957)		0.656 (0.471-0.841)	0.95 (0.901-1.000)	0.951 (0.902-1.000)	0.753 (0.581-0.925)	0.677 (0.506-0.848)	0.95 (0.900-1.000)	0.801 (0.684-0.918)	0.658 (0.512-0.805)	0.56 (0.345-0.774)
CTR. 4	0.775 (0.608-0.941)	0.597 (0.385-0.809)		0.819 (0.668-0.970)	0.685 (0.429-0.942)	0.64 (0.378-0.901)	0.426 (0.148-0.704)	0.818 (0.667-0.970)	0.728 (0.554-0.902)	0.688 (0.433-0.943)	0.464 (0.202-0.725)
CTR. 1	0.827 (0.729-0.926)	0.657 (0.468-0.846)	0.622 (0.385-0.858)		0.759 (0.527-0.991)	0.793 (0.567-1.000)	0.669 (0.456-0.883)	1.000 (1.000-1.000)	0.896 (0.821-0.970)	0.556 (0.365-0.747)	0.589 (0.339-0.839)
CTR. 10	0.87 (0.778-0.963)	0.828 (0.625-1.000)	0.656 (0.435-0.877)	0.957 (0.913-1.000)		0.741 (0.527-0.955)	0.619 (0.421-0.818)	0.956 (0.913-1.000)	0.826 (0.724-0.929)	0.616 (0.449-0.783)	0.572 (0.331-0.813)
CTR. 11	0.721 (0.585-0.857)	0.604 (0.430-0.779)	0.563 (0.350-0.777)	0.92 (0.844-0.995)	0.682 (0.466-0.898)		0.705 (0.453-0.956)	1.000 (1.000-1.000)	0.92 (0.844-0.995)	0.527 (0.353-0.701)	0.486 (0.243-0.729)
CTR. 6	0.754 (0.610-0.899)	0.636 (0.449-0.824)	0.452 (0.210-0.694)	1.000 (1.000-1.000)	0.695 (0.469-0.921)	0.877 (0.791-0.964)		1.000 (1.000-1.000)	0.938 (0.876-1.000)	0.457 (0.260-0.654)	0.635 (0.452-0.819)
CTR. 2	0.773 (0.663-0.884)	0.614 (0.427-0.801)	0.581 (0.355-0.806)	0.935 (0.873-0.996)	0.709 (0.484-0.935)	0.806 (0.594-1.000)	0.617 (0.407-0.828)		0.902 (0.833-0.972)	0.52 (0.330-0.709)	0.55 (0.308-0.793)
CTR. 3	0.699 (0.576-0.823)	0.535 (0.337-0.734)	0.534 (0.299-0.769)	0.866 (0.786-0.945)	0.633 (0.396-0.871)	0.766 (0.545-0.987)	0.557 (0.348-0.767)	0.933 (0.885-0.980)		0.471 (0.273-0.669)	0.503 (0.254-0.752)
CTR. 7	0.885 (0.776-0.993)	0.771 (0.631-0.911)	0.885 (0.804-0.966)	0.942 (0.884-1.000)	0.828 (0.645-1.000)	0.77 (0.578-0.962)	0.603 (0.392-0.813)	0.942 (0.884-1.000)	0.826 (0.702-0.950)		0.543 (0.299-0.787)
CTR. 5	0.793 (0.655-0.931)	0.588 (0.384-0.793)	0.536 (0.257-0.814)	0.896 (0.798-0.994)	0.69 (0.426-0.954)	0.637 (0.368-0.907)	0.635 (0.325-0.946)	0.895 (0.797-0.994)	0.791 (0.668-0.914)	0.488 (0.271-0.704)	

Chapter VI

Population distribution of β-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands

Guido M. Voets[1 *], Tamara N. Platteel[1, 2], Ad C. Fluit[1], Jelle Scharringa[1], Claudia M. Schapendonk[1], James Cohen Stuart [1], Marc J.M. Bonten[1], Maurine A. Leverstein-van Hall [1, 3] on behalf of the National ESBL Surveillance Working Group

[1] Department of Medical Microbiology, University Medical Centre Utrecht, The Netherlands

[2] SALTRO, Department of Medical Microbiology, Utrecht, The Netherlands

[3] National Institute for Public Health and the Environment, Bilthoven, The Netherlands

PLoS One; 2012; 7; e52102

Abstract

There is a global increase in infections caused by *Enterobacteriaceae* with plasmid-borne β-lactamases that confer resistance to third-generation cephalosporins. The epidemiology of these bacteria is not well understood, and was, therefore, investigated in a selection of 636 clinical *Enterobacteriaceae* with a minimal inhibitory concentration >1 mg/L for ceftazidime/ceftriaxone from a national survey (75% *E. coli*, 11% *E. cloacae*, 11% *K. pneumoniae*, 2% *K. oxytoca*, 2% *P. mirabilis*). Isolates were investigated for extended-spectrum β-lactamases (ESBLs) and *ampC* genes using microarray, PCR, gene sequencing and molecular straintyping (Diversilab and multi-locus sequence typing (MLST)). ESBL genes were demonstrated in 512 isolates (81%); of which 446 (87%) belonged to the CTX-M family. Among 314 randomly selected and sequenced isolates, $bla_{\text{CTX-M-15}}$ was most prevalent (n=124, 39%), followed by $bla_{\text{CTX-M-1}}$ (n=47, 15%), $bla_{\text{CTX-M-14}}$ (n=15, 5%), $bla_{\text{SHV-12}}$ (n=24, 8%) and $bla_{\text{TEM-52}}$ (n=13, 4%). Among 181 isolates with MIC ≥16 mg/L for cefoxitin plasmid encoded AmpCs were detected in 32 and 27 were of the CMY-2 group. Among 102 *E. coli* isolates with MIC ≥16 mg/L for cefoxitin *ampC* promoter mutations were identified in 29 (28%). Based on Diversilab genotyping of 608 isolates (similarity cut-off >98%) discriminatory indices of bacteria with ESBL and/or *ampC* genes were 0.994, 0.985 and 0.994 for *E. coli, K. pneumoniae* and *E.* cloacae, respectively. Based on similarity cut-off >95% two large clusters of *E. coli* were apparent (of 43 and 30 isolates) and 21 of 21 that were typed by belonged to ST131 of which 13 contained $bla_{\text{CTX-M-15}}$. Our findings demonstrate that $bla_{\text{CTX-M-15}}$ is the most prevalent ESBL and we report a larger than previously reported prevalence of *ampC* genes among *Enterobacteriaceae* responsible for resistance to third-generation cephalosporins.

Introduction

The increasing prevalence of plasmid-borne β-lactamases in *Enterobacteriaceae* that confer resistance to third-generation cephalosporins is a world-wide problem. The most prevalent amongst these acquired β-lactamases are the Ambler class A ESBLs of the CTX-M, TEM and SHV families. [1] These ESBLs are capable of hydrolyzing penicillins, cephalosporins (except cephamycins), and monobactams and are inhibited by clavulanic acid. [2] An emerging class of β-lactamases are the plasmid-borne Ambler class C cephalosporinases (pAmpCs). [3] AmpC enzymes are capable of hydrolyzing penicillins, cephalosporins (although fourth-generation cephalosporins only weakly), and monobactams and are not inhibited by clavulanic acid. [3] The molecular epidemiology of these resistance mechanisms is largely unknown, as most large-scale molecular surveys were limited, either to certain species (e.g., *Escherichia coli* or *Klebsiella pneumoniae*), a specific environment (either hospital or general practice) or specimen type (e.g. urine or faeces). [4-9]

In the Netherlands the proportions of urine samples and blood cultures with *E. coli* (intermediate) resistant to third-generation cephalosporins increased from 2.6% and 2.6%, respectively in 2008 to 3.4% and 4.7%, respectively, in 2010 [10].

The aim of this study was to determine the population distribution of beta-lactamase conferring resistance to third-generation cephalosporins in an unbiased, cross-sectional, large and nation-wide sample of clinical isolates in the Netherlands.

Materials and Methods

Isolates

From February 1, 2009 until May 1, 2009, 31 Dutch microbiology laboratories were asked to submit all isolates of *E. coli*, *K. pneumoniae, Klebsiella oxytoca*, *Proteus mirabilis* and *Enterobacter cloacae* with a positive ESBL screen test (minimal inhibitory concentration (MIC)>1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek-2 expert system). The need for written consent of patients was waived by the ethical committee because of the retrospective nature of the study, the use of fully anonimized patient data only and because of the absence of any study related procedures. From each laboratory the first 25 consecutive isolates, if available, were included in this study, allowing for only 1 isolate per patient.

In a central laboratory screen tests were repeated using broth microdilution (BMD) (Merlin Diagnostic GmbH, Rüsselsheim, Germany) and only isolates with a confirmed positive test were included in this study. Susceptibility testing was performed for amikacin, cefotaxime +/- clavulanic acid, ceftazidime +/- clavulanic acid, chloramphenicol, ciprofloxacin, fosfomycin, gentamicin, nitrofurantoin, piperacillin/tazobactam, tobramycin and trimethoprim/sulfamethoxazole using Sensititre microbroth dilution plates (TREK Diagnostic Systems, East-Grinstead, England). MICs were interpreted according to EUCAST criteria.

For each isolate the following epidemiological data were collected: age (0-19, 20-59 and ≥60 years) and gender of the patient, specimen type (urine, faeces, wounds/skin, respiratory tract, blood and other (e.g., ascites, gynecological cultures)) and institution (hospital (university, non-university), general practitioner (GP), or long term care facility (LTCF)). The participating laboratories are geographically dispersed over the Netherlands and represent a mixture of secondary and tertiary care hospitals, LTCFs and GPs. The 31 laboratories serve 58 hospitals, covering approximately 45% of all hospital beds in the Netherlands.

Molecular characterization of beta-lactamase genes

The presence of ESBL genes was determined by Check-KPC ESBL microarray analysis (Cat. No. 10-0018, CheckPoints, Wageningen, The Netherlands), which detects single nucleotide polymorphisms (SNPs) and reports the presence of TEM or SHV SNPs associated with an ESBL phenotype and specifies CTX-M groups (CTX-M group 1, 2, 9, or combined 8/25) [1]. As the assay cannot provide a type number for TEM, SHV and CTX-M genes (http://www.lahey.org/Studies/), PCR and gene sequencing was performed for definite determination of ESBL genes as previously described [11-13]. From all screen-positive isolates a random sample of 314 isolates was taken for sequence-based confirmation of resistance genes. Isolates with a negative array result were first investigated using TEM, SHV, and CTX-M group-specific PCRs and, if negative, with multiplex PCRs for detecting other Ambler A class ESBL families (GES, PER and VEB) [14]. All PCR products were sequenced. In isolates with an AmpC phenotype (cefoxitin MIC ≥16 mg/L) the presence of pAmpC was determined by PCR and sequencing [14]. For *Enterobacter* spp., PCR results for plasmid ACT-1 and MIR-1,-2,-3 were not included because the primers used for these PCRs are based on primers that may also detect the chromosomal *ampC* of *Enterobacter* spp. [15]. If negative and no other β-lactamase was detected, the promoter of the chromosomal *ampC* of *E. coli* was sequenced to identify mutations associated with derepression [16-18]. For this PCR the following primers were designed: ECC-GS-F: GATCGTTCTGCCGCGTG and ECC-GS-R: GGGCAGCAAATGTGGAGCAA.

Isolate typing

E. coli, K. pneumoniae, K. oxytoca and *Enterobacter* spp. isolates were typed using DiversiLab (bioMérieux, Marcy l'Etoile, France) [19]. Representative *E. coli* isolates from dominant patterns identified by Diversilab were also analyzed by multi-locus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli). Discriminatory index calculations were preformed using Ridom EpiCompare as previously described. [19]

Statistical Analysis

Statistical analysis (Mann-Whitney) was performed using SPSS 15.0 (IBM, Nieuwegein, The Netherlands). Associations were considered statistically significant in case of a p-value ≤ 0.02.

Results

ESBL and AmpC distribution

In the three-month study period, 1,427 ESBL screen-positive isolates were collected

in the 31 participating laboratories. The first 25 isolates per laboratory, if available, comprised 723 isolates, of which 31 were excluded because of lack of viable cells or contamination with other strains, and 56 because positive screen tests could not be confirmed, leaving 636 isolates for further investigation: 479 *E. coli* (75%), 68 *E. cloacae* (11%), 67 *K. pneumoniae* (11%), 11 *K. oxytoca* (2%), and 11 *P. mirabilis* (2%). Sources and specimens of these isolates are listed in the supplementary information (Table S1).

ESBL genes were detected in 512 of 636 isolates (81%): in 416 of 479 (87%) *E. coli,* in 64 of 67 (96%) *K. pneumoniae,* in 35 of 68 (51%) *E. cloacae*, in 6 of 11 (54%) *K. oxytoca,* and in 3 of 11 (27%) *P. mirabilis* (Table 1). Two ESBL genes were detected in 26 isolates (Table S2). Genes from the CTX-M-groups were detected most frequently (in 446 isolates), followed by SHV-genes (in 56 isolates) and TEM (in 46 isolates). Sequencing of 314 isolates revealed 16 CTX-M-variants, 3 SHV-variants, 4 TEM-variants, one GES-1 and one PER-5 (Table 2). Sequencing could not discriminate between CTX-M-15 and CTX-M-28 in 12 isolates. Nor could it discriminate between CTX-M-1 and CTX-M-61 in 2 isolates. Overall, CTX-M-15 was most prevalent, followed by CTX-M-1 and CTX-M-14 (Table 2).

In total 181 (28%) of 636 isolates had an AmpC phenotype; a cefoxitin MIC ≥16 mg/L (102 *E. coli* (56%), 63 *E. cloacae* (35%), 10 *K. pneumoniae* (6%), 6 *P. mirabilis* (3%)). A pAmpC gene was detected in 32 isolates: 25 of 102 (24%) *E. coli*, 3 of 10 (30%) *K. pneumoniae* and in all 4 *P. mirabilis*. These 32 isolates represented 5% of the 636 isolates (Table 3). Five different types of pAmpC β-lactamases were identified: CMY-2-group, ACT-5, MIR-1/2/3, DHA-1, and ACT-like. Sequencing did not allow for discrimination in the CMY-2 group, and MIR-1 and -2 and -3.

The remaining forty-two *E. coli* isolates with AmpC-resistance phenotype and without either an ESBL or pAmpC were further investigated. Sequencing of the promoter region of the chromosomal *ampC* revealed different mutations that have been linked to resistance to third-generation cephalosporins in 29 isolates [16-18]. These mutations were found at the positions –1, –18,–42, –82, –88, and +58, and an insertion of an amino acid between the –10 and –35 region of the promoter. No mechanism was elucidated for the remaining 13 isolates.

Isolate Typing

All *Klebsiella* spp., *E. coli*, and *E. cloacae* isolates were analyzed by DiversiLab (n=625), and seven isolates appeared non-typable. For the following analysis only isolates with a detectable ESBL- or *ampC* gene were included (n=608). When using a similarity >98% for pattern definition, 253 (53%) of 414 ESBL-producing *E. coli* had unique patterns, and cluster sizes ranged from 2 isolates (44 patterns) to 25 isolates (1 pattern) (Table 4). The overall discriminatory index of ESBL-producing *E. coli* was 0.994 (95% confidence interval (CI) 0.991-0.996). When using a similarity of >95%

two large clusters emerged, one of 43 isolates (comprising 3 patterns (n=25, n=4, n=4)) and one of 30 isolates (comprising 4 clusters (n=17, n=6, n=5, and n=2)). MLST typing of 21 (15 (71%) with CTX-M-15, 4 (19%) with CTX-M-1, 1 (5%) CTX-M-52, 1 (5%) with TEM-52) randomly selected isolates (11 and 10 from the cluster of 43 and 30 isolates, respectively) revealed that all belonged to ST131.

Forty-one (64%) of 64 *K. pneumoniae* had unique patterns, and clusters of identical patterns ranged from 2 (n=4) to 7 (n=1), and the discriminatory index was 0.985 (95% CI 0.969-1.0). All isolates of this cluster of 7 contained a CTX-M-1 group ESBL and sequencing of 3 of these genes revealed CTX-M-15. Forty-eight (71%) of 68 *E. cloacae* had unique patterns. There were eight clusters with 2 (n=6) or 3 isolates (n=2) and a discriminatory index of 0.994 (95% CI 0.989-0.999).

Inclusion of the 22 isolates without detectable ESBL or *ampC* genes did not change interpretation (data not shown).

Association between β-lactamase gene and susceptibility

CTX-M-15 isolates were – on average – susceptible to 4.5 of 8 antibiotics tested, which was lower than isolates harboring TEM-52, CTX-M-1, or CTX-M-14 (mean susceptibility to 6.6, 6.0 and 5.7 antibiotics, respectively; p<=0.004 Mann Whitney U-test) and a similar co-resistance pattern as isolates harboring SHV-12 (Table 5). There were no significant associations between β-lactamase genes and age, gender, specimen type, and institution (data now shown).

Discussion

The population structure of third-generation cephalosporin resistant *Enterobacteriaceae* in the Netherlands is characterized by predominance of *E. coli* with CTX-M-15 ESBL genes, a high level of bacterial genotypic diversity, although clusters of genotypes, often *E. coli* belonging to MLST131, were observed in individual laboratories. AmpC type resistance was observed in 53 *E. coli* isolates (11% of all *E.* coli) and resulted from pAmpC genes, mostly being bla_{CMY-2}, or *ampC* promoter mutations in equal frequencies.

The predominance of the CTX-M-gene family among the ESBL genes in Dutch isolates has also been observed in Belgium, France, Italy, Poland, Spain and Canada. [6-8,20-22] As in the Netherlands, CTX-M-15 was most prevalent within the CTX-M family in all these countries, except in Poland and Spain were CTX-M-3 and CTX-M-14 were more prevalent. [7,21]

E. coli ST131 carrying IncFII plasmids with CTX-M-15 is considered the most important disseminator of CTX-M-15 worldwide. Our finding of clusters of ST131

isolated within a short time-frame in single laboratories suggests the occurrence of clonal dissemination of ESBL-producing ST131 *E. coli*. In our study most of the ST 131 *E. coli* contained CTX-M-15, and presence of this gene was associated with – on average – higher levels of resistance. Whether the clonal spread of ST131 occurs in health care-facilities or in the community remains to be determined as in the present study ST131 was in equal numbers obtained from samples submitted from health care institutes or by GPs. As such we agree with current literature that the origin ST131 remains unclear. [23]

The high prevalence of CTX-M-1 and TEM-52 in *E. coli* in the Netherlands may result from food-borne exposure, as poultry and retail meat are frequently contaminated with *E. coli* harbouring these genes on identical plasmids as found in human isolates. [24] Similar findings (albeit with lower prevalence of TEM-52) have been reported from Belgium, where retail meat was also frequently contaminated with ESBL-producing *E. coli* and which country shares food distributors with the Netherlands, and north Italy. [20,22] In contrast, TEM-19 rather than TEM-52 is the most prevalent TEM β-lactamase in Spain and Poland. [7,21]

Five percent of *E. coli* and 4% of *K. pneumoniae* conferring resistance to third generation cephalosporins carried pAmpC beta-lactamases. Little is known about the epidemiology of CMY-group beta-lactamases. In Poland CMY-12 and CMY-15 were predominant in *E. coli* and in the UK and Ireland pAmpCs of the CIT group, which includes the CMY-2 group, and genes belonging to the FOX and ACC family were detected in *E. coli* and *K. pneumoniae* [7,25]. The source of CMY-2 is unknown. Although CMY-2 genes have been identified in poultry and poultry meat in Belgium, Spain and the Netherlands [26-28], more detailed studies are needed to demonstrate the relevance and frequency of gene or strain transmission between both reservoirs.

All *E. coli*, except three isolates, resistant to cefoxitin in which no pAmpC beta-lactamases or mutated promoters were detected, contained ESBL genes, which may explain their resistance phenotype. In the other three isolates the increased MIC for cefoxitin may have resulted from mechanisms not investigated in this study, e.g. porin mutations, alteration in the expression of efflux pumps and/or porins, and mutations in the target of β-lactamases.

In this study, derepressed chromosomal *ampC* genes and pAmpC beta-lacmtase genes were equally prevalent in *E. coli.* This has also been observed in a French study [29], but a higher prevalence of derepressed chromosomal *ampC* genes was detected in Belgium. [30] This difference could result from differences in selection of isolates.

In 636 isolates with phenotypic resistance to third-generation cephalosporins 551 ESBLs and 82 AmpCs (including 53 assumed chromosomal *ampC* genes in *E.*

cloacae) were detected in 610 (96%) isolates. In the remaining 26 isolates (14 *E. coli*, 5 *K. oxytoca*, 4 *P. mirabilis*, and 3 *K. pneumoniae*) resistance may have been caused by mechanisms not investigated in this study, such as the presence of OXA genes, hyperproduction of chromosomal OXY genes in *K. oyxtoca*, porin mutations, alteration in the expression of efflux pumps and/or porins, and mutations in the target of the β-lactamases.

Although we consider the selected isolates to be representative for the Netherlands, there may have been some selection bias as we did not adjust isolate selection on the size of the catchment populations of the different laboratories.

Our findings demonstrate that in the Netherlands $bla_{\text{CTX-M-15}}$ is the most prevalent cause of third-generation cephalosporin resistance in the Netherlands and that resistance due to either hyperproduction of chromosomal *ampC* or plasmid-borne AmpC beta-lactamases occurs more frequently than previously reported.

Acknowledgements

We like to thank all participants of the National ESBL Surveillance Working Group: Prof. M.J.M. Bonten, MD PhD (University Medical Center Utrecht, Dept. of Med. Microbiology, Utrecht), A.P. van Dam, MD PhD (Onze Lieve Vrouwe Gasthuis, Dept. of Med. Microbiology, Amsterdam), G. Andriesse, MD PhD (Lievensberg Hospital, Lab. of Med. Microbiology, Bergen op Zoom), J.A.J.W. Kluytmans, MD PhD (Amphia Hospital, Dept. of Med. Microbiology, Breda), R.W. Vreede, MD PhD (Diagnostic Center SSDZ, Dept. of Med. Microbiology, Delft), F.W. Sebens, MD (Deventer Hospital, Dept. of Med. Microbiology and Infection Control Infectious Dis., Delft), L.J.M. Sabbe, MD PhD (Admiraal De Ruyter Hospital, Dept. of Med. Microbiology and Immunology, Goes), J.F.P. Schellekens, MD PhD, and W.H.M.Vogels MD (Laboratory for Infectious Diseases Groningen, Groningen), J.P. Arends, MD (University Medical Center Groningen, Lab. of Medical Microbiology, Groningen), J.W. Dorigo-Zetsma, MD PhD (Central Bact. and Ser. Laboratory Hilversum/Almere, Dept. of Med. Microbiology, Hilversum), K. Waar, MD PhD (Izore, Center for Infect. Diseases Friesland, Leeuwarden), B.J.M. Vlaminckx, MD PhD (St. Antonius Hospital, Dept. of Med. Microbiology, Nieuwegein), A.M. Horrevorts MD PhD (Canisius Wilhelmina Hospital, Dept. of Med. Microbiology, Nijmegen), P. Sturm, MD PhD (University Medical Center St. Radboud, Dept. of Med. Microbiology, Nijmegen), F.S. Stals, MD (Laurentius Hospital, Dept. of Med. Microbiology, Roermond), R.G.F. Wintermans, MD (Franciscus Hospital, Lab. of Med. Microbiology, Roosendaal), B. Moffie, MD (Vlietland Hospital, Dept. of Med. Microbiology, Schiedam), B.G.A. Hendrickx, MD PhD (ZorgSam Hospital Zeeuwsvlaanderen, Lab. of Med. Microbiology, Terneuzen), A.G.M. Buiting, MD PhD (Streek lab. voor de Volksgezondheid, Tilburg), L. Verhoef, MD PhD (SALTRO, Primary Health Care Laboratory, Dept. of Med. Microbiology , Utrecht), H.T. Tjhie, MD PhD (Stichting PAMM, Lab. Of Med. Microbiology, Veldhoven), M.J.H.M. Wolfhagen, MD PhD (Isala Clinics, Lab. of Med. Microbiology and Inf. Diseases), B.M.W. Diederen, MD PhD (Streeklab. voor de Volksgezondheid Kennemerland, Haarlem), S.F.T. Thijssen, MD PhD (Diakonessenhuis, Dept. of Med. Microbiology, Utrecht), E.M. Mascini, MD PhD, and A. van Griethuysen, MD PhD (AlysisGroep, Dept. of Med. Microbiology and Immunology, Arnhem), P.C. Wever, MD PhD (Jeroen Bosch Hospital, Reg. Lab. of Med. Microbiology and Inf. Control, Den Bosch), A. Fleer, MD PhD (Gelre Hospitals, Dept. of Med. Microbiology, Apeldoorn), J.H.T. Wagenvoort, MD PhD (Atrium Medisch Centrum Parkstad, Dept. of Med. Microbiology, Heerlen), A.T. Bernards, MD PhD (University Medical Center Leiden, Dept. of Med. Microbiology, Leiden)

References

1. Gniadkowski M (2008) Evolution of extended-spectrum beta-lactamases by mutation. Clin Microbiol Infect 14 Suppl 1: 11-32.

2. Paterson DL, Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18: 657-686.

3. Jacoby GA (2009) AmpC beta-lactamases. Clin Microbiol Rev 22: 161-182.

4. Luzzaro F, Mezzatesta M, Mugnaioli C, Perilli M, Stefani S, et al. (2006) Trends in production of extended-spectrum beta-lactamases among Enterobacteriacae of medical interest: report of the second Italian nationwide survey. J Clin Microbiol 44: 1659-1664.

5. Mulvey MR, Bryce E, Boyd DA, Ofner-Agostini M, Land AM, et al. (2005) Molecular characterization of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. Antimicrob Agents Chemother 49: 358-365.

6. Galas M, Decousser JW, Breton N, Godard T, Allouch PY, et al. (2008) Nationwide study of the prevalence, characteristics, and molecular epidemiology of extended-spectrum-beta-lactamase-producing *Enterobacteriaceae* in France. Antimicrob Agents Chemother 52: 786-789.

7. Empel J, Baraniak A, Literacka E, Mrowka A, Fiett J, et al. (2008) Molecular survey of beta-lactamases conferring resistance to newer beta-lactams in *Enterobacteriaceae* isolates from Polish hospitals. Antimicrob Agents Chemother 52: 2449-2454.

8. Zhanel GG, Decorby M, Adam H, Mulvey MR, McCracken M, et al. (2010) Prevalence of Antimicrobial-Resistant Pathogens in Canadian Hospitals: Results of the Canadian Ward Surveillance Study (CANWARD 2008). Antimicrob Agents Chemother 54: 4684-4693.

9. Sturm PD, Bochum ET, van Mook-Vermulst SV, Handgraaf C, Klaassen T, et al. (2010) Prevalence, molecular characterization, and phenotypic confirmation of extended-spectrum beta-lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* at the Radboud University Nijmegen Medical Centre in The Netherlands. Microb Drug Resist 16: 55-60.

10. Wiegand I, Geiss HK, Mack D, Sturenburg E, Seifert H (2007) Detection of extended-spectrum beta-lactamases among *Enterobacteriaceae* by use of semiautomated microbiology systems and manual detection procedures. J Clin Microbiol 45: 1167-1174.

11. Olesen I, Hasman H, Aarestrup FM (2004) Prevalence of beta-lactamases among ampicillin-resistant *Escherichia coli* and *Salmonella* isolated from food animals in Denmark. Microb Drug Resist 10: 334-340.

12. Naiemi NA, Duim B, Savelkoul PH, Spanjaard L, de Jonge E, et al. (2005) Widespread transfer of resistance genes between bacterial species in an intensive care unit: implications for hospital epidemiology. J Clin Microbiol 43: 4862-4864.

13. Paauw A, Fluit AC, Verhoef J, Leverstein-van Hall MA (2006) *Enterobacter cloacae* outbreak and emergence of quinolone resistance gene in Dutch hospital. Emerg Infect Dis 12: 807-812.

14. Voets GM, Fluit AC, Scharringa J, Cohen Stuart J, Leverstein-van Hall MA (2011) A set of multiplex PCRs for genotypic detection of extended-spectrum beta-lactamases, carbapenemases, plasmid-mediated AmpC beta-lactamases and OXA beta-lactamases. Int J Antimicrob Agents 34: 356-359.

15. Rottman M, Benzerara Y, Hanau-Bercot B, Bizet C, Philippon A, et al. (2002) Chromosomal ampC genes in *Enterobacter* species other than *Enterobacter cloacae*, and ancestral association of the ACT-1 plasmid-encoded cephalosporinase to Enterobacter asburiae. FEMS Microbiol Lett 210: 87-92.

16. Caroff N, Espaze E, Berard I, Richet H, Reynaud A (1999) Mutations in the *ampC* promoter of *Escherichia coli* isolates resistant to oxyiminocephalosporins without extended spectrum beta-lactamase production. FEMS Microbiol Lett 173: 459-465.

17. Haldorsen B, Aasnaes B, Dahl KH, Hanssen AM, Simonsen GS, et al. (2008) The AmpC phenotype in Norwegian clinical isolates of *Escherichia coli* is associated with an acquired ISEcp1-like ampC element or hyperproduction of the endogenous AmpC. J Antimicrob Chemother 62: 694-702.

18. Siu LK, Lu PL, Chen JY, Lin FM, Chang SC (2003) High-level expression of AmpC beta-lactamase due to insertion of nucleotides between -10 and -35 promoter sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-spectrum-cephalosporin treatment. Antimicrob Agents Chemother 47: 2138-2144.

19. Fluit AC, Terlingen AM, Andriessen L, Ikawaty R, van Mansfeld R, et al. (2010) Evaluation of the DiversiLab system for detection of hospital outbreaks of infections by different bacterial species. J Clin Microbiol 48: 3979-3989.

20. Brigante G, Luzzaro F, Perilli M, Lombardi G, Coli A, et al. (2005) Evolution of CTX-M-type beta-lactamases in isolates of *Escherichia coli* infecting hospital and community patients. Int J Antimicrob Agents 25: 157-162.

21. Paniagua R, Valverde A, Coque TM, Baquero F, Canton R (2010) Assessment of prevalence and changing epidemiology of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* fecal carriers using a chromogenic medium. Diagn Microbiol Infect Dis 67: 376-379.

22. Rodriguez-Villalobos H, Bogaerts P, Berhin C, Bauraing C, Deplano A, et al. (2011) Trends in production of extended-spectrum beta-lactamases among *Enterobacteriaceae* of clinical interest: results of a nationwide survey in Belgian hospitals. J Antimicrob Chemother 66: 37-47.

23. Platell JL, Johnson JR, Cobbold RN, Trott DJ (2011) Multidrug-resistant extraintestinal pathogenic *Escherichia coli* of sequence type ST131 in animals and foods. Vet Microbiol 153: 99-108.

24. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, et al. (2011) Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect 17: 873-880.

25. Woodford N, Reddy S, Fagan EJ, Hill RL, Hopkins KL, et al. (2007) Wide geographic spread of diverse acquired AmpC beta-lactamases among *Escherichia coli* and *Klebsiella* spp. in the UK and Ireland. J Antimicrob Chemother 59: 102-105.

26. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, et al. (2008) Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. Antimicrob Agents Chemother 52: 1238-1243.

27. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D (2010) Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet Microbiol 145: 273-278.

28. Cortes P, Blanc V, Mora A, Dahbi G, Blanco JE, et al. (2010) Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Appl Environ Microbiol 76: 2799-2805.

29. Corvec S, Cremet L, Leprince C, Dauvergne S, Reynaud A, et al. (2010) Epidemiology of *Escherichia coli* clinical isolates producing AmpC plasmidic beta-lactamase during a 5-year period in a French teaching Hospital. Diagn Microbiol Infect Dis 67: 277-281.

30. Bogaerts P, Rodriguez-Villalobos H, Bauraing C, Deplano A, Laurent C, et al. (2010) Molecular characterization of AmpC-producing *Escherichia coli* clinical isolates recovered at two Belgian hospitals. Pathol Biol (Paris) 58: 78-83.

Table 1. Identification of ESBL-groups as determined by ESBL array and PCR in 3rd generation cephalosporin resistant *Enterobacteriaceae*.

ESBL-group	*E. coli* N=479	*E. cloacae* N=68	*K. pneumoniae* N=67	*K. oxytoca* N=11	*P. mirabilis* N=11	*All species* N=636
CTX-M-1	301	16	48	3	2	370
CTX-M-2	2					2
CTX-M-8/25	2					2
CTX-M-9	59	11	1	1		72
SHV-2	2		4			6
SHV-4	23	14	11			48
SHV-31	1	1				2
TEM-3	28	1	2			31
TEM-4	1					1
TEM-5	2					2
TEM-17	2					2
TEM-19	10					10
GES				2		2
PER					1	1
No ESBL-gene detected	63	33	3	5	8	112

Note: 26 isolates contained 2 ESBLs

Table 2. Identification of ESBL β-lactamase genes in 3rd generation cephalosporin resistant *Enterobacteriaceae*.

ESBL-group	ESBL-gene	*E. coli* n=235 (75%)	*E. cloacae* n=33 (11%)	*K. pneumoniae* n=32 (10%)	*K. oxytoca* n=8 (3%)	*P. mirabilis* n=6 (2%)	Species n=314
CTX-M-1	CTX-M-1	47			1		47
	CTX-M-15	80	4	20			104
	CTX-M-15/28	9		3			12
	CTX-M-22	3					3
	CTX-M-79	2					2
	Other	2				1	3
CTX-M-9	CTX-M-9	3	3				6
	CTX-M-14	15					15
	CTX-M-17	3					3
	CTX-M-27	4					4
	Other	4		1		1	6
	All CTX-M Variants	172	7	24	1	2	206
SHV-2	SHV-2	1		1			2
SHV-4	SHV-5			2			2
	SHV-12	13	8	3			24
	All SHV Variants	14	8	6			28
TEM-3	TEM-19	2					2
	TEM-52	13					13
TEM-5	TEM-12	1					1
TEM-19	TEM-19	1					1
	All TEM Variants	17					17
	GES-1				1		1
	PER-5					1	1
	All Variants				1	1	2
None of the above genes detected		37	19	2	5	3	66

Table 3. Presence of AmpC β-lactamase genes in isolates with a MIC ≥16 mg/L for cefoxitin.

AmpC-gene	*E. coli* n=102 (56%)	*E. cloacae* n=63 (35%)	*K. pneumoniae* n=10 (6%)	*P. mirabilis* n=6 (3%)	Species n=181
CMY-2 group	22		1	4	27
ACT-5	1				1
ACT-like *	1				1
MIR-1/2/3	1		1		2
DHA-1			1		1
Chromosomal	29	53 **			82
None of the above genes detected	48	10	7	2	67

* GenBank Number = EF125014.1, ** = presumed

Table 4. Number of clusters for each cluster size in DiversiLab using >98% similarity per species.

Species (n=isolates)	Diversilab cluster size										not typable	DI*	95% CI
	1	2	3	4	5	6	7	14	17	25			
E. cloaca (n=68)	48	6	2								2	0.994	0.989 - 0.999
E. coli (n=465)	253	44	14	2	2	1		1	1	1	2	0.994	0.991- 0.996
K. pneumoniae (n=64)	41	4	2				1				2	0.985	0.969 - 1.0
K. oxytoca (n=11)	7		1								1	0.911	0.801 - 1.0

* DI = Discriminatory Index

Table 5. Co-susceptibility according to EUCAST breakpoints in *E. coli* harboring the five most common ESBL genes.

Antibiotic	CTX-M-15 (n=77) (%, n)	CTX-M-1 (n=44) (%, n)	SHV-12 (n=7) (%, n)	CTX-M-14 (n=15) (%, n)	TEM-52 (n=12) (%, n)
ciprofloxacin	9 (7)	57 (25)	43 (3)	60 (9)	53 (7)
tobramycin	21 (16)	86 (38)	43 (3)	67 (10)	100 (12)
trimethoprim/ sulfamethoxazole	35 (27)	25 (11)	14 (1)	53 (8)	33 (4)
gentamicin	56 (43)	86 (38)	57 (4)	67 (10)	92 (11)
amikacin	60 (46)	96 (42)	74 (5)	93 (14)	100 (12)
chloramphenicol	69 (53)	64 (28)	29 (2)	33 (5)	75 (9)
nitrofurantoin	99 (76)	96 (42)	100 (7)	100 (15)	100 (12)
fosfomycin	99 (76)	98 (43)	100 (7)	100 (15)	100 (12)
mean no. of co-susceptible antibiotics (range)	4.5 (2-8)	6.1 (3-8)	4.6 (3-7)	5.7 (3-8)	6.6 (5-8)

Supplementary Table 1. Material and provider of the isolates grouped by species.

	E. cloacae (n=68)	*E. coli* (n=479)	*K. oxytoca* (n=11)	*K. pneumoniae* (n=67)	*P. mirabilis* (n=11)
Material					
Ascites	3	5		1	
Blood	1	10		2	
Gynaecological		3		15	
Pulmonary	8	29	5		2
Rectum/faeces	12	58	3	18	
Urine	33	333	2	25	7
Wound/Abscess/Skin	11	39	1	5	2
Other/Unknown		2		1	
Provider					
Academic Hospital	1	23	2	14	1
Non-Academic Hospital	52	259	8	35	7
Long-term Care Facility	3	26		4	2
General Practitioner	10	147		12	1
Other/Unknown	2	24	1	2	

Supplementary table 2. Combinations of β-lactamases expressed in one isolate in the primary selection and random sample.

ESBL-ESBL combinations	Species	n	β-lactamase genotypes
CTX-M-1 gr. & CTX-M-9 gr.	*E. cloacae*	2	Not Typed (n=2)
	E. coli	1	CTX-M-15 & CTX-M-27 (n=1)
CTX-M-1 gr. & CTX-M-8/25 gr.	*E. coli*	1	CTX-M-15 (CTX-M-8/25-gr. Not Typed) (n=1)
CTX-M-1 gr. & SHV-4 gr.	*E. cloacae*	2	CTX-M-15 & SHV-12 (n=1)
			Not typed (n=1)
	E. coli	6	CTX-M-1/61 & SHV-12 (n=2)
			CTXM-15 & SHV-12 (n=2)
			Not typed (n=2)
CTX-M-9 gr. & SHV-4 gr.	*E. cloacae*	3	CTX-M-9 & SHV-12 (n=1)
			SHV-12 (CTX-M-9-gr. not typed) (n=1)
			Not typed (n=1)
	E. coli	1	CTX-M-9 & SHV-12 (n=1)
	K. pneumoniae	1	Not typed (n=1)
CTX-M-1 gr. & TEM-3 gr.	*E. coli*	2	CTX-M-1/61 & TEM-52 (n=1)
			Not typed (n=1)
CTX-M-1 gr. & TEM-19 gr.	*E. coli*	2	CTX-M-15 (TEM-19-gr. not typed) (n=1)
			TEM-19 (CTX-M-1-gr. not typed) (n=1)
CTX-M-1 gr. & TEM-4 gr.	*E. coli*	1	Not typed (n=1)
SHV-2 gr. & TEM-19 gr.	*E. coli*	1	Not typed (n=1)
SHV-31 gr. & TEM-3 gr.	*E. cloacae*	1	Not typed (n=1)
	E. coli	1	Not typed (n=1)
SHV-4 gr. & TEM-25 gr.	*E. coli*	1	SHV-12 & TEM-25 (n=1)

ESBL-AmpC combinaties	Species	n	ESBL Genotypes
CTX-M-1 gr. & CIT	*E. coli*	4	CTX-M-1 (CMY not typed) (n=1)
			CTX-M-15 (CMY not typed) (n=2)
			Not typed (n=1)
	K. pneumoniae	1	Not typed (n=1)
CTX-M-1 gr. & MIR	*E. coli*	1	CTX-M-15 & MIR-1/2/3 (n=1)
SHV-4 gr. & MIR	*K. pneumoniae*	1	MIR-1/2/3 (SHV-4-gr. not typed) (n=1)

ESBL-ESBL-AmpC combinaties	Species	n	ESBL Genotypes
CTX-M-1 gr. & SHV-4 gr. & DHA	*K. pneumoniae*	1	DHA-1

Chapter VII

Identical plasmid AmpC *Enterobacteriaceae* β-lactamase genes and plasmid types in *E. coli* isolates from Dutch patients and poultry meat

Guido M. Voets, Ad C. Fluit, Jelle Scharringa, Thijs van den Munckhof, Maurine A. Leverstein-van Hall, James Cohen Stuart

Department of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands

Accepted pending revisions

Abstract

Objectives: The increasing prevalence of third-generation cephalosporin-resistant *Enterobacteriaceae* is a worldwide problem. Recent studies showed that poultry (meat) and humans share identical Extended-Spectrum Beta-Lactamase (ESBL) genes, plasmid types, and *Eschericia coli* strain types, suggesting that transmission from poultry to humans may occur, possibly through handling or consumption of ESBL-contaminated meat. The aim of this study was to compare plasmid borne Ambler class C beta-lactamase (pAmpC) genes, their plasmids, and bacterial strain types between *E. coli* isolates from Dutch retail chicken meat and clinical isolates representative for the Dutch patient population from the same period.

Methods: In total, 98 Dutch retail chicken meat samples, and 479 third-generation cephalosporin non-susceptible human clinical *E. coli* isolates collected in a Dutch national surveillance study from the same period were screened for pAmpC production. The presence of pAmpC was confirmed using PCR and sequencing. Plasmid typing was performed using PCR-based replicon typing (PBRT). *E coli* strains were compared using the DiversiLab system.

Results: On 12 of 98 chicken meat samples (12%), a pAmpC producing *E. coli* was detected (all bla_{CMY-2}). Of the 479 human *E. coli*, 25 (5.2%) harboured a pAmpC (bla_{CMY-2} n=22, bla_{ACT} n=2, bla_{MIR} n=1). PBRT was performed in 11 of 12 bla_{CMY-2} producing *E. coli,* and showed that 10 (91%) of poultry meat isolates harboured bla_{CMY-2} on an IncK plasmid, and one isolate (9%) on an IncI1 plasmid. Of 19 human bla_{CMY-2} producing isolates available for PBRT, 8 (42%) also harboured bla_{CMY-2} on an IncK plasmid, and 9 (47%) on an IncI1 plasmid. Thus, 17 of 25 pAmpC producing human *E. coli* (68%) have the same AmpC gene (bla_{CMY-2}) and plasmid type (IncI1 or IncK) as found in poultry meat. DiversiLab isolate typing showed one cluster of 2 isolates containing one human isolate and one retail poultry meat isolate, but each with a different plasmid.

In conclusion, this study shows that bla_{CMY-2} on identical plasmid types was detected in *E. coli* isolates from Dutch patients and poultry meat, implying that a food-borne transmission route of bla_{CMY-2} harbouring plasmids cannot be excluded.

Introduction

The increasing prevalence of plasmid-borne β-lactamases conferring resistance to third-generation cephalosporins in *Enterobacteriaceae* is a world-wide problem. Recent studies have suggested that transmission of Extended-Spectrum Beta-Lactamase (ESBL) genes, their plasmids, and the *Escherichia coli* isolates harbouring those plasmids may occur from poultry to humans, possibly through handling or

consumption of contaminated meat (1,9,11).

Like ESBLs, plasmid-borne Ambler class C ß-lactamases (pAmpCs) confer resistance to penicillins and cephalosporins up to the third generation, and have been demonstrated throughout the world (8). Although the presence of pAmpCs, mostly bla_{CMY-2}, has been demonstrated in poultry (5,7), poultry meat (3,4,13,15), and humans, no comparison has been made between pAmpC genes in humans and poultry meat from the same period and region. If pAmpC genes and plasmids from those two compartments are different, poultry meat would be an unlikely source of pAmpC in humans. If identical pAmpC genes are found on the same plasmid types and strains, a transmission route of pAmpCs from poultry meat to humans cannot be excluded, either by transmission of pAmpC producing bacterial strains or via horizontal transfer of AmpC encoding plasmids. We therefore compared the pAmpC genes, their plasmids, and the bacterial strain types between *E. coli* isolates obtained from Dutch retail chicken meat, and clinical isolates representative for the Dutch patient population.

Materials & Methods

Human *E. coli* isolates were obtained in a Dutch national surveillance study as described previously (12). Briefly, in February, March and April 2009, 31 microbiology laboratories throughout the Netherlands submitted all *E. coli, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis*, and *Enterobacter cloacae* isolates with a positive ESBL screen test, i.e. a minimal inhibitory concentration (MIC) >1 mg/L of cefotaxime or ceftazidime or an expert system ESBL alert from Phoenix (Becton Dickinson, USA) or Vitek-2 (Biomerieux, France), as described previously (3). Per participating laboratory, the first 25 consecutive isolates, if available, were included in this study. Only one isolate per patient was included. In the central study laboratory, the MIC determination was repeated using broth microdilution (BMD) (Merlin Diagnostic GmbH, Rüsselsheim, Germany). All *E. coli* isolates with an MIC of >1 mg/L for cefotaxime or ceftazidime and ≥16mg/L for cefoxitin using BMD were included in this study.

As described previously (3), retail poultry meat *E. coli* isolates were obtained from 98 raw chicken breasts collected in April 2010 from 12 stores in Utrecht, a city in the centre of the Netherlands. The meat samples were representative for the poultry meat being sold in the Netherlands, as they were collected from the 5 largest national super market chains, with a combined Dutch market share of 90%. *E. coli* isolates producing pAmpC were detected as part of a study investigating the prevalence of ESBLs on poultry meat. The phenotypic detection of ESBL producing micro-organisms on the meat samples was performed by homogenizing 25 g meat in 225 mL peptone water in a stomacher. The homogenate was used for overnight

pre-enrichment. This was followed by inoculation of an ESBL selective plate with 0.1 mL of the pre-enriched homogenate. In case of growth on the ESBL selective plate, species identification of each morphologically different colony was performed by MALDI-TOF (Bruker Daltonics, Germany). An ESBL Etest was performed on all (n=163) isolates (BioMérieux, Marcy l'Etoile, Lyon, France) growing on the selective plates. Minimum inhibitory concentrations (MICs) of antibiotics were determined by broth micro-dilution (Merlin, Bornheim-Hersel, Germany) of isolates growing on the selective plates. Isolates without synergy between cefotaxime or ceftazidime and clavulanic acid in the ESBL Etest (off range) were suspected of AmpC production, and these isolates were further evaluated using a multiplex PCR (3,14), with sequencing of the amplicons.

To distinguish all members of the CIT AmpC family, the following primers were used: CIT-F: GCAGGAGCAGGCTATTCC, CIT-F2: ATGATGAAAAAATCGTTATGCT, CIT-F3: GCTCCAGCAGGGCATTG, and CIT-R: ATTGCAGCTTTTCAAGAATGCG.

To compare plasmids harbouring bla_{CMY-2} in humans and retail poultry meat, PCR-based replicon (PBRT) (2) was performed on the bla_{CMY-2} producing *E. coli* isolates from both compartments. The association between plasmid AmpC genes and plasmids was determined by transformation [12]. *E. coli* strain typing was performed using DiversiLab (BioMérieux, France). A similarity of >95% was used of cut-off for relatedness.

Results

A total of 479 human *E. coli* isolates with an MIC of >1 mg/L for cefotaxime and/or ceftazidime were included, of which 102 (21%) with a cefoxitin MIC ≥16 mg/L. PCR and sequencing showed that 25 of these isolates (5.2%) harboured a pAmpC, of which 22 (88%) harboured a bla_{CMY-2}, two (8%) an bla_{ACT}, and one isolate (4%) contained a bla_{MIR} MIR AmpC beta-lactamase.

Of the 98 poultry meat samples, 92 (94%) were contaminated with one or more ESBL-positive *E. coli* isolates (3,9). On 12 meat samples (12%), a bla_{CMY-2} producing *E. coli* isolate was detected, of which 10 also produced an ESBL. No other pAmpC genes were detected on the meat samples.

Eleven of 12 poultry meat isolates, and 19 of 22 human isolates were available for PBRT analysis. Of the 11 retail poultry meat isolates, 10 (91%) encoded bla_{CMY-2} on an IncK plasmid, and one isolate (9%) encoded bla_{CMY-2} on an IncI1 plasmid (Table 1), which also encoded a CTX-M-1 group ESBL gene. Of the 19 human clinical isolates, 8 (42%) encoded bla_{CMY-2} on an IncK plasmid, 9 of the human isolates (47%) encoded bla_{CMY-2} on an IncI1 plasmid, and two isolates (11%) harboured untypeable plasmids. Thus, 17 of the 25 pAmpC producing human *E. coli* have the same AmpC gene

(*bla*$_{CMY-2}$) and plasmid type (IncI1 or IncK) as found in 91% of the pAmpC-positive poultry meat isolates.

DiversiLab isolate typing using a similarity of >95% showed one cluster of two human *E. coli* isolates. Two clusters were identified among the poultry meat isolates (one cluster of five, one cluster of two isolates). There was one cluster with one human isolate and one retail poultry meat isolate, but each with a different plasmid.

Discussion

This is the first study comparing plasmid-borne AmpC genes, their plasmids, and the *E. coli* strains harbouring those plasmids from retail poultry meat and human clinical *E. coli* isolates from the same region and year. The *bla*$_{CMY-2}$ gene on IncK or IncI1 plasmids was the only pAmpC beta-lactamase identified in *E. coli* contaminating poultry meat. *Bla*$_{CMY-2}$ was also the most prevalent pAmpC among human *E.* coli, and in 68% of pAmpC producing human isolates, *bla*$_{CMY-2}$ was located on the same plasmid type as detected on poultry meat. Based on these findings, transmission of *bla*$_{CMY-2}$ harbouring plasmids from poultry meat to humans cannot be excluded. On the other hand, the finding of identical resistance genes and plasmid types on poultry meat and in humans may also reflect a much more complex dissemination route than food-borne transmission, e.g., via contamination of the environment or parallel independent micro-evolution (6).

This study provides several new insights. First, although IncI1 and IncK plasmids harbouring *bla*$_{CMY-2}$ have been detected previously in live poultry and ICU-patients (5,10), this is the first study showing that *bla*$_{CMY-2}$ on those plasmids can be found on poultry retail meat, representative for the meat being sold in the Netherlands. This implicates potential exposure of the general population to *bla*$_{CMY-2}$ beta-lactamase during handling or consumption of poultry meat. Furthermore, we show that although identical *bla*$_{CMY-2}$ harbouring plasmids have been detected in retail meat and humans, the *E. coli* strains harbouring those plasmids were not the same. This may be explained by plasmid transfer from poultry adapted *E. coli* strains to human adapted *E. coli* strain types. This is in contrast to the ESBL data, where similar *E. coli* strain types producing an ESBL were found on both poultry meat and humans (9,11), and a study from Canada comparing *bla*$_{CMY-2}$ producing *E. coli* from the environment and ICU patients (10).

This study has several limitations. First, the number of isolates is relatively low. Second, the prevalence of AmpC beta-lactamase positive isolates on poultry meat may have been underestimated, because an ESBL selective agar containing cloxacillin was used, which has an inhibitory effect on AmpC beta-lactamases. This selective medium was used because the original aim of the poultry meat study was

to detect ESBL producing *Enterobacteriaceae* (3).

In conclusion, this study shows that bla_{CMY-2} on identical plasmid types was detected in *E. coli* isolates from Dutch patients and poultry meat, implying that a food-borne transmission route of bla_{CMY-2} harbouring plasmids cannot be excluded.

Reference List

1. **Calbo, E., N. Freixas, M. Xercavins, M. Riera, C. Nicolas, O. Monistrol, M. M. Sole, M. R. Sala, J. Vila, and J. Garau**. 2011. Foodborne nosocomial outbreak of SHV1 and CTX-M-15-producing *Klebsiella pneumonia:* epidemiology and control. Clin.Infect.Dis. **52**:743-749.

2. **Carattoli, A., A. Bertini, L. Villa, V. Falbo, K. L. Hopkins, and E. J. Threlfall**. 2005. Identification of plasmids by PCR-based replicon typing. J.Microbiol. Methods **63**:219-228.

3. **Cohen, S. J., M. T. van den, G. Voets, J. Scharringa, A. Fluit, and M. L. Hall**. 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int.J.Food Microbiol. **154**:212-214.

4. **Dhanji, H., N. M. Murphy, M. Doumith, S. Durmus, S. S. Lee, R. Hope, N. Woodford, and D. M. Livermore**. 2010. Cephalosporin resistance mechanisms in *Escherichia coli* isolated from raw chicken imported into the UK. J.Antimicrob.Chemother. **65**:2534-2537.

5. **Dierikx, C., A. van Essen-Zandbergen, K. Veldman, H. Smith, and D. Mevius**. 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet.Microbiol. **145**:273-278.

6. **Ewers, C., A. Bethe, T. Semmler, S. Guenther, and L. H. Wieler**. 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin.Microbiol.Infect. **18**:646-655.

7. **Glenn, L. M., M. D. Englen, R. L. Lindsey, J. F. Frank, J. E. Turpin, M. E. Berrang, R. J. Meinersmann, P. J. Fedorka-Cray, and J. G. Frye**. 2012. Analysis of antimicrobial resistance genes detected in multiple-drug-resistant *Escherichia coli* isolates from broiler chicken carcasses. Microb. Drug Resist. **18**:453-463.

8. **Jacoby, G. A.** 2009. AmpC beta-lactamases. Clin.Microbiol.Rev. **22**:161-82, Table.

9. **Leverstein-van Hall, M. A., C. M. Dierikx, S. J. Cohen, G. M. Voets, M. P. van den Munckhof, A. van Essen-Zandbergen, T. Platteel, A. C. Fluit, d. S.-B. van, J. Scharinga, M. J. Bonten, and D. J. Mevius**. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin.Microbiol.Infect. **17**:873-880.

10. **Mataseje, L. F., P. J. Baudry, G. G. Zhanel, D. W. Morck, R. R. Read, M. Louie, and M. R. Mulvey**. 2010. Comparison of CMY-2 plasmids isolated from human, animal, and environmental *Escherichia coli* and *Salmonella* spp. from Canada. Diagn.Microbiol.Infect.Dis. **67**:387-391.

11. **Overdevest, I., I. Willemsen, M. Rijnsburger, A. Eustace, L. Xu, P. Hawkey, M. Heck, P. Savelkoul, C. Vandenbroucke-Grauls, Z. K. van der, X. Huijsdens, and J. Kluytmans**. 2011. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg.Infect. Dis. **17**:1216-1222.

12. **Platteel, T. N., J. W. Cohen Stuart, A. J. de Neeling, G. M. Voets, J. Scharringa, S. N. van de, A. C. Fluit, M. J. Bonten, and M. A. Leverstein-van Hall**. 2013. Multi-centre evaluation of a phenotypic extended spectrum beta-lactamase detection guideline in the routine setting. Clin.Microbiol. Infect. **19**: 70-76.

13. **Sheikh, A. A., S. Checkley, B. Avery, G. Chalmers, V. Bohaychuk, P. Boerlin, R. Reid-Smith, and M. Aslam**. 2012. Antimicrobial resistance and resistance genes in *Escherichia coli* isolated from retail meat purchased in Alberta, Canada. Foodborne.Pathog.Dis. **9**:625-631.

14. **Voets, G. M., A. C. Fluit, J. Scharringa, S. J. Cohen, and M. A. Leverstein-van Hall**. 2011. A set of multiplex PCRs for genotypic detection of extended-spectrum beta-lactamases, carbapenemases, plasmid-mediated AmpC beta-lactamases and OXA beta-lactamases. Int.J.Antimicrob.Agents **37**:356-359.

15. **Zhao, S., K. Blickenstaff, S. Bodeis-Jones, S. A. Gaines, E. Tong, and P. F. McDermott**. 2012. Comparison of the prevalences and antimicrobial resistances of *Escherichia coli* isolates from different retail meats in the United States, 2002 to 2008. Appl.Environ.Microbiol. **78**:1701-1707.

Table 1: Distribution of plasmid mediated AmpC beta-lactamase genes and plasmids from human and poultry meat isolates

Source	pAmpC gene	Plasmid type	Number of PBRT typed isolates	ESBL on same plasmid
Poultry meat	CMY-2	IncK	10 (91%)	none
	CMY-2	IncI1	1 (9%)	CTX-M-1
	Total	**-**	**11 (100%)**	
Human	CMY-2	IncK	8 (42%)	none
	CMY-2	IncI1	9 (47%)	none
	CMY-2	untypeable	2 (11%)	none
	Total	**-**	**19 (100%)**	

Chapter VIII

Poultry-derived *Escherichia coli* can grow in an *ex vivo* model with the human gut microbiota

Guido M. Voets [1], Jorg Brunner [2], Maurine A. Leverstein-van Hall [3], James Cohen Stuart [1], Jelle Scharringa [1], Roy C. Montijn [2], Frank H.J. Schuren [2], Ad C. Fluit [1]

[1] Department of Medical Microbiology, University Medical Centre Utrecht, Utrecht, The Netherlands

[2] Microbiology Department, TNO Quality of Life, Zeist, The Netherlands

[3] Department of Medical Microbiology, Bronovo, Den Haag, The Netherlands

Abstract

Introduction The prevalence of human infections caused by Extended-Spectrum ß-Lactamase (ESBL) positive *Escherichia coli* is increasing. Previously we have shown that part of the third-generation cephalosporin resistance genes, plasmids and strains from poultry and human origin were indistinguishable. These findings were suggestive for transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain.

Aim. The first aim of this study is to determine whether poultry- and retail poultry meat-derived *E. coli* may grow in human microbiota. The second aim was to determine the relative transfer rate from ESBL-positive plasmids from poultry to human *E. coli* strains in human microbiota.

Methods. Human-derived, poultry-derived, and poultry meat-derived ESBL-positive *E. coli* were included. A human faeces culture was used to simulate the human microbiota. Dilution series of the *E. coli* isolates were added to the human faeces culture and grown in diluted SIEM medium buffered by 1M MES pH 6.0 under anaerobic conditions with and without antibiotics. Conjugation was also tested *in vitro* using a known *E. coli* K12 acceptor strain. Conjugants were identified using selective plates and confirmed by PCR and sequencing of resistance genes, plasmid-based replicon typing and isolate typing with DiversiLab.

Results. Poultry and retail poultry meat-derived *E. coli* did grow in the human microbiota. No marked difference in growth was detected between poultry-derived, retail poultry meat-derived or human-derived *E. coli*. One conjugation event occurred from a human-derived *E. coli* to the human gut microbiota in the absence of antibiotic pressure. No conjugation event between a poultry-derived isolate to human *E. coli* strains in human microbiota was identified, though conjugation was observed *in vitro* between a poultry-derived isolate and a control K12 *E. coli* acceptor strain.

Conclusion. Poultry and retail poultry meat-derived ESBL-positive *E. coli* did grow well in human microbiota but no transfer was observed of plasmid encoded ESBLs from the poultry derived *E. coli* to the human microbiota.

Introduction

Human infections with ESBL-positive *Enterobacteriaceae* show a global increase in prevalence. The presence of third-generation cephalosporin-resistant *Escherichia coli* has been demonstrated in several reservoirs, e.g., companion animals, livestock, and humans. [1-4] This is facilitated by a high degree of genome plasticity due to, e.g., horizontal gene transfer. Specifically, the core genome consists of less than 2,000 genes, but the pan genome encompasses 10,000 genes. [5]

Several studies showed that the genes conferring resistance to third-generation cephalosporins are indistinguishable between different reservoirs. [2, 4, 6] Furthermore, these genes are present on indistinguishable plasmids according to plasmid-based replicon typing (PBRT), plasmid multi-locus sequence typing (pMLST) and recently whole-plasmid sequencing (personal communication Dik Mevius). [5, 7] In addition, part of the *E. coli* from these different reservoirs is indistinguishable by multi-locus sequence typing (MLST). [5]

Therefore, it was hypothesised that poultry and retail-poultry meat, may act as a reservoir for Extended-Spectrum ß-Lactamase (ESBL) producing *E. coli* due to the transmission of isolates, plasmids and genes. [4]

A Belgian study demonstrated that *E. coli* from a broiler harboring an IncI plasmid carrying a $bla_{\text{TEM-52}}$ gene could be maintained in a continuous flow culture system with fresh faeces from a healthy volunteer to simulate the microbiota. Transfer of the plasmid to *E. coli* of the microbiota took place in the system in the absence of antimicrobial treatment. [8] Unfortunately, only one isolate was tested.

The first aim of this study is to determine whether poultry- and retail poultry meat-derived *E. coli* can grow in the presence of human microbiota and compare their growth to human-derived *E. coli*. The second aim was to determine the relative transfer rate from ESBL-positive plasmids from poultry to human *E. coli* strains in human microbiota in the presence or absence of antibiotics.

Materials and Methods

Human microbiota and isolates

Faeces samples from seven healthy individuals were collected and mixed as previously described. [9] Aliquots were prepared and stored at –70°C.

Four human ESBL-positive *E. coli* isolates were obtained from a study of the population distribution of ß-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands (Table 1). [3]

Four ESBL-positive poultry isolates were derived from the Dutch surveillance program on antibiotic resistance in bacteria isolated in food-producing animals in 2006 (Table 1). [4]

One ESBL-positive retail poultry meat-derived *E. coli* was obtained from a study into the presence of ESBL-positive *Enterobacteriaceae* on poultry retail meat in the Netherlands (Table 1). [7]

The isolates were typed using multi-locus sequence typing (MLST) and the plasmids harbouring the ESBL-genes were typed using plasmid-based replicon typing (PBRT) and plasmid multi-locus sequence typing (pMLST). [10, 11] The poultry or poultry-derived isolates were paired with an isolate of human origin based on MLST, plasmid type and *bla* gene (see Table 1).

A known *E. coli* K12 conjugation acceptor strain was used for *in vitro* experiments. [12]

Growth experiments

The faeces culture was pre-cultured in diluted SIEM (Standard Ileal Efflux Medium) buffered by 1M MES (2-(-*N*-morpholino)ethanesulfonic acid) pH 6.0 under anaerobic conditions at 37°C. The human-derived, poultry-derived, and poultry meat derived isolates were pre-cultured in LB medium under anaerobic conditions at 37°C. A thousand-fold dilution of the faeces pre-culture was added to fresh diluted SIEM buffered by 1M MES pH 6.0.

In all experiments, including conjugation experiments, aliquots of 1 ml diluted buffered SIEM were distributed in a 96-deep-wells plate. Aliquots of 100 µl of $5x10^{-3}$, $5x10^{-4}$ and $5x10^{-5}$ dilutions of the strains were grown under anaerobic conditions in the presence of the faeces culture as well as in the absence of the faeces culture for the lowest inoculum.

Conjugation experiments

Experiments were performed with and without the addition of antibiotics to investigate the effect of low levels of antibiotics on conjugation. The *Enterobacteriaceae* of the faeces culture could grow in ≤0.08 µg/ml cefotaxime, ≤0.125 µg/ml ciprofloxacin, or <12.5 µg/ml rifampicin. One isolate of each source (38.16, 39.47, and 1407; Table 1) was randomly selected for the experiments with antibiotics. Antibiotic concentrations were chosen to allow at least 80% of maximum growth for the three chosen strains as well as the faeces culture. Concentrations of 0.01 or 0.03 µg/ml cefotaxime or 0.04 or 0.08 µg/ml ciprofloxacin were used.

The number of CFUs of the isolates was determined at 0 h on VRBDA plates supplemented with 1 µg/ml cefotaxime. The number of CFUs of the third-generation cephalosporin-resistant portion of the culture was determined at 24 h

and 48 h using VRBDA plates supplemented with 1 µg/ml cefotaxime. These plates were copied to VRBDA plates supplemented with 1 µg/ml cefotaxime and 100 µg/ml rifampicin to determine the number of CFUs derived from the donor isolates among the third-generation cephalosporin-resistant colonies.

As a control donor strains and the ciprofloxacin-resistant K12 *E. coli* isolate as acceptor were added in a 1:1 ratio in LB. The occurrence of transfer was determined on LB agar plates supplemented with 1 µg/ml cefotaxime and 40 µg/ml ciprofloxacin.

Differentiation between donor and acceptor E. coli

To determine whether rifampicin could be used as a chromosomal marker for the nine ESBL donor isolates the faeces culture was checked for rifampicin resistance using Violet Red Bile Dextrose Agar (VRBDA) (Oxoid, Badhoevedorp, NL) with 0, 25, 100, and 400 µg/ml rifampicin. No growth of faecal flora was observed on agar plates containing rifampicin. Therefore, rifampicin resistance was chosen for induction of resistance in the nine donor isolates. The isolates were inoculated in 2 ml Luria-Bertani medium (LB) with a dilution series of rifampicin (Sigma, Zwijndrecht, NL) (0, 12.5, 25, 50, 100, 200, 400 µg/ml). Daily, samples with 80% growth compared to a control without rifampicin at OD_{720} were used to inoculate 200 µl in 2 ml of a new dilution series. After one week all isolates were able to grow in 400 µg/ml rifampicin. The isolates were grown for an additional three to four days in LB supplemented by 400 µg/ml rifampicin to allow compensatory mutations to develop. [13] Resistance induction was confirmed by sequencing using previously described primers. [14]

To demonstrate transfer of third-generation cephalosporin resistance to *Enterobacteriaceae* in the faecal culture isolates the *rpoB* gene of possible conjugant-derived colonies was sequenced. Isolates with wild-type *rpoB* genes or with mutations not linked to resistance that were not previously sequenced were considered isolates from the faecal culture.

To exclude the possible reversion of the induced rifampicin mutation in the donor strain DiversiLab was used to confirm that conjugants were faecal isolates. PCR for $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$ was performed as described earlier [15] to confirm the presence of an ESBL gene. Possible conjugant were analysed using plasmid-based replicon typing (PBRT) to determine whether the gene was transferred as part of the original plasmid. [9]

Results

Growth of E. coli in the presence of human gut microbiota

All poultry-derived, retail poultry meat-derived, and human-derived isolates were

able to grow in the presence of the flora of the human faeces culture in the absence of antibiotics (poultry-derived and retail poultry meat-derived isolates in Figure 1A and human-derived isolates in Figure 1B). The size of the initial inoculum of the isolates had only a minor influence on the maximum number of CFUs that was reached. In most cases $1x10^6$ to $1x10^8$ CFU were reached. Control wells containing the isolates in the same medium but without the human faeces culture showed a 1 to 2 log higher number of CFUs compared to wells with the same initial inoculum in the presence of the faeces culture. No significant difference in the number of CFUs after 24 and 48 h between isolates from different origins was detected.

Conjugation experiments

Only one ESBL-positive conjugant was detected in the experiments without antibiotics. The human-derived isolate 1407 was the donor. No conjugants were obtained from poultry or poultry meat-derived isolates.

To investigate whether low levels of antibiotics could enhance conjugation cefotaxime and ciprofloxacin were used. The poultry-derived, retail poultry meat-derived, and human-associated isolates were able to grow in the faeces culture in the presence of 0.01 and 0.03 µg/ml cefotaxime (Figure 2). A similar number of CFUs was reached in the presence of cefotaxime in comparison to the number of CFUs in the absence of antibiotics. The poultry-derived isolate reached a lower number of CFUs in comparison to the human-derived isolates in 0.04 µg/ml of ciprofloxacin and became undetectable at 0.08 µg/ml, suggesting a decrease from the original inoculum level. The retail poultry meat-derived isolate only showed lower numbers of CFUs at the highest concentration of ciprofloxacin. The human-derived isolate was insensitive to the presence of ciprofloxacin. No conjugant-derived colony was detected.

In order to further investigate conjugation with poultry and poultry meat-derived isolates in the presence of human microbiota experiments with an *E. coli* K12 as acceptor were performed. Unfortunately, the K12 *E. coli* isolate did not grow in the SIEM medium. Therefore, a conjugation control experiment in the presence of human faecal flora was not possible. In an *in vitro* control experiment in LB medium with the different donor isolates and the ciprofloxacin-resistant K12 *E. coli* isolate as acceptor yielded conjugants.

Discussion

Genotypic data from several studies suggested that poultry and retail poultry meat derived isolates may be a source of β-lactamases for humans. [4, 6, 8, 16, 17] This study shows that poultry-derived and retail poultry meat-derived *E. coli* harbouring ESBL-carrying plasmids can grow in human faecal flora containing *Enterobacteriaceae* including *E. coli*. These isolates could grow in the absence of antibiotics despite the presence of the human microflora and no significant difference in the number of CFUs was found compared to the human-derived isolates. This shows that *in vitro* the establishment of poultry-derived and retail poultry meat-derived isolates in human gut flora does not require the creation of a niche by the usage of antibiotics. These data are in agreement with a study of a single broiler-derived isolate that was used in a continuous flow system containing human faecal flora. [8] Although only one conjugant-derived colony was detected in our study in the absence of antibiotics, this indicated that transfer of ESBL-harbouring plasmids to faecal human *E. coli* is possible. A conjugation with a single broiler-derived ESBL-positive isolate in a continuous flow system yielded higher numbers of conjugants, but this may be due to a different experimental set-up and/or different bacterial isolates. [8] Taken together our results suggest that poultry or poultry meat-derived ESBL-positive *E. coli* may colonize the human gut. These bacteria may subsequently become either the cause of an infection or form a reservoir of ESBL-encoding plasmids that can be transferred to other strains or species.

We also evaluated whether the presence of antibiotics would enhance transfer of ESBL-carrying plasmids. Low concentrations of some antibiotics, e.g., ciprofloxacin, initiate a bacterial SOS-response that can increase horizontal transfer. [18] We could establish sub-MIC growth conditions in the model where the *Enterobacteriaceae* in the human faecal flora were not influenced by cefotaxime and only to a limited extent by ciprofloxacin. However, the ciprofloxacin concentrations used for induction were lower than the optimal concentration for the induction of the SOS response. This was required as the number of CFUs of *Enterobacteriaceae* in the human culture dropped below 80% in comparison to the growth in the absence of ciprofloxacin when concentrations higher than 0.08 µg/ml ciprofloxacin were used. [18] However, no conjugants were obtained suggesting that the concentrations used did not elicit a markedly increased rate of transfer from the plasmid or ß-lactamase gene resulting in the detection of conjugant-derived colonies.

Proper estimates of the rates of transfer, however, cannot be made as only one conjugant-derived colony was detected in absence of antibiotics. This can be explained by the limitations of the detection of transfer. Firstly, the experimental setup had a lower detection limit of around $1x10^4$ CFU. So, any conjugant that had not reached at least $1x10^4$ CFU was most likely not detected. Secondly, we were required to adopt a negative selection technique involving the copying of plates, because the *Enterobacteriaceae* in the human flora did not harbour a

suitable resistance marker. This technique limits the number of colonies per plate to a maximum of 100 colonies. This effectively means that theoretically the rate of transfer must be at least 1% to detect one conjugant-derived colony. This is partially remedied by the use of multiple plates yielding an estimated 500 colonies per experimental condition. Therefore the rate of transfer must be at least 2‰ to detect one conjugant-derived colony.

Due to the inability of a positive control, a previously described K12 *E. coli* isolate that can accept plasmids by conjugation, to grow in SIEM medium a conjugation control experiment in the presence of human flora was not possible. However, control experiments in LB medium with the ciprofloxacin-resistant K12 *E. coli* isolate as acceptor yielded conjugant-derived colonies. Therefore we conclude that the poultry and retail poultry meat isolates are able to transfer their ESBL-carrying plasmids to other *E. coli*. The fact that we cannot replicate this with our model may be related to the limitations of the model or inhibition by the presence of the faeces culture, although we could show transfer from a human-derived isolate.

Concluding, these findings demonstrate that poultry- and retail poultry meat-derived *E. coli* can grow in the presence of the human microbiota containing other *Enterobacteriaceae* including *E. coli*. No differences in the ability to compete with an existing flora between poultry-derived and human-derived *E. coli* were found. We did not detect a conjugation event between a poultry-derived or retail poultry meat-derived isolate and an *Enterobacteriaceae* from the human culture, although transfer was shown from an human-derived isolate.

Acknowledgments

We would like to thank Claudia Schapendonk and Norbert Sedee for their work in this study. We would also like to thank Dik Mevius and Cindy Dierikx of the CVI for their generous donation of the poulty-derived strains.

References

1. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D. 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Vet Microbiol* **145:**273-278.

2. Ewers C, Grobbel M, Stamm I, Kopp PA, Diehl I, Semmler T, Fruth A, Beutlich J, Guerra B, Wieler LH, Guenther S. 2010. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. *J Antimicrob Chemother* **65:**651-660.

3. Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, Bonten MJ, Hall MA. 2012. Population distribution of Beta-lactamase conferring resistance to third-generation cephalosporins in human clinical *Enterobacteriaceae* in the Netherlands. *PLoS One* **7**:e52102.

4. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, Platteel T, Fluit AC, van de Sande-Bruinsma N, Scharinga J, Bonten MJ, Mevius DJ. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect* **17:**873-880.

5. Clermont O, Olier M, Hoede C, Diancourt L, Brisse S, Keroudean M, Glodt J, Picard B, Oswald E, Denamur E. 2011. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect Genet Evol* **11:**654-662.

6. Cohen Stuart J, van den Munckhof T, Voets G, Scharringa J, Fluit A, Hall ML. 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. *Int J Food Microbiol* **154:**212-214.

7. Voets GM, Fluit AC, Schapendonk CM, Sharringa J, Leverstein-van Hall M, Cohen Stuart J. 2013 Identical AmpC beta-lactamase genes on identical plasmids in Dutch patients and poultry meat. [accepted pending modification]

8. Smet, A., G. Rasschaert, A. Martel, D. Persoons, J. Dewulf, P. Butaye, B. Catry, F. Haesebrouck, L. Herman, and M. Heyndrickx. 2010. In situ ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *J Appl Microbiol* **110:**541-9.

9. Ladirat SE, Schols HA, Nauta A, Schoterman MH, Keijser BJ, Montijn RC, Gruppen H, Schuren FH. 2013. High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition. *J Microbiol Methods* **92:**387-397.

10. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. *J Microbiol Methods* **63:**219-228.

11. Garcia-Fernandez, A., G. Chiaretto, A. Bertini, L. Villa, D. Fortini, A. Ricci, and A. Carattoli. 2008. Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. J Antimicrob Chemother 61:1229-33.

12. Leverstein-van Hall MA, Box AT, Blok HE, Paauw A, Fluit AC, Verhoef J. 2002. Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting. *J Infect Dis* **186:**49-56.

13. Reynolds MG. 2000. Compensatory evolution in rifampin-resistant *Escherichia coli*. *Genetics* **156:**1471-1481.

14. Xu M, Zhou YN, Goldstein BP, Jin DJ. 2005. Cross-resistance of *Escherichia coli* RNA polymerases conferring rifampin resistance to different antibiotics. *J Bacteriol* **187:**2783-2792.

15. Cohen Stuart J, Dierikx C, Al Naiemi N, Karczmarek A, Van Hoek AH, Vos P, Fluit AC, Scharringa J, Duim B, Mevius D, Leverstein-Van Hall MA. 2010. Rapid detection of TEM, SHV and CTX-M extended-spectrum beta-lactamases in *Enterobacteriaceae* using ligation-mediated amplification with microarray analysis. *J Antimicrob Chemother* **65:**1377-1381.

16. Carattoli, A. 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clin *Microbiol Infect* **14 Suppl 1:**117-23.

17. Cortes, P., V. Blanc, A. Mora, G. Dahbi, J. E. Blanco, M. Blanco, C. Lopez, A. Andreu, F. Navarro, M. P. Alonso, G. Bou, J. Blanco, and M. Llagostera. 2010. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. *Appl Environ Microbiol* **76:**2799-805.

18. Dorr T, Lewis K, Vulic M. 2009. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genet* **5:**e1000760.

Table 1: Characteristics of the isolates used.

Isolate No.	Origin	Genes	IncI1 pMLST-Type	Plasmid size (kb)	ST isolate
38.16	Poultry	CTX-M-1	ST7	100	58
1190901365	Human	CTX-M-1	ST7	100/95	58
38.27	Poultry	CTX-M-1	ST7	88	10
1190900148	Human	CTX-M-1	ST7	100	10
38.52	Poultry	CTX-M-1	ST7	100	117
39.47	Poultry meat	CTX-M-1	Non-typable	97	117
1190901407	Human	CTX-M-1	ST7	100	117
38.34	Poultry	TEM-52c	ST10	97	10
1190900320	Human	TEM-52c	ST36	95	10

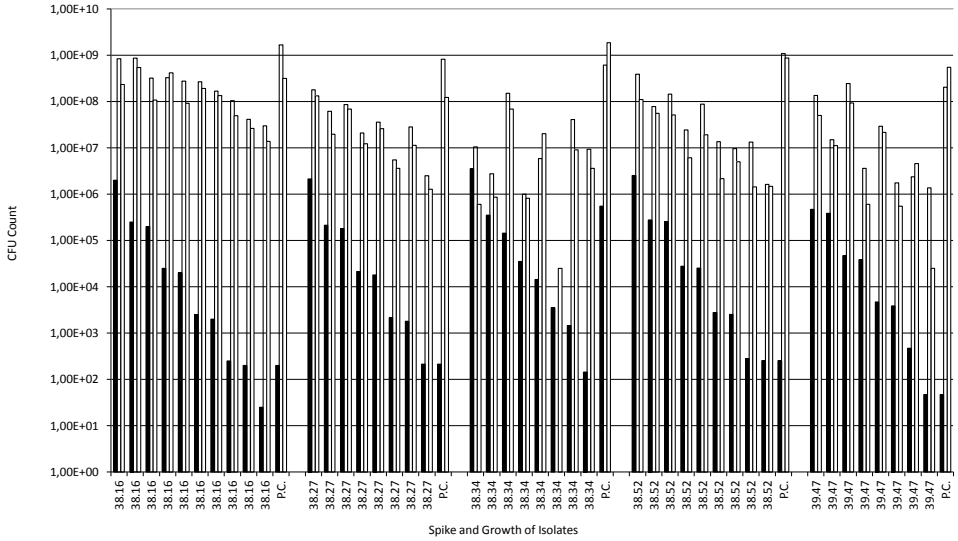


Figure 1A: Growth of the poultry-derived and retail poultry meat-derived isolates in the presence of human faeces culture in the absence of antibiotics. Each bar represents the average of three individual counts of the same experiment. The black bars represent the number of CFUs of the initial inoculum of the isolate at 0 h. The hatched bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 24 h. The white bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 48 h. At the X-axis the specific isolates are indicated. P.C.: positive control of the isolate represents the growth of the isolate under the same conditions but in absence of the faeces culture.

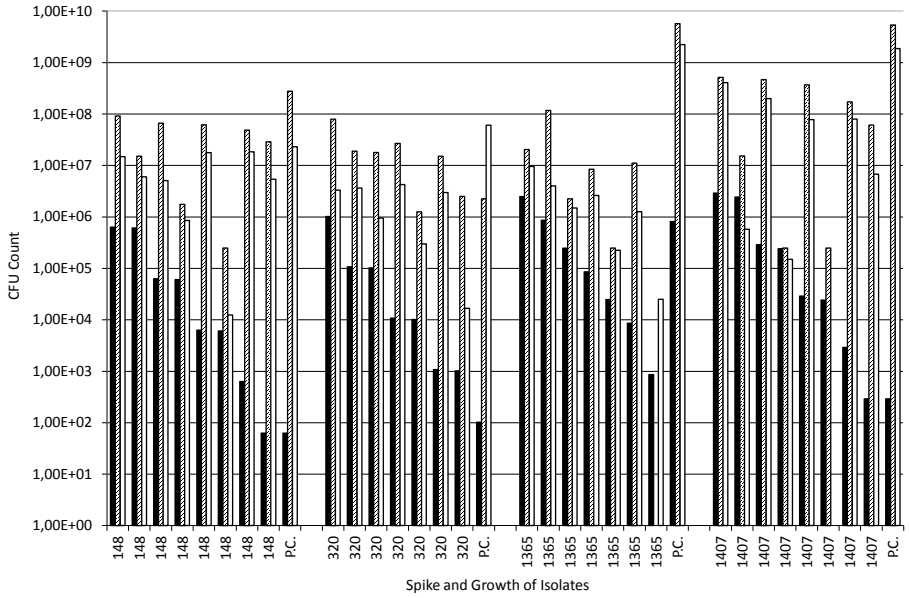


Figure 1B: Growth of the human-derived isolates in the presence of human faeces culture in the absence of antibiotics. Each bar represents the average of three individual counts of the same experiment. The black bars represent the number of CFUs of the initial inoculum of the isolate at 0 h. The hatched bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 24 h. The white bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 48 h. At the X-axis the specific isolates are indicated. P.C.: positive control of the isolate represents the growth of the isolate under the same conditions but in absence of the faeces culture.

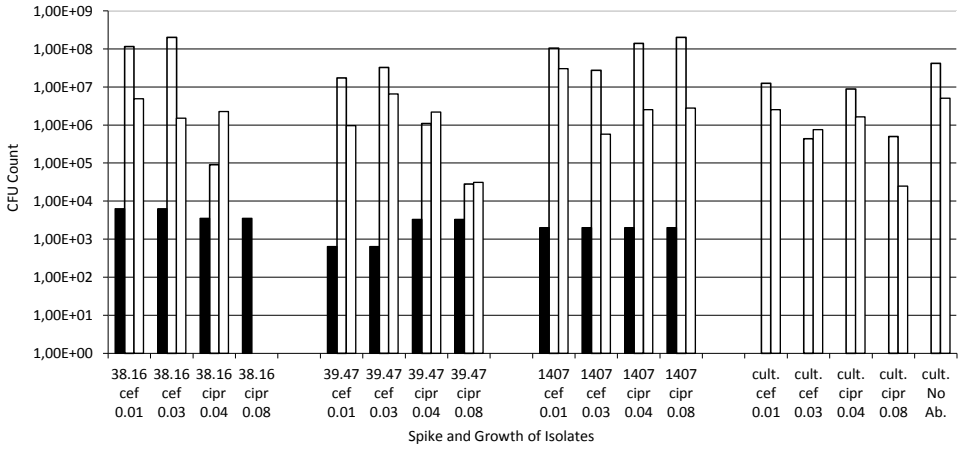


Figure 2: Influence of antibiotics on the growth of the poultry-derived (38.16), retail poultry meat-derived (39.47), human-derived (1407) isolates and *Enterobacteriaceae* of the human faeces culture. CFUs for the faeces culture were counted on VRBA plates without antibiotics. All bars represent the average of three individual counts of the same experiment. The black bars represent the number of CFUs of the initial inoculum of the isolate at 0 h. The hatched bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 24 h. The white bars represent the number of CFUs of third-generation cephalosporin-resistant isolates after 48 h. The inoculum of the culture was the standard thousand-fold dilution. On the X-axis the specific isolates are indicated. cef = cefotaxime, cipr = ciprofloxacin The numbers below the antibiotic abbreviations indicate the concentration in µg/ml.

Chapter IX

Summary and General Discussion

Enterobacteriaceae can cause a wide variety of infections ranging from gastrointestinal syndromes to wound infections and from respiratory to urinary tract infections. These infections have significant mortality rates. Many classes of antibiotics are used to treat these infections. In particular, third-generation cephalosporins are used as part of empiric treatment world-wide in case of severe infections that may be caused by *Enterobacteriaceae*. The emergence of multi-resistant *Enterobacteriaceae*, especially those carrying extended-spectrum beta-lactamases (ESBLs) and carbapenemases pose a threat to public health.

Adequate detection of resistant isolates and typing of these isolates are required for treatment, outbreak management, and monitoring the increasing prevalence of clinical relevant strains. Next to the detection of isolates the detection of the plasmids harboring resistance genes is required for studying the prevalence of resistance. Several studies have shown that outbreaks were caused by the spread of plasmids harboring resistance genes rather than the spread of a particular isolate. [1] Therefore, methods to detect and type isolates and/or plasmids are crucial. The data obtained by these methods provide the first step that may allow the discovery of the sources of antibiotic resistance for human isolates. This thesis aims to provide this insight into the genetic background of the most prevalent mechanisms of third-generation cephalosporin resistance in the Netherlands.

Currently, automated systems determine the minimal inhibitory concentrations, e.g. Vitek-2 and Phoenix, of clinical isolates. Based on the phenotype of the MIC determination, or the combination of phenotypes, an automated system may produce an alarm for “ESBL” or “carbapenemase”. However, the actual presence of this needs to confirmed. Naturally, this also holds true for screening of ESBL- or carbapenemase-producing micro-organisms. Confirmation of ESBL production is commonly performed using ESBL Etests and combination disc tests. Some labs employ molecular techniques for their routine diagnostics to detect the presence of resistance. The confirmation of carbapemenases is commonly performed using the Modified Hodge test, combination disc test or at times molecular techniques. Routine diagnostics end at this step, as it provides the required information for the treatment of a patient. However, to obtain a better understanding of the molecular epidemiology of these resistant bacteria more information is required, such as determination of the mechanism (which type of beta-lactamase) causing resistance.

Combination disc tests using several inhibitors already give insights into the Ambler class of beta-lactamase, e.g., synergy with clavulanic acid indicates an Ambler A class beta-lactamase. However, these methods do not provide information about which gene family within the Ambler class is causing the resistance. To this end additional tests are required to determine the type of beta-lactamase harbored by an isolate. For additional information on the genes present PCRs have been developed that target specific beta-lactamase families or groups within families, e.g., groups within the CTX-M, TEM, and SHV families. This can be followed by either specific PCRs or DNA sequencing.

In chapter 2 the Oxoid *Brilliance*™ CRE Agar plate was evaluated for its sensitivity and specificity in detecting carbapenemase-producing *Enterobacteriaceae*. The CRE plate had high sensitivity (≥90%) for several carbapenemases, i.e. KPC, NDM, GIM, and VIM. Sensitivity for the OXA-48 was lower at 84%, and the specificity for carbapenemase-producing *Enterobacteriaceae* was only 71%.

However the 'modified carbapenem' used in this agar was not specified, which precludes suggestions to improve the confirmation of OXA-48 or the specificity of the plates. Alternative specific screening methods use carbapenemases in combination with dipicolinic acid or boronic acid. They tend to have higher sensitivity but lower specificity.

The detection of OXA-48 using a temocillin Etest combined with meropenem double disc diffusion, as described by Hartl et al., yielded excellent results and all ESBLs/AmpC harboring isolates could be distinguished from OXA-48 producers. [2] However, in another study all 13 OXA-48 carrying isolates were resistant when tested with temocillin discs (30 µg), as were 57/153 other carbapenemase-positive isolates, implying low specificity of this testing method. [3]

Thus in regions where OXA-48 is not prevalent the *Brilliance*™ CRE Agar plate is, based on these studies, most appropriate, because of its simplicity of interpretation and better sensitivity and specificity than other screening methods. In areas where OXA-48 is highly prevalent or in outbreaks additional and/or different screening methods may be more prudent.

In chapter 3 the check-MDR CT102 DNA microarray was evaluated. The microarray can detect the most prevalent prevalent ESBL gene families (SHV, TEM and CTX-M) and carbapenemases (NDM, VIM, KPC, OXA-48 and IMP). However, the array can only detect families or groups within families in the case of ESBLs. As such, it can give a first impression about the prevalence of genetic resistance mechanisms guiding which PCRs to use for further analysis. The microarray had a high sensitivity (97% and 100% for ESBLs and carbapenemases, respectively) and specificity (100% and 98%, for ESBLs and carbapenemases, respectively). This makes it a rapid and accurate tool for the detection of carbapenemase and ESBL genes. It should be

noted that the microarray does occasionally suffer from incorrectly categorizing SHV-12 genes into the SHV-2 group instead of the SHV-4 group. The reason for this erroneous allocation of SHV-group remains unknown.

Although, the microarray is costly and time consuming it is an important tool for epidemiological research. Unlike beta-lactam family PCRs, which have one PCR for one family, the array is able to screen an isolate for several resistance mechanisms simultaneously, as well as providing data for groups of some families. Thus, it fits both in the mechanism detection as well as type specification techniques.

In chapter 4 a set of seven multiplex PCRs is described to detect 17 different carbapenemase and AmpC families using one amplification protocol. In addition, it was demonstrated that primers for the detection of ESBL genes, described in another study could be amplified with the same protocol. [4]

In comparison to the microarray, described in chapter 3, the multiplex PCRs can detect more families and some groups within families without significant cross-reactions. Thus strictly it is a beta-lactamase type determination method. It, however, lacks an internal control and requires a significant number of positive controls, which limit its usage. Moreover, without prior knowledge via, e.g., phenotypic detection or microarray, the set of 11 multiplex PCRs on itself becomes time-consuming to perform on a single sample. Next to its greater discriminatory power, the PCR products can be sequenced to obtain the exact type of ESBL or carbapenemase providing even more knowledge. Sequencing of a sample is time-consuming, however.

The multiplex PCR might be improved by modifying it into a real-time PCR. This will eliminate the need to put a PCR product on a gel, which will save time, manpower, and possible contamination, e.g., of other amplifications. To lower the costs the number of PCRs can be limited to the most prevalent and clinical relevant beta-lactamase families, e.g., CTX-M, TEM, SHV, NDM, KPC, and OXA-48 for the Netherlands.

At present it will depend on individual laboratory circumstances which methods are implemented, but the future of diagnostics and research lies with molecular techniques. In the near future it is likely that after a “ESBL” warning by an automated system isolates will undergo whole genome sequencing, which will yield resistance mechanisms, virulence genes, e.g., toxins, and typing data with a ‘super’ multi-locus sequence typing (MLST) software pipeline.

The reproducibility of DiversiLab (bioMérieux) isolate typing system, using an automated repetitive-sequence-based PCR (rep-PCR) technique, was evaluated for *Escherichia coli* and *Klebsiella* spp. in chapter 5. Repetitive-sequence-based PCR uses species-specific repeat sequences. The size of the repeat sequences for each repeat locus is conserved, but the number of repeat loci, as well as the number repeats

at a particular locus, differ per isolate, creating unique finger-prints. Advantages of DiversiLab are that it uses automated BioAnalyzer and standardized kits, avoiding the need for quality control of the chemicals used. This facilitates in theory, easier implementation in diagnostic laboratories. However, the concordance between the laboratories performing local analysis was low for the clusters and only reached acceptable levels for unique isolates. Central analysis significantly improved the concordance for both groups. However, the discriminatory power remained lower in comparison to pulsed-field gel electrophoresis (PFGE). Effectively, unique isolates in DiversiLab are most likely not related, whereas isolates that are clustered by DiversiLab should raise a red flag that requires additional tests or epidemiological evidence to consider them as a true cluster.

In this study PFGE, which is commonly used, had a higher discriminatory power than DiversiLab. However, it is very time consuming and requires proper training. Even then reproducibility of the banding patterns is limited. Furthermore, as a fingerprinting technique, the addition of more isolates may hamper the identification of unique clusters due to blurring of the borders of said clusters. This holds true for other finger printing techniques as well, e.g., amplified fragment length polymorphism.

Besides the lower discriminatory power, DiversiLab suffers from the lack of an international database, like Pulse-Net for PFGE. This makes it currently impossible to track international clones using this technique. We, therefore, suggested strict adherence to an improved amplification protocol. With the suggested changes of better DNA isolation methods, more reproducible DNA amplification and strict adherence to protocols, reproducibility and interpretation will improve. When the discrepancies are reduced to acceptable levels it might be prudent to organize an international databank in order to identify potential clones. But even with an international databank, DiversiLab suffers from its restricted use of specific species only.

In short, although DiversiLab is not a perfect typing method, at present, it has some benefits for epidemiological purposes. In the near future, finger-printing techniques are likely to be replaced by whole-genome sequencing, though. This allows comparison of data across the globe. Furthermore, the discriminatory power of whole-genome sequencing is unparalleled which maximizes cluster identification. Using a software pipeline to screen house-hold genes one could perform a ‘super’ MLST in order to type isolates.

The techniques described in the previous chapters, among others, were used to determine the population distribution of beta-lactamase genes that confer resistance to third-generation cephalosporins in clinical *Enterobacteriaceae*. The results of this study are described in chapter 6.

Like other nation-wide studies in other countries, the most prevalent ESBL family in the Netherlands was CTX-M. In fact, the most common ESBL was CTX-M-15, which is also the most common ESBL type in western and northern European countries and the USA. In eastern Europe, e.g., Poland, CTX-M-3 is more common and in southern Europe, e.g., Spain, CTX-M-9. However, these countries also report a rise in prevalence of CTX-M-15. Of the SHV and TEM family SHV-12 and TEM-52 were the most prevalent. SHV-12 has also been reported by other nations, but TEM-52 has not been frequently reported in clinical studies. In fact, the second most prevalent CTX-M, CTX-M-1, has not been reported frequently in clinical studies. These two ESBLs combined account for a large proportion (30%) of the ESBLs in Dutch human isolates. CTX-M-1 and TEM-52 were commonly reported in poultry, however.

The last finding of chapter 6 led to an investigation into the poultry and retail poultry meat as a source for ESBL-harboring human *Enterobacteriaceae* isolates. The study concluded that up to 35% of human ESBL-positive *E. coli* contained ESBL genes and in 19% contained ESBL-genes located on plasmids that were genetically indistinguishable from those obtained in poultry isolates. The predominating genes were $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ (86% humans, 77% poultry, 75% retail poultry meat). Although these findings did not unequivocally prove that poultry and retail poultry meat is a source of infection for humans it was suggestive that this might be a route of transfer. In chapter 7 poultry and retail poultry meat were investigated as a possible source for AmpC beta-lactamases. We, therefore, compared the pAmpC genes, their plasmids, and bacterial strain types between *E. coli* isolates from retail chicken meat and clinical isolates in the Netherlands.

In this study similarities between the isolates, ESBL-harbouring plasmids, and resistance genes were detected. The combination of these isolates, plasmids, and genes have been rarely reported in human isolates. This does not, however, provide conclusive evidence for poultry and retail-poultry meat as a source for clinical *Enterobacteriaceae*. Contamination by a shared third source for both humans and poultry or the dissemination of a successful plasmid to both human and poultry isolates are alternative hypotheses that can explain these findings.

The conclusions of chapter 7 are weakened by the fact that the study is an ecological study rather than an epidemiological study for it is based on two observations in two reservoirs which are linked due to molecular characterization. Furthermore, the human and meat samples come from different years and different locations. Studies that have collected samples from one location and one time point that support the hypothesis have been performed, however. For instance, there has been a study on broiler farms where broiler farmers acquired beta-lactamase-harboring isolates with the same MLST type as their flock, as well as a plasmid harboring the same beta-lactamase as described by Dierikx et al. [5] It also showed that the strain carried changed after an incubation period when a new flock arrived. However, this did only occur in a minority of broiler farms.

As stated, in chapter 7 the hypothesis of poultry and retail poultry meat as a source for beta-lactamase harboring *Enterobacteriaceae* was based on genetic similarities on the level of isolate type, plasmid type, and beta-lactamase gene. In chapter 8 a model is used to test the ability of ESBL-harboring poultry-derived and retail poultry meat-derived *E. coli* to grow in the presence of human microbiota, including *E. coli* microbiota-derived, as well as the possibility of transfer of the ESBL-harboring plasmid from these *E. coli* to the *E. coli* from the microbiota. Only one case of transfer was detected. This transfer took place from a control human-derived *E. coli* to a microbiota-derived *E. coli*. More transfer may have taken place, however, for the experimental setup had a lower detection limit of around $1x10^4$ colony forming units (CFU). All poultry-derived and retail poultry meat-derived isolates were able to grow in the presence of microbiota, even in the absence of antibiotics. The ability of poultry-derived and retail-poultry meat-derived isolates to grow in the presence of human microbiota, however, is not conclusive proof that poultry acts as a source for clinical *Enterobacteriaceae*. It 'merely' shows the potential of isolates associated with a poultry source to thrive among the human microbiota.

The model used in this chapter is not the perfect model for the human intestinal tract. In fact, a more accurate *ex vivo* model is available at TNO called the TIM-model. The TIM-model mimics the bowel movements, including the turn-over of the microbiota. When a follow-up study is to be performed with an *ex vivo* model the TIM-model would be preferable. Moreover, qPCR can be used to better track the growth of the donor strains and the *E. coli* of the human microbiota. Alternatively, either by induction or by the transformation of a plasmid with a selection gene the donor strain can be easily distinguished from the acceptor isolates lowering the chance of missing transfer events.

As discussed, the study described in chapter 7, as well as the study suggesting transfer of ESBL from poultry to humans, do not unequivocally prove that poultry and retail poultry meat are sources of infections for humans. Yet, these findings were suggestive for such a route of transfer. The results of chapter 8, showing that poultry- and retail poultry meat-derived *E. coli* can grow *ex vivo* in the presence of the human microbiota or transfer plasmids to *E. coli* from the human microbiota, also lend support to this theory. However, none of these studies can unequivocally demonstrate that ESBL-producing bacteria or ESBL-containing plasmids are transferred from poultry to humans via food, to subsequently cause infection. Moreover, the potential role of other reservoirs for humans has not been determined, let alone that the relative contributions of different reservoirs have been quantified.

It will be difficult to prove definitely that poultry is a source of third-generation cephalosporins in human isolates. The best achievable study would use whole genome sequencing of isolates from different reservoirs. With whole genome sequencing the maximum discriminatory power will be achieved as single nucleotide

changes can be detected. This includes plasmids. So, both isolates and plasmids can be compared in far more detail than MLST, DiversiLab, PBRT and pMLST allow. When the isolates do not differ in sequence or show only a few single nucleotide polymorphisms (SNPs) it is highly likely that the isolates are closely related and that transfer has occurred recently. The same is true for plasmids. When there a numerous SNPs present in isolates or plasmids from the different reservoirs recent transfer between the reservoirs is unlikely. In the proposed study preferably samples of soil, poultry, poultry-meat and humans would be collected at the same location within a limited time frame.

Although the actual impact of the poultry reservoir on the human reservoir, or vice versa, could not be determined with the data obtained in this thesis, it is safe to say that poultry is certainly not the only source for ESBL-harboring *Enterobacteriaceae* for humans, though. In fact, studies examining other reservoirs, like companion animals, livestock or vegetables, also find similarities with the human reservoir. The amount of contribution of each reservoir to the human reservoir remains unknown, however. Nor that every piece of poultry meat, despite that 90-100% of meat, depending on the study, is contaminated with beta-lactamase harboring *E. coli*, will lead to the acquisition by humans of poultry-derived *E. coli* harboring beta-lactamases.

Certainly other forms of transmission may be important in the spread of beta-lactamases, whether this is from other non-human sources or due to, e.g., nosocomial spread. For instance people traveling abroad, who come in contact with the local medical facilities, have been shown by several studies to contribute to the spread of multi-drug resistant isolates from areas with a high incidence to areas with lower incidence. Moreover, people travelling abroad also have been shown to more frequently carry resistant isolates after return, even without exposure to medical facilities. The route of acquisition, however, remains unknown for these patients and could come from any of the mentioned routes of transmission and more.

It would unrealistic, however, to believe that antibiotic resistance would quickly vanish even if all possible sources would be detected and contained. As more patients are infected with multi-drug resistant isolates the question becomes how to treat these patients. These multi-drug resistant isolates are still rare, luckily. Future treatment options may include strain specific medication administrated, e.g., within 24 hours after hospitalization. Such strains might require new combinations or rarely used drugs. In case of an outbreak of a highly resistant strain isolate typing by whole genome sequencing may be the key in the future. Molecular techniques are becoming ever more present in diagnostic labs and the price of whole genome sequencing is diminishing every year. With improved software pipe lines that can identify these resistant isolates within a day, as well as alerting to the presence of which particular genes are present that are associated with toxins, virulence, and

resistance, proper and adequate medication can be administrated to a patient that is tailored to combat the specific strain the patient suffers from.

Referenties

1. Ruiz-Garbajosa P, Curiao T, Tato M, Gijón D et al. Multiclonal disperal of KPC genes following the emergence of non-ST258 KPC-producing *Klebsiella pneumoniae* clones in Madrid, Spain. *J Antimicrob Chemother.* 2013; [Epub ehead of print]

2. Hartl R, Widhalm S, Kerschner H, Apfalter P. Temocillin and meropenem to discriminate resistance mechanisms leading to decreased carbapenem susceptibility with focus on OXA-48 in *Enterobacteriaceae*. *Clin Microbiol Infect.* 2013; **19**: E230-232

3. Day KM, Pike R, Winstanley TG, Lanyon C et al. Use of faropenem as inidicator of carbapenemase activity in the *Enterobacteriaceae*. *J Clin Microbiol.* 2013; **51**: 1881-1886

4. Dallenne C, Da Costa A, Decre D, Favier C et al. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae. J Antimicrob Chemother.* 2010; **65**: 490-495

5. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A et al. Extended-spectrum-beta-lactamase- and Ampc-beta-lactamase-producin *Escherichia coli* in Dutch broilers and broiler famers. *J Antimicrob Chemother.* 2013; **68**: 60-67

Nederlandse samenvatting

Enterobacteriaceae kunnen een breed scala aan infecties en ziektes veroorzaken. Deze variëren van infecties van wonden, ademhalingsstelsel tot aan infecties van de urine wegen. Enkele van deze infecties zijn geassocieerd met een hoog sterftecijfer. Voor het behandelen van deze infecties worden verschillende klassen van antibioticagebruikt. In het bijzonder, maken derde-generatie cefalosporines deel uit van de empirische behandeling van zware infecties die mogelijk het gevolg zijn van *Enterobacteriaceae*. Het verschijnen van multi-resistente *Enterobacteriaceae*, met name isolaten die extended-spectrum β-lactamases (ESBLs) en carbapenemases met zich meedragen, is daarom een significante bedreiging voor de volksgezondheid.

Accurate detectie en typering van deze resistente isolaten zijn nodig voor de behandeling van patiënten, signalering van de stijgende prevalentie van klinisch relevante isolaten en uitbraak management. Daarnaast is de detectie van de plasmides die de resistentie genen met zich mee dragen van belang. Verschillende studies hebben aangetoond dat sommige uitbraken eerder gekoppeld zijn aan de verspreiding van plasmides dan de verspreiding van een specifiek isolaat. Het detecteren en typeren van isolaten en plasmides is daarom cruciaal. Daarnaast kan, dankzij de kennis die verkregen wordt met behulp van epidemiologische en prevalentie studies, een start gemaakt worden met de zoektocht naar de mogelijke bron(nen) van de antibiotica resistentie genen. Het doel van dit proefschrift is om inzicht ter verschaffen in de genetische achtergrond van de meest prevalente derde-generatie cefalosporine resistentie mechanisme in Nederland.

Momenteel wordt het testen van isolaten voor resistentie voornamelijk gedaan met behulp van automatische systemen in Nederland, bijv. Vitek-2 en Phoenix. Op basis van de fenotype van de MIC determinatie, of the de combinatie van fenotypes, kan een automatisch systeem een alarm genereren voor “ESBL” of “carbapenemase”. Dit resultaat moet echter worden bevestigd. Dit geldt ook voor screening methodes voor ESBL of carbapenemase producerende bacteriën. ESBL confirmatie wordt met name gedaan door het gebruik van een ESBL Etest of de Combination Disc test. Enkele laboratoria in de routine diagnostiek maken gebruik van moleculaire technieken voor het detecteren van resistentie. De confirmatie van carbapenemases wordt voornamelijk gedaan met de Modified Hodge test, Combination Disc test of moleculaire technieken. Routine diagnostiek eindigt na deze stap omdat dit voldoende informatie oplevert voor een adequate behandeling van de patiënt. Voor het begrijpen van de moleculaire epidemiologie van deze resistente bacteriën is echter meer kennis nodig. Het vaststellen van het mechanisme (welk type beta-lactamase) dat de resistentie veroorzaakt is bijvoorbeeld van belang. Combination

Disc testen die gebruik maken van een variëteit aan remmers geven al inzicht in de Ambler klasse van de beta-lactamase. Echter, deze informatie is niet afdoende aangezien ze geen informatie geeft over welk gen binnen de Ambler klasse de resistentie veroorzaakt. Hiervoor zijn additionele testen nodig, zoals PCRs die specifieke beta-lactamase families of groepen binnen beta-lactamase families kunnen detecteren, zoals groepen binnen de CTX-M, TEM en SHV families. Dit kan gevolgd worden door gen specifieke PCRs of DNA sequencen.

In hoofdstuk 2 wordt de *Brilliance*™ CRE Agar geëvalueerd voor de sensitiviteit en specificiteit voor het detecteren van carbapenemase producerende *Enterobacteriaceae*. De CRE Agar had een hoge sensitiviteit (≥90%) voor verscheidene carbapenemases, bijv. KPC, NDM, GIM en VIM. De sensitiviteit voor het detecteren van OXA-48 producerende *Enterobacteriaceae* was maar 84% en de specificiteit voor carbapemenase producerende *Enterobacteriaceae* was slechts 71%.

In hoofdstuk 3 wordt de check-MDR CT102 DNA microarray geëvalueerd. Deze microarray kan een breed scala aan ESBL en carbapenemase families en soms zelfs groepen binnen deze familie detecteren, maar niet individuele beta-lactamase types. Het kan dus een eerste indruk geven over de prevalentie van de genetische resistentie mechanismes en daarmee hulp bieden of en welke PCRs uitgevoerd moeten worden voor verdere analyses. De microarray had een hoge sensitiviteit (respectievelijk 97% en 100% voor ESBLs en carbapenemases) en specificiteit (respectievleijk 100% en 98%, voor ESBLs en carbapenemases). Dit maakt het een snelle en gevoelige test voor de detectie van carbapenemase en ESBL genen. Echter, de microarray karakteriseert soms isolaten die SHV-12 bezitten als lid van de SHV-2 groep in plaats van de SHV-4 groep. We konden helaas niet achterhalen waarom deze fout optreedt.

In hoofdstuk 4 wordt een set van zeven multiplex PCRs beschreven. Deze set biedt de mogelijkheid voor het detecteren van 17 verschillende carbapenemase en AmpC families met behulp van één amplificatie protocol. Daarnaast tonen we aan dat de primers die ontwikkeld waren voor het detecteren van ESBL genen en beschreven in een andere studie, ook gebruikt kunnen worden met het amplificatie protocol beschreven in dit hoofdstuk.

De reproduceerbaarheid van het DiversiLab isolaat typerings system (bioMérieux), dat gebruik maakt van een automatische repetitive-sequence-based PCR (rep-PCR) techniek, wordt geëvalueerd voor *Escherichia coli* en *Klebsiella* spp. in hoofdstuk 5. Repetitive-sequence-based PCR maakt gebruik van species-specifieke sequenties die een aantal malen achter elkaar herhaald worden. De lengte van de sequentie die herhaald wordt, of te wel het aantal base paren, is voor iedere serie van herhaalde sequentie geconserveerd. Echter, zowel het aantal loci als het aantal malen dat de sequentie herhaald wordt binnen een locus verschilt per isolaat. Dit creëert voor isolaten die genetisch onderling verschillen unieke sets van amplificatie producten.

Dit wordt vervolgens weergegeven als zogenoemde 'finger-prints'. DiversiLab heeft als voordelen dat het gebruik maakt van de geautomatiseerde BioAnalyzer en gestandaardiseerde kits, die de noodzaak voor kwaliteitscontroles voor de componenten van deze kits overbodig maakt. Dit maakt het in theorie makkelijker om de techniek in gebruik te nemen op diagnostiek laboratoria. Wanneer de methode echter uitgevoerd werd door verschillende laboratoria was de reproduceerbaarheid van de 'finger-prints' laag voor de clusters van verwante isolaten en bereikte slechts een acceptabel niveau voor de unieke isolaten. Door het uitvoeren van een centrale analyse verbeterde de concordantie voor beide groepen. In vergelijking tot pulsed-field gel electrophoresis (PFGE) had DiversiLab een lager resolutie niveau om isolaten te onderscheiden. Ongeacht van de uitkomst van DiversiLab, hetzij een uniek isolaat dan wel behorend tot een cluster, kunnen aanvullende testen nodig zijn, is vooral het wel of niet aanwezig zijn van een epidemiologisch verband tussen nodig om deze uitkomst te ondersteunen.

Verschillende van de boven beschreven techieken werden in hoofdstuk 6 gebruikt om de populatie distributie van beta-lactamase genen die resistentie tegen derde-generatie cefalosporines geven in klinische *Enterobacteriaceae* te beschrijven.

De meest prevalente ESBL familie die gevonden werd, was de CTX-M familie, waarvan CTX-M-15 het meest voorkwam. Deze resultaten zijn in overeenstemming met resultaten voor west Europa, noord Europa, en Amerika. Dit in tegenstelling tot oost Europa, zoals in Polen, waar CTX-M-3 meer voorkomt, en in zuid Europa, bijvoorbeeld Spanje, waar CTX-M-9 domineert. Ook in oost en zuid Europa wordt echter de opmars van CTX-M-15 gesignaleerd. Van de SHV en TEM families waren SHV-12 en TEM-52 het meest prevalent. SHV-12 wordt ook vaak gesignaleerd in nationale studies in andere landen, maar TEM-52 wordt niet vaak gevonden in klinische studies. Ook de tweede meest voorkomende CTX-M, CTX-M-1, wordt niet vaak gerapporteerd in studies van klinische isolaten. Deze twee zeldzamere ESBLs vormden echter een ongewoon groot gedeelte (30%) van de in deze studie gevonden ESBLs. CTX-M-1 en TEM-52 worden echter wel vaak gerapporteerd in pluimvee.

Deze laatste bevindingen van hoofdstuk 6 waren de aanleiding om onderzoek te doen naar kip en kippenvlees als mogelijke bron voor ESBL-dragende *Enterobacteriaceae* voor mensen. Uit de resultaten werd geconcludeerd dat maximaal 35% van de humane ESBL-positieve *E. coli* ESBL genen bezaten die genetisch niet te onderscheiden waren van de ESBL genen die gevonden werden in pluimvee en kippenvlees. In 55% van deze isolaten lag het gen op een plasmides die met de gebruikte metohden niet genetisch te onderscheiden waren van de plasmides in pluimvee die ook deze ESBL genen dragen. In alle drie compartimenten waren dezelfde twee genen dominant ($bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ (86% humane, 77% pluimvee, 75% kippenvlees isolaten)). Deze bevindingen zijn geen onomstotelijk bewijs dat pluimvee en kippenvlees als bron dienen voor mensen, maar zijn wel suggestief

dat dit een mogelijke route is van infectie. In hoofdstuk 7 werd onderzocht of pluimvee en kippenvlees ook als mogelijke bron voor AmpC beta-lactamases voor de mens kon dienen. Hiervoor werden Nederlandse klinische en kippenvlees *E. coli* isolaten vergeleken voor de pAmpC genen, de plasmides waar deze op lagen en de overeenkomst tussen de isolaten zelf.

Er werden overeenkomsten op het niveau van isolaat, plasmide en pAmpC gen gevonden tussen *E. coli* isolaten afkomstig van kippenvlees en mensen. De gevonden combinatie van isolaat type, plasmide en resistentie gen is zelden in isolaten afkomstig van de mens gerapporteerd. Dit is echter geen onomstotelijk bewijs dat kip en kippenvlees als een bron dienen voor klinische *E. coli*.

De bevindingen in hoofdstuk 7 waren gebaseerd op de genetische overeenkomsten die waren gevonden op het niveau van isolaat type, plasmide en resistentie gen tussen kippenvlees en mensen isolaten. Ondanks dat dit geen omstotelijk bewijs is blijft het suggestief dat pluimvee en kippenvlees mogelijk als bron dienen voor verscheidende beta-lactamases voor mensen. De resultaten van hoofdstuk 8 tonen aan dat *E. coli* afkomstig van pluimvee- en kippenvlees kunnen groeien in de aanwezigheid van de humane microbiota in een *ex vivo* model. Daarnaast werd er suggestief bewijs verkregen dat plasmides kunnen worden overdragen. Dit ondersteund de hypothese dat kip en kippenvlees een potentiële bron voor ESBL genen bij humane isolaten zijn. Het gepresenteerde onderzoek kan echter niet zonder twijfel aantonen dat ESBL producerende *E. coli* of ESBL dragende plasmides worden overgedragen van pluimvee naar mens via de voedselketen en vervolgens eventueel een infectie veroorzaken. Daarnaast is de potentiële rol van andere reservoirs voor ESBL of pAmpC dragende isolaten bij mensen niet bepaald als onderdeel van dit proefschrift.

Concluderend, dit proefschrift beschrijft enkele technieken die van dienst kunnen zijn in de opsporing van en onderzoek naar *Enterobacteriaceae* die resistent zijn voor derde-generatie cefalosporines. Verder beschrijft dit proefschrift de prevalentie van derde-generatie cefalosporine resistente *Enterobacteriaceae* in Nederland en beschrijft onderzoek naar de mogelijkheid dat kip en kippenvlees een bron zijn van ESBL-producerende isolaten en/of ESBL-dragende plasmides voor klinische isolaten. Onomstotelijk bewijs dan wel voor dan wel tegen deze hypothese werd echter niet verkregen. Hiervoor is aanvullend onderzoek nodig. Dit zal een whole genome sequencing onderzoek vereisen gezien de benodigde resolutie die nodig is op zowel het niveau van de isolaten als wel de plasmiden.

Dankwoord

Hoewel mij vaak genoeg is verteld dat dit mijn boekje is, is dat eigenlijk een leugen. Er is een enorme scala aan mensen die in meer of mindere mate heeft bijgedragen aan dit boekje. Om er voor te zorgen dat ik iedereen benoem en ook wegens het milieu aspect zal ik mijn dankwoord erg algemeen houden en bijzondere individuen eruit lichten.

Op de eerste plaats van dit dankwoord horen natuurlijk mij co-promotoren Ad Fluit en James Cohen Stuart.

Ad, oorspronkelijk was je er bij gehaald om van de zijlijn er voor te zorgen dat de technische kant van mijn werk enigszins soepel verliep. Echter, naar mate de tijd vorderde werd jouw rol als maar groter. Zeker toen James en Maurine vertrokken naar nieuwe uitdagingen. Ik heb me al vaker uitgedrukt over mijn dank, maar laat dat ook hier zwart op wit staan. Dank voor je ervaring, nuchterheid, en scherpe inzichten.

James, ondanks dat je in mijn derde jaar vertrok naar het MCA, bleef je tot het einde een belangrijke bijdrage leveren voor dit boekje, zowel in praktische zin als wel op het vlak van het schrijven. Zeker jou onuitputtelijke energie om zinnen scherp te formuleren en hier over na te denken zal mij bijblijven.

De volgende op mijn lijst is natuurlijk mijn promoter, Marc Bonten. Marc, ik kon altijd mijn eigen weg gaan, maar als ik problemen had stond jij voor me klaar. Ook als we samen aan een stuk werkte was dat een prettige ervaring waar jij de knoop kon doorhaken waar het moest en we snel en efficient het stuk naar het volgende niveau tilde.

Verder mag ik niet Maurine Leverstein-van Hall ongenoemd laten. Maurine, in de eerste twee jaar heb ook jij flink wat energie gestoken in dit project en zonder jou was het nooit mogelijk geweest deze PhD te doen. Hiervoor ben ik je dank verschuldigd.

Ook de andere (ex-)leden van de ESBL groep wil ik hier graag noemen: Tamara, Thijs, Wouter, Claudia, Karin, Anna, en in het bijzonder mijn student, Arian, en ‘gouden handjes’ Jelle. Zeker de laatst genoemde heeft mij vaak geholpen vanaf de eerste dag met zowel zijn ‘gouden handjes’ als wel met het opvijzelen van moreel. Ik kon het dan ook niet laten om Jelle als paranimph te vragen.

Verder zijn er meerdere kamergenoten de revue gepasseerd: Nicole, Bart, Alex, Lydia, Laurens en Amin. Bedankt voor de lol, de wetenschappelijke discussies, maatschappelijke discussies, en de algemene discussies. Ik heb er flink wat herinneringen aan over gehouden.

Verder acht ik de hele microbiologie afdeling iets te groot om allemaal hier individueel te noemen ondanks dat ik zeker met flink wat personen leuke gesprekken

en tijden heb gehad. Twee mensen wil ik nog wel even graag er uitlichten: Marco (je spreadsheets zijn er nog altijd) en Machiel (interessante gesprekken als wel de praktische hulp).

Ook heb ik een flink tijdje (hoe vaak is het vertrek wel niet uitgesteld?) bij TNO Zeist op de MBT afdeling mogen vertoeven. Ook hier ben ik bang dat als ik ieder persoon zou opnoemen ik er een vergeet en dus de torn van die persoon over mij afroep. Echter om er een paar uit te lichten: Norbert (bedankt voor alle hulp tijdens de proeven ook als deze me gek dreven), Frank (voor het met Ad en mij bekokstoven van hoofdstuk 7) en Roy (voor het geven van de mogelijkheid om dit te werk te doen bij TNO).

Ook het samenwerkingsverband met het CVI (Dik, Cindy, en Alieda) vond ik erg leuk om te doen. Ondanks dat er daar van weinig in dit boekje te zien is, is het wel degelijk onderdeel geweest van mijn PhD.

Ook de samenwerking met het bedrijfsleven, die mij ook hoofdstukken opleverde, waren een ervaring opzich. Mijn dank gaat uit naar Vivianne Monte (bioMerieux) en Peter Boleij (CheckPoints).

Naast werk is ontspanning ook belangrijk. Hiervoor zijn meerdere vrije tijds bestedingen de revue gepasseerd. Zo heb ik flink wat pijltjes en plezier beleeft bij mijn boogschutters-vereniging, Geronimo.

Ook is het zien van de leden van mijn jaarclub, Entropia, altijd weer erg gezellig. Ik ben blij weer terug te keren in jullie gezelschap en dank jullie voor jullie geduld tijdens de laatste maanden waar ik nauwelijks ergens bij aanwezig kon zijn. Bedankt voor jullie gezeligheid: Anja, Bram, Enna, Fedor, Irene, Joris, Marcus, Teije, Tijs, Ylvie.

I also spend quiet a few hours behind the computer for relaxation. Though most games I play are single-player RPGs and Strategy games, there is one important exception. According to the statistics I have currently over 2000 hours of my life spend on Team Fortress 2, a first-person cartoonesque shooter. This is certainly, in no small part to the many friends I have in the game. I thank the guys and girls from German Headquarters, my former competitive team ‘Ducky Liberation Forces’, the old SilentForces, and all the people I met randomly through this game.

Een hobby die ik weigerde op te geven is die van mijn roleplaying D&D groepen. Ongeveer om de twee weken kon ik mijn weer heer en meester achten en mij wanen in een wereld zonder al die PhD problemen voor een wereld met slechts hier een daar een dodelijk encounter. Linda, Richard, Nico, Paulien, Erik, Remko, en Bas ontzettend bedankt voor al die leuke tijd al spelende wijze dan wel al discussierende wijze. De laatst genoemde, die mijn tweede paranimph is, wil ik er even uitlichten, want sinds de tijd dat ik met hem samen in een huis woonde, zijn trouwerij in Polen en daarna is hij een geweldig voorbeeld van hoe twee tegenpolen uitstekend door een deur kunnen. Bas bedankt je humor en je heerlijk relaxte houding naar alles toe.

De andere die er even uitgelicht moet worden is Richard, gezien dankzij zijn talent ik een unieke kaft heb.

Verder wil ik ontzettend mijn ouders bedanken die altijd voor me klaar staan wat er ook maar aan de hand was. Meerdere keren hebben ze me in huis geholpen, van toen ik naar Zeist verhuisde tot tijdens de PhD tijd, als wel met mij gepraat over van alles wat me maar dwars zat. Eveneens mijn zus, die mij reeds voor ging in het PhD halen, als wel haar vriend, Thijs, die in hetzelfde traject zit, waren goede gesprekspartners tijdens moeilijke tijden.

Last but not least, ondanks dat ze niet kan lezen, wil ik hier Jedi melden. In mijn tweede jaar geadopteerd op de leeftijd van 11 heeft deze lieve poes mij veel plezier en liefde geschonken. Hoewel ze misschien niet altijd de handigste tijd kiest om perse bij mij in de buurt te willen zijn zou ik voor geen goud mijn PhD tijd zonder haar hebben gedaan.

Publication List

These papers that have appeared in scientific journals but are not presented in this thesis.

1. van Dijk K, **Voets GM**, Scharringa J, Voskuil S, Fluit AC, Rottier WC, Leverstein-Van Hall MA, Cohen Stuart JW. A disc diffusion assay for detection of class A, B and OXA-48 carbapenemases in Enterobacteriaceae using phenyl boronic acid, diplicolinic acid and temocillin. *Clin Microbiol Infect.* 2013; [Epub ahead of print]
2. Platteel TN, Leverstein-Van Hall MA, Cohen Stuart JW, **Voets GM**, van den Munckhof MP, Scharringa J, van de Sande N, Fluit AC, Bonten MJ, ESBL National Surveillance Working Group. Differences in the antibiotic susceptibility of human Escherichia coli with poultry-associated and non-poultry-associated extended-spectrum beta-lactamases. *Eur J Clin Microbiol Infect Dis.* 2013; **32**: 1091-1095
3. Platteel TN, Cohen Stuart JW, de Neeling AJ, **Voets GM**, Scharringa J, van de Sande N, Fluit AC, Bonten MJ, Leverstein-van Hall MA, ESBL national surveillance working group. Multi-centre evaluation of a phenotypic extended spectrum β-lactamase detection guideline in the routine setting. *Clin Microbiol Infect.* 2013; **19**: 70-76
4. Platteel TN, Stuart JW, **Voets GM**, Scharringa J, van de Sande N, Fluit AC, Leverstein-Van Hall MA, ESBL national surveillance working group. Evaluation of a commercial microarray as a confirmation test for the presence of extended-spectrum β-lactamases in isolates from the routine clinical setting. *Clin Microbiol Infect.* 2011; **17**: 1435-1438
5. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, **Voets GM**, van den Munckhof MP, van Essen-Zandbergen A, Platteel T, Fluit AC, van de Sande-Bruinsma N, Scharinga J, Bonten MJ, Mevius DJ, National ESBL surveillance group. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clin Microbiol Infect.* 2011; **17**: 873-880
6. Leverstein-Van Hall MA, Stuart JC, **Voets GM**, Versteeg D, Tersmette T, Fluit AC. Global spread of New Dehi metallo-β-lactamase 1. *Lancet Infect Dis.* 2010; **10**: 830-831
7. Leverstein-van Hall MA, Stuart JC, **Voets GM**, Versteeg D, Roelofsen E, Fluit AC. Carbapenem-resistant Klebsiella pneumoniae following foreign travel. *Ned Tijdschr Geneeskd.* 2010; **154**: A2013.

Curriculum Vitae

Guido Maarten Voets was born on October 20, 1982 in Leiden, in the Netherlands. He graduated from high school, Stedelijk Gymnasium Leiden, on June 29, 2001. In the same year he started his study Biotechnology at the Wageningen University and Research Center. He performed his first internship under the supervision of Dr. E. H. M. Limpens and Prof. Dr. A. H. J. Bisseling at the department of Experimental Plant Sciences at Wageningen University and Research Center. His second internship performed at Intercell in Vienna. He was supervised by Ir. Birgit Noiges and Ir. Andreas Meike, both affiliated with Intercell, and by prof. dr. ir. H. F. J. Savelkoul at the department of Celbiology and Immunology at Wageningen University and Research Center. He graduated on June 19, 2008. In July 2009, he started his PhD training the department of Medical Microbiology at the University Medical Center Utrecht under supervision of prof. dr. M. J. M. Bonten, dr. A. C. Fluit, and dr. J. W. T. Cohen Stuart. The results of this study are described in this thesis, and are published in different scientific journals.

Guido Maarten Voets

gmvoets@gmail.com

DON'T PANIC