TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 875

MEDICA - ODONTOLOGICA

GENETIC BASIS AND DIAGNOSTICS OF EXTENDED-SPECTRUM B-LACTAMASES AMONG *ENTEROBACTERIACEAE* IN FINLAND

by

Sofia Forssten
(née Nyberg)

TURUN YLIOPISTO
UNIVERSITY OF TURKU
Turku 2009

From the Department of Medical Microbiology, University of Turku, Turku, Finland and the Department of Infectious Disease, Division of Health Protection, National Institute for Health and Welfare (former Laboratory of Microbial Ecology, Department of Bacterial and Infectious Diseases, National Public Health Institute) in Turku, Finland.

Supervised by

Docent Jari Jalava
National Institute for Health and Welfare
Division of Health Protection
Department of Infectious Disease
Surveillance and Control
Turku, Finland

Research Professor Pentti Huovinen
National Institute for Health and Welfare
Division of Health Protection
Turku, Finland

Reviewed by

Professor Mikael Skurnik
Department of Bacteriology and Immunology
The Haartman Institute
University of Helsinki
Helsinki, Finland

Docent Jaakko Uksila
KESLAB Laboratory
Central Finland Health Care District
Jyväskylä, Finland

Dissertation Opponent

Professor Jukka Pelkonen
Department of Clinical Microbiology
University of Kuopio
Kuopio, Finland

ISBN 978-951-29-4071-4 (PRINT)
ISBN 978-951-29-4072-1 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

To Henka

SOFIA FORSSTEN

Genetic Basis and Diagnostics of Extended-Spectrum β-Lactamases among *Enterobacteriaceae* in Finland

Department of Medical Microbiology, University of Turku, Turku, Finland and the Department of Infectious Disease, Division of Health Protection, National Institute for Health and Welfare (former Laboratory of Microbial Ecology, Department of Bacterial and Infectious Diseases, National Public Health Institute) in Turku, Finland.
Annales Universitatis Turkuensis, 2009, Turku, Finland
Painosalama Oy

ABSTRACT

Since the introduction of antibiotic agents, the amount and prevalence of β-lactam resistant enterobacteria has become an increasing problem. Many enterobacteria are opportunistic pathogens that easily acquire resistance mechanisms and genes, which make the situation menacing. These bacteria have acquired resistance and can hydrolyse extended spectrum cephalosporins and penicillins by producing enzymes called extended-spectrum β-lactamases (ESBLs). ESBL-producing bacteria are most commonly found in the gastro-intestinal tract of colonised patients. These resistant strains can be found in both health-care associated and community-acquired isolates. The detection and treatment of infections caused by bacteria producing ESBLs are problematic.

This study investigated the genetic basis of extended-spectrum β-lactamases in *Enterobacteriaceae*, especially in *Escherichia coli* and *Klebsiella pneumoniae* isolates. A total of 994 Finnish *Enterobacteriaceae* strains, collected at 26 hospital laboratories, during 2000 and 2007 were analysed. For the genetic basis studies, PCR, sequencing and pyrosequencing methods were optimised. In addition, international standard methods, the agar dilution and disk diffusion methods were performed for the resistance studies, and the susceptibility of these strains was tested for antimicrobial agents that are used for treating patients.

The genetic analysis showed that $bla_{\text{CTX-M}}$ was the most prevalent gene among the *E. coli* isolates, while $bla_{\text{SHV-12}}$ was the most common β-lactamase gene in *K. pneumoniae*. The susceptibility testing results showed that about 60% of the strains were multidrug resistant. The prevalence of ESBL-producing isolates in Finland has been increasing since 2000. However, the situation in Finland is still much better than in many other European countries.

Keywords: *Enterobacteriaceae, Escherichia coli*, *Klebsiella* spp. extended-spectrum β-lactamases, antibiotic resistance

SOFIA FORSSTEN

Genetisk bas och diagnostik av β-laktamaser med utvidgat spektrum bland *Enterobacteriaceae* i Finland

Medicinsk mikrobiologi, Åbo universitet, Åbo, Finland och Avdelningen för infektionssjukdomar, Verksamhetsområdet för hälsoskydd, Institutet för hälsa och välfärd (tidigare Mikrobekologilaboratoriet, Avdelningen för bakterie- och infektionssjukdomar, Folkhälsoinstitutet) Åbo, Finland.
Annales Universitatis Turkuensis, 2009, Åbo, Finland
Painosalama Oy

ABSTRAKT

Förekomsten och mängden av enterobakterier som är resistanta mot β-laktamer har ökat sedan antibiotikaeran började. Många enterobakterier är opportunistiska patogener som lätt skaffar resistensmekanismer och gener, vilket leder till en hotande situation. Dessa bakterier har skaffat resistensmekanismer med vars hjälp de kan hydrolysera penicillin och cefalosporiner med utvidgat spektrum genom produktion av enzymer kallade β-laktamaser med utvidgat spektrum (ESBL). ESBL-producerande bakterier förekommer i mag-tarmkanalen hos koloniserade patienter. De resistenta stammarna förekommer både bland kliniska och öppenvårdsisolat. Detektering av ESBL-producerande bakterier och behandling av infektioner orsakade av dessa bakterier är problematiska.

Denna studie undersökte ESBL-enzymernas genetiska bas hos *Enterobacteriaceae*, speciellt hos *Escherichia coli*- och *Klebsiella pneumoniae*-isolat. Totalt analyserades 994 finländska *Enterobacteriaceae*-stammar, samlade vid 26 sjukhuslaboratorier, mellan åren 2000 och 2007. För de genetiska studierna optimiserades PCR, sekvensering och pyrosekvensering. De internationella standardmetoderna agarutspädning och lappdiffusion användes för att studera antibiotikaresistensen, och suskeptibiliten hos bakterierna testades med antibiotika som används för behandling av patienter.

De genetiska analyserna visade att $bla_{\text{CTX-M}}$ var den mest prevalenta genen hos *E. coli*, medan $bla_{\text{SHV-12}}$ var den vanligaste hos *K. pneumoniae*. Suskeptibilitetstestningen visade att ca 60% av stammarna var mångresistenta. Förekomsten av ESBL-producerande isolat har ökat i Finland sedan början av 2000-talet, men tillsvidare är situationen bättre i Finland i jämförelse med många andra europeiska länder.

Nyckelord: *Enterobacteriaceae, Escherichia coli*, *Klebsiella* spp., β-laktamaser med utvidgat spektrum, antibiotikaresistens

SOFIA FORSSTEN

Laajakirjoisten β-laktamaasien genetiikka ja diagnostiikka *Enterobacteriaceae* –kannoissa Suomessa

Lääketieteellinen tiedekunta, Turun yliopisto ja Tartuntatautien seurannan ja torjunnan osasto, Terveyden ja hyvinvoinnin laitos (aikaisemmin Mikrobiekologian laboratorio, Bakteeri- ja tulehdustautien osasto, Kansanterveyslaitos), Turku, Suomi.
Annales Universitatis Turkuensis, 2009, Turku, Finland
Painosalama Oy

TIIVISTELMÄ

Bakteerien lääkevastustuskyky eli resistenssi on levinnyt kaikkialla maailmassa bakteerilääkkeiden laajan käytön seurauksena. Resistenssi on lisääntynyt erityisesti beetalaktaameille, jotka kuuluvat eniten käytettyihin bakteerilääkkeisiin. Beetalaktaamiresistenssi on yleistä varsinkin Enterobacteriaceae-heimon bakteereissa. Resistenssi beetalaktaamilääkkeille on useimmiten beetalaktamaasi-välitteistä. Nämä entsyymit hajottavat vaihtelevasti niin penisilliini- kuin kefalosporiiniryhmän lääkeaineita. Laajakirjoisia beetalaktamaaseja eli ESBL-entsyymejä (extended spectrum beta-lactamaase) tuottavia bakteereja löydetään niin sairaala- kuin avohoitopotilailtakin. Enterobakteerit kolonisoivat nimensä mukaisesti suolistoa, ja aiheuttavat virtsatieinfektioita ja henkeä uhkaavia yleisinfektioita.

Tässä väitöskirjatyössä tutkittiin ESBL-kantojen esiintyvyyttä ja resistenssitekijöitä *Escherichia coli* ja *Klebsiella pneumoniae* bakteeriessa. Tutkimuksessa analysointiin 994 suomalaista *Enterobacteriaceae*-kantaa, jotka kerättiin 26 sairaalalaboratorioista vuosien 2000 ja 2007 välillä. ESBL-resistenssitekijöiden tunnistamisessa käytettiin molekulaarisia menetelmiä, kuten PCR-geenimonistusta, sekvensointia ja pyrosekvensointia. Lisäksi tutkimuksessa kerättyjen bakteerien herkkyydet määritettiin hoidossa yleisesti käytettäville bakteerilääkkeille maljalaimennos- ja kiekkoherkkyysmenetelmillä kansainvälisiä standardeja seuraten.

$bla_{\text{CTX-M}}$ oli yleisin beetalaktamaasigeeni *E. coli* -bakteerikannoissa ja $bla_{\text{SHV-12}}$ *K. pneumoniae* -kannoissa. Herkkyysmäärityksissä todettiin, että peräti 60 % tutkituista kannoista oli resistenttejä monelle eri bakteerilääkkeelle samanaikaisesti. ESBL-tuottavien kantojen määrä on noussut Suomessa 2000-alusta lähtien, mutta tilanne on silti vielä parempi kuin monissa muissa Euroopan maissa.

Avainsanat: *Enterobacteriaceae, Escherichia coli*, *Klebsiella* spp., laajakirjoiset β-laktamaasit, antibiottiresistenssi, bakteerilääke

CONTENTS

ABBREVIATIONS

AES	automated expert system
AmpC	class C β-lactamases
ARI-1	imipenem-resistant *Acinetobacter baumannii*
ATCC	American Type Culture Collection
bp	base pair
BES	Brazil extended-spectrum β-lactamase
BSAC	the British society for Antimicrobial Chemotherapy
CLSI	Clinical and Laboratory Standards Institute
CMY	cephamycin-hydrolyzing β-lactamase
CphA	cephalosporin A
CTX-M	cefotaximase
Dice	a coefficient used to analyze the similarities of the PFGE banding patterns
EARSS	the European Antimicrobial Resistance Surveillance System
EHEC	Enterohemorrhagic *E. coli*
ESBL	extended-spectrum β-lactamase
ETEC	enterotoxigenic *E. coli*
EUCAST	the European Committee on Antimicrobial Susceptibility Testing
FiRe	the Finnish Study Group for Antimicrobial Resistance
FOX	plasmid-mediated AmpC-type β-lactamase, resistant to cephamycins
GES	Guiana extended spectrum
GIM	German imipenemase
IBC	integron-associated β-lactamase
ICU	intensive care unit
IMI	imipenem-hydrolyzing β-lactamase
IMP	active on imipenem
IS	insertion sequence
K1	β-lactamase from *Klebsiella oxytoca*, also called OXY
KPC	*Klebsiella pneumoniae* carbapenemase
LPS	lipopolysaccharide
Mbl-1	metallo β-lactamase-1
MDR	multidrug resistant
MFP	membrane fusion protein
MIC	minimal inhibitory concentration
MOX	plasmid-mediated AmpC-type β-lactamase, moxalactam resistant
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NMC	Not Metalloenzyme Carbapenemase
npv	negative predictive value
OMP	outer membrane protein
OXA	oxacillin-hydrolyzing, oxacillinase
OXY	β-lactamase from *Klebsiella oxytoca*, also called K1
PBP	penicillin-binding protein

PER	extended-spectrum cephalosporin-hydrolyzing β-lactamase
PCR	polymerase chain reaction
PDR	pandrug resistance
PFGE	pulsed field gel electrophoresis
PPi	pyrophosphate
ppv	positive predictive value
RND	resistance-nodulation-division
SFO	a β-lactamase found in *Enterobacter cloacae,* with a high degree of homology to the β-lactamase of *Serratia fonticola*
SHV	sulfhydryl variable
SIM	Seoul imipenemase
SME	*Serratia marcescens* enzyme
spp.	species (plural)
SRGA	the Swedish Reference Group for Antibiotics
TEM	a β-lactamase named after a Greek patient Temoneira
TLA	a plasmid-mediated class A β-lactamase
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UTI	urinary tract infection
VIM	Verona integron-encoded metallo β-lactamase
XDR	extensive drug resistance

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following articles, referred to in the text by Roman numerals I-IV. In addition, some unpublished results have also been included in the Results-section. The original communications are reproduced with the permission of the copyright holders.

I Nyberg S. D., Österblad M., Hakanen A. J., Huovinen P., Jalava J. and the Finnish Study Group for Antimicrobial Resistance. Detection and Molecular genetics of Extended-Spectrum Beta-Lactamases among cefuroxime- resistant Escherichia coli and Klebsiella spp. isolates from Finland, 2002-2004. *Scand J Infect Dis* 2007;39: 417-424.

II Nyberg S. D., Meurman O., Jalava J. and Rantakokko-Jalava K. Evaluation of detection of extended-spectrum beta-lactamases among Escherichia coli and Klebsiella spp. isolates by VITEK 2 AST-N029 compared to the agar dilution and disk diffusion methods. *Scand J Infect Dis* 2008;40: 355-362.

III Haanperä M.*, Forssten S. D.*, Huovinen P. and Jalava J. Typing of SHV extended-spectrum beta-lactamases by pyrosequencing in *Klebsiella pneumoniae* strains with chromosomal SHV β-lactamase. *Antimicrob Agents Chemother* 2008;52: 2632-2635.

IV Forssten S. D., Kolho, E., Lauhio, A., Lehtola, L., Mero, S., Oksaharju, A., Jalava, J., Tarkka, E., Vaara, M. and Vuopio-Varkila, J. Emergence of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates during the years 2000 and 2004 in Helsinki, Finland. *Clin Microbiol Infect*. In Press.

*These two authors contributed equally to the work.

1. INTRODUCTION

One of the most important contributions to medical science in recent history is the introduction of β-lactam antibiotics into the health care system during the latter period of the World War II. The availability of antibiotics to treat infectious diseases improved the wellbeing of humans and animals. However, since the introduction of antibiotic agents during the middle of the 20th century, antibiotic resistance has emerged and resulted in impaired treatment of human diseases (Neu 1992).

The β-lactam antibiotics are one of the most widely used group of antimicrobial agents, due to their comparatively high effectiveness. The β-lactams represent by weight 60% of all the antimicrobial agents used and are typically used to treat infections caused by Gram-negative bacteria (Livermore and Woodford 2006). Thus, due to the wide use, resistance to the β-lactam antibiotics has emerged quickly. Enzymes called β-lactamases are produced by Gram-negative organisms, especially by *Enterobacteriaceae.*

Enterobacteriaceae is a large family belonging to the γ-proteobacteria. This family includes many pathogens, such as *Escherichia coli*. *Enterobacteriaceae* are capable of resisting many antimicrobial agents by e.g. producing enzymes called β-lactamases. The first β-lactamase was detected during the 1960s (Datta and Kontomichalou 1965). Many Gram-negative bacteria naturally possess a chromosomal β-lactamase. Since the β-lactamases show sequence homology with the penicillin-binding proteins (PBPs), they are thought to have evolved from them. This development was likely due to the selective pressure exerted by β-lactam-producing soil organisms found in the environment.

The β-lactamases were relatively rare until β-lactam antibiotics were introduced into the market of medicine and agriculture half a century ago. During the last 20 years, many new β-lactam antibiotics have been developed that were specifically designed to be resistant to the hydrolytic action of β-lactamases. (Medeiros 1997; Henriques Normark and Normark 2002). Most probably, the selective pressure of the use of new antibiotics in the treatment of patients has selected for new variants of β-lactamases. During the mid-1980s, the first β-lactamase with an extended-spectrum was detected in Germany in 1983 (Kliebe *et al*. 1985). The ESBL (extended-spectrum β-lactamase) phenomenon began in the western parts of Europe, probably because expanded-spectrum β-lactam antibiotics were first used there clinically. Today, over 610 β-lactamases exist (Fisher *et al.* 2005; Jacoby and Bush 2009).

The detection of ESBL producing strains is complicated due to the large variety of enzymes. Depending on the amount of enzyme or on which enzymes are present, different resistance phenotypes occur. Spreading of ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* creates a microbiological threat comparable to methicillin resistant *Staphylococcus aureus*, MRSA. The spread of these strains should be minimised. Therefore new time saving detection methods are needed. The aim of this project was to study the genetics of the ESBLs and to study the phenotypical methods that are used to detect and type ESBL producers. Hence, the results of the genetic basis and the prevalence of antibiotic resistance among Finnish bacterial isolates have been compiled in this work.

2. REVIEW OF THE LITERATURE

2.1. The family *Enterobacteriaceae*

2.1.1. Typical characteristics

The Gram-negative bacteria belonging to *Enterobacteriaceae* represent a large and heterogeneous group of many genera (more than 100 species) of facultatively-anaerobic, rod-shaped (0.3-1.0 x 1.0-6.0 μm) bacteria (Eisenstein and Zalenik 2000). Their optimal growth is between 22 and 37°C and they will grow on most simple bacteriological media and most can grow on D-glucose as the sole source of carbon, even though some require amino acids and/or vitamins. Hence they are able to use various carbohydrates and glucose both in the presence and absence of oxygen. Both acid and gas are usually formed from glucose. Enterobacterial species are oxidase negative and catalase positive. Most enterobacteria are able to move with peritrichous flagella; flagella over the entire surface or along the sides. The only exception is *Tatumella* that has lateral flagella. Many bacteria, e.g. *E. coli,* have two distinct modes of movement: forward movement (swimming), and tumbling. The GC% is 38-60% (Krieg and Holt 2005).

2.1.2. Natural habitat of enterobacteria

The natural habitat of *Enterobacteriaceae* is soil and water, but also the human and animal intestines. Some species are insect- or plant-associated (Janda and Abbott 1998). They are a major component of the normal human intestinal microbiota but relatively uncommon at other body sites (Balows *et al.* 1992). However, they are also common in our homes, e.g. transferred via food, bacteria can be found in the sink and on the cutting boards in the kitchen. *Enterobacteriaceae* represent a minority in faecal microbiota. The greatest number and the greatest species variation of bacteria are present in the colon. The total number of obligate anaerobes like *Bacteroides* and *Clostridium* is enormous (10^{10} - 10^{11} cells/gram of intestinal content), while facultative anarobes like *Enterobacteriaceae* constitute generally less than 10^{7} cells/gram of intestinal content (Guarner and Malagelada 2003).

2.1.3. Structure of the cell wall

The cell wall of enterobacteria consists of an inner cytoplasmic membrane and outer layer consisting of lipopolysaccharides (LPS) and lipoproteins. LPS consists of lipid A, core polysaccharide, and O antigen (Figure 1). This cell wall is relatively thin, in comparison with the Gram-positive cell wall. The O-polysaccharide, a side-chain of LPS, is species-specific in bacteria and carries the serologically determinant groups of the respective O antigen. These specific polysaccharides often consist of a large number (up to eight) of monosaccharides. The cell-wall LPS represent the endotoxins of the bacteria (Westphal *et al.* 1981). The periplasm is a space between the inner

cytoplasmic membrane and outer lipid membrane. This space contains a loose network of peptidoglycan chains. This layer contains a basic repeating unit of an alternating disaccharide — *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic (NAM) acid. The shape and rigidity of the cell is maintained by the heavily cross-linked peptidoglycan layer. This space may constitute up to 40% of the total cell volume in a Gram-negative bacteria (Stock *et al.* 1977).

The individual peptidoglycan cell wall units are produced inside the cell, while the final cross-linking is catalysed outside the cytoplasmic membrane by a group of membrane-anchored bacterial enzymes known as the cell-wall transpeptidases. In the cross-linking reaction, a peptide bond is formed between the D-alanine on one chain and the free amino end of a diamino pimelic acid on the other chain. A linkage is formed with the D-alanine, causing the terminal d-alanine to be cleaved (Wilke *et al.* 2005). Transpeptidases and carboxypeptidases cross-link glycopeptide polymers in the synthesis of the bacterial cell wall. Energy for transpeptidases to cross-link, is produced when carboxypeptidase cleaves D-alanine from the glycopeptide. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as PBPs (Wise 1996).

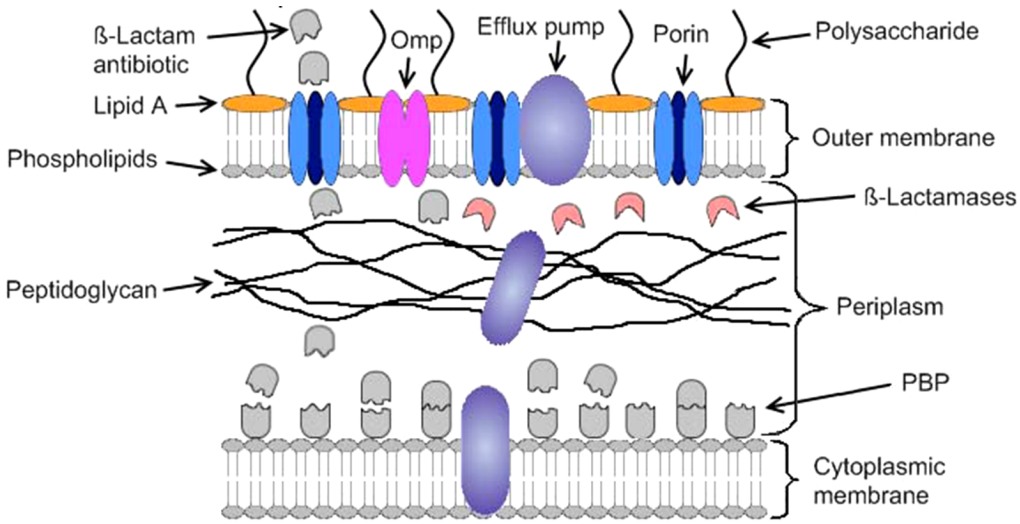


Figure 1. A schematic representation of the cell wall of Gram-negative bacteria and the action of β-lactams. This membrane consists of a bilayer containing phospholipids, lipopolysaccharides (consisting of lipid A and polysaccharide), and outer membrane proteins (Omp). A β-lactam must diffuse across the outer membrane of the cell using pores, before crossing the periplasm, which can contain any type of β-lactamase before it reaches its PBP targets, which lie on the outer surface of the cytoplasmic membrane. Modified from Madigan and Martiko 2006; Livermore and Woodford 2006.

2.1.4. Genera belonging to the family Enterobacteriaceae

2.1.4.1. ***Escherichia***

The genus *Escherichia* consists of five species; *E. blattae*, *E. coli, E. fergusonii, E. hermanii,* and *E. vulneris* (Bruckner *et al.* 1999). *E. coli* is the most numerous aerobic commensal inhabitant of the large intestine in humans (Janda and Abbott 1998). *E. coli* can be commonly found in animal faeces and the lower intestines of mammals, and they possess adhesive fimbriae that promote binding to intestinal epithelial cells. *E. coli* can also be found in environments at a higher temperature, such as on the edge of hot springs. They prefer to live at a high temperature rather than low. *E. coli* can be distinguished from most other coliforms by its ability to ferment lactose at 44°C (Janda and Abbott 1998).

In our intestines, *E. coli* helps our body to break down the food we eat as well as assist with digestion processes, waste processes, vitamin K production, and food absorption (Krieg and Holt 2005).

E. coli is probably the most famous member of the *Enterobacteriaceae* group, since it is a model organism and lots of our knowledge of biochemical processes and genetics derive from this species. *E. coli* is also commonly used as an indicator of faecal contamination (Janda and Abbott 1998).

2.1.4.2. ***Klebsiella***

The genus *Klebsiella* is among the oldest known genera in the *Enterobacteriaceae* described for the first time by Trevisan in 1885. *Klebsiella* consist of three species (*K. oxytoca, K. pneumoniae* and *K. variicola*) and five pneumoniae subspecies (*K.pneumoniae* spp. *aerogenes, granulomatis, ozaenae, pneumoniae,* and *rhinoscleromatis*) (Janda and Abbott 1998; Bruckner *et al.* 1999). *Klebsiella* species are non-motile and have a prominent mucoid polysaccharide-based capsule (K antigen**)**. The capsule protects from phagocytosis and aids in adherence, and due to the capsule *Klebsiella* form large moist colonies on solid culture media. *K. pneumoniae* are widely distributed in the environment where they contribute to biochemical and geochemical processes. Like *E. coli, Klebsiella* are colonisers of the gastrointestinal tract (Eisenstein and Zalenik 2000).

Klebsiella are ubiquitous and may colonize the skin, pharynx, or gastrointestinal tract in humans. *K. pneumoniae* and *K. oxytoca* are both opportunistic pathogens found in the environment and on mammalian mucosal surfaces; they are commonly passed by hands of hospital personnel.

2.1.4.3. ***Enterobacter***

The *Enterobacter* genus includes at least eleven species (*E. aerogenes, E. agglomerans, E. amnigenus, E. asburiae, E.cancerogenus, E. cloacae, E. gergoviae, E. hormaechei, E. intermedius, E. kobei* and *E. sakazakii*) of highly motile bacteria. They are biochemically similar to *Klebsiella*, but unlike *Klebsiella*, they are able to decarboxylate ornithine, in other words ornithine positive. The *Enterobacter* strains are fimbriate and slime-forming. *E. sakazakii* may be confused with *E. cloacae*, but can be

distinguished by yellow pigment production. *Enterobacter* spp. can be found on human skin and plants as well as in soil, water, sewage, intestinal tracts of humans and animals (Eisenstein and Zalenik 2000).

2.1.4.4. ***Other enterobacterial genera***

Other enterobactarial species most commonly encountered are *Citrobacter* spp., *Hafnia alvei*, *Proteus mirabilis*, *Salmonella* spp., *Serratia* spp. and *Shigella. Citrobacter* genus consists of twelve species and can be isolated from water, sewage, soils and food, as well as from the faeces of man and other animals, where they may be part of the normal microbiota of the large intestine. The genus *Hafnia* contains a single species, *H. alvei*, and it is generally motile. *H. alvei* can biochemically resemble non-motile salmonella (Janda and Abbott 1998).

The *Yersinia* genus contains 14 species, and these are relatively slow growers among the *Enterobacteriaceae*. *Y. pestis, Y. pseudotuberculosis* and *Y. enterocolitica* are very well documented human pathogens, while the remaining eleven species *Y. intermedia*, *Y. massiliensis, Y. similis, Y. aleksiciae, Y. frederiksenii*, *Y. kristensenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. aldovae*, *Y. rohdei* and *Y. ruckeri* (a fish pathogen causing redmouth disease) have generally been termed as "*Y. enterocolitica*-like" species, even though each is a distinct species (Martin *et al.* 2009). *Yersinia* is most commonly isolated from fresh water and food, and has been infrequently recovered from human clinical specimens (Sulakvelidze *et al.* 2000). *Yersinia* display their biochemical characteristics most reliably at temperatures between 25 and 32°C (Eisenstein and Zalenik 2000).

The *Proteus* genus is highly motile and does not form regular colonies. Instead, swarming colonies are formed when plated on a non-inhibitory media. *P. mirabilis* is considered to be the most important member of this genus.

The genus *Salmonella* consists of several serotypes and most of them are motile. Salmonella species produce hydrogen sulphide, and all except *S. typhi* produce gas from glucose. The *Serratia* genus contains ten species and two subspecies. However, only two species are clinically commonly isolated. They are *S. liquefaciens* and *S. marcescens*. *S. marcescens* often produce prodigiosin, a characteristic red pigment, when grown at 20°C. Most of the species are motile. *Serratia* strains can be distinguished from other enterobacterial species by their unique production of three enzymes: DNase, lipase, and gelatinase. The *Shigella* genus consisting of four species is closely related to *Escherichia,* and considered to be a subspecies of *E. coli*. *Shigella* is non-motile and anaerogenic, i.e. it does not produce gas from glucose. However, about 5% of ordinary *E. coli* strains are also anaerogenic (Janda and Abbott 1998).

2.1.5. Clinical significance of enterobacteria

Enterobacterial infections can be produced by bacteria that normally live in the human digestive tract without causing serious disease or by bacteria that enter from the outside. In many cases these infections are nosocomial, which means that they are acquired in the hospital. Enterobacterial strains are the most common cause of urinary tract infections but also cause pneumonia, sepsis, infections of wounds and of the

intestine. Many *Escherichia* spp. are harmless commensals. However, some strains of *E. coli* can cause severe infections in humans and many animals, e.g. sheep, horses, and dogs. Some particular strains are human pathogens, and are known, significant sources of gastrointestinal disease, ranging from simple diarrhoea to dysentery-like conditions (Janda and Abbott 1998; Kaper *et al.* 2004). Some of the main diarrhoeagenic *E. coli* are enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) (Kaper *et al.* 2004). ETEC has caused occasional outbreaks in industrialized countries, but has been a common agent for tourist diarrhoea (Naimi *et al.* 2003). EHEC causes severe infections and the low infection dose enables effective spreading of this pathogen via e.g. food or water (Bell 2008).

The *Klebsiellae* are rarely associated with diseases in the normal host, but they are a major cause of nosocomial and opportunistic infection (Eisenstein and Zalenik 2000). Common sites for *Klebsiella* infections include the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites. Both *E. coli* and *K. pneumoniae* are recognised as frequent causes of urinary tract infections. *Klebsiella* sometimes cause pneumonia, ear and sinus infections. *K. oxytoca* is an occasional cause of wound, bloodstream and urinary tract infections (Janda and Abbott 1998; Struve and Krogfelt 2004).

Enterobacter species are rarely pathogenic in a healthy individual. Several species cause opportunistic infections of the urinary tract as well as other body sites. *E. aerogenes* and *E. cloacae* are two such pathogens that do not cause diarrhoea, but that are sometimes associated with urinary tract and respiratory tract infections (Janda and Abbott 1998).

Of the *Yersinia* genus, three species are pathogenic in humans, *Y. pestis, Y. enterocolitica* and *Y. pseudotuberculosis*. *Y. pestis* is the bacterial agent of plague, while *Y. enterocolitica* and *Y. pseudotuberculosis* can cause e.g. gastroenteritis, but may also cause infections at wounds, joints, and the urinary tract. All three biovariants of *Y. pestis* have been responsible for high mortality rates in epidemics throughout human history, including the Black Death (Janda and Abbott 1998; Linde *et al.* 1999; Wren 2003).

Citrobacter spp. can sometimes be opportunistic pathogens especially in immunocompromised patients or in infants (Pepperell *et al.* 2002). *H. alvei* are occasionally implicated in both intestinal and extraintestinal infections in humans. The *Salmonella* genus contains species that cause typhoid fever, paratyphoid fever, and enteric infections. *Proteus* includes pathogens responsible for many human urinary tract infections. The most common species in the *Serratia* genus, *S. marcescens*, is normally the only pathogen and usually causes nosocomial infections. A *Shigella* infection may lead to diarrhoea, sometimes associated with bloody and mucous excretions, a disease known as bacillary dysentry (Janda and Abbott 1998).

2.2. β-lactam antibiotics

2.2.1. General structure and function

β-lactam is a generic name for all β-lactam antibiotics that contain a β-lactam ring, a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom, see Figure 2. The β-lactam bactericidal antibiotics consist of four main antibiotic groups. These main groups are penicillins, cephalosporins, carbapenems and monobactams. The principal classification of β-lactams is based upon the structure (Wilke *et al.* 2005).

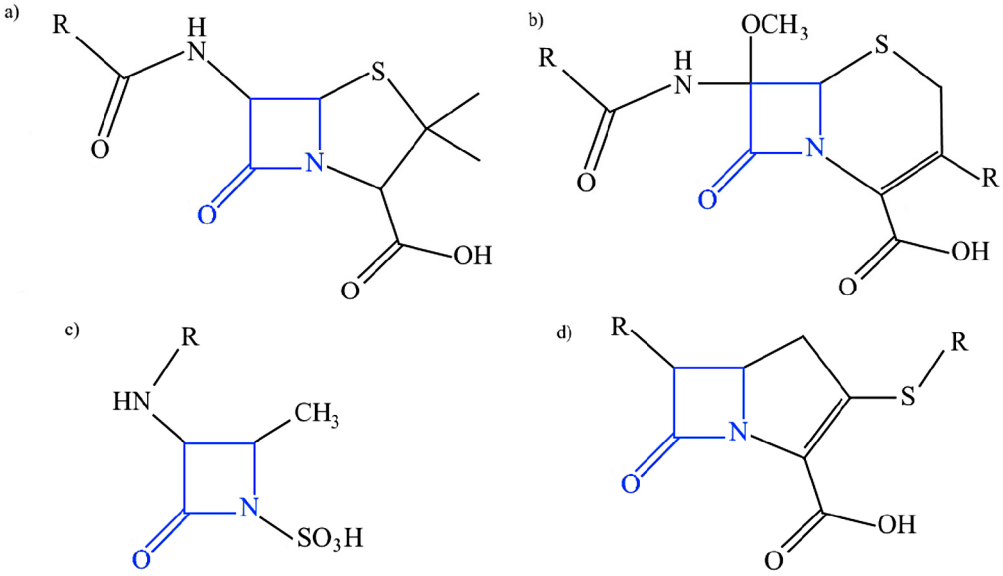


Figure 2. Structure of the β-lactam antibiotics are represented by a) penicillin and b) cephalosporins, c) monobactam and d) carbapenems. The β-lactam ring is shown in blue. The R-group symbolises the side-chains. The methoxy-group (-OCH_3) in the cephalosporin structure is present in the methoxycephalosporins but not in the aminocephalosporins. Modified from Livermore and Willams 1996.

The β-lactam antibiotics are analogues of the terminal amino acid (D-alanyl-D-alanine) residues on the precursor NAM/NAG-peptide subunits of the peptidoglycan layer. The nucleus of the β-lactam molecule irreversibly binds to the Ser403 residue of the PBP active site. This prevents the transpeptidation of the peptidoglycan layer, and hence disrupts the synthesis of the cell wall (Tipper and Strominger 1965; Livermore and Williams 1996). The action of β-lactam antibiotics is shown in Figure 3. The

effectiveness of β-lactam antibiotics relies on the ability to reach the PBP intact and the ability to bind to the PBP. The activity also depends on the growth rate; slow growing cells are killed more slowly than rapidly growing ones (Livermore and Williams 1996).

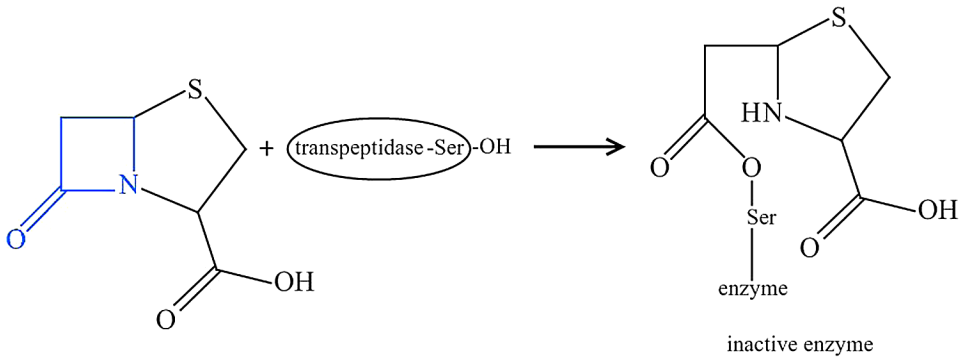


Figure 3. Action of β-lactam antibiotics. The β-lactam ring is shown in blue. Transpeptidase is also known as PBP. Modified from Machebouef *et al.* 2007.

2.2.2. Penicillins

Penicillin was discovered by Sir Alexander Fleming in 1928, and this is the original group of β-lactam antibiotics. Several penicillins have been developed from the benzyl-linked compound that was first used (Livermore and Williams 1996). The penicillins contain a β-lactam ring attached to a thiazolidine ring (Figure 2). The side chain of different penicillins is modified in order to change the activity (Yao and Moellering 1995). The term "penicillin" is often used in the generic sense to refer to one of the narrow-spectrum β-lactams, in particular, benzylpenicillin. Benzylpenicillin was the first penicillin to enter clinical use. Phenoxymethylpenicillin is commonly known as penicillin V. Examples of aminopenicillins are ampicillin and amoxicillin. The penicillins are all sensitive to the β-lactamases, however, temocillin, is resistant to most of these enzymes (Livermore *et al.* 2006).

Penicillins bind to the PBPs. Ureido and piperazine-penicillins are derived from ampicillin and these have increased activity for PBP-3. Carboxypenicillins that include carbenicillin and ticarcillin have a wider spectrum: they have activity against *Pseudomonas aeruginosa*, and *Proteus* and *Enterobacter* strains that are resistant to ampicillin. The carboxypenicillins differ from other penicillins in that they contain a carboxylate moiety in the sidechain of the molecule. These molecules are more effective against Gram-negative bacteria, presumably because they penetrate the outer cell wall more extensively than other penicillins. Mecillinam or amdinocillin binds to PBP-2 rather than PBP-1 and -3 that are targets for other penicillins (Neu 1985).

2.2.3. Cephalosporins

The first isolation of cephalosporin compounds were from cultures of *Cephalosporium acremonium* in 1948. Modifications resulted in the development of useful antibiotic agents, and the first agent cephalothin was launched by Eli Lilly in 1964. Cephalosporins are bactericidal and have the same mode of action as other β-lactam antibiotics, such as penicillins (Wise 1996).

The cephalosporins contain an aminocephalosporanic acid nucleus consisting of a β-lactam ring fused to a dihydrothiazine ring. Different substitutions alter the antibacterial activities and pharmacokinetic properties (Wise 1996). The cephalosporins are chemically divided into two groups: oxyimino- and methoxycephalosporins. Cefotaxime, ceftriaxone and ceftazidime are examples of oxyiminocephalosporins. The cephems, e.g. cephamycins like cefotetan and cefoxitin, are closely related to cephalosporins but have an α–methoxy (-OCH_3) group called oxacephem group instead of the 7-aminocephalosporanic acid in oxyiminocephalosporins (Figure 2, page 19)[1]. The cephems are highly resistant to various β-lactamases (Bush 1996; Yao and Moellering 2003).

Cephalosporins are classified by grouping into four generations based on their antibacterial activity. The first generation consists of narrow-spectrum drugs, like cephalothin and cefazolin, and these have a relatively modest activity to Gram-negative bacteria. Wild type *E. coli* and *Klebsiella* spp. are susceptible to these. The second generation cephalosporins are stable to certain β-lactamases and have hence an increased activity against Gram-negative bacteria. Cefuroxime and cephamycins, e.g. cefotetan and cefoxitin, are examples of the 2nd generation cephalosporins. The third generation cephalosporins are much more active against *Enterobacteriacea* than narrow-spectrum drugs. These antibiotics are more stable to β-lactamases and are able to pass through the outer cell envelopes of Gram-negative bacteria. Cefotaxime and ceftriaxone are examples of drugs belonging to this generation. The fourth generation cephalosporins, like cefepime, developed in 1994, reduce the affinity for and increase the stability for chromosomally but also some plasmid-mediated β-lactamase enzymes (Yao and Moellering 2003).

2.2.4. Monobactams

In monobactams the β-lactam ring is alone, and not fused to another ring, hence they lack the double ring structure found in traditional β-lactam antibiotics and they can easily be synthesised. The monobactams like aztreonam have different side-chains affixed to a monocyclic nucleus. Monobactams have a wide range of activity to aerobic Gram-negative bacteria (Bush 1996).

[1] The original cephalosporins, named before 1975 are spelled with 'ph', and were often trademarked. Hence, the World Health Organization suggested by the "International Nonproprietary Names" the use of "f" for the generic drug name of all cephalosporins.

2.2.5. Carbapenems

The carbapenems are structurally very similar to the penicillins, but the sulphur atom of the structure has been replaced with a carbon atom (Figure 2, page 19), and hence the name of the group, the carbapenems. This class of β-lactams consists of imipenem, meropenem, doripenem, ertapenem, panipenem and biapenem. This group has the widest spectrum of antibacterial activity of the antibiotic agents that are currently available. The carbapenems bind to PBP-1 and -2 (Spratt *et al.* 1977).

2.2.6. β-lactamase inhibitors

β-lactamase inhibitors are designed to inhibit or destroy the effectiveness of β-lactamase enzymes. They have a poor activity on their own against PBPs and are hence co-administered with β-lactam antibiotics. The β-lactamase inhibitors are so called suicide inhibitors; they form an irreversible acyl enzyme complex by a covalent bond during the catalysis reaction with the β-lactamase, which leads to activity loss of the enzyme (Figure 4). The β-lactamase inhibitors are divided into two groups: clavulanic acid and penicillanic acid sulfones. Clavulanic acid acts synergistically with different penicillins and cephalosporins against Gram-negative bacteria that produce β-lactamases. The penicillanic acid sulfones, sulbactam and tazobactam are structurally related and sulbactam is combined with ampicillin, while tazobactam is combined with piperacillin (Moosdeen *et al.* 1988).

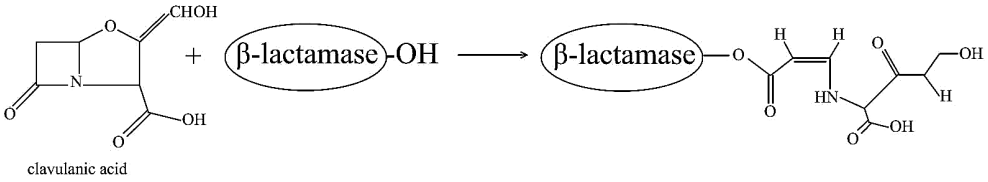


Figure 4. Action of β-lactamase inhibitors. The inhibitor binds and inactivates the β-lactamase. Modified from http://homepage.ntlworld.com/diamonddove/08BLInhibitors/BLInhibitors.htm.

2.3. Resistance to β-lactams

The resistance to β-lactams can be inherent or acquired. Inherent resistance in a Gram-negative bacterium is due to an outer membrane that establishes a permeability barrier against the antibiotic. For example, Gram-negative bacteria are intrinsically resistant to penicillin G by virtue of their double membrane structure which prevents the antibiotic from accessing the cell wall target. Intrinsic resistance is not considered

an important clinical problem since antimicrobial agents are or were not intended for use against intrinsically resistant bacteria (2006). It is the acquired resistance that is of clinical importance.

Acquired resistance to β-lactams operates through different mechanisms: production of β-lactamases, changes in the outer membrane permeability or alterations to the PBPs (Wilke *et al.* 2005). β-lactamases are a group of enzymes that are most likely originally targeted for synthesis of the cell wall, but have also evolved to degrade and inactivate β-lactam antibiotics. These flexible enzymes have been detected in both Gram-positive and Gram-negative bacteria, but these enzymes are especially important in Gram-negative bacteria as they are the most common cause of β-lactam resistance in this group of bacteria (Bush *et al.* 1995; Livermore 1995).

The outer membrane of Gram-negative bacteria plays an important role serving as a diffusion barrier to extracellular solutes and interacts with the bacterial environment (Russell and Chopra 1996). Before a β-lactam reaches its PBP targets which are on the outer surface of the cell membrane (Figure 1, page 15), it must diffuse across the outer membrane of the cell, by using the pores that are formed by porins, and then cross the periplasm. The porins, which represent one family of outer membrane proteins (Omps), form channels to permit diffusion of small hydrophilic solutes through the outer membrane. The porins are divided into two classes: specific and non-specific. In *E. coli,* OmpC and OmpF represent the nonspecific porins that permit the general diffusion of small polar molecules. A loss of either of these porins has been related to antibiotic resistance (Nikaido 1989).In *Klebsiella* spp. OmpK36 and OmpK35 are the homologues of OmpC and OmpF (Nikaido 1992; Domenech-Sanchez *et al.* 1999). Usually, *K. pneumoniae* strains express OmpK35 and OmpK36, while the ESBL-producing strains commonly express only one of these, normally OmpK36, or no porin at all (Martinez-Martinez *et al.* 1996; Hernandez-Alles *et al.* 2000).

Alterations of PBPs may contribute to β-lactam resistance, since their affinity for the drugs is reduced. Alterations may occur either by point mutations in PBP genes, by remodeling of PBP genes with foreign DNA, or by acquisition of a resistant PBP (Spratt 1994; Essack 2001).

Multidrug efflux systems that contribute to antimicrobial resistance have also been described in a broad range of Gram-negative pathogens. These efflux systems are capable of transporting captured antibiotics from the periplasmic space, cytoplasmic membrane, and/or cytoplasmic space by so called the resistance-nodulation-division (RND) exporter proteins. The RND transporters work in conjunction with the membrane fusion proteins (MFP) present in the periplasm, while the outer membrane porin proteins (OMP) serve as the final step in removal of the antibiotic from the cell (Poole 2001). The presence of drug-efflux pumps leads to a similar phenotype as β-lactamases. Genes that encode multidrug resistance (MDR) pumps are normal constituents of bacterial chromosomes. Hence, an intrinsic potential is provided to bacteria to develop a MDR phenotype without acquisition of antibiotic resistance genes (Nikaido 1998b; Nikaido 1998a; Poole 2004).

The resistance is often due to impermeability or the presence of a β-lactamase alone. However, these factors work together - for any given external β-lactam concentrations, the β-lactam concentration in the periplasm maintains a steady-state level (Zimmermann and Rosselet 1977). Reduced permeability through porin loss

reduces the steady-state periplasmic drug concentrations and thereby reduces PBP inactivation. Decreased permeability may act synergistically with the expression of β-lactamases or active efflux to confer higher levels of resistance (Essack 2001; Livermore and Woodford 2006). In some cases, β-lactamases that have only low activity *in vitro* can confer resistance in a suitably impermeable host strain (Livermore and Woodford 2006).

The focus in this work will be on 3rd generation cephalosporin resistance caused by β-lactamases.

2.4. β-lactamases

2.4.1. Classification and nomenclature of β-lactamases

Different classifications based on phenotype, gene or amino acid protein sequences and function have been attempted since the beginning of the 1970s (Richmond and Sykes 1973; Ambler *et al.* 1991; Bush *et al.* 1995; Hall and Barlow 2005). One of the most used classification schemes is Ambler's (Ambler *et al.* 1991) based upon amino acid sequences. By this classification the β-lactamases are divided into four molecular classes, A, B, C and D, Figure 5.

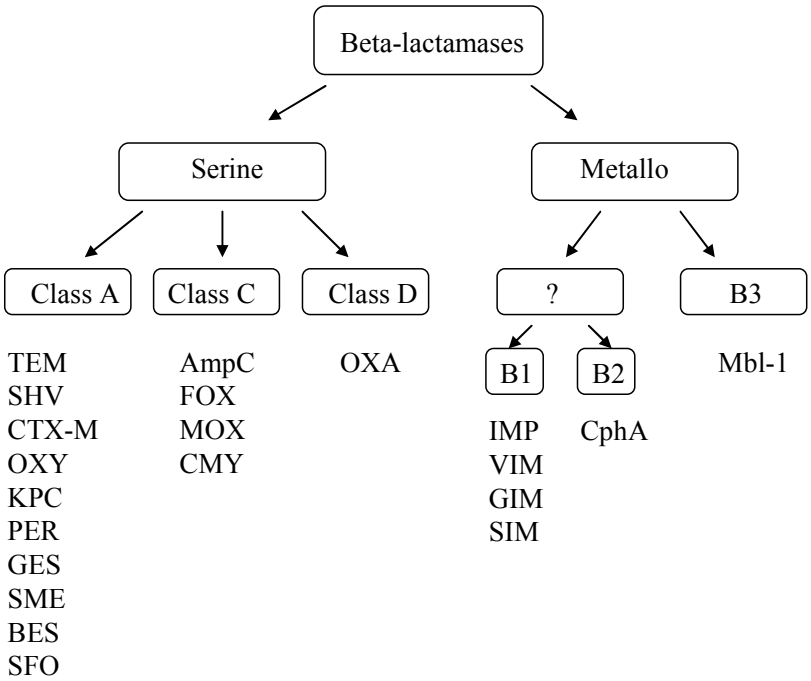


Figure 5. Classification based on phylogeny of the serine and metallo β-lactamases. Modified from Ambler 1980; Hall and Barlow 2003; Hall and Barlow 2005.

Originally, only the serine and metallo β-lactamase classes were designated, but when a new serine β-lactamase group was discovered that lacked sequence homology with class A and class B enzymes, it was designated Class C (Jaurin and Grundstrom 1981). When other serine ß-lactamases were found with no sequence homology with the existing classes, they were designated as class D (Ouellette *et al.* 1987). Hence, class A, C and D have serine moieties in their active site, while class B enzymes contain the metallo enzymes that have zinc molecules in the active site (Ambler 1980). The serine and metallo β-lactamases do not share sequence or structural homology. The phylogeny of serine-β-lactamases represented by 17 bacterial DNA is presented in Figure 6.

In addition to the Ambler classification, β-lactamases can be classified based on their substrate specificity. Penicillinases, extended-spectrum β-lactamases (ESBLs), carbapenemases and so on are examples of classification of β-lactamases by they preferred substrate specificity. This classification does not follow the phylogeny of these enzymes. For example, ESBLs exist among several different phylogenetically defined β-lactamase groups. For this work, ESBLs are the most important group of β-lactamases. The exact definition of the ESBLs is not clear (Livermore 2008). In this study ESBLs are defined as acquired, transferable β-lactamases that can significantly hydrolyse 3rd generation cephalosporins and are inhibited by β-lactamase inhibitors.

The nomenclature of β-lactamases is not logical. They are named in different ways, some after their preferred substrate, like IMP (active on imipenem) and OXA (oxacillinase), others according to biochemical properties, like SHV, while others are named according to bacteria, or patient or hospitals (Bush and Jacoby 1997).

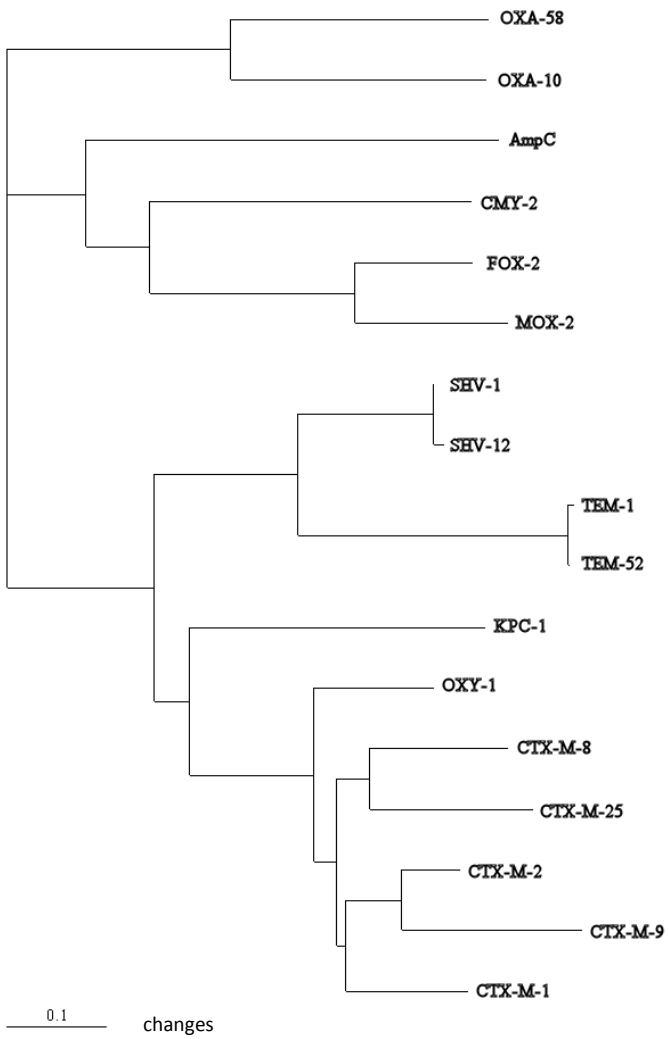


Figure 6. The phylogeny of serine-β-lactamases represented by 17 bacterial DNA sequences. The sequences were obtained from Genbank and were aligned by BioEdit, while ClustalW was used for the dendrogram. The tree was visualised by Treeview. The following DNA sequences were used: AF189721, AF518567, X92506, AF252622, AY750915, AJ871864, AB282997, AY883411, EF035567, EF035556, EF653401, Y10282, AJ276453, EU162133, F297554, EU131095 and DQ098648.

2.4.2. Structure and action of β-lactamases

The β-lactamases are divided into two classes; serine and metallo β-lactamases that do not share sequence or structural homology. However, both β-lactamase classes hydrolyse the amide bond of the four-membered β-lactam ring (Figure 7). The serine β-lactamases have a serine in the middle of their structure. The covalent bond that is formed during hydrolysis between the enzyme and the β-lactam ring is typical of these enzymes. The three classes of serine ß-lactamases, A, C and D share similarity on the protein structure level, which proves that they descended from a common ancestor (Hall and Barlow 2004). The metallo β-lactamases need a bivalent cation, usually zink, to be able to hydrolyse the β-lactam ring (Garau *et al*. 2005a).

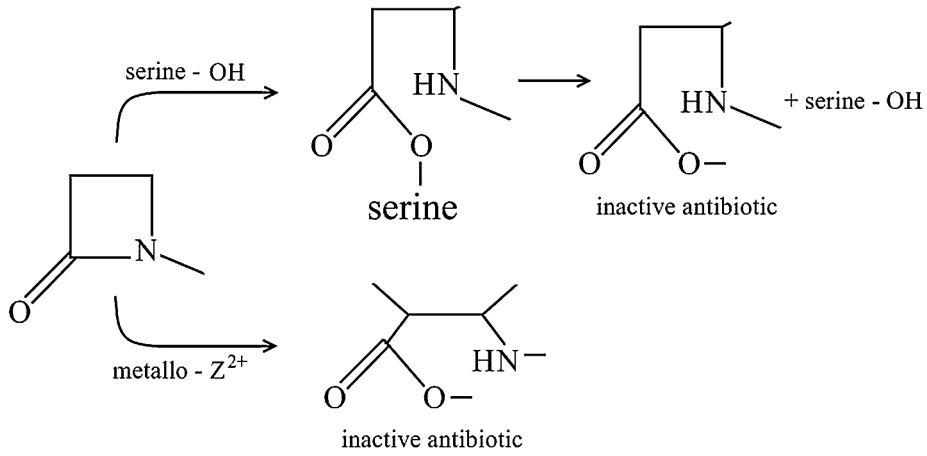


Figure 7. Action of β-lactamases. The upper cascade illustrates the action of serine β-lactamases, while the lower cascade illustrates the metallo β-lactamases. The serine β-lactamases open the β-lactam bond via a covalent acyl enzyme intermediate, hence the antibiotic is inactivated. The metallo β-lactamases use zinc to activate a water molecule and catalyse its direct addition to the β-lactam ring. Modified from Livermore and Williams 1996; Garau *et al*. 2005a.

2.5. Genetics of β-lactamases

The β-lactamases can be both chromosomal and plasmid-encoded. Most of the *Enterobacteriaceae* species have at least one chromosomal β-lactamase. The expression of these enzymes occurs often only at a very low level. Chromosomal β-lactamases typically confer resistance to penicillins but not to other β-lactams like

extended-spectrum cephalosporins or carbapenems that plasmid-encoded β-lactamases can inactivate (Sanders 1987).

2.5.1. Inducible or constitutive expression

The chromosomally encoded enzymes can be inducible or constitutively expressed. An inducible gene is a gene whose expression is responsive to e.g. an environmental change, while a constitutively expressed gene is transcribed continually. The β-lactamase production, which can be induced by the presence of certain antibiotics, is usually encoded by the bacterial chromosome. This occurs e.g. in species expressing chromosomal *ampC*; like in *Serratia, Pseudomonas, Acinetobacter, Citrobacter* and *Enterobacter*. In *E. coli* no obvious regulators are present, and therefore the AmpC expression is uninducible (Jacobs *et al.* 1997; Schmidtke and Hanson 2006). *E. cloacae* is inherently resistant to the first and second generation cephalosporins and to aminopenicillins, a mechanism mediated by the production of chromosomal AmpC (Ambler 1980). The inducible *ampC* expression in enteric bacteria, e.g. *E. cloacae*, involves a signalling system where several regulators such as AmpG, AmpR and AmpD are involved. AmpG allows entry of muropeptides into the cytoplasm from the periplasmic space as part of the cell wall recycling pathway, while AmpR is required for regulation of chromosomal *ampC* gene expression. Normally AmpR binds a precursor of the peptidoglycan biosynthesis, and hence inhibits transcription of *ampC* (Honoré *et al.* 1986). When one or several PBPs are inactivated by a β-lactam, a higher amount of peptidoglycan degradation products enter the cell. The induction is normally stopped when the β-lactam is removed, and hence the β-lactamase production returns to basal limits. However, a spontaneous mutation within the bacterial genome may result in a stably derepressed state in which β-lactamase is permanently hyperproduced even in the absence of an inducer (Garau 1994). The constitutive hyperproduction of AmpC makes a bacterial isolate resistant to most β-lactam antibiotics, except carbapenems and 4th generation cephalosporins (Nelson and Elisha 1999; Fernandez-Cuenca *et al.* 2005), and consequently is of great concern.

2.5.2. Transferable resistance

Transferable β-lactamase genes can be spread on plasmids, transposons, insertion sequences and integrons, by conjugation, transduction or transformation. During conjugation the donor bacterium extends a rod-like conjugation pilus that connects with the recipient bacteria. The plasmid is transferred via this extension. Transduction is the transfer of DNA from one cell to another via a replicating virus, while transformation is the process in which a recipient cell takes up DNA from the environment. A plasmid is an extra-chromosomal double-stranded DNA molecule that occurs naturally in bacteria. Plasmids are capable of autonomous replication within a suitable host (Madigan and Martinko 2006). Transposons are mobile genetic elements that can move around to different positions within the genome of a single cell. Transposons were first discovered in maize in 1948, and are short linear DNA segments. Class II transposons move directly from one position to another within the

genome using a transposase to "cut and paste" them within the genome. Hence, class II transposons allow transfer and permanent addition of genes e.g. encoding antibiotic resistance. When the transposable elements lack additional genes, they are known as insertion sequences. Hence, insertion sequences are small, around 700 to 2500 bp in length (Madigan and Martinko 2006). Insertion sequences participate in rearrangement of chromosomes and plasmid integration (Mahillon and Chandler 1998). Integrons are genetic elements that contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. The majority of the known gene cassettes encode resistance to antibiotics (Fluit and Schmitz 1999).

Most of the β-lactamases are integrated within plasmids and transposons that enable the rapid transfer of these resistance genes between microbes. The association of insertion sequences with these β-lactamase genes are also involved in their dissemination and expression (Bennett and Hawkey 1991; Bradford 2001).

The β-lactamase genes are often found within integrons as part of multi-drug resistance cassettes that confer resistance to several other antibiotic classes such as aminoglycosides, macrolides, sulphonamides and chloramphenicol (Medeiros 1997; Weldhagen 2004; Wilke *et al.* 2005). The gene cassettes are mobile units, and each comprises a gene, normally an antibiotic resistance gene and a recombination site. Integrons are translocatable by themselves. Transfer of resistance genes occur when integron containing one or several genes is incorporated into a plasmid (Hall and Collis 1995; Roy 1995).

2.6. The clinically most important β-lactamases

2.6.1. Class A β-lactamases

The class A β-lactamases is a large family consisting of TEM, SHV and CTX-M β-lactamases, but also a number of other rarer enzymes that often exhibit ESBL activity. Class A β-lactamases is the most common group (Bush *et al.* 1995).

2.6.1.1. TEM β-lactamases

The TEM-1 (named after a Greek patient Temoneira) was first reported in 1965 from an *E. coli* strain and represents today one of the most prevalent β-lactamase in *Enterobacteriaceae* (Datta and Kontomichalou 1965). Isolates harbouring TEM-1 ß-lactamases are resistant to ampicillin. Although TEM-type β-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found with increasing frequency in other Gram-negative species (Bradford 2001). The TEM-1 β-lactamase encoded by the $bla_{\text{TEM-1}}$ gene is present on Tn2 and Tn3 transposons (Datta and Kontomichalou 1965). TEM-3 reported in 1987 was the first TEM variant with increased activity against extended-spectrum cephalosporins.

The TEM ESBL phenotype is produced by single amino acid substitutions at positions 104, 164, 238, and 240, but ESBLs usually have more than a single amino acid substitution (Bradford 2001). Currently, more than 165 bla_{TEM} gene variants exist

including the gene variants that are resistant to inhibitors like clavulanic acid (Jacoby and Bush 2009).

The classical TEM β-lactamases can mutate so that they become resistant to inhibitors, but these mutations tend to reduce the activity against cephalosporins. Several studies have shown that single amino acid substitutions at position 244 render resistance to β-lactamase inhibitor combinations (Oliphant and Struhl 1989; Delaire *et al*. 1992; Vedel *et al*. 1992). These enzymes were first called inhibitor resistant TEM (IRT) (Bonomo and Rice 1999) but have been renamed with numerical TEM labels. The IRT β-lactamases are not ESBLs, even if they are often discussed with ESBLs because they are also derivatives of the classical TEM enzymes. Inhibitor-resistant TEM β-lactamases have mainly been found in clinical isolates of *E. coli*, but also in other enterobacterial species (Bradford 2001).

2.6.1.2. SHV β-lactamases

SHV stands for sulphydryl variable and SHV-1 was first described in 1972 as Pit-2 (Pitton 1972), while SHV-2 was detected in Germany in the beginning of the 1980s (Kliebe *et al*. 1985). SHV is most commonly found in *K. pneumoniae* (Tzouvelekis and Bonomo 1999). The SHV β-lactamases have a similar structure to TEM, with which they share 68% of their amino acids (Tzouvelekis and Bonomo 1999). Both chromosomal, e.g. $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-11}}$, and plasmid-mediated bla_{SHV} genes are found (Livermore 1995). The amino acid changes at positions 238 and 240 according to the Ambler classification (Ambler *et al.* 1991) are present in the SHV variant with ESBL activity (Huletsky *et al.* 1993; Hujer *et al.* 2001; Paterson *et al.* 2003). The serine residue at position 238 is crucial for effective hydrolysis of ceftazidime, while the lysine residue at position 240 is crucial for hydrolysis of cefotaxime (Huletsky *et al*. 1993). Presently, 120 SHV varieties have been detected and they are found worldwide (Jacoby and Bush 2009). The SHV ESBLs were the predominant ESBL type in Europe during the 1990s and were predominant in the United States during the beginning of the 21st century. The SHV-2, SHV-5 and SHV-12 variants are the most common ones (Paterson *et al*. 2003).

2.6.1.3. CTX-M β-lactamases

In 1986, a non-TEM and a non-SHV ESBL cephalosporinase was discovered in a cefotaxime-resistant *E. coli* (Matsumoto *et al.* 1988). A few years later in 1989, similar cefotaxime-resistant strains were found in Germany, France and Argentina (Bauernfeind *et al.* 1990; Bauernfeind *et al.* 1992). This cefotaxime resistance was due to enzymes named cefotaximases (CTX-M), and these CTX-Ms showed a much higher degree of activity to cefotaxime than to ceftazidime (Walther-Rasmussen and Hoiby 2004). In Table 1, examples of the first detection of a CTX-M in some countries are presented. The CTX-Ms represent plasmid acquisition of β-lactamase genes that can normally be found on the chromosome of *Kluyvera* species (Humeniuk *et al*. 2002). *Kluyvera* is a infrequent, opportunistic pathogen of the *Enterobacteriaceae* family (Farmer *et al.* 1981). The $bla_{\text{CTX-M}}$ gene variants show less than 40% identity to bla_{SHV} and bla_{TEM}, and are hence not closely related. Different properties are involved in the

cefotaxime-hydrolysing activity of the *bla*$_{CTX-M}$ genes. The most important amino acids residues for the ESBL activity of the CTX-M β-lactamases are Asn104, Ser237, Asp240 and Arg276 (Ishii *et al.* 1995; Gazouli *et al*. 1998; Shimamura *et al*. 2002; Bonnet *et al*. 2003).

Table 1. The first occurrence of CTX-M β-lactamase in countries representing the different geographical regions in the world.

	country (year[a])	CTX-M sub group [b]	species	reference[c]
Scandinavia	Sweden (2002)	1 (*bla* $_{CTX-M-15}$)	*E. coli*	Fang *et al.* 2004
	Norway (2003)	1 (*bla* $_{CTX-M-15}$)	*E. coli*	Tofteland *et al.* 2004
Europe	United Kingdom (2000)	1 and 9 (*bla* $_{CTX-M-15}$)	*E. coli*	Alobwede *et al* . 2003
	France (1989)	1, 2 and 9	*E. coli*	Bernard *et al.* 1992
	Spain (2000)	9 (*bla* $_{CTX-M-9}$ and *bla* $_{CTX-M-14}$)	*K. pneumoniae and Enterobacter* spp.	Canton *et al.* 2002,
North America	the United States (2002)	1	*E. coli*	Moland *et al* . 2003
	Canada (2000)	1 and 9 (*bla* $_{CTX-M-15}$ and *bla* $_{CTX-M14}$)	*E. coli*	Mulvey *et al.* 2004
South America	Argentina (1990)	2, 8 and 9	nontyphoid *Salmonella*	Bauernfeind *et al.* 1992
Asia	Japan (1986)	2	*E. coli*	Matsumoto *et al.* 1988

[a] when the first CTX-M was reported

[b] the most common sub group and (gene variant)

[c] for the first reported CTX-M

In the late 1990s, seven different *bla*$_{CTX-M}$ genes had been described but have since grown rapidly and now over 80 different variants have been found (Jacoby and Bush 2009). The CTX-M β-lactamases can be grouped into five subgroups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTXM-25, on the basis of amino acid sequence similarity. The clusters differ by approximately 20%, when the identity within a cluster is approximately 98%. CTX-M-8 and CTX-M-9 probably originate from *Kluyvera georgiana* (Poirel *et al.* 2002; Olson *et al.* 2005), while the CTX-M-1 and -2 family evolved from *K. ascorbata* (Rodriguez *et al.* 2004).

The CTX-M enzymes have been detected in a variety of *Enterobacteriaceae* species, from widely separated geographical regions. However, the CTX-M variants are mostly found in *E. coli, S. typhimurium*, *K. pneumoniae* and *Proteus mirabilis*. CTX-M-1 (especially the *bla*$_{CTX-M-15}$ gene belonging to the CTX-M-1 sub group), *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-14}$ are the most common gene types in Europe (Woodford *et al.* 2004; Livermore *et al*. 2007). CTX-M-14 and CTX-M-15 have recently emerged in the United States (Castanheira *et al.* 2008).

A chromosomally mediated β-lactamase, OXY/K1, is produced by most strains of *K. oxytoca.* This enzyme has a 70 to 75% similarity with the CTX-M enzymes (Tzouvelekis *et al*. 2000). K1 hydrolyses penicillins, ceftriaxone, cefotaxime, and aztreonam, while it is inhibited by clavulanic acid, like the plasmid-mediated CTX-Ms. A mutational hyperproduction of the oxy/K1 in a strain express susceptibility to ceftazidime but resistance to piperacillin, cefuroxime, and aztreonam. The K1 β-lactamases are usually not regarded as ESBLs (Gheorghiu *et al*. 1997) but they produce a level of resistance similar to ESBLs.

2.6.1.4. Class A carbapenemases

The molecular class A carbapenemases, including the plasmid-mediated serine β-lactamases KPC (named after *K. pneumoniae* carbapenemase) and GES (Guiana extended spectrum) and the chromosomally encoded SME (named after *Serratia marcescens* enzyme) and IMI/NMC (imipenem-hydrolyzing β-lactamase/not metalloenzyme carbapenemase) enzymes, are effective carbapenemases. KPC was first detected in North Carolina 2001 (Fridkin *et al.* 1999; Yigit *et al.* 2001; Yigit *et al.* 2008) and has become common in the North Eastern part of the United States and Israel, but is also emerging in China and Europe (Bradford *et al.* 2004; Lomaestro *et al.* 2006; Leavitt *et al.* 2007; Wei *et al.* 2007; Cuzon *et al.* 2008; Woodford *et al.* 2008). So far six variants are known (Jacoby and Bush 2009), distinguished by one or two amino-acid substitutions. KPC-1 was found in North Carolina, KPC-2 in Baltimore and KPC-3 in New York. The revised sequence of KPC-1 has shown that it is identical to that of KPC-2 (Yigit *et al.* 2008). The GES/IBC family of β-lactamases is an infrequently encountered family that was first described in 2000 (Poirel *et al.* 2000). The SME and IMI/NMC enzymes occur among *Serratia* and *Enterobacter* isolates (Livermore and Paterson 2006). The hydrolytic mechanism of these enzymes requires an active-site serine at amino acid position 70 according to Ambler numbering (Ambler *et al.* 1991). The class A carbapenems have the ability to hydrolyze a broad spectrum of antibiotics, including carbapenems, cephalosporins, penicillins, and aztreonam, and all are inhibited by clavulanic acid and tazobactam (Queenan and Bush 2007).

2.6.2. Metallo β-lactamases

The metallo β-lactamases represent the class B β-lactamases and were found 40 years ago. They are able to degrade all classes of β-lactams except monobactams. Additionally, they have an efficient activity against carbapenems. The metallo β-lactamases are resistant to therapeutic β-lactamase inhibitors. The metallo β-lactamases are zinc-dependant and so called EDTA-inhibited enzymes and are mostly found in *Pseudomonas* sp. and *Serratia* sp. (Bebrone 2007). The metallo β-lactamases are predominantly chromosomally encoded but can also be found on plasmids. The metallo β-lactamases are subdivided on the basis of sequence alignments into three subclasses B1, B2 and B3 (Garau *et al*. 2005b), but phylogenetic analysis have revealed that subgroup B3 lacks detectable sequence similarity with B1 and B2 (Hall *et al.* 2003). The subclasses have different zink binding sites. B1 and B3 are able to bind one or two zink ions, while B2 are mono-Zn enzymes that have evolved specificity toward carbapenems (Heinz and Adolph 2004; Garau *et al.* 2005a; Gonzalez *et al.* 2007). Subgroup B1 represents carbapenemases, IMP (active on imipenem) and VIM (Verona integron-encoded metallo β-lactamase).

The class B carbapenemases, e.g. IMP and VIM, are clinically the most important metallo β-lactamases. The IMP-type carbapenemases currently consisting of 24 varieties (Jacoby and Bush 2009), became established in Japan in the 1990s in both enteric Gram-negative organisms and in *Pseudomonas* and *Acinetobacter* species (Rasmussen and Bush 1997). The IMP enzymes spread slowly to other countries in the Far East, and the first IMP was reported in Europe in 1997 (Cornaglia *et al*. 1999). The

VIM family, a second growing family of carbapenemases, was first discovered in *P. aeruginosa* in Italy in 1996 (Cornaglia *et al*. 2000) and includes now 22 members (Jacoby and Bush 2009), which have a wide geographic distribution in Europe, South America, the Far East and the United States. Both IMP and VIM are integron-associated, sometimes within plasmids and they hydrolyse all β-lactams except monobactams, and evade all β-lactamase inhibitors. GIM-1 (German imipenemase) was isolated in Germany in 2002 (Castanheira *et al.* 2004). The enzyme SIM-1 (Seoul imipenemase) has the closest amino acid identity to the IMP family (64 to 69%). Since their initial discoveries, SPM, GIM, and SIM metallo β-lactamases have not spread beyond their countries of origin. However, VIM and IMP continue to be detected worldwide, with an overall trend of these two metallo- β-lactamases moving beyond *P. aeruginosa* and into the *Enterobacteriaceae* (Queenan and Bush 2007).

2.6.3. Class C β-lactamases

Class C β-lactamases (AmpC) are an important group of proteins that are broadly distributed. This is the second most common β-lactamase group (Bush *et al.* 1995). AmpC is typically encoded on the chromosome of Gram-negative bacteria including *Citrobacter*, *Serratia* and *Enterobacter* species where its expression is usually inducible. About 20 years ago, the inducible chromosomal genes were detected on plasmids and were transferred to organisms, typically not expressing these types of β-lactamase, like *Klebsiella* spp., *E. coli*, or *Salmonella* spp. (Philippon *et al.* 2002; Hanson 2003). CMY, MOX and FOX are examples of plasmid-mediated (also called transferable) AmpC β-lactamases (Bauernfeind *et al.* 1996a; Doi and Paterson 2007; Adler *et al.* 2008). In *E. coli* AmpC is poorly expressed, while in *Klebsiella* and *Salmonella* species the *ampC* gene is missing from the chromosome. AmpC is constitutively overexpressed either by deregulation of the *ampC* chromosomal gene or by acquisition of a transferable *ampC* gene on a plasmid.

AmpC β-lactamases, in contrast to ESBLs, hydrolyse broad and extended-spectrum cephalosporins but are resistant to inhibition by β-lactamase inhibitors such as clavulanic acid (Sanders 1987; Thomson 2001; Hanson 2003). Strains only producing AmpC can be detected but organisms producing plasmid-encoded AmpC and ESBLs are difficult to distinguish by phenotypical testing. Cefoxitin (a cephamycin) resistance may indicate the possibility of AmpC-mediated resistance but it can also be an indication of reduced outer membrane permeability (Philippon *et al.* 2002). Cefepime resists hydrolysis by AmpC and can hence be used as a possible screening agent (Tzouvelekis *et al.* 1999). Almost 100 different AmpC enzymes exist today and are commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria (Jacoby and Bush 2009).

2.6.4. Class D β-lactamases

The OXA β-lactamases differ from the TEM and SHV enzymes and they belong to class D according to Ambler (Livermore and Paterson 2006). The OXA group mainly

occur in *Acinetobacter* and *Pseudomonas* species. Originally, the family of OXA β-lactamase was created as a phenotypic group for a few β-lactamases that had a specific hydrolysis profile (Ambler 1980). The first OXA β-lactamase (ARI 1) with carbapenemase activity was described by Paton *et al.* in 1993.

The OXA β-lactamases attack the oxyimino-cephalosporins and have a high hydrolytic activity against oxacillin and cloxacillin. Various OXA derivatives that are not ESBLs have also been described (Paton *et al.* 1993). Some OXAs, like OXA-51, are chromosomally encoded and are found in *A. baumannii*. Even though a variation in activity can be seen, these are not considered to be ESBLs (Afzal-Shah *et al.* 2001). Substitutions at one of two amino acid positions are probably required for the ESBL phenotype; an asparagine for serine at position 73, or an aspartate for glycine at position 157 (Heritier *et al.* 2005). In particular, the Gly157Asp substitution may be necessary for high-level resistance to ceftazidime. The OXA-type β-lactamases are poorly inhibited by clavulanic acid. OXA carbapenemases hydrolyse carbapenems very slowly *in vitro*. Many of the bacterial isolates carrying OXA β-lactamases have been found in Turkey and France (Philippon *et al.* 1997; Danel *et al.* 1999) and since there are frequently located on plasmids or integrons they can be widely spread (Naas and Nordmann 1999; Bradford 2001). Currently, 139 OXA-type β-lactamases have been detected (Jacoby and Bush 2009).

2.6.5. Other β-lactamases

In addition to the clinically most important β-lactamases mentioned above, there are hundreds of β-lactamases of which some are clinically important ESBLs. Several uncommon ESBLs can be found in *Enterobacteriaceae* (Naas *et al.* 2008), like BES-1 (Brazil extended-spectrum β-lactamase), (Bonnet *et al.* 2000), IBC (integron-associated β-lactamase), PER-2 (Bauernfeind *et al.* 1996b), SFO-1 (homolog to the β-lactamase of *S. fonticola*) (Matsumoto and Inoue 1999), and TLA (Silva *et al.* 2000). These uncommon ESBLs have been found in a limited number of geographic sites. However, new gene variants are detected all the time in different enterobacterial species.

2.7. Detection of ESBLs

Since these β-lactamase enzymes have rapidly evolved, not only a major therapeutic dilemma has raised, but also problems to detect these enzymes. Especially important is the detection of plasmid encoded ESBLs. Antibiotic resistance among bacteria can in general be detected either phenotypically or genotypically. For clinically important bacteria, diagnostic laboratories perform phenotypic-based analyses using standardised susceptibility testing methods, the disk diffusion, E-test or agar dilution method. However, for ESBLs standard MIC or disk diffusion based methods may not be enough.

2.7.1. Minimal inhibitory concentration

In a dilution-based growth inhibition assay, the minimal inhibitory concentration (MIC) can be determined for a bacterial isolate and the tested antibiotics, thereafter it is interpreted as susceptible, intermediate, or resistant. The MIC category "breakpoints" are based on an evaluation of the clinical efficacy of the drug, and a comparison of MICs of micro-organisms from a variety of sources.

The breakpoints do not always differentiate strains with or without resistance mechanisms. This is especially true with present CLSI cephalosporin breakpoints. It is a well-known fact that *Enterobacteriaceae* species (*E. coli*, *Klebsiella* spp or *Proteus mirabilis*) can have a functional ESBL gene and still have 3rd generation cephalosporin MIC so low that the strain would be classified as susceptible to that antibiotic if CLSI breakpoints were used (Clinical and Laboratory Standards Institute 2007a). This kind of strains is problematic, since they can be clinically resistant despite of the low MICs. There are two different approaches to overcome this problem. Firstly, different kind of phenotypic and genetic methods can be used to detect production of ESBLs or the precence of ESBL gene (see 2.8.2 and 2.8.3). Secondly, new better and clinically more relevant breakpoints for antibiotics can be defined.

EUCAST (the European Committee on Antimicrobial Susceptibility Testing, http://www.escmid.org/sites/index_f.aspx?par=2.4) was set up in 1996, in order to standardise the antimicrobial breakpoints and susceptibility testing in Europe so that comparable results and interpretations are produced. EUCAST utilize modern multidisciplinary approach for setting breakpoints for antibiotics. This procedure includes, for example, microbiological data (wild type MIC distributions), Pharmacokinetic/Pharmacodynamic data, clinical data and Monte Carlo simulation to obtain clinically relevant breakpoints. These so called clinical breakpoints can be used in everyday clinical laboratory work to advise correct therapy for patients. EUCAST already have new breakpoint for cephalosporins, however, not all European countries are using them yet.

2.7.2. Extended-spectrum β-lactamase tests

All clinically relevant ESBLs are serine β-lactamases. This means that their function can be, at least in theory, blocked by β-lactamase inhibitors (e. g. clavulanic acid). The most common ESBLs (TEM, SHV, CTX-M) are clavulanic acid susceptible. This property can be utilized in ESBL diagnostics. If the strain produces ESBL, 3rd generation cephalosporin combined with clavulanic acid inhibit bacterial growth more efficiently than the cephalosporin alone. Because the problems with the detection of ESBL producing strains with MIC breakpoints, CLSI have proposed diagnostic tests that utilise this synergy between β-lactamase inhibitors and cephalosporins. According to the CLSI criteria, five different antimicrobial agents (cefotaxime, ceftazidime, ceftriaxone, aztreonam and cefpodoxime) can be used for screening for ESBLs in *E. coli* and *Klebsiella* spp. that may indicate an isolate to be ESBL producer. Subsequently a confirmatory test, based on synergism, is required. A $\geq$ 5-mm increase in zone diameter or a $\geq$ 3 twofold concentration decrease in an MIC, for e.g. cefotaxime tested in combination with clavulanic acid versus tested alone confirms

production of ESBL. These CLSI criteria for screening and confirmation of ESBL producers have been the golden standard also in Finland. In this CLSI ESBL screening and confirmatory method, the isolates are first examined for resistance with an indicator cephalosporin, and secondly the synergy between an oxyimino-cephalosporin and clavulanate is tested, distinguishing the strains with a positive synergy (i. e. ESBLs) from the strains without synergy (i. e. non-ESBL β-lactamases). In general, it is recommended to use several cephalosporins for the detection of ESBLs. Ceftazidime works well with TEM and SHV, while cefotaxime is adequate with CTX-M enzymes. Cefotaxime and ceftazidime should be used together, while cefpodoxime, which is also considered a good indicator, can be used alone (Clinical and Laboratory Standards Institute 2005b; Clinical and Laboratory Standards Institute 2007a).

The screening and confirmation methods only indicate the possible presence of ESBLs (Livermore and Brown 2005). Since the amount of different β-lactamases has increased rapidly and also the combination of different β-lactamases is more common in different species the detection and verification of presence of ESBLs have become more and more difficult. The large amount and variety of ESBL genes are not enough for making the ESBLs problematic. Different resistance phenotypes may be expressed by the same enzyme, depending on the bacterial host and the test conditions. In TEM and SHV ESBLs the expanded spectrum is accompanied by a loss of intrinsic hydrolytic activity. However, this can be compensated by an increase of the gene copy number. This increase can be due to gene duplication, carriage on a multicopy plasmid or the presence of a promoter with increased activity (Nordmann 1998). In *K. pneumoniae,* ESBL production is often followed by a decreased expression of Omps. This leads to cefepime resistance in TEM and SHV ESBLs or allows an AmpC to express resistance to imipenem (Bush and Singer 1989; Queenan *et al.* 2004).

2.7.3. Molecular based ESBL detection methods

The only way to define and identify the presence of a β-lactamase gene is by genetic methods, e.g. PCR and sequencing. By these methods it is possible to identify the different point mutations that can cause an ESBL. However, the clinical meaning of the different β-lactamases is the most important issue and clinically relevant for the outcome of the patient, and not the mechanisms or gene behind the resistance (Queenan and Bush 2007).

Pyrosequencing is a sequencing method based on the principle "sequence-by-synthesis" (Ronaghi *et al.* 1998). The method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it. It is possible to detect sequences up to 60 bp. The reaction cascade of enzymes produces an impulse of light when a nucleotide is added to a complementary base in the single stranded template DNA and a base pair is formed. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Then ATP sulphurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP and the number of incorporated nucleotides. The light signal is detected and presented by the device as a so called pyrogram. The technology of

pyrosequencing is ideal for genetic analysis and if using a multiplex pyrosequencing assay, several polymorphisms in a single well can be detected (I; III; Haanperä *et al.* 2005).

2.8. Emerging problems with extended-spectrum β-lactamases

The ESBLs are a heterogenous group. The enzymes have differencies in the activity level against the β-lactams. Additionally, the co-existence of several β-lactamases, variation of the catalytic efficiencies of the β-lactamases, as well as the penetration rates of β-lactams into bacterial cells modifies the gene expression. For example, TEM and SHV have similar activity against β-lactams, but some variants like TEM-12 have only minor ESBL activity (Livermore and Paterson 2006). Rapid and accurate tests that identify bacterial infections, and which antimicrobial agents are suitable for treatment, are important. Even though the β-lactam antibiotics are used worldwide, the prevalence and distribution is not consistent. Today most antibiotics are used in the community in day care centers, long term facilities and local practices. Today no detection test for the presence of ESBL is completely reliable. By correct ESBL detection, prescription of unnecessary antibiotics would be avoided.

The antibiotic resistance does not identify borders between clinics, humans or animals. About half of all antibiotics that are produced worldwide are used in animals (Greenwood *et al.* 2007). The same antibiotics that are used in humans are also used in animals. Most of the antibiotics are used in food animals to improve production and to prevent infections. Consequently, development of resistant bacteria in animals can pass the resistance genes to bacteria infecting humans (Sturenburg and Mack 2003). Transmission of genes may also be due to contact with companion animals, since ESBLs have been detected among companion animals (Millar *et al.* 2001; Costa *et al.* 2004; Moreno *et al.* 2008), and $bla_{\text{CTX-M-1}}$-type genes have also been detected among bacterial isolates from Finnish companion animals (Nyberg *et al.* 2006). ESBLs have also been detected among farm animals, for example, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ subgroups have been found among poultry, e.g. in France and Denmark (Weill *et al.* 2004b; Meunier *et al.* 2006; Wu *et al.* 2008),.

Antibiotics generate evolution also outside hospitals. On farms that use antibiotics in livestock production, resistant strains are common and have been found in soils and groundwater affected by farm effluents (Jacoby and Medeiros 1991). Lettuce or vegetables might be one source, since it is possible to isolate *E. coli* from vegetable specimens that have been contaminated with human or animal faeces (Hamilton-Miller and Shah 2001). ESBL producing *E. coli* has also been detected in cooked food (Lavilla *et al.* 2008). Another reservoir for antibiotic resistant bacteria is the soil microbiome; representing the proteobacteria phylum that contains bacterial species closely related to clinically significant pathogens. Hence antibiotic resistant genes could be obtained by clinical pathogens (Palumbi 2001). River water has also been indicated to be a reservoir for antimicrobial resistant bacteria (Kim *et al.* 2008). Sanitizers are being used during food manufacturing, and various cleaning products are also used by consumers. This use has not been proven to be an emerging risk factor for carriage of antimicrobial drug-resistant bacteria on hands (Aiello *et al.* 2005; 2006) but it might provide a suitable environment for emergence of resistant species.

There are several risk factors or problems with organisms producing ESBLs. These organisms are important causes of nosocomial infections and limited therapeutic options are available (Dantas *et al.* 2008). For young healthy adults an ESBL infection is not life threatening, and young adults do not acquire infections as easily as older adults. With an increasing age and decreasing health the risk is increased. The length of hospital stay, severity of the illness, time in the intensive care unit (ICU), urinary or arterial catheterization, and previous exposure to antibiotics are risk factors associated with UTI caused by ESBL- producing bacteria (Pitout *et al.* 2005a).

Even though, antibiotic resistance is mostly a nosocomial problem, resistant bacteria are also spread in the community, especially in day care centers and nursing homes (Schiappa *et al.* 1996; Lautenbach *et al.* 2001; Bisson *et al.* 2002; Pena *et al.* 2006). From being most prevalent in *K. pneumoniae* strains at hospitals, the prevalence of ESBLs has changed and they are now present in community-acquired *E. coli* strains. Due to the increase of different resistance mechanisms the treatment options are narrowing against Gram-negative bacteria.

3. AIMS OF THE STUDY

The main purpose of this work was to study the resistance genetics of ESBL-producing *Enterobacteriaceae* in Finland and to evaluate the diagnostic methods used in clinical laboratories.

The specific aims of the study were:

1. To study ESBL genetics in Finland and to compare the situation to other European countries (I, IV, unpublished).

2. To evaluate the automated VITEK 2 AST-N029 susceptibility testing and the existing screening and confirmation methods for detection of ESBL-producing *Enterobacteriaceae* (I, II).

3. To develop pyrosequencing methods for rapid identification of SHV ESBL genes (III).

4. MATERIALS AND METHODS

4.1. Bacterial isolates

A total of 994 *Enterobacteriaceae* strains arrived during the period of 2000-2007 to the former Laboratory of Human Microbial Ecology at the National Public Health Institute, Turku, Finland (nowadays known as Department of Infectious Disease, Division of Health Protection, the National Institute for Health and Welfare) (Table 2). All the strains were not included in the original publications, since the strains in the publications were selected based on a specific criterion in different studies. Therefore the strains that were included in the original publications are described below.

(I) The material consisted of 402 cefuroxime-resistant *E. coli* and *Klebsiella* spp. clinical isolates. These isolates were collected from 26 different laboratories included in the Finnish Study Group for Antimicrobial Resistance (FiRe) network, between January 2002 and December 2004. The strains were mainly isolated from urine, but also from pus, bile, mucous and wound.

(II) The material consisted of 123 non-duplicate clinical isolates. Of these, 62 were *E. coli*, 36 *K. pneumoniae* and 25 *K. oxytoca* isolates, representing ESBL and non-ESBL-producers, selected from the cefuroxime-resistant *E. coli* and *Klebsiella* spp and from the collection of the Clinical microbiology laboratory of the Turku University Hospital.

(III) The isolates included in this study were 106 *K. pneumoniae* and ten *E. coli* with SHV gene. Sixty-six *K. pneumoniae* isolates represented different SHV variants from the FiRe collection, while in 40 *K. pneumoniae* isolates, the SHV-type had not been confirmed by cyclic sequencing due to heterogeneous sequences corresponding to the amino acid positions 35, 238 and/or 240. The ten SHV-positive *E. coli* isolates were selected as controls.

(IV) The base study population consisted of all patients in Helsinki City public health care system from which *E. coli* or *K. pneumoniae* isolates with an ESBL implying phenotype were isolated from the clinical samples. Only the ESBL producing isolates (ESBL positive by the CLSI criteria), isolated from patients for the first time during the study period from January 2000 to December 2004 were included in the material. A total of 746 patients with *E. coli* ESBL colonisation or infection and 91 with *K. pneumoniae* ESBL colonisation or infection were identified during the surveillance. From the 91 patients with *K. pneumoniae* infection or colonisation, 84 isolates were available for further analysis while only those patients with *E. coli* blood isolates (n=36) were included in the study. Of the *E. coli* isolates only the blood isolates were included, since most of the urine isolates collected represented possible clusters and hence would affect the clonality analysis. An isolate was defined to be health-care associated if it was nosocomial (isolation obtained >48h after admission) or the patient had been hospitalised within a year prior to culture (excluding clinic visits) or if the patient was a resident in a long-term care

facility at the time of the index isolation. The isolate was defined to be community-acquired if none of the above mentioned criteria was fulfilled.

Table 2. Source of the 994 strains included in this study.

	source					
	urine	blood	faeces	wound	nd	total
Citrobacter spp.	1	1				2
E. cloacae					1	1
E. coli	332	55	2	31	248	668
K. ornithinolytica				1	1	2
K. oxytoca	11			1	31	43
K. pneumoniae	113	6	5	25	126	275
K. terrigena					1	1
Salmonella spp.					2	2
total	457	62	7	58	410	994

4.2. Identification of isolates

The bacterial isolates were identified by biochemical methods. Most of the isolates were identified by the Api 20E identification system (bioMérieux, Lyon, France), while some strains were identified by conventional biochemical methods, where the initial identification was based on Gram staining and negative oxidase test. The Gram-negative and oxidase negative rods were further screened for β-glucuronidase activity with a PGUA disc (Rosco, Taastrup, Denmark) and checked for indole production.

4.3. Antimicrobial susceptibility testing (I-III)

4.3.1. The agar dilution method (I,II and IV)

MICs were determined according to the CLSI (former NCCLS) by a standard agar dilution method (Yao and Moellering 2003) on Mueller-Hinton II medium containing antimicrobial agents (Becton Dickinson Microbiology systems, Cockeysville, MD, USA) with a Denley multipoint Inoculator (Denley Instruments Ltd, Billinghurst, UK). The plates were incubated at 35˚C for 18 hours. The following antibiotics were tested: amoxicillin- clavulanic acid (0.5-64 µg/ml and 0.25-32 µg/ml respectively), ampicillin (0.25-256 µg/ml), aztreonam (0.03-64 µg/ml), aztreonam- clavulanic acid (1-128 µg/ml and 4 µg/ml respectively), cefepime (0.008-32 µg/ml), cefotaxime (0.008-64 µg/ml), cefotaxime-clavulanic acid (0.008-64 µg/ml and 4 µg/ml respectively), cefoxitin (0.125-128 µg/ml), ceftazidime (0.062-64 µg/ml), ceftazidime- clavulanic acid (1-128 µg/ml and 4 µg/ml respectively), cefuroxime (1-64 µg/ml), cephalothin

(0.5-64 µg/ml), chloramphenicol (1-128 µg/ml), ciprofloxacin (0.008-16 µg/ml), gentamicin (0.06-64 µg/ml), imipenem (0.06-64 µg/ml), meropenem (0.06-64 µg/ml), nalidixic acid (0.5-128 µg/ml), nitrofurantoin (0.125-256 µg/ml), piperacillin-tazobactam (0.5-128 µg/ml and 4 µg/ml respectively), streptomycin (0.25-128 µg/ml), sulfamethoxazole (0.25-1024 µg/ml), tetracycline (0.25-164 µg/ml), tobramycin (0,016-256 µg/ml), trimethoprim (0.03-1024 µg/ml) and trimethoprim-sulfamethoxazole (0,002-32 µg/ml), all from Sigma Chemical Co., St. Louis, MO, USA. *E. coli* American Type Culture Collection (ATCC) 25922, *E. coli* ATCC 35218, EARSS *E. coli* U2A1526 (an ESBL producer, CTX-M-1), *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were used as control strains for determination of MIC values.

4.3.2. The disk diffusion method (I-III)

Disk diffusion test was performed on Mueller-Hinton II agar (agar thickness 4 mm) with an inoculum equal to 0.5 McFarland turbidity according to CLSI (Clinical and Laboratory Standards Institute 2005a). The plates were incubated at 35°C for approximately 18 hours. The following Oxoid antimicrobial discs were tested: amoxicillin-clavulanic acid (30 µg), aztreonam (30 µg), cefotaxime (30 µg), cefpodoxime (10 µg), ceftazidime (30 µg), ceftriaxone (30 µg), CD01; cefpodoxime/clavulanic acid (10/1 µg), CD02; ceftazidime/clavulanic acid (30/10 µg), CD03; cefotaxime/clavulanic acid (30/10 µg); piperacillin-tazobactam (110 µg) all from Berner Oy, Helsinki, Finland. *E. coli*, ATCC 25922, *E. coli* ATCC 35218, EARSS *E. coli* U2A1526, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were used as control strains.

4.3.3. The VITEK 2 method (II)

The VITEK 2 (BioMérieux) is an automated system performing bacterial identification and rapid antimicrobial susceptibility testing method from a manually prepared inoculum (Clinical and Laboratory Standards Institute 2007b). The bacterial growth is monitored in a few antibiotic concentrations by the instrument which gives an estimation of the MIC by comparing the growth curves to those of reference strains with known MICs. A comment is provided by the Automated Expert System (AES) on the results and also a suggestion of the resistance mechanisms that are consistent with the susceptibility profile of the tested strain may be obtained. This is accomplished by comparison to a database with susceptibility profiles of a number of strains of the same species with known resistance mechanisms. AES may also suggest corrections to the primary results on the basis of the detected mechanisms, e.g. edit the interpretations for all cephalosporins and aztreonam as resistant once it has recognised the tested strain as a probable ESBL producer. The following cards were used: AST-N029, a card designed for antibiotics used in the Nordic countries and AST-N041, which is designed to detect ESBL producers.

4.4. Detection and sequencing of β-lactamase genes (I-IV)

4.4.1. DNA isolation and PCR primers

The DNA was liberated from pure cultures of each strain by heating a bacterial suspension for ten minutes at 95˚C. The specific primers designed for the PCR amplification, DNA sequencing and pyrosequencing reactions are described in Table 3. The primers were synthesized by Thermo-electron (Thermo Electron GmbH, Ulm, Germany).

4.4.2. Amplification of DNA by PCR

All isolates were screened for the resistance genes bla_{SHV}, bla_{TEM}, and $bla_{CTX\text{-}M}$ by PCR, with specific primers (Table 3) that were designed on the basis of published sequences of TEM, SHV and CTX-M groups. The PCR reaction for the metallo and OXA β-lactamases were preformed as described earlier (Ellington *et al.* 2006; Woodford *et al.* 2006).

Table 3. PCR and sequencing primers.

primer	sequence 5´-3´	application	reference
CTX-con-PCR-f	ATGTGCAGYACCAGTAARGTKATGGC	PCR	I
CTX-con-PCR-r	CDCCGCTGCCGGTYTTATCVCC	PCR	I
CTX_rev_2_bio_mod	TCGCCGGGAATGGCGGT	PCR	I
CTX_pyro_660	GCGYTRCAGTAYAGCG	sequencing primer	I
CTX_pyro_687	ATGAATAARYTGATTGC	sequencing primer	I
SHV_plasmid_141f	TGTCGCTTCTTTACTCGCCTTT	PCR	I
SHV_plasmid_1064r	CGGGTTAGCGTTGCCAGTGCT	PCR	I
SHV_seq_209f	TTCGCCTGTGTATTATCTMCCT	sequencing primer	I
SHV_seq_495f	GGACTACTCGCCGGTCAGCGAA	sequencing primer	I
SHV_seq_595r	ATTGGCGGCGCTGTTATCGCTC	sequencing primer	I
SHV_seq_805f	CTGCTGCAGTGGATGGTGGAC	sequencing primer	I
SHV_seq_971r	TAAATCACCACAATGCGCTCT	sequencing primer	I
SHV_No-PCR	ATGCGTTATATTCGTCTGTGCAT	PCR	Tofteland *et al* . 2007
SHV_pyro_35_Rbio	CCGCACAGCACCACTTTA	PCR	III
SHV_seq_35	CGCAGCCGCTGGAACAGATTA	sequencing primer	III
SHV_cod238_1	CTGGTTTATCGCCGATAAGA	PCR	III
SHV_pyro_238_Rbio	TTGCCAGTGCTCGATCAG	PCR	III
SHV_seq_238_240	TATCGCCGATAAGACCGGAG	sequencing primer	III
Tem_kok_f2	ATTTYCGTGTCGCCCTTATTCC	PCR	I
Tem_kok_r2	ATGCTTAATCAGTGAGGCACCTA	PCR	I
Tem_seq_17f	TYCGTGTCGCCCTTATTCCCTT	sequencing primer	I
Tem_seq_361f	TGCAGTGCTGCCATAACCATGA	sequencing primer	I
Tem_seq_396f	TGCCAACTTACTTCTGACAAC	sequencing primer	I
Tem_seq_503r	CGCTCGTCGTTTGGTATGGCTTC	sequencing primer	I
Tem_seq_851r	ATCAGTGAGGCACCTATCTCAG	sequencing primer	I

The 25 µl PCR reaction volume for TEM contained 0.4 µM of each primer, 0.03 U/µl AmpliTaqGold-LD polymerase (Applied Biosystems, Bridgewater, NJ, USA), 10 x AmpliTaqGold-LD buffer (Applied Biosystems), 2 mM $MgCl_2$ and 0.2 mM DNA

polymerisation mix (Amersham Biosciences, Piscataway, NJ, USA). The 25 µl reaction mix for CTX-M contained 0.4 µM of each primer, 0.03 U/µl AmpliTaqGold-polymerase (Applied Biosystems), 10 x AmpliTaqGold-buffer (Applied Biosystems), 2 mM $MgCl_2$ and 0.2 mM DNA polymerisation mix (Amersham Biosciences). The 25 µl SHV reaction mix contained 0.2 µM of each primer, 0.02 U/µl Taq-polymerase, 10 x PCR buffer ($MgCl_2$ included) both from Roche, Espoo, Finland, 6% DMSO and 0.2 mM DNA polymerisation mix (Amersham Biosciences). All reactions contained 5 µl of bacterial suspension as the template. The bla_{TEM} genes were amplified by 40 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 1 min, and extension at 72˚C for 1 min. The bla_{SHV} genes were amplified by 40 cycles of denaturation at 94˚C for 30 s, annealing at 60˚C for 1 min, and extension at 72˚C for 2 min. The CTX-M PCR reaction was submitted to 38 cycles of denaturation at 94˚C for 30 s, annealing at 61˚C for 1 min, and extension at 72˚C for 90 s. The Peltier Thermal Cycler PTC-200 (MJ Research, Inc., Watertown, MA, USA) was used for all PCR amplification reactions. The PCR products were analysed by electrophoresis in 1% SeaKem LE agarose gel (Cambrex Bio Science, Rockland, ME, USA).

4.4.3. Purification and sequencing of DNA

The PCR products were purified with High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Espoo, Finland) according to the manufacturer's instructions. The sequencing primers that were used for bla_{TEM} and bla_{SHV} genes are shown in Table 3. The sequencing reactions were done by using an ABI BigDye™ Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) on an iCycler iQ5 (Bio-Rad, Hercules, CA, USA) and analysed with an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems).

4.4.4. Data analysis of DNA sequences

The DNA sequences were analysed, edited and translated into amino acids sequences with Vector NTI software (Informax, Inc, No. Bethesda, MD, USA). The amino acid sequences were compared to known published TEM and SHV variants by Fasta - protein similarity search - through the European Bioinformatics Institute (http://www.ebi.ac.uk/fasta33/index.html) and also manually compared to known sequences (K. Bush and G.A. Jacoby, 2005-2007, http://www.lahey.org/studies/webt.htm).

The pyrosequencing method was used for the grouping of CTX-M positive isolates. A large amount of different bla_{CTX-M} genes (K. Bush and G.A. Jacoby, 2005, http://www.lahey.org/studies/webt.htm) representing the five main groups; CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, were aligned, and a specific part of 15 bp located at bp 702-717 in the DNA sequence of AF189721 was chosen in order to separate the main groups from each other by pyrosequencing, see Figure 8.

Figure 8. Alignment of the 15 bp DNA sequence that was chosen for separation of the five main CTX-M subgroups by pyrosequencing.

For separation of *bla*$_{SHV}$ genes, the nucleic acid sequences corresponding to the amino acid positions 35 and 238-240 were included to the SHV pyrosequencing assays.

4.4.5. CTX-M pyrosequencing

A specific biotinylated reverse primer, CTX-_rev_2_bio_mod (Table 3), was designed for PCR, while the same forward primer was used as before in this study. The 25 µl PCR reaction for CTX-M contained 0.4 µM of each primer, 0.03 U/µl AmpliTaqGold-polymerase, 10 x AmpliTaqGold-buffer, 2 mM $MgCl_2$, 4% DMSO and 0.2 mM DNA polymerisation mix. A specific sequencing primer, CTX_pyro_687 (3), was designed for the pyrosequencing reaction to be able to distinguish the different groups of CTX-M. All CTX-M positive isolates were sequenced by pyrosequencing according to the instructions of the manufacturer (Biotage Ab, Uppsala, Sweden).

4.4.6. Pyrosequencing of the bla$_{SHV}$ genes

The PCR reactions for amino acid position 35 and 238-240, contained 0.4 µM primers, 0.030 U/ml Taq polymerase, 1x PCR Buffer with $MgCl_2$ (Roche), 6% DMSO and 0.2 mM dNTPs (GE Healthcare UK Ltd, Buckinghamshire, UK). The temperature cycling consisted of 10 min at 95ºC followed by 40 cycles of 30 sec at 94ºC, 30 sec at 54ºC and 30 sec at 72ºC. The temperature cycling was performed on an iCycler iQ5 (Bio-Rad).

4.5. Genotyping by Pulsed Field Gel Electrophoresis

In order to investigate genetic relatedness between isolates, the Pulsed Field Gel Electrophoresis (PFGE) was performed. Bacterial suspensions, with optical density at 550 nm of 0.50-0.70, corresponding to a turbibidity of 2-2.5 McFarland, were used. The PFGE-analysis of the *E. coli* and *Klebsiella* spp. isolates was performed as previously described (Kaufmann 1998). For digestion, the restriction endonuclease *Xba*I (New England BioLabs, Inc., USA) was used and the DNA fragments were separated in 1.2% PFGE-agarose gel with a CHEF DR^R III system (Bio-Rad, Hercules, CA, USA). A CHEF DNA size standard, Lambda ladder (Bio-Rad) was used as a molecular size standard and was included in every run. Banding patterns were analysed according to Dice (a coefficient used to analyze the similarities of the PFGE banding patterns) and UPGMA (Unweighted Pair Group Method with Arithmetic mean) with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The banding patterns were categorized according to Tenover *et al.* 1995.

5. RESULTS

5.1. Genetic basis of ESBLs in Finland (I-IV, unpublished)

PCR primers were designed and the reactions were optimized for the *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$ genes in order to study the genetic basis of β-lactam resistance among the *Enterobacteriaceae* strains. Additionally, pyrosequencing assays for the *bla*$_{CTX-M}$ and *bla*$_{SHV}$ genes were developed. The CTX-M pyrosequencing assay separates the five main subgroups from each other, while the SHV assay distinguishes the classical β-lactamases from the broad and extended-spectrum β-lactamases.

5.1.1. Distribution of ESBL genes

The material from the original publications with the unpublished data represents 994 human clinical *Enterobacteriaceae* isolates. Of these, 986 represented *E. coli*, *K. pneumoniae* and *K. oxytoca* and 784 (78.9%) carried one or several of the above mentioned ESBL genes. The prevalence of β-lactamase genes in all of these isolates is shown in Table 4. Fifty-one different gene or gene combinations were detected among these isolates. In addition, eight human clinical isolates representing other enterobacterial species two *Citrobacter* spp., one *Enterobacter cloacae*, one *Klebsiella terrigena*, two *Klebsiella ornithinolytica and* two *Salmonella* spp. were analysed. The following genes were detected among these isolates: one *Citrobacter* isolate carried a *bla*$_{CTX-M}$ gene, one *K. ornithinolytica* carried *bla*$_{TEM-1}$, one *K. terrigena* had *bla*$_{CTX-M-1}$, *bla*$_{SHV-1}$ and *bla*$_{TEM-1}$, one *Salmonella* had *bla*$_{CTX-M-1}$ and *bla*$_{TEM-1}$, while one *Salmonella* had a *bla*$_{TEM-63}$. A small amount of strains that had a decreased susceptibility to carbapenems were tested for metallo and OXA β-lactamases, but no positive strains were found.

5.1.1.1. ESBLs among cefuroxime resistant strains (I)

In the study of the molecular genetics of ESBLs in Finland (I), a total of 464 isolates were collected from the FiRe laboratories. However, only 402 of these were resistant to cefuroxime and hence included in the study. Of these 402 strains, 274 were *E. coli*, 97 *K. pneumoniae* and 31 *K. oxytoca* isolates. In all, 200 (73.0%) of the *E. coli* strains and 67 (69.1%) of the *K. pneumoniae* carried an ESBL gene. None of the 31 *K. oxytoca* strains had an ESBL gene. They all carried the chromosomal oxy/K1. The distribution of the ESBL genes among *E. coli* and *K. pneumoniae* is shown in Table 5. The non-ESBL TEM-1 was common and detected in 161 (40.0%) *E. coli* and 49 (50.5%) *K. pneumoniae* strains. A *bla*$_{CTX-M}$ - type gene (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-9}$) alone or in addition with a classical β-lactamase gene was detected in 184 (92.0%) of the *E. coli* strains. Of these strains, a *bla*$_{CTX-M-1}$-type gene from two strains and a *bla*$_{CTX-M-9}$-type gene from nine strains were sequenced. The *bla*$_{CTX-M-1}$-type gene represented a *bla*$_{CTX-M-15}$ gene, while of the nine strains with a *bla*$_{CTX-M-9}$-type gene four had a *bla*$_{CTX-M-9}$ gene and five a *bla*$_{CTX-M-14}$ gene. A *bla*$_{SHV}$ ESBL gene was detected in

Table 4. The prevalence of β-lactamases among the 986 *E. coli, K. pneumoniae* and *K. oxytoca* strains included in this study. The $bla_{\text{CTX-M}}$ genes were only typed on the subgroup level.

	E. coli		*K. pneumoniae*		*K. oxytoca*		total	
genes	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
ESBLs								
CTX-M-1	86	12.9	2	0.7	2	4.7	90	9.12
CTX-M-2	1	0.1	0	0.0	0	0.0	1	0.10
CTX-M-9	42	6.3	2	0.7	1	2.3	45	4.56
CTX-M-1 and CTX-M-9	2	0.3	0	0.0	0	0.0	2	0.20
CTX-M-1 and SHV-2	0	0.0	3	1.1	0	0.0	3	0.30
SHV-2/-2A	3	0.4	3	1.1	0	0.0	6	0.61
SHV-5	2	0.3	0	0.0	0	0.0	2	0.20
SHV-12	15	2.2	2	0.7	0	0.0	17	1.72
SHV-60	0	0.0	1	0.4	0	0.0	1	0.10
SHV-5 and SHV-12	0	0.0	1	0.4	0	0.0	1	0.10
TEM-3 and SHV-28	0	0.0	1	0.4	0	0.0	1	0.10
TEM-20	3	0.4	0	0.0	0	0.0	3	0.30
TEM-40 (IRT-11)	3	0.4	0	0.0	0	0.0	3	0.30
TEM-52	1	0.1	0	0.0	0	0.0	1	0.10
ESBLs and broad-spectrum								
CTX-M-1 and SHV-1/-11	0	0.0	7	2.5	0	0.0	7	0.70
CTX-M-1, SHV-1 and SHV-11	0	0.0	1	0.4	0	0.0	1	0.10
CTX-M-1, SHV-1, SHV-2 and TEM-1	1	0.1	0	0.0	0	0.0	1	0.10
CTX-M-1, SHV-2 and SHV-1	2	0.3	2	0.7	0	0.0	4	0.41
CTX-M-1, SHV-2/12 and TEM-1	1	0.1	0	0.0	0	0.0	1	0.10
CTX-M-1 and SHV-12	1	0.1	0	0.0	0	0.0	1	0.10
CTX-M-1, SHV-1, SHV-2 and TEM-1	0	0.0	1	0.4	0	0.0	1	0.10
CTX-M-1, SHV-1/11 and TEM-1	50	7.5	56	20.4	0	0.0	106	10.74
CTX-M-1, SHV-11, SHV-12 and TEM-1	0	0.0	1	0.4	0	0.0	1	0.10
CTX-M-9 and SHV-11	0	0.0	1	0.4	0	0.0	1	0.10
CTX-M-9, SHV-1/11 and TEM-1	3	0.4	3	1.1	0	0.0	6	0.61
CTX-M-9, SHV-12 and TEM-1	2	0.3	0	0.0	0	0.0	2	0.20
CTX-M-9, SHV-11,-12 and TEM-1	0	0.0	1	0.4	0	0.0	1	0.10
CTX-M-1 and TEM-1	245	36.7	4	1.5	0	0.0	249	25.23
CTX-M-2 and TEM-1	2	0.3	0	0.0	0	0.0	2	0.20
CTX-M-9 and TEM-1	35	5.2	3	1.1	0	0.0	38	3.85
CTX-M-1, CTX-M-9 and TEM-1	3	0.4	0	0.0	0	0.0	3	0.30
SHV-2 and SHV-1	0	0.0	1	0.4	0	0.0	1	0.10
SHV-2a and SHV-11	0	0.0	1	0.4	0	0.0	1	0.10
SHV-2a, SHV-1 and TEM-1	0	0.0	1	0.4	0	0.0	1	0.10
SHV-5 and SHV-1	0	0.0	6	2.2	0	0.0	6	0.61
SHV-5 and SHV-11	0	0.0	2	0.7	0	0.0	2	0.20
SHV-5 and TEM-1	1	0.1	0	0.0	0	0.0	1	0.10
SHV-5, SHV-1 and TEM-1	0	0.0	1	0.4	0	0.0	1	0.10
SHV-12 and SHV-1/-11	0	0.0	6	2.2	0	0.0	6	0.61
SHV-12 and TEM-1	7	1.0	45	16.4	1	2.3	53	5.37
SHV-12, SHV-1 and TEM-1	0	0.0	4	1.5	0	0.0	4	0.41
SHV-12, SHV-11 and TEM-1	0	0.0	37	13.5	0	0.0	37	3.75
broad-spectrum								
SHV-1	0	0.0	12	4.4	0	0.0	12	1.22
SHV-11	0	0.0	12	4.4	0	0.0	12	1.22
SHV-1 and SHV-11	0	0.0	2	0.7	1	2.3	3	0.30
SHV-1/SHV-11 and TEM-1	2	0.3	10	3.6	0	0.0	12	1.22
SHV-27	0	0.0	2	0.7	0	0.0	2	0.20
SHV-36	0	0.0	1	0.4	0	0.0	1	0.1
TEM-1	59	8.8	4	1.5	1	2.3	64	6.48
others								
LEN-16	0	0.0	1	0.4	0	0.0	1	0.10
oxy/K1	0	0.0	0	0.0	36	86.0	36	3.65
no gene	69	10.3	9	3.3	1	2.3	79	8.00
undefined variants of TEM or CTX-M	27	4.0	23	8.4	0	0.0	50	5.07
total	668		275		43		986	100

15 of the *E. coli* strains and of these, two had an SHV-2, one with SHV-2A, three with SHV-5 and nine with SHV-12. Only one strain with a bla_{TEM} ESBL gene, TEM-52, was detected.

Among the 67 *K. pneumoniae* strains with an ESBL gene, 44 (65.7%) had a bla_{CTX-M}-type gene, while a bla_{SHV} ESBL gene was carried by 27 (40.3%) strains. Five strains (7.4%) had both a bla_{CTX-M}-type gene and a bla_{SHV} (Table 5). The following bla_{SHV} ESBLs were detected; four SHV-2 (two in combination with CTX-M), one SHV-2a (in combination with an undefined CTX-M), three SHV-5, 17 SHV-12 (two in combination with CTX-M-1), one SHV-28, and one with SHV-60. The strain harbouring SHV-28 had also a bla_{TEM} ESBL gene, bla_{TEM-3}. One strain had an unknown bla_{TEM}. The most common gene combination among the *K. pneumoniae* strains, were the bla_{SHV}, bla_{TEM-1} and a bla_{CTX-M}-type gene. Thirty (30.9%) strains had all these three genes present, while 17 (17.5%) strains had the combination of a bla_{SHV} and a bla_{TEM} gene.

5.1.1.2. ESBLs among the Helsinki outbreak strains (IV)

In the material collected from the Helsinki region (IV), a total of 36 blood *E. coli* and 84 *K. pneumoniae* isolates had been ESBL positive during the initial screening at the hospitals and of these 35 (97.2%) *E. coli* and 73 (86.9%) *K. pneumoniae* carried an ESBL gene (Table 5). Of the *E. coli* strains, 33 (94.3%) carried a bla_{CTX-M}-type gene, while two (5.7%) carried a bla_{SHV-12} gene. Twenty-three (69.7%) *E. coli* strains had a bla_{CTX-M}-type gene, in combination with TEM-1. Among the *K. pneumoniae* strains, 11 (15.1%) carried a bla_{CTX-M}-type gene, while 63 (86.3%) had an SHV ESBL. The following SHV ESBLs were detected: two strains had SHV-2, two with SHV-2A, five with SHV-5 and 54 with SHV-12. Eleven *K. pneumoniae* carried a non-ESBL β-gene, three strains with bla_{SHV-11}, seven with the combination of bla_{SHV-11} and bla_{TEM}, and one strain with bla_{SHV-1}.

Table 5. Distribution of the ESBLs among the ESBL-producing *E. coli* and *K. pneumoniae* strains collected from the FiRe laboratories (I) and the Helsinki outbreak (IV).

	cefuroxime resistant strains (I)				Helsinki ESBL outbreak (IV)			
	E. coli		*K. pneumoniae*		*E. coli*		*K. pneumoniae*	
ESBL	nr	%	nr	%	nr	%	nr	%
CTX-M-1	132	66.0	33	49.3	27	77.1	8	11.0
CTX-M-2	3	1.5						
CTX-M-9	45	22.5	4	6.0	2	5.7	1	1.4
CTX-M-1and CTX-M-9					1	2.9		
TEM-3 and SHV-28			1	1.5				
TEM-52	1	0.5						
SHV-2	2	1.0	2	3.0			2	2.7
SHV-2A	1	0.5					2	2.7
SHV-2 and CTX-M-1			1	1.5				
SHV-2/2a and unknown CTX-M			2	3.0				
SHV-5	3	1.5	3	4.5			5	6.8
SHV-12	9	4.5	15	22.4	2	5.7	53	72.6
SHV-12 and CTX-M-1			2	3.0			1	1.4
SHV-60			1	1.5				
undefined CTX-M	4	2.0	2	3.0	3	8.6	1	1.4
undefined TEM			1	1.5				
total	200		67		35		73	

5.1.2. Clonality analysis of ESBL-producing E. coli and K. pneumoniae (IV)

This study was performed in order to characterise the citywide epidemiology of ESBLs in Helsinki. A total of 36 *E. coli* and 84 *K. pneumoniae* isolates, collected from the hospitals in Helsinki between 2000 and 2004, were available for clonality analysis. All of these isolates were ESBL positive during screening in the hospitals.

Of the 36 *E. coli* isolates, 33 were successfully analysed with PFGE (Figure 9). One large clonal cluster A_E consisting of 13 (39.4%) strains was detected among these *E. coli* isolates. The most prevalent gene was *bla*$_{\text{CTX-M}}$, detected in all of the A_E strains. Eleven of these carried a *bla*$_{\text{CTX-M-1}}$–type gene and seven of these had the combination of a *bla*$_{\text{CTX-M-1}}$-type gene and a *bla*$_{\text{TEM}}$ gene. Two *bla*$_{\text{CTX-M}}$–type genes could not be defined by pyrosequencing. The smaller PFGE cluster B_E consisted of eight strains, and of these, six strains carried a *bla*$_{\text{CTX-M-1}}$–type gene and one a *bla*$_{\text{CTX-M-9}}$-type gene, while one strain carried *bla*$_{\text{SHV-12}}$. The 12 strains that did not belong to cluster A_E or B_E represented individual PFGE profiles and 10 of these carried a *bla*$_{\text{CTX-M}}$-type gene. Eight strains had a *bla*$_{\text{CTX-M-1}}$–type gene, one strains had both a *bla*$_{\text{CTX-M-1}}$–type gene and a *bla*$_{\text{CTX-M-9}}$–type gene, while one strain had an undefined *bla*$_{\text{CTX-M}}$–type gene. One strain carried *bla*$_{\text{SHV-12}}$ and in one strain no ESBL gene was detected.

Of the 84 *K. pneumoniae* strains, 81 were successfully analysed with PFGE, and one major clonal cluster A_K was identified consisting of 60 (74.1%) strains (Figure 9). The strains in this clonal complex were collected from 16 different hospitals and 59 were health care associated, while one was community-acquired. Of the strains in cluster A_K, 50 (83.3%) strains carried a *bla*$_{\text{SHV-12}}$. Of these, 44 (88.0%) had a *bla*$_{\text{SHV-12}}$ in combination with *bla*$_{\text{TEM}}$. Of these *bla*$_{\text{TEM}}$ positive strains, 37 (84.1%) were sequenced and they all had *bla*$_{\text{TEM-1}}$. The combination of *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{SHV-11}}$ was detected in 23 strains. Nine (15.0%) strains in cluster A_K carried only a chromosomal *bla*$_{\text{SHV-11}}$ gene in combination with or without a *bla*$_{\text{TEM-1}}$, while two strains had a *bla*$_{\text{CTX-M-1}}$-type gene, in combination with a *bla*$_{\text{SHV}}$ (one strain had a *bla*$_{\text{SHV-1}}$ and the other had a *bla*$_{\text{SHV-12}}$ and a *bla*$_{\text{SHV-11}}$).

The index case with an ESBL-positive *K. pneumoniae* isolate belonged to this complex A_K and had been transferred to HUCH in 2001 from a hospital located in Central Africa. This case had ten contact patients who were also colonised with an ESBL producing *K. pneumoniae*. Of the ten isolates from these patients, eight belonged to the same clonal cluster A_K, while two represented individual PFGE profiles. However, they all carried a *bla*$_{\text{TEM-1}}$ in combination with *bla*$_{\text{SHV-11}}$ or *bla*$_{\text{SHV-12}}$.

The remaining 21 *K. pneumoniae* strains not belonging to cluster A_K represented small PFGE strain types, or individual banding patterns. A SHV ESBL was detected in 11 (52.4%) of these strains; two *bla*$_{\text{SHV-2}}$, two had *bla*$_{\text{SHV-2A}}$, three had *bla*$_{\text{SHV-5}}$ and four had *bla*$_{\text{SHV-12}}$. A *bla*$_{\text{CTX-M}}$-type gene was detected in eight (38.1%) strains, six with a *bla*$_{\text{CTX-M-1}}$-type gene, one with a *bla*$_{\text{CTX-M-9}}$-type gene and one with an undefined *bla*$_{\text{CTX-M}}$-type gene. Two of the strains carried only a chromosomal or a chromosomal variant of *bla*$_{\text{SHV}}$, no ESBL could be detected. Of the three *K. pneumoniae* strains that could not be analysed with PFGE, two had a *bla*$_{\text{SHV-5}}$, while one had a *bla*$_{\text{CTX-M-1}}$–type gene.

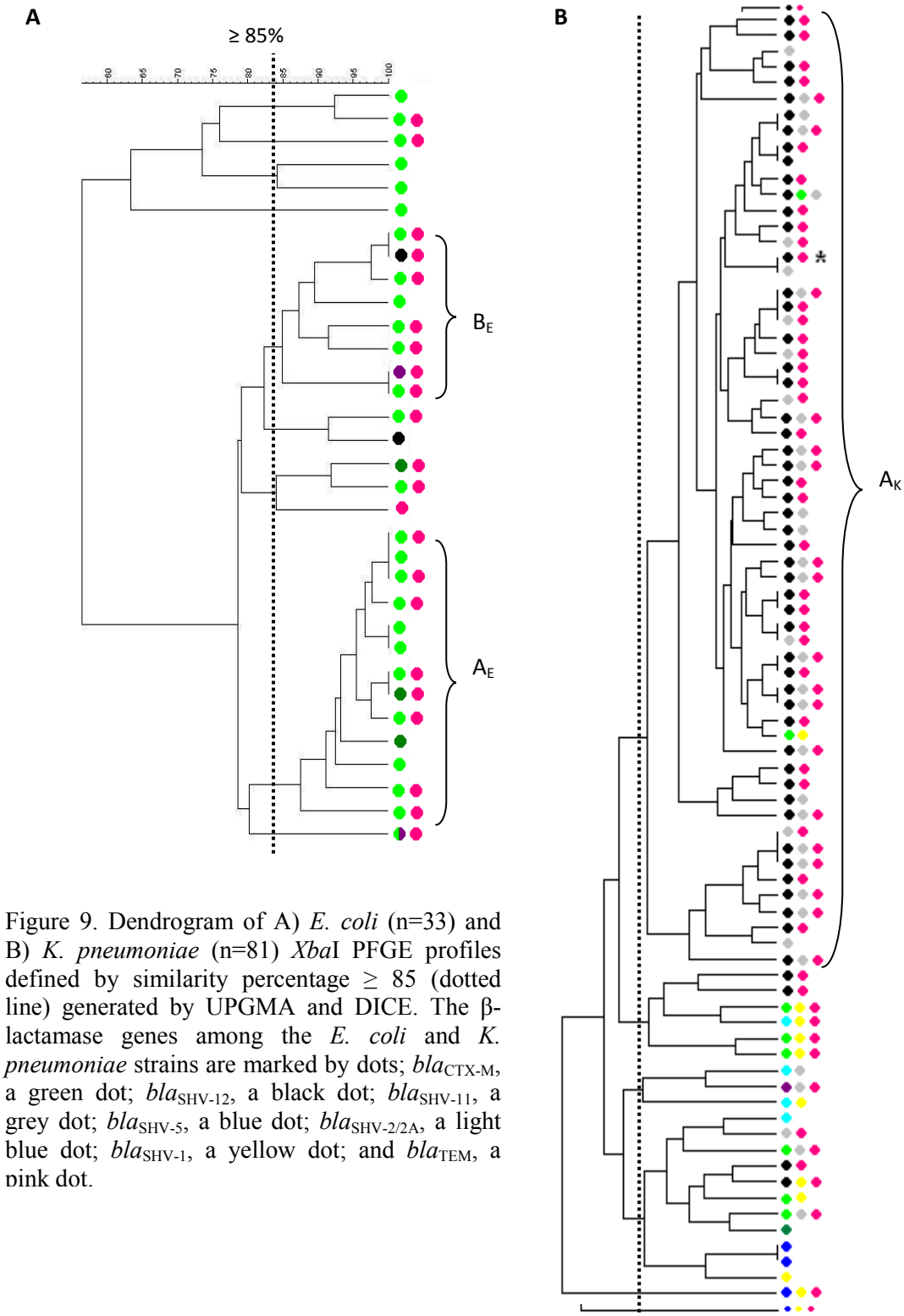


Figure 9. Dendrogram of A) *E. coli* (n=33) and B) *K. pneumoniae* (n=81) *Xba*I PFGE profiles defined by similarity percentage ≥ 85 (dotted line) generated by UPGMA and DICE. The β-lactamase genes among the *E. coli* and *K. pneumoniae* strains are marked by dots; $bla_{\text{CTX-M}}$, a green dot; $bla_{\text{SHV-12}}$, a black dot; $bla_{\text{SHV-11}}$, a grey dot; $bla_{\text{SHV-5}}$, a blue dot; $bla_{\text{SHV-2/2A}}$, a light blue dot; $bla_{\text{SHV-1}}$, a yellow dot; and bla_{TEM}, a pink dot.

5.2. MDR among *E. coli* and *K. pneumoniae* ESBL isolates (I, IV)

A strain is considered a multidrug resistant (MDR), if an isolate is resistant to representatives of three or more classes of antibiotics (penicillins, cephalosporins, aminoglycosides, monobactams, fluoroqinolones, sulfoamides, tetracyclines and carbapenems), while the definition of extensive drug resistance (XDR) is an isolate that is resistant to all but one or two classes. A pandrug resistant (PDR) isolate is resistant to all available classes of antimicrobial agents (Falagas and Karageorgopoulos 2008). The strains that were included in the MDR analysis were susceptibility tested by the agar dilution method for β-lactam antibiotics, fluoroqinolones, tetracycline and aminoglycosides.

A total of 197 *E. coli* and 58 *K. pneumoniae* cefuroxime-resistant ESBL strains could be included in the MDR analysis. Of these, 113 (57.4%) *E. coli* and 28 (48.2%) *K. pneumoniae* were MDRs. The most common phenotype among both species was resistance to cefotaxime, gentamicin and ciprofloxacin. This phenotype was found in 16.2% of the *E. coli* and in 48.3% of the *K. pneumoniae* strains. The most effective drug *in vitro* among both the *E. coli* and *K. pneumoniae* isolates was imipenem (100% susceptible).

Of the 35 *E. coli* and 74 *K. pneumoniae* ESBL isolates from the IV study, 32 (91.4%) *E. coli* and 70 (94.6%) *K. pneumoniae* strains were MDRs. Many of the *E. coli* and *K. pneumoniae* strains were resistant to ciprofloxacin, 31 (86.1%) and 65 (77.4%), respectively. Among the *E. coli* isolates 19 (54.2%) were resistant to ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole (resistance to ampicillin and 3rd generation cephalosporins included), while 50 (67.6%) of the *K. pneumoniae* isolates were resistant to cefoxitin, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole (resistance to 3rd generation cephalosporins included). Among the most effective drugs for the *E. coli in vitro* were carbapenems (100% susceptible), piperacillin-tazobactam (94.4%) and nitrofurantoin (72.2%), and for *K. pneumoniae,* carbapenems (97.6%) and cefepime (76.2%).

In both these studies (I and IV) no PDR isolates could be detected among the *E. coli* or *K. pneumoniae* strains. Only one strain among the *E. coli* had an elevated MIC to meropenem (4 μg/ml), while two of the *K. pneumoniae* isolates had an increased MICs (2 and 4 μg/ml) for meropenem, while one strain were resistant to meropenem (32 μg/ml) and hence was an XDR strain. This XDR strain was resistant (in addition to 3rd generation cephalosporins and meropenem) to cefoxitin, trimethoprim-sulfamethoxazole, nitrofurantoin, tobramycin, ciprofloxacin, tazobactam-piperacillin, and aztreonam, while susceptible to tetracycline and chloramphenicol.

5.3. Evaluation of ESBL detection methods (I-IV, unpublished)

Methods for detection of ESBL producing strains are usually based on phenotypical tests. In this work, the functionality of the agar dilution and disk diffusion methods and the VITEK2 method were evaluated. The phenotypical data was compared to the genotypical data that had been confirmed by PCR and hence was considered as “the golden standard”.

5.3.1. Screening and confirmatory test for ESBLs

Of all the 986 strains included in this study, a total of 540 *E. coli* and 238 *Klebsiella* spp. strains (I, IV + unpublished) were screened and verified according to the CLSI criteria, i.e with cefotaxime and cefotaxime in combination with clavulanic acid. Of the verified *E. coli* and *Klebsiella* spp. strains, 79 strains were falsely identified as ESBL negatives, when compared to the genotypic data confirmed by PCR. Of the false negative strains, 42 strains were verified by the agar dilution method and 37 strains by the disk diffusion method. By the agar dilution 20 *E. coli* and 22 *K. pneumoniae* were false negatives, and by the disk diffusion 16 *E. coli* and 21 *K. pneumoniae* strains. Of these false negative strains, four *E. coli* and three *K. pneumoniae* strains were negative with both methods. The genotypic and phenotypic data of these strains are shown in Table 6. Accordingly, 17 *E. coli* and 14 *K. pneumoniae* were confirmed as false positive with the CLSI criteria by agar dilution. These strains had none of the tested genes or only broad-spectrum β-lactamase genes. Sixteen *E. coli* and four *K. pneumoniae* strains carrying only broad-spectrum β-lactamases were considered false positives with disk diffusion. Of the strains that were tested with both methods, eight *E. coli* and three *K. pneumoniae* were confirmed as false positives (Table 7), when comparing the CLSI confirmation results with the PCR results.

In study I, a total of 402 cefuroxime resistant strains collected between 2002 and 2004 were included. Of these 402 cefuroxime resistant strains, 274 were *E. coli* and 128 *Klebsiella* spp. (31 *K. oxytoca* and 97 *K. pneumoniae*). By disk diffusion, 194 (70.8%) *E. coli* and 59 (60.8%) *K. pneumoniae* harbouring an ESBL gene were verified as ESBL positive by the CLSI criteria. Five of the *K. oxytoca* strains not having an ESBL gene were considered false positives according to the CLSI criteria. Three of the *E. coli* strains with a $bla_{\text{CTX-M}}$, were detected by the disk diffusion method, however, they were considered false negatives by the agar dilution method. For the *K. oxytoca* strains carrying an oxy/K1, 13 strains were considered ESBL positive by the CLSI criteria by the disc diffusion method.

By the agar dilution method 192 (47.8%) *E. coli* harbouring an ESBL gene, were also verified as ESBL positive by the CLSI criteria, while 59 (60.8%) of the *K. pneumoniae* were verified. Twenty-one of the *K. oxytoca* strains not harbouring an ESBL gene, were falsely identified as ESBL positive by the agar-dilution method.

The specificity and sensitivity of the CLSI criteria (Table 8) for cefotaxime and ceftazidime were calculated for cefuroxime resistant *E. coli* and *K. pneumoniae* isolates (I). Susceptibility results were obtained for all 97 *K. pneumoniae* isolates for both the agar dilution and disk diffusion methods, while of the *E. coli* isolates susceptibility results were obtained for all 274 isolates with the disk diffusion method, while 264 isolates with the agar dilution method.

Table 6. MIC results for the *E. coli* and *K. pneumoniae* isolates that were false negatives with both disk diffusion and agar dilution methods.

species	gene	AMP	AMC	TZP	FEP	CTX	FOX	CAZ	CXM	ATM	CAZ/C	CTX/C
E. coli	CTX-M-1	>256	64	32	0.5	16	>128	16	>64	32	16	8
E. coli	CTX-M-1	>256	32	4	4	32	64	32	>64	32	16	16
E. coli	CTX-M-9	>256	>64	4	8	64	>128	64	>64	8	32	16
E. coli	TEM-1, CTX-M-9	>256	>64	4	1	8	16	0.5	>64	1	1	8
K. pneumoniae	CTX-M-1	>256	32	>128	4	4	8	2	>64	64	1	4
K. pneumoniae	TEM-1, SHV-1, CTX-M-1	>256	32	16	0.5	0.5	64	1	64	0.5	1	1
K. pneumoniae	TEM-1, SHV-1, CTX-M-9	>256	>64	2	1	8	2	0.5	>64	1	1	8

AMP, ampicillin; AMC, amoxicillin; TZP, tazobactam; FEP, cefepime; CTX, cefotaxime; FOX, cefoxitin; CAZ, ceftazidime; CXM, cefuroxime; ATM, aztreonam; CAZ/C, ceftazidime/clavulanic acid, and CTX/C, cefotaxime/clavulanic acid.

Table 7. The MIC results for the *E. coli* and *K. pneumoniae* isolates that were confirmed as false positives by both agar dilution and disk diffusion methods

species	gene	AMP	AMC	TZP	FEP	CTX	FOX	CAZ	CXM	ATM	CAZ/C	CTX/C
E. coli	-	257	64	4	2	16	32	4	64	4	1	1
E. coli	TEM-1	257	8	2	2	16	4	1	65	2	1	0.1
E. coli	TEM-1	257	32	8	32	64	16	64	65	32	1	0.25
E. coli	TEM-1	257	32	16	16	64	16	32	65	32	1	0.25
E. coli	TEM-1	257	8	2	2	32	4	1	65	2	1	0.1
E. coli	TEM-1	257	16	8	16	64	16	16	65	32	1	0.25
E. coli	TEM-1, SHV-11	257	32	2	1	4	32	65	64	16	1	0.5
E. coli	TEM-1	257	16	4	0.5	0.25	1	65	8	16	1	0.06
K. pneumoniae	TEM-1	257	32	128	2	8	16	65	64	65	1	0.1
K. pneumoniae	TEM-1, SHV-11	257	32	129	32	64	64	65	65	65	2	0.5
K. pneumoniae	TEM-1	257	32	128	8	16	16	65	65	65	2	0.1

Table 8. The calculated sensitivity (%) and specificity (%) of the CLSI ESBL verification test for CTX, CAZ and cefpodoxime (CPD).

	agar dilution				disk diffusion					
	E. coli		*K. pneumoniae*		*E. coli*			*K. pneumoniae*		
	CTX	CAZ	CTX	CAZ	CTX	CAZ	CPD	CTX	CAZ	CPD
sensitivity	98	65	88	75	96	74	97	84	77	76
specificity	82	95	78	85	92	95	97	82	81	92
ppv	94	98	91	93	97	97	98	92	91	93
npv	93	47	72	58	90	57	95	67	58	75
accuracy	94	72	94	72	95	80	80	83	77	85

ppv= positive predictive value, npv= negative predictive value

Only a part of the cefuroxime resistant isolates were screened by cefpodoxime according to the CLSI criterion by the disk diffusion method; 176 *E. coli* and 59 *K. pneumoniae* isolates. Cefotaxime had a higher sensitivity for both the *E. coli* and *K. pneumoniae* isolates than ceftazidime, with both the agar dilution and disk diffusion methods.

The accuracy of the agar dilution and disk diffusion methods, i.e. the percentage of strains that were correctly designated as ESBL-positive or -negative using the genotype data as reference, was also calculated for the cefuroxime resistant strains, Table 8. Cefotaxime had the highest accuracy for the *E. coli* and *K. pneumoniae* isolates with the agar dilution method. Cefotaxime had also the highest accuracy for *E. coli* by disk diffusion method, while cefpodoxime had the highest accuracy for the *K. pneumoniae*.

5.3.2. Detection of ESBLs by VITEK 2 (II)

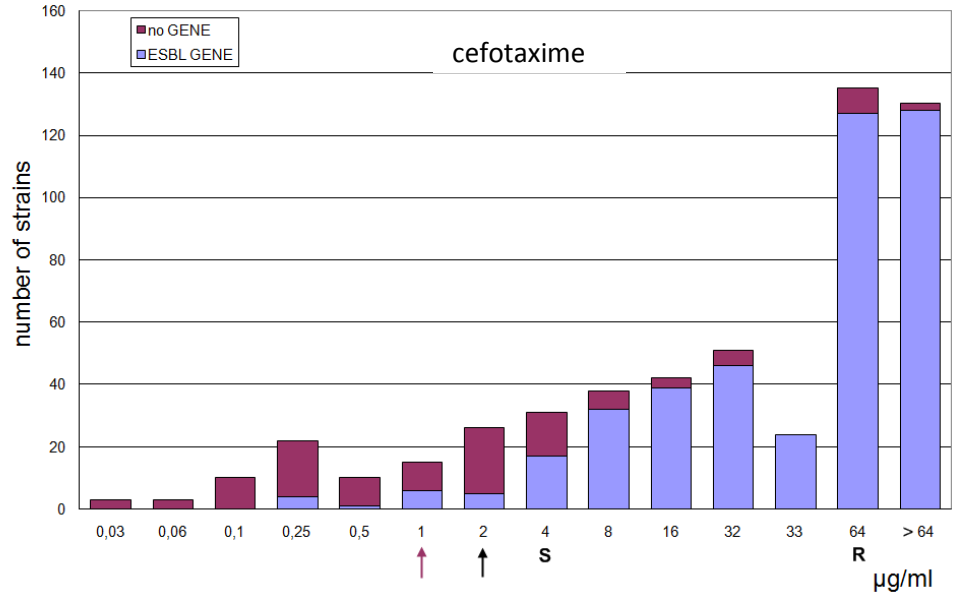
The VITEK 2 is an automated method for detection of antibiotic resistance. By VITEK 2, 123 strains were studied for ESBL production. The results from the VITEK 2 were compared with the resistance gene data and the results from the disk diffusion and agar dilution methods. The isolates represented *E. coli*, *K. pneumoniae* and *K. oxytoca* species and 47.2% carried a $bla_{\text{CTX-M}}$, bla_{SHV} or bla_{TEM} ESBL gene. Two different analysing cards were used for evaluation of VITEK; the Nordic card AST-N029 and the AST-N041 card. The AST-N041 is a card for detection of ESBL producing isolates. With VITEK 2 the phenotypic results - either an ESBL-positive or -negative strain was compared to the genotypic results. A strain was considered ESBL positive by Vitek, if a strain was either positive or an alternative by AST N029 and then verified by AST N041. The results from the VITEK 2 were then compared with the results from the disk diffusion and agar dilution methods, which are the most common methods used for ESBL detection. Hence, the performance of VITEK 2 could be evaluated in comparison to these methods. Taking together all the studied strains, VITEK 2 had the highest sensitivity 96%. The agar dilution method had a sensitivity of

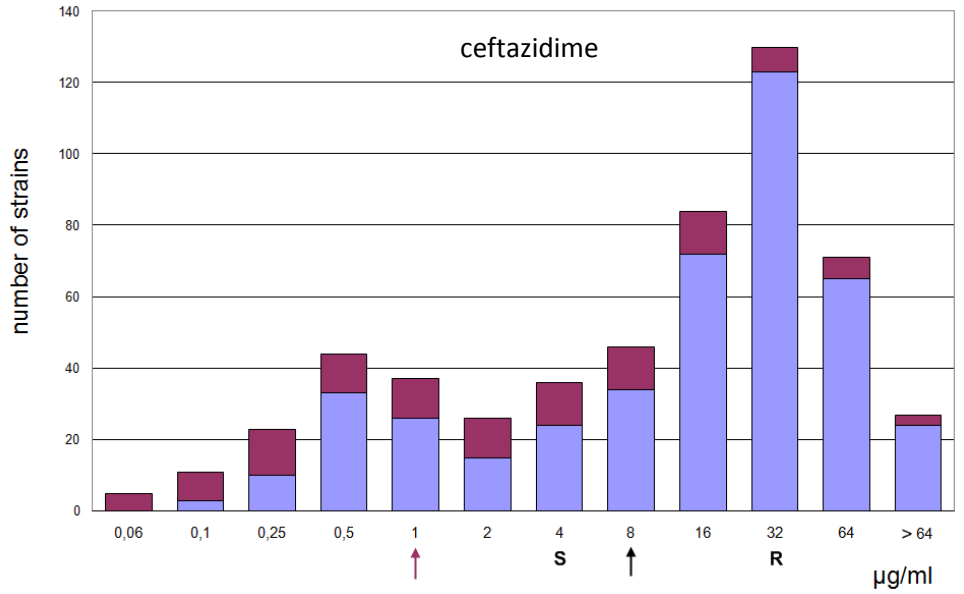
93%, while the sensitivity for the disk diffusion method was 83%. The specificity was lower with VITEK 2 (66%) than with the disk diffusion (94%) or agar diffusion (75%) methods. VITEK 2 had the highest accuracy for the *K. pneumoniae* and the *K. oxytoca* strains, 94% and 84%, respectively, while the agar dilution method had the highest accuracy (99%) for the *E. coli* strains.

5.3.3. Comparison of CLSI versus EUCAST

In order to compare the CLSI screening values with the EUCAST clinical breakpoints, the distribution of cefotaxime and ceftazidime MIC for 540 *E. coli* and 238 *K. pneumoniae* strains is shown, Figure 10. Of these, 21 *E. coli* and 21 *K. pneumoniae* strains carrying an ESBL gene were undetected by cefotaxime and ceftazidime, by the agar dilution method, according to the CLSI criteria. However, by EUCAST, only two *E. coli* and seven *K. pneumoniae* of the strains would have been considered susceptible for cefotaxime, while the rest of the strains were resistant. With ceftazidime eight *E. coli* and eight *K. pneumoniae* were susceptible, while the rest of the strains were either intermediate or resistant.

a) *E. coli*


no GENE
ESBL GENE
cefotaxime
number of strains
160
140
120
100
80
60
40
20
0
0,03
0,06
0,1
0,25
0,5
1
2
4
8
16
32
33
64
> 64
S
R
µg/ml


ceftazidime
number of strains
140
120
100
80
60
40
20
0
0,06
0,1
0,25
0,5
1
2
4
8
16
32
64
> 64
S
R
µg/ml

b) *K. pneumoniae*

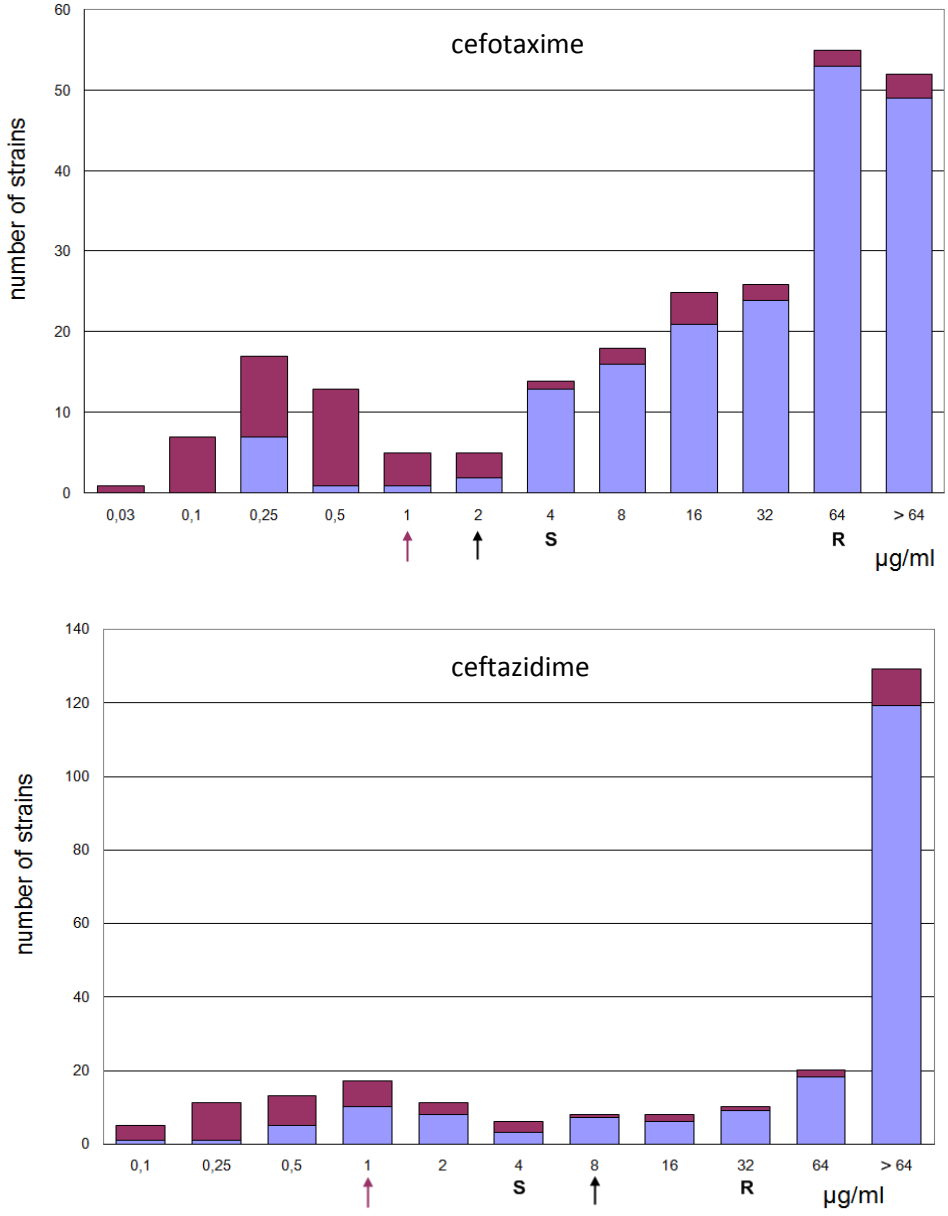


Figure 10. Comparison of the CLSI breakpoints and EUCAST clinical breakpoints by the agar dilution method for cefotaxime and ceftazidime for a) *E. coli* and b) *K. pneumoniae*. Both the genetically verified ESBLs (ESBL gene) and the non-ESBLs (no gene) are included in the histogram. The CLSI screening breakpoint for cefotaxime and ceftazidime is 2 µg/ml. The susceptible (S) and resistant (R) breakpoints are ≤4 µg/ml and ≥32 µg/ml, respectively for ceftazidime and ≤4 µg/l and ≥ 64µg/ml for cefotaxime. The EUCAST clinical breakpoints (S≤/R>, indicated with arrows) are 1/2 for cefotaxime and 1/8 for ceftazidime.

5.3.4. *Typing of SHV β-lactamases by pyrosequencing (III)*

A SHV pyrosequencing assay was developed in order to detect and separate the chromosomal and plasmid-mediated bla_{SHV} genes, since heterogeneous peaks were detected in some *K. pneumoniae* strains by traditional sequencing, Figure 11 (III). For this study, a total of 116 strains were selected, 106 *K. pneumoniae* and ten *E. coli,* that had arrived during the period of 2000-2005 to the Laboratory of Human Microbial Ecology, at the National Public Health Institute.

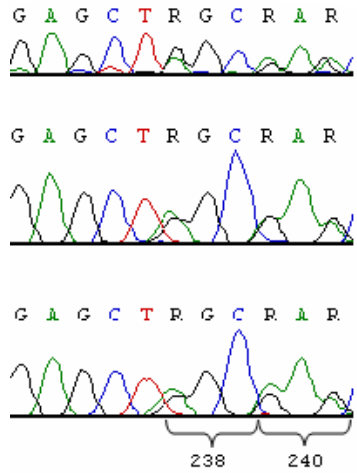


Figure 11. An example of an electropherogram for the three different sequencing primers of an ambiguous position for the amino acid position 238 and 240[2]. Three positions are indistinct; the first position of codon 238 is R (both an A and a G gave a signal) and the first and third position of codon 240 are R with sequences obtained with three different sequencing primers (III).

This pyrosequencing assay consisted of two assays (35 and 238-240), and by combining the results from these assays, the chromosomal SHV-1 or SHV-11 could be differentiated from plasmid-mediated ESBL SHV genotypes, since the DNA sequences corresponding to the studied amino acid positions are known. The dispensation orders designed for the applications of this study generated distinct pyrograms allowing easy differentiation between wild-type SHV and ESBL SHV variants. In Figure 12, examples of pyrograms for the 238-240 assay are shown.

In the ten *E. coli* isolates that were selected as control strains, the following genes were found with cyclic sequencing and confirmed by pyrosequencing: bla_{SHV-2}, bla_{SHV-2A}, bla_{SHV-5}, bla_{SHV-11}, and bla_{SHV-12}. By pyrosequencing, the 40 *K. pneumoniae* isolates that had not been typed by cyclic sequencing were found to have divergent sequences at the nucleotides corresponding to the Ambler positions 35, 238 and/or 240[2]. The exact sequence combinations of the isolates containing two SHV sequences could be resolved by pyrosequencing, because the most probable sequences present at the analysed positions are known and because the pyrosequencing peaks are quantitative.

[2] The standard numbering for class A β-lactamases was established by Ambler *et al.* in 1991 by aligning 20 bacterial sequences, therefore codon 239 that exist in e.g. *Staphylococcus aureus* and *Streptomyces aerofaciens*, does not exist in e.g. *K. pneumoniae.*

Only 34 of the 66 *K. pneumoniae* isolates which SHV-type could be determined by cyclic sequencing were found to contain only one SHV variant by pyrosequencing. The pyrosequencing results of these strains were in agreement with the results from the cyclic sequencing.

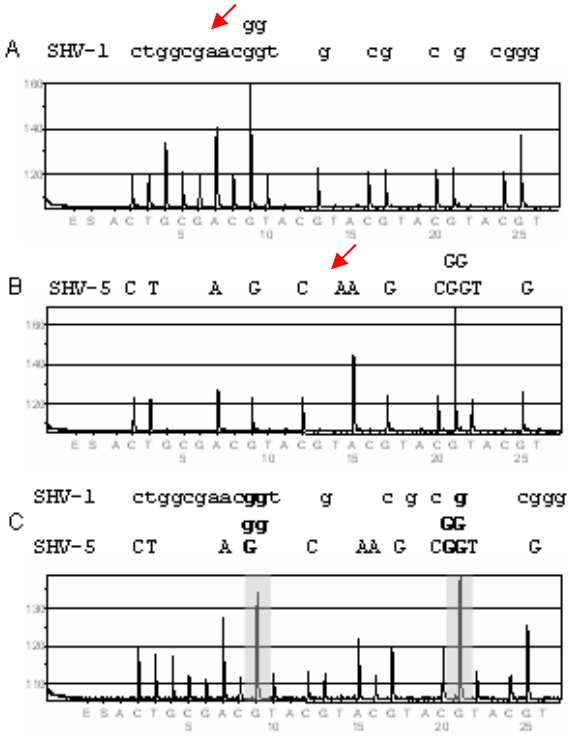


Figure 12. Three pyrograms representing the SHV assay for amino acid position 238-240 (the positions are indicated by the red arrows). The obtained sequences are indicated above the pyrograms. A) a pyrogram for an isolate harbouring SHV-1 (gly:GGC and glu:GAA), B) an isolate harbouring SHV-5 (ser:AGC and lys:AAG) and C) an isolate harbouring both SHV-1 (sequence indicated by lower cases) and SHV-5 (sequence indicated by upper cases). In C, the proportion of SHV 1 is lower than that of SHV-5 since the G peak at dispensation 9 deriving mainly from SHV-1 is lower than the peak of dispensation 21 that is mainly from the mutated SHV copy.

Consequently, 32 strains were found to carry more than one bla_{SHV} gene by pyrosequencing and had erroneously been typed by cyclic sequencing to carry only one bla_{SHV} gene. When the cyclic sequencing electropherograms of these isolates were reanalysed, heterogeneous peaks could be detected at the nucleotide positions corresponding to the heterogeneous positions detected by pyrosequencing. Surprisingly, one *K. pneumoniae* isolate was found to apparently carry three bla_{SHV} gene copies: two different bla_{SHV-1} copies (more than one sequences can be detected for

the sequence at amino acid position 240) and one bla_{SHV-2} copy. The SHV-type of this isolate was determined as SHV-2 by cyclic sequencing.

Of the *K. pneumoniae* strains, 101 (95.3%) of the 106 were probable chromosomal SHV carriers. The most common SHV combinations in this study were pyrosequencing type SHV-12 combined with SHV-11 (n=35) or SHV-1 (n=12).

6. DISCUSSION

6.1. Genetic basis of ESBL-producing *Enterobacteriaceae* in Finland (I, IV, unpublished)

6.1.1. Resistance to 3rd generation cephalosporins

The resistance to 3rd generation cephalosporins among *E. coli* and *K. pneumoniae* varies between different geographic locations (EARSS 2007) and in several countries the resistance to 3rd generation cephalosporins has increased since the 1990s (Manninen ***et al.*** 1997; Potz ***et al.*** 2006; Livermore 2007). According to the FinRes (FinRes - Statistics on antimicrobial resistance in clinical bacteria in Finland) the resistance to 3rd generation cephalosporins in 2007 in Finland was 1.3% for *E. coli* and 1.7% for *K. pneumoniae* blood isolates, and 0.9% for *E. coli* and 0.6% for *K. pneumoniae* for urine isolates (www.finres.fi). A similar resistance situation is found in Sweden, and Norway, however the resistance in *E. coli* was somewhat higher (~2.2 %) (EARSS data. Available at: http://www.earss.rivm.nl. Data retrieved on December, 2008). In France a similar resistance among *E. coli* can be found, while about 10% of the *K. pneumoniae* are resistant. In the United Kingdom and Spain the resistance is about 10% for both species (EARSS data. Available at: http://www.earss.rivm.nl. Data retrieved on December, 2008). However, especially in the eastern parts of Europe the resistance to 3rd generation cephalosporins among *E. coli* is much higher, between 20-30% in Bulgaria, 28% in Romania, and over 40% in Turkey (Coque *et al.* 2008; EARSS 2008). In the United States during 1999-2004 a lower resistance rate was found among all *Enterobacteriaceae* compared with Europe. The susceptibility rate of ceftazidime in the Unites States was 75-100% during year 1999-2004 (Goossens and Grabein 2005). In South America, especially the amount of highly resistant *Klebsiella* spp. has increased and 37% are resistant to 3rd generation cephalosporins (Hicks *et al.* 2008).

6.1.2. Prevalence of ESBL genes (I, IV, unpublished)

This study was carried out in order to detect the most common ESBL genes in Finland. The amount of ESBL producing *E. coli* and *K. pneumoniae* was very low during the 1990s in Finland (Manninen *et al.* 1997), but since then the prevalence has increased. The distribution of the most common β-lactamases from the first and the fourth study are similar to the molecular results for all isolates included in this thesis, even though, the sampling in both the first and fourth study was in some way restricted. In both materials, the CTX-M β-lactamases were the most common among the *E. coli*, while among the *K. pneumoniae* strains; the CTX-M β-lactamases were most common in study I and SHV β-lactamases in the IV study. In all studies the material has to be collected according to some restrictions, so that the size of the material is workable and possible to analyse. Since the prevalence of ESBL producing strains in Finland had been so low during the 1990s, it was hence decided to collect all

the cefuroxime resistant strains from the FiRe laboratories for the first study. All the possible ESBL producers were probably not detected by this sampling criterion and likewise non-ESBL strains only resistant to cefuroxime, could be included. Cefuroxime is a poor indicator substrate for ESBLs since e.g. non-ESBL *K. oxytoca* hyperproducing K1 are highly resistant to cefuroxime (Potz *et al.* 2004), and biochemical tests like API 20 may fail to differentiate between *K. pneumoniae* and *K. oxytoca* (Kovtunovycha *et al.* 2003). However, cefuroxime was used by most hospital laboratories and hence did not require extra work in the laboratories. In the fourth study, the aim was to characterise the citywide molecular epidemiology of ESBLs in Helsinki, and the bacterial isolates represented hospital clusters of suspected ESBLs, and since the strains had a probable connection to a possible cluster, it may have influenced the genetic basis detected among the strains.

During the beginning of the 21st century, the prevalence of ESBLs in Finland has increased (I). According to the PCR and sequencing results, the CTX-M β-lactamases or the combination of a CTX-M β-lactamases, especially the CTX-M-1 subgroup and TEM-1 were the most prevalent among the Finnish *E. coli* isolates. The prevalence of CTX-M β-lactamases was 92% among the *E. coli* ESBLs in study I. Of these, CTX-M-1-type β-lactamases was detected in 66%, CTX-M-9-type β-lactamases in 23% and CTX-M-2-type β-lactamases in 2%. Similar results to those in the I study was found in Sweden during the period from 2001 to 2006, when 92% of ESBL-positive *E. coli* isolates expressed a CTX-M-type enzyme, $bla_{\text{CTX-M-1}}$ being the most prevalent gene type represented by 80%, while the CTX-M-9-type and CTX-M-2-type β-lactamases represented 16% and 4% respectively. Strains harbouring CTX-M-9-type β-lactamases emerged in Sweden in 2004 (Fang *et al.* 2008), but a similar increase could not be detected among the Finnish isolates harbouring CTX-M-type β-lactamases. In Norway about 90% of the isolates had a CTX-M-type β-lactamases, where CTX-M-1 represented 64%, CTX-M-9 33% and CTX-M-2 2% (Tofteland *et al.* 2007). In a study from Denmark, the occurrence of ESBL producers was below 1%, with dominance of CTX-M and SHV enzymes (Kjerulf *et al.* 2008). An ESBL SHV was detected in 17 *E. coli* strains (I and IV), and of these, SHV-12 was the predominant present in eleven (64.7%) strains. SHV β-lactamases are not common among *E. coli.* Only a handful of studies of *E. coli* harbouring SHV-12 have been reported (Nuesch-Inderbinen *et al.* 1997; Nakamura *et al.* 2000; Teshager *et al.* 2000; Brinas *et al.* 2003; Valverde *et al.* 2007).

A TEM-52 was detected in an *E. coli* isolate in this study. TEM-52 has been found e.g. in *E. coli* strains from clinical sample and samples from wild and healthy animals in Portugal (Costa *et al.* 2004; Costa *et al.* 2006; Machado *et al.* 2007; Poeta *et al.* 2008), but has mostly been recorded among *Salmonella* and *Shigella* spp. strains in e.g. Korea (Jeong *et al.* 2003; Weill *et al.* 2004a; Yates *et al.* 2004; Vázquez *et al.* 2006).

Among the *K. pneumoniae* ESBL isolates in this study, the bla_{SHV} genes were the predominant, and especially $bla_{\text{SHV-12}}$, carried by 50.7% of the *K. pneumoniae* strains from the I (17 of the 67 strains) and the IV (54 of the 73 strains) study. One explanation for the high prevalence of SHV-12 is probably the clonal spread of *K. pneumoniae* strains. The $bla_{\text{SHV-12}}$ has also been detected in other studies e.g. a nation wide study in Spain (23.9% of the strains carried $bla_{\text{SHV-12}}$), (Hernandez *et al.* 2005), a multicenter study from Portugal (18.2%) (Machado *et al.* 2007), a multicenter study

from Korea (51.5%) (Bae *et al.* 2007) and from a one center study in southern Taiwan (50.0%) (Yan *et al.* 2000). In other countries, like Belgium, Tunisia, Turkey and South Africa, the bla_{SHV-5} has been the most prevalent bla_{SHV} gene (Chanawong *et al.* 2001; Gruteke *et al.* 2003; Paterson *et al.* 2003; Ben-Hamouda *et al.* 2004; Jeong *et al.* 2004), while it was quite rare among the Finnish *K. pneumoniae* strains. In a study from Norway by Tofteland *et al*. 2008 the dominating genes in *K. pneumoniae* were bla_{SHV-5} (47.4%) and bla_{SHV-2} (21.0%). However, in all studies the DNA sequences for the ESBL strains have not been manually analysed and hence all studies probably do not take in account the presence of both a chromosomal and a plasmid-mediated bla_{SHV} gene (study III, chapter 5.3.3). The differences in the amino acid mutations that are present in the e.g. bla_{SHV-5} and bla_{SHV-12} genes are very small, and hence the results may not be truly correct in all studies, therefore the prevalence of the bla_{SHV-12} might be higher than reported.

6.1.3. CTX-M ESBLs

Bacterial strains carrying CTX-M β–lactamases have spread rapidly, since the first CTX-Ms were detected in Japan, Germany and South America in the end of the 1980s (Matsumoto *et al.* 1988; Bauernfeind *et al.* 1990; Bauernfeind *et al.* 1992). The first CTX-M in Europe was described in 1989 in Germany in an *E. coli* isolate and almost simultaneously in a *E. coli* in France (Bauernfeind *et al.* 1990; Bernard *et al.* 1992). Since then, an increase in the CTX-M β-lactamases has been seen in many countries in Europe and Asia (Cantón and Coque 2006; Novais *et al.* 2006; Livermore *et al.* 2007; Cantón *et al.* 2008; Freeman *et al.* 2008; Rossolini *et al.* 2008). In the United Kingdom isolates with CTX-M-1 co-existed initially with isolates harbouring CTX-M-9, CTX-M-14, and SHV-12, and to some extent with TEM (Livermore *et al.* 2007). CTX-Ms are now also common among Finnish *E. coli* and *K. pneumoniae* isolates (I, IV). Of the ESBL isolates from the study I (Table 5, page 48), a CTX-M β–lactamase was detected in 92% of the *E. coli* and 58% of the *K. pneumoniae* strains. CTX-M-15 has become the most prevalent enzyme among UTI causing *E. coli* strains during the last couple of years in the United Kingdom (Livermore *et al.* 2007), and likewise, the CTX-M-1 subgroup is the predominant among the *E. coli* in Finland. Only a small amount of the Finnish bla_{CTX-M} genes were sequenced, however the $bla_{CTX-M-15}$, $bla_{CTX-M-9}$ and $bla_{CTX-M-14}$ variants were detected.

The prevalence of ESBLs in Europe is not shared by all regions or countries. In Spain, the first CTX-M β–lactamases was detected in *K. pneumoniae* and *Enterobacter* spp. a few years after the report in Germany (Canton *et al.* 2002; Coque *et al.* 2002). However, no dramatic increase in the CTX-Ms was seen until the beginning of the 21th century when the CTX-M-9 sub group was reported in faecal *E. coli* and *Salmonella* spp. In a multicenter study (Hernandez *et al.* 2005), enzymes of the CTX-M-9 subgroup were detected in about 40% of the *E. coli* strains and in about 10% of the *K. pneumoniae*. A high local prevalence of CTX-M-9 and CTX-M-14 has been reported in several studies (Valverde *et al.* 2004; Hernandez *et al.* 2005; Valverde *et al.* 2008). The CTX-M-1 subgroup is predominantly reported in most western European countries (Livermore *et al.* 2007). However, in Western Austria, the $bla_{CTX-M-1}$-type genes are still sporadic (Prelog *et al.* 2008), while the CTX-M-2 subgroup is common in Belgium

(Rodriguez-Villalobos *et al.* 2006). In the eastern European countries $bla_{CTX\text{-}M\text{-}3}$ is the predominant genotypes (Edelstein *et al.* 2003; Korten *et al.* 2007; Empel *et al.* 2008).

The CTX-M-1 subgroup has since it was first reported increased dramatically in the United Kingdom and in France (Brigante *et al.* 2005; Livermore *et al.* 2007). Earlier ESBLs in these countries were mostly seen as bla_{TEM} or bla_{SHV} (Livermore *et al.* 2007). The CTX-M-15 producing *E. coli* has also increased in Spain and Portugal (Hernandez *et al.* 2005; Romero *et al.* 2005; Oteo *et al.* 2006; Mendonça *et al.* 2007).

In Canada, the first CTX-M was detected in 2000 (Mulvey *et al.* 2004) and now a similar CTX-M prevalence to the European countries can be found with the CTX-M-9 (Bush 2008) and the CTX-M-1 subgroup as the most common ones, with a high frequency among the community (Pitout *et al.* 2007). It has been reported from a local community-based study that 70% of the *E. coli* ESBLs are CTX-M-producers (Pitout *et al.* 2004). In the United States, CTX-M ESBLs were first reported in 2002 in five states (Ohio, Texas and Idaho, Virgina and Washington) during the investigation of the ESBLs types in a U.S. hospital surveillance study (Moland *et al.* 2003). However, during the last years they have been scattered all over the country (Lewis *et al.* 2007; Castanheira *et al.* 2008; Hanson *et al.* 2008). In South America, nontyphoid *Salmonella* strains resistant to cefotaxime with CTX-M-2 (Bauernfeind *et al.* 1992) were spread from Argentina to neighbouring countries during the beginning of the 1990 (Rossi *et al.* 1995). Thereafter this CTX-M ESBL has been found in e.g. *E. coli* and *Shigella* (Bauernfeind *et al.* 1990; Radice *et al.* 2002). CTX-M-2 is the most common and has been broadly found in Argentina, Uruguay and Paraguay, but also CTX-M-8 and -9 have been found e.g. in Brazil (Bonnet 2004).

It is unclear why the spread of CTX-M has been more extensive than the spread of TEM and SHV mutants. Even though different antibiotic policies exist in various countries, this seems to have no affect on the prevalence of β-lactamases. The Nordic countries have a similar prevalence of resistance to 3rd generation cephalosporins among invasive strains as other countries in Western Europe, e.g. France and Luxembourg (EARSS, http://www.rivm.nl/earss/result/), even though the antibiotic policies, e.g. use and availability of antibiotics differ (Gould and Meer 2004). Some risk factors of the acquisition of $bla_{CTX\text{-}M}$ genes may be pressure from the surroundings by antimicrobial agents e.g. contact with health care facilities (Paterson and Bonomo 2005). A selective pressure is created, by the use of antibiotics as feed additives in animal farming and agriculture (Woodford *et al.* 2004). Fortunately, the European Commission banned the additive of antibiotics to feed in 2006 (Anadón 2006), so this selective pressure is hopefully declining. The increase of CTX-M-type β-lactamases may also be due to insertion sequences (ISs) e.g. IS*Ecp1* which appear to enable the mobilization of $bla_{CTX\text{-}Ms}$. The IS*Ecp1* are often located in multidrug resistance regions containing different transposons and ISs (Poirel *et al.* 2003).

6.1.4. Community-acquired ESBLs

Enterobacteria causing community-acquired infections are usually less resistant than health care acquired infections. However, they are of a great concern, since multi-resistant *E. coli* have emerged (Livermore and Paterson 2006). During a survey in France in 1993 where over 2500 *E. coli*, *K. pneumoniae* and *P.* mirabilis isolates from

non-hospitalised patients were included, no community-acquired ESBLs were detected (Goldstein *et al.* 1995). However, since then the ESBL-producing *Enterobacteriaceae* have increased among community-acquired strains and several reports from e.g. Spain, the United Kingdom, France, Canada and Hong Kong have been published (Rodriguez-Bano *et al.* 2004; Woodford *et al.* 2004; Pitout *et al.* 2005a; Pitout *et al.* 2005b; Ho *et al.* 2007). The first community-acquired ESBL strain was found in Ireland towards the end of the 1990s (Cormican *et al.* 1998). In general, the patients have developed UTI with an *E. coli* harbouring a CTX-M. Especially *E. coli* with CTX-M-1 β–lactamases have been observed among community-acquired strains (Pitout *et al.* 2004; Pitout *et al.* 2005b; Livermore *et al.* 2007). A population-based surveillance CTX-M *E. coli* study in Canada showed that ESBL-producing *E. coli* is predominantly a community-onset pathogen (Pitout *et al.* 2004).

During the collection of the material for the IV study, a small material consisting of community-acquired UTI CTX-M *E. coli* strains could be found (unpublished). The first (to our knowledge) community-acquired CTX-M in Finland was detected in January 2004 (unpublished). Hence, it can be concluded that since community-acquired ESBLs are found in Finland, the prevalence will probably increase as seen in e.g. the United Kingdom and Spain (Valverde *et al.* 2004; Woodford *et al.* 2004; Valverde *et al.* 2008).

The increase in community-acquired ESBL infections in e.g. the United Kingdom has not yet been explained, the reasons are probably the same as for the emergence of CTX-M producers. Recently, a study was published that confirmed that age, female gender, and presence of other medical conditions increase the risk for community-acquired ESBLs (Laupland *et al.* 2008). In the same Canadian study, foreign travel was found to be a novel major risk factor for developing community-acquired ESBL-producing *E. coli* infections. However, further studies are needed so that the specific cause or exposures that lead to an increased risk for community-acquired ESBLs can be charted.

6.1.5. Epidemiology of ESBL producers (IV)

Prevention of emergence and spread of ESBL-producing *Enterobacteriacae* are major challenges to infection control teams. Especially, *E. coli* and *K. pneumoniae* strains are of great concern, since these pathogens are the most common causative Gram-negatives in both community- and health care-acquired infections. In Finland, and especially in the Helsinki region, the number of new ESBL-cases has increased steadily during the beginning of the 21th century (IV). From 2000 to 2004 clusters of potent ESBL-producing *E. coli* and *K. pneumoniae* were recognised in the Helsinki region. This urged an epidemiological analysis and a retrospective investigation of the previously collected *E. coli* and *K. pneumoniae* strains that had been ESBL positive during the initial screening. Hence, it was investigated whether the isolates were genetically related, and the genetic basis of the strains was characterised.

Among the *E. coli* strains in the Helsinki outbreak (IV) one major and one minor clonal complex were detected, whilst the reminders represented sporadic strains. This is consistent with several other studies where the majority of the strains are represented by one or two larger clonal clusters in an ESBL outbreak, while the rest of the strains

are sporadic (Gniadkowski *et al.* 1998; Mena *et al.* 2006; Woodford *et al.* 2007).) The major clonal *E. coli* complex represented about 40% of the strains in the Helsinki study (IV). CTX-M was the most prevalent ESBL among this *E. coli* clonal complex, and major CTX-M outbreaks has been described among nosocomial *E. coli* in Norway (Naseer *et al.* 2007) and among *E. coli* and *K. pneumoniae* in Sweden (Fang *et al.* 2004; Fang *et al.* 2008; Lytsy *et al.* 2008), but also in studies from the United Kingdom, Italy, Canada, and Hong Kong (Woodford *et al.* 2004; Brinas *et al.* 2005; Pitout *et al.* 2005a; Bagattini *et al.* 2006; Woodford *et al.* 2007; Lau *et al.* 2008). The percentage of strains belonging to the major clonal cluster is usually much higher in short-term studies than in studies where the material has been collected over several years, which could also be seen in the Helsinki study (IV). In comparison, in a study by Fang *et al.* 2008 with a time period similar to the Helsinki study (IV), 22.1% of the strains represented the major clonal complex. Likewise, in an *E. coli* study from England the major clonal cluster represented 20.3% of the strains (Woodford *et al.* 2007), while in a short term study by Fang *et al.* 2004, 55.6% of the *E. coli* CTX-M-1 strains represented the major clonal complex.

The major clonal cluster represented 74.1% of the *K. pneumoniae* strains in the Helsinki study (IV). In other *K. pneumoniae* studies, similar proportions have been found. In a study of a intensive care unit from Spain, the major clone represented 98.1% of the strains (Mena *et al.* 2006) and in a University hospital study from Sweden, all the *K. pneumoniae* strains belonged to the same clonal cluster (Lytsy *et al.* 2008). Among the *K. pneumoniae* strains from the Helsinki outbreak (IV), the $bla_{\text{SHV-12}}$ gene was the most common, and this ESBL has also been reported from a *K. pneumoniae* outbreak in Korea (Roh *et al.* 2008). However, the SHV-5, found worldwide (Medeiros 1997), has been more predominant among *K. pneumoniae* outbreaks (Gniadkowski *et al.* 1998; Brinas *et al.* 2004; Guadalupe *et al.* 2004; Kassis-Chikhani *et al.* 2006; Cagnacci *et al.* 2008). The prevalence of SHV-12 among these studies might be higher since the presence of both a chromosomal and a plasmid-mediated bla_{SHV} gene is probably not taken in account during sequencing analysis (see Chapter 5.3.4).

Among the *E. coli* strains the same ESBL genes were found both in strains belonging to the major cluster and in the sporadic strains, hence a genotypic difference could not be detected among the major clonal complexes or the individual PFGE patterns. Still, differences could be detected among the *K. pneumoniae* strains between the major clonal cluster and the sporadic strains. The SHV-12 was predominant among the major cluster, while only a few of the sporadic strains carried an SHV-12 gene.

A clonal spread of genotypically related ESBLs among several hospitals was detected in the Helsinki study, and this kind of transfer has been described before (Yuan *et al.* 1998; Bisson *et al.* 2002). Since the ESBLs are often encoded on plasmids that are easily transferred between isolates, strains that are not genotypically related may produce the same ESBL due to plasmid transfer from species to species (Fiett *et al.* 2000). The plasmids were not characterized in the Helsinki study, and therefore it can not be concluded that a specific plasmid caused the outbreak. Plasmid-mediated outbreaks are seldom documented due to difficulties following the plasmid-encoding ESBLs, since a strain can carry multiple plasmids and this together with recombination or deletion, change the size of the plasmids that encode ESBLs (Oteo *et al.* 2006).

6.1.6. Source of the Helsinki ESBL outbreak

It might be difficult to identify the source of an ESBL outbreak. It is known that *E. coli* and *K. pneumoniae* outbreaks usually start in one ward and then spread to other parts of the hospital (Knothe *et al.* 1983; Brun-Buisson *et al.* 1987; Pena *et al.* 2001; Fang *et al.* 2004; Mamlouk *et al.* 2006; Mena *et al.* 2006). However, the source might also be related to food, environment or direct contact with animals (Sabota *et al.* 1998; Mirelis *et al.* 2003; Brinas *et al.* 2005; Mesa *et al.* 2006; Warren *et al.* 2008). In the Helsinki Study (IV) the source of the *E. coli* outbreak was not identified, while the source of the *K. pneumoniae* outbreak could be identified. A patient had been transferred to a hospital in Helsinki from a hospital in Central Africa. This case had ten known contact patients who were colonised with an ESBL-producing *K. pneumoniae*. Many of the ESBLs isolates from the Helsinki region came from young females with UTI and UTI should not be easily spread. One explanation may be conjugal transmission within the same household (Lietzau *et al.* 2006; Lietzau *et al.* 2007), as was seen with one *K. pneumoniae* strain in the IV study. Another theory considered to be the cause for the spread of *E. coli* ESBLs in Helsinki was the transmission of ESBL genes "from a food source". Since ESBLs have also been found among farm animals in several studies (Liebana *et al.* 2004; Brinas *et al.* 2005; Mesa *et al.* 2006; Riano *et al.* 2006; Jouini *et al.* 2007; Machado *et al.* 2008; Smet *et al.* 2008) and the consumption of broiler has increased in Finland during the last years, a minor study of Finnish and foreign poultry meat was performed (unpublished). However; no *E. coli*, or other possible ESBL-producing strains were detected.

6.2. Antibiotic resistance and ESBLs (I, IV)

The production of ESBLs in *Enterobacteriaceae* causes resistance to 3rd generation cephalosporins. Genes that encode ESBLs are often found on plasmids together with genes encoding resistance to aminoglycosides, sulphonamides, tetracyclines and fluoroqinolones (Paterson 2006), and therefore ESBLs are often MDR producers. The prevalence of MDR and resistance to fluoroqinolones e.g. ciprofloxacin (Cormican *et al.* 1998; Kang *et al.* 2004), is increasing among ESBL producers (Paterson *et al.* 2000; Tolun *et al.* 2004; Livermore *et al.* 2008).

The Finnish ESBL producing strains were susceptibility tested for several different antibiotic classes. Of all the *E. coli* and the *K. pneumoniae* ESBL strains from the I and IV study, about 60% were MDRs. MDR ESBL strains have also been reported from other studies (Hyle *et al.* 2005; Wyllie *et al.* 2005; Cagnacci *et al.* 2008). A common phenotype among the Finnish ESBLs showed resistance to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole and similar results have been found in other studies (Mena *et al.* 2006; Naseer *et al.* 2007).

Carbapenems are considered the most potent agents for treating MDR Gram-negative bacteria. ESBLs have been reported as carbapemen susceptible, for example, in the United Kingdom the MDR *E. coli* and *K. pneumoniae* strains are so far not resistant to carbapenems (Livermore *et al.* 2008). Unfortunately, during the last years carbapenem resistant strains has been reported. For example ertapenem resistant CTX-

M-producing *K. pneumoniae* (Elliott *et al.* 2006; Girlich *et al.* 2009), and in Greece and Italy, cases of carbapenem resistant MDR *Klebsiella* spp. strains producing MBLs have been reported (Falagas *et al.* 2007; Cagnacci *et al.* 2008). The XDR resistance is mainly caused by MBL strains (Souli *et al.* 2008), but also by strains with carbapenemases, e.g. KPC. KPC was initially isolated from a *K. pneumoniae* strain in the United States (Yigit *et al.* 2001), but have also been detected in Israel (Navon-Venezia *et al.* 2006) and Greece (Cuzon *et al.* 2008). Among the *E. coli* and *Klebsiella* strains in this study, reduced susceptibility to carbapenems was uncommon and no MBL-producing strains were found (analysed by PCR), not even in the meropenem resistant *K. pneumoniae* strain. All three *K. pneumoniae* strains that had increased MICs for meropenem were also resistant to cefoxitin, and hence the increased resistance may be due to loss of OmpK36 combined with production of an AmpC β-lactamase (Martinez-Martinez *et al.* 1996; MacKenzie *et al.* 1997; Kassis-Chikhani *et al.* 2006; Cagnacci *et al.* 2008). No PDR-producers could be found among the Finnish strains, and the prevalence of PDR resistance in *Enterobacteriaceae* is also rare in Europe (Souli *et al.* 2008).

6.3. Detection of ESBL-producing *E. coli* and *Klebsiella* spp. isolates (I, II)

The recommended methods for detection of susceptibility among bacterial strains vary between laboratories and countries. The ESBL detection methods can be roughly divided into two groups: phenotypic methods, which detect the ability of the ESBL enzymes to hydrolyse different cephalosporins, and genotypic (molecular techniques) methods, which detect the gene responsible for the production of the ESBL. The phenotypic methods are commonly used by clinical diagnostic laboratories since these tests are easy to perform and they are also cost effective. In addition, the incorporation to automated susceptibility systems, have made them easily accessible (Wiegand *et al.* 2007).

Different recommendations for detection of ESBLs exist. Some countries follow the CLSI recommendations (Clinical and Laboratory Standards Institute 2007b), while others have national bodies like SRGA (the Swedish Reference Group for Antibiotics) in Sweden and BSAC (the British society for Antimicrobial Chemotherapy) in the United Kingdom. In Finland the national body called FiRe is a coalition of the Finnish clinical microbiology laboratories and the bacteriology units of the former National Public Health Institute. FiRe's primary goal has been to standardise the antimicrobial susceptibility testing methodology in Finland and as the basis for the standard methodology the CLSI standard method was accepted. Now EUCAST is trying to unify the methods and breakpoints for the *Enterobacteriaceae* for all European countries.

6.3.1. Existing detection tests

Commercial manufacturers have developed different tests for ESBL detection to be used along with MIC test methods in the clinical laboratory. One test is the Etest ESBL strips (AB Biodisk, Solna, Sweden), a two-sided strips containing a gradient of ceftazidime on one end and ceftazidime plus clavulanic acid on the other end. Other methods are the disk diffusion test (ROSCO or OXOID) and MicroScan ESBL plus panel (Dade Behring, Schwalbach, Germany). The CLSI screening and confirmation criteria rely on the MIC difference test in which a β-lactamase inhibitor is used to protect the activity of an indicator antimicrobial drug against an ESBL-producing strain. Lowering of ≥3 two-fold dilution steps is considered a positive result in its MICs of ceftazidime or cefotaxime in the presence of clavulanic acid (4 mg/L), versus its MIC when tested alone (Clinical and Laboratory Standards Institute 2007b). In the study of the cefuroxime resistant isolates (I), this test worked quite well with the CTX-M, SHV and TEM β-lactamases; however, all resistance causing β-lactamases are not inhibited by clavulanic acid. For example, AmpC, the metallo β-lactamases and some OXA-enzymes are not inhibited and one strain often carries multiple β-lactamases (Liu *et al.* 1992; Livermore 1995; Coudron *et al.* 2000; Paterson *et al.* 2003). The presence of these, can easily lead to false-negative results. There is not one single detection method that covers all different β-lactamase resistance mechanisms (Drieux *et al.* 2008), and the sensitivity and specificity of a susceptibility test to detect ESBLs vary with the cephalosporin tested (De Gheldre *et al.* 2003; Navon-Venezia *et al.* 2003; Tenover *et al.* 2003; Tofteland *et al.* 2007). The clinical breakpoints by EUCAST does not identify a specific resistance, moreover it divides the strains into susceptible "wild-type" strains and non-susceptible "non-wild-type" strains. By comparing the EUCAST clinical breakpoints with the CLSI screening values among the Finnish strains in this study, a very small amount of ESBLs, would have been considered susceptible.

Compared to a recent study on the sensitivities of the ESBL confirmatory test by disk diffusion among cefuroxime resistant strains (Navon-Venezia *et al.* 2003), a somewhat higher sensitivity value was reported for cefotaxime (96% vs. 94%) for the Finnish cefuroxime resistant strains *E. coli* isolates, while a lower sensitivity was reported for the *K. pneumoniae* isolates (84% vs. 95%) (I). A similar sensitivity value was reported with ceftazidime for the Finnish *E. coli* (76% vs 74%) while a lower sensitivity value was reported for *K. pneumoniae* (84% vs 77%). Cefpodoxime was also evaluated and here a difference was found among the sensitivity values. A higher sensitivity was reported for the Finnish *E. coli* (88% vs 97%), while a lower sensitivity was found for *K. pneumoniae* (95% vs 76%). However, in the study by Navon-Venezia *et al.* 2003, the ESBL verification was not confirmed with genotypic data, and hence the proportion of false positives and negatives cannot be calculated. The overall sensitivity of the CLSI phenotypical test was also calculated in the II study, and similar sensitivity values as for the study I was reported. From the results of these studies, it can hence be concluded that the combination of cefotaxime and ceftazidime or the use of cefpodoxime are the best cephalosporins for screening and confirmation of ESBLs.

Failures with both MIC and disk diffusion have been described (I; II; Jacoby and Han 1996; Vercauteren *et al.* 1997; Hageman *et al.* 2003; Sturenburg *et al.* 2004; Bedenic *et al.* 2007). Also other mechanisms than the presence of AmpC or metallo β-

lactamases, or the combination of mechanisms may lead to a false positive result for strains not carrying ESBL genes. The high-level expression of SHV-1 in *K. pneumoniae* can cause the MIC of ceftazidime to rise to levels at which an ESBL would be suspected (Miro *et al.* 1998; Rice *et al.* 2000). The outer membrane proteins (Omps) have also a great role. ESBL production combined with a modification of Omps e.g. OmpF (OmpK35) and OmpC (OmpK36) have been associated with increased cephalosporin and quinolone MICs (Martinez-Martinez *et al.* 1996; Ardanuy *et al.* 1998; Martínez-Martínez 2008). The size of the Omps varies e.g. OmpF is a wider channel than OmpC, thereby allowing larger molecules to pass through the outer membrane (Medeiros 1997). A change in a porin combined with an SHV-1 or TEM-1 has led to false positive results in *K. pneumoniae* (Wu *et al.* 2001). In the present study, K. oxytoca strains were incorrectly verified as ESBLs according to the CLSI criteria for both the agar dilution and the disc diffusion method. This was probably due to the overproduction of oxy/K1. Oxy/K1 can be overproduced several-fold, due to point mutations in their promoter sequence (Fournier *et al.* 1996; Fournier and Roy 1997). Hence, the *K. oxytoca* strains can be resistant to aztreonam, cefuroxime, ceftriaxone and cefpodoxime but not to ceftazidime, and among the cefuroxime resistant strains, the false positive *K. oxytoca* strains were all susceptible to ceftazidime.

6.3.2. Automated systems for susceptibility testing (II)

Different automated or semiautomated systems have been used for identification of species and for susceptibility testing. The instrumentation ranges in complexity from the simple optical reading of zones of inhibition to more complicated devices. In Finland the use of automated systems is quite small. In a questionnaire, done by the Laboratory of Microbial Ecology at the National Public Health Institute, only 5 of 20 Finnish hospital laboratories that took part in the questionnaire use the VITEK 2 system. However, the usage is increasing and hence we wanted to test the reliability of the system.

VITEK 2 is an automated system that includes a highly advanced software system that then interprets the identified bacteria's antibiotic resistance patterns. VITEK 2 has been evaluated for its ability to detect ESBL strains and has shown a good sensitivity in comparison with molecular identification and phenotypical methods. Hence, it is a reliable tool when detecting possible ESBL producers (Sanders *et al.* 1996; Sanders *et al.* 2001; Leverstein-van Hall *et al.* 2002; Livermore *et al.* 2002; Linscott and Brown 2005; Robin *et al.* 2008).

6.3.3. Verification of VITEK 2 for β-lactamase diagnostics (II)

In the Nordic countries, the VITEK 2 AST-N029 card is used for susceptibility testing of antibiotics and the aim of this study was to test the abilities of the VITEK 2 AST-N029 and to compare it with the agar dilution and disk diffusion methods. The strains that gave a positive result with AST-N029 were further tested with the ESBL test card, AST-N041. The AST-N029 card would be a timesaving method, compared to the disk diffusion or agar dilution methods, for the hospital laboratories to detect

ESBLs. This was the first study of the AST-N029 card and ESBL detection, and so far no other studies have been reported. In this study (II), the *E. coli*, *K. pneumoniae* and *K. oxytoca* strains, both ESBL-producers and non-ESBLs, were chosen to identify the strength and limitation of the test card.

In this study, VITEK 2 had the highest sensitivity for all the species that were tested, compared to the other phenotypic methods. The sensitivity, specificity and accuracy of VITEK 2 and AST-N029 for the *E. coli, K.pneumoniae* and *K. oxytoca* in this study, are comparable to the VITEK 2 results with other cards in previous studies (Sanders *et al.* 1996; Leverstein-van Hall *et al.* 2002; Livermore *et al.* 2002; Sorlozano *et al.* 2005; Spanu *et al.* 2006). The accuracy differences between the phenotypic methods were not as remarkable with the *E. coli* and *K. pneumoniae,* as with the *K. oxytoca*. *K. oxytoca* is considered a problematic organism, in consideration to discriminate between ESBLs and hyperproduction of oxy/K1 (Leverstein-van Hall *et al.* 2002; Potz *et al.* 2004; Paterson and Bonomo 2005). However, VITEK 2 worked adequately and had the highest accuracy and specificity. *K. oxytoca* isolates that produce TEM or SHV-type ESBLs may be distinguished from isolates hyperproducing K1, since TEM and SHV β-lactamases usually have MIC for ceftazidime ≥2 µg/ml, while oxy/K1 hyperproducers do not. Strains producing oxy/K1 confers reduced susceptibility or are resistant to cefuroxime and aztreonam. These strains are usually also resistant to ceftriaxone and cefpodoxime, while typically susceptible to ceftazidime. However, in this study the Nordic card AST-N029 only recorded two strains carrying oxy/K1 as an ESBL or possible ESBL. All the other oxy/K1 strains were correctly considered ESBL negatives, while eight of these were considered ESBL positive by the ESBL card, AST-N041.

The AST-N029 card had a high accuracy in identification of the ESBL-producing isolates; however, an attachment of a formal confirmation test to the AST-N029 card would save reporting time. The reporting time for microbiological laboratory using VITEK 2 AST-N029 for susceptibility testing could be shortened with a direct ESBL confirmation. An ESBL-positive isolate could be reported during the first 48 h of the treatment when using VITEK 2 AST-N029, which is difficult to achieve if another overnight cultivation is required for a separate confirmation test.

E. coli or *K. pneumoniae* strains with acquired AmpC or other non-classical ESBLs are not included in the database of VITEK 2, and hence VITEK 2 is currently not suitable for identification of such strains. However, since both cefoxitin and cefpirome are included on the AST-NO29 card, detection of suspicious strains, for example those resistant to cefoxitin or susceptible to cefpirome, is possible (Nasim *et al.* 2004; Pfaller and Segreti 2006).

6.3.4. SHV and pyrosequencing (III)

The first molecular method for identification of β-lactamases was a hybridisation method. This method was developed by Ouellette *et al.* 1988 and used to characterise β-lactamases e.g. TEM-1. To be able to define the exact SHV variants within an isolate harbouring more than one bla_{SHV} gene, the isolates have to be cloned and sequenced, which is a laborious method. Other methods, like isoelectric focusing (REF), PCR-SSCP (M'Zali *et al.* 1998), minisequencing (Howard *et al.* 2002) and real time PCR

assays (Randegger and Hächler 2001; Szabo *et al.* 2005) have been developed, but problems exist also with these and more appropriate methods are required for working in routine laboratories.

By cyclic sequencing the determination of the SHV-type in isolates carrying more than one bla_{SHV} gene is almost impossible. Careful analyses are required and also the sequence quality values should be taken into consideration (al Naiemi *et al.* 2006). However, it is difficult to resolve the exact SHV combination, as the combination of the variable nucleotides of the different gene copies is hard to determine. By traditional sequencing of PCR amplified bla_{SHV} genes in this study, ambiguous bla_{SHV} nucleotide sequences were not always detected for the DNA sequence corresponding to the amino acid position 238 and 240 (Figure 11, page 59). The pyrosequencing technique was tested whether it could be used to resolve these ambiguous bla_{SHV} sequences.

6.3.4.1. Chromosomal SHV and pyrosequencing

Most *Enterobacteriaceae* have chromosomal β-lactamases, e.g. AmpC in *E. coli* or SHV-1 or SHV-11 in *K. pneumoniae* (Babini and Livermore 2000; Caroff *et al.* 2000; Chaves *et al.* 2001; Corvec *et al.* 2007). The presence of the chromosomal β-lactamases may interfere when using molecular methods to detect ESBLs.

The dispensation orders that were designed for the SHV pyrosequencing assay for amino acid positions 35, 238 and 240, generated distinct pyrograms allowing easy differentiation between chromosomal $bla_{SHV\text{-}1}$ and ESBL bla_{SHV} variants. By combining the results from the two assays, it is possible to differentiate the chromosomal $bla_{SHV\text{-}1}$ or $bla_{SHV\text{-}11}$ from plasmid-mediated ESBL bla_{SHV} genotypes or isolates carrying more than one bla_{SHV} gene, since the DNA sequences corresponding to the studied amino acid positions are known. The strains that carried more than one bla_{SHV} gene could not be identified by cyclic sequencing due to the ambigous peaks. However, by the pyrosequencing method, these peaks could be resolved.

The majority of the *K. pneumoniae* strains that were studied by the pyrosequencing assays carried more than one bla_{SHV} gene, indicating presence of both a chromosomal and plasmid-mediated type. A remarkable difference between carriage of ESBL genes in *K. pneumoniae* isolates with $bla_{SHV\text{-}1}$ or $bla_{SHV\text{-}11}$ was not detected, in contrast to another study but likewise the co-carriage of $bla_{SHV\text{-}12}$ and $bla_{SHV\text{-}11}$ was more common than co-carriage of $bla_{SHV\text{-}12}$ and $bla_{SHV\text{-}1}$ (Lee *et al.* 2006). Nevertheless, the results of this study agree with the hypothesis that most ESBL bla_{SHV} are plasmid-mediated and are present in high copy numbers in bacteria, whereas $bla_{SHV\text{-}1}$ and $bla_{SHV\text{-}11}$ are usually chromosomally encoded and present only in low copies.

6.3.4.2. Comparison of pyrosequencing to other methods

The SHV strains in this study were usually phenotypically correctly identified, hence the problem is not the detection. Moreover, the SHV pyrosequencing method brings value to epidemiological studies where the presence of both a plasmid mediated and a chromosomally mediated variant is relevant. A PCR coupled with pyrosequencing, provide for rapid detection and identification of SHV variants. Isolates can rapidly (within four hours) be distinguished from the wild type. The

pyrosequencing method have also been successfully used for differentiation of the CTX-M to the subgroup level as in the I study and Naas et al. 2006a, to distinguish the wild type TEM-1 from the ESBL variants (unpublished), OXA (Naas *et al.* 2006b) and for identification of GES-type ESBLs (Poirel *et al.* 2006).

Also other methods have been used for a rapid detection of SHV ESBL, like microarray, and RT-PCR. Also these methods are based on detection of a single or a few mutations. While it is possible to distinguish the presence of both a chromosomal and plasmid-mediated gene by pyrosequencing, neither of these methods is able to do that. However, the actual SHV-type must still be determined by using other methods, covering also other amino acid positions, i.e. having the complete sequence of the corresponding gene type.

6.3.5. Guidelines and prevention of ESBLs in the future

The confirmatory test does not always work properly with the existing ESBL genes (Drieux *et al.* 2008; Kahlmeter 2008). New β-lactamase genes and gene combinations are found continually, and hence new guidelines are needed. The CLSI breakpoints have been used as the common guidelines in Finland, but since the breakpoints for cephalosporins are too high, other guidelines, like EUCAST, should be used. Recently, a new simplified classification nomenclature scheme for the ESBLs, from a clinical perspective, was presented. This classification expands the definition of ESBL and according to the authors would engage health care professionals in prevention of the spread of ESBLs (Giske *et al.* 2008). However, the prevention of ESBL will not be solved by changing the classification schemes, moreover, in order to limit the ESBLs, all health care professionals should be aware of the ESBL threat, so that possible outbreaks can be prevented and the selective pressure from the surroundings should be minimised.

7. SUMMARY AND CONCLUSIONS

In this work, the molecular genetics and the diagnostic methods of β-lactamases in Finland was studied. During the last 20 years, the ESBLs have evolved from an interesting scientific observation to a great worldwide medical problem. The enterobacterial species have evolved resistance to many antibiotics e.g. to 3rd generation cephalosporins. Some of these resistance mechanisms occur on low levels in natural populations, but the widespread use of carbapenems, cephamycins and oxyimino-cephalosporins in the past few decades has led to the enrichment of a new generation of β-lactamases with an extended substrate spectrum.

Based on the genetic basis in this study, it is possible to conclude that the distribution of β-lactamase genes in Finland is very similar to the gene variants found elsewhere in Europe. However, the ESBL situation in Finland is still quite good in comparison to many other European countries. As seen in many other countries, the *bla*$_{\text{CTX-M-1}}$-type genes are the most prevalent ESBLs also in *E. coli* in Finland. The ESBL study from Helsinki is the first report of an ESBL outbreak from Finland. *E. coli* harbouring CTX-M-1 caused the *E. coli* outbreak, while SHV-12 caused the outbreak of *K. pneumoniae*. One major clone harbouring CTX-M-1 among the *E. coli* and SHV-12 among the *K. pneumoniae* strains were detected.

The resistance to 3rd generation cephalosporins has increased in most countries, including Finland, during the last ten years. However, the resistance percentage is still quite low in Finland in comparison to the rest of the European countries, but is probably increasing due to the growing amount of ESBLs. Bacteria can have a multifaceted resistance profile due to the presence of multiple β-lactamases e.g. ESBLs in combination with non-ESBL enzymes. In this study, about 60% of the ESBL strains were MDR.

The phenotypic methods, the agar dilution and disk diffusion method worked nicely in this study. Most of the strains were correctly detected. However, the success of the present diagnostic methods is debatable, since part of the strains is falsely identified by the agar dilution and disk diffusion methods according to the CLSI criteria and thereby jeopardizes the treatment success of a patient. In this study it was shown that VITEK 2 AST-N029 is, even without a formal ESBL confirmation test, an accurate and time-saving tool for detection of ESBLs in *E. coli, K. pneumoniae* and *K. oxytoca*, in comparison to the agar dilution and disk diffusion methods. A rapid and correct detection that distinguishes between wild-type and non wild-type strains, is necessary to treat patients accordingly, with the right choice of antimicrobial agents.

Since the ESBL producing strains are such a large and complex group, confirmation with easily performed molecular biological assays is not possible, and the existing assays are not designed for large scale use. The pyrosequencing method assays that were developed in this work for detection of *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{SHV}}$ genes are rapid methods that can be further developed and could be introduced for larger use.

The prevalence of antibiotic resistance continues to increase and is unavoidable, since it represents a characteristic of the general evolution of bacteria. It is difficult, if not impossible, to predict the development of ESBLs and antimicrobial resistance in the future. Therefore, to conclude, since limited therapeutic options are already a fact for some of the strains expressing ESBLs, the ESBLs present a host of challenges.

ACKNOWLEDGEMENTS

This study was carried out at the National Institute for Health and Welfare, Department of Infectious Diseases, Division of Health Protection in Turku and registered at the Faculty of Medicine at the Department of Medical Microbiology and Immunology, University of Turku.

I thank my supervisors Docent Jari Jalava and Research Professor Pentti Huovinen for excellent guidance and support throughout this project and for the chance to do my thesis project in the Laboratory of Microbial Ecology. I express my thanks to Professor Matti Viljanen for accepting my thesis work to be published from the Department of Medical Microbiology and Immunology, University of Turku.

Professor Mikael Skurnik and Docent Jaakko Uksila are acknowledged for the thorough review of this work and for their valuable and constructive comments.

I wish to thank my co-authors for their contribution to the manuscripts, especially Dr Kaisu Rantakokko-Jalava, Dr Olli Meurman, Dr Jaana Vuopio-Varkila, Professor Martti Vaara and Dr Elina Kolho. Merja Rantala is acknowledged for presenting the animal and veterinary side of microbiology to me.

Monica Österblad is thanked for introducing the microbial world to me and also for interesting discussions. I am also grateful to Antti Hakanen for interesting discussions and for the advice they provided during the writing processes of the papers.

I want to thank those who have helped me with the practical work, for excellent technical assistance: Minna Lamppu, Marja-Liisa Lindgren, Anna-Liisa Lumiaho, Tuula Randell, Mari Virta and Tarja Laustola. I am grateful to Erkki Nieminen and Anne Nurmi for assistance with data dilemmas.

The ladies in the office are thanked for assistance and help with bureaucratic questions: Pirjo Ketonen, Virpi Aaltonen, Paula Kiiskilä and Jaana Halme.

I would also like to thank those at the Molecular Medicine Sequencing Laboratory in Helsinki for the help with “ready to run” sequencing: Pekka Ellonen and Heli Keränen.

I thank my past and present room-mates: Marjo Haanperä-Heikkinen, Marianne Gunell, Miika Bergman, Laura Lindholm, Mirva Lehtopolku and Anna-Kaisa Suominen for a cheerful atmosphere with both scientific and unscientific discussions.

I would also like to thank all senior and junior researchers at the former National Public Health Institute in Turku.

Aila Palomäki is thanked for the linguistic revision of this thesis.

I want to thank all my friends for sharing joys and troubles both during this project and everyday life.

I wish to thank my parents Rita and Henrik, my brother Wille and my sister-in-law Johanna for the support and encouragement over the years.

Finally, I wish to express gratitude and appreciation to Henka for always being there, and for the help with the figures of this thesis.

Financial support was provided by the Microbes and Man research programme, Academy of Finland, by the Ministry of Social Affairs (Special Epidemiology Grants), the Swedish Cultural Foundation in Finland, the Turku University Foundation, the Valto Takala foundation, the Maud Kuistila Foundation and by a Special Government Grant (EVO-grant).

Turku, October 2009
Turku, 2009

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