**From Clinical Microbiology**
**Department of Microbiology, Tumor and Cell Biology - MTC**
**Karolinska Institutet, Stockholm, Sweden**

# ANTIBIOTIC RESISTANCE IN GRAM-NEGATIVE BACILLI

## - WITH FOCUS ON CEPHALOSPORIN RESISTANCE MECHANISMS IN *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

Owe Källman


Karolinska Institutet

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# ABSTRACT

**Objectives.** The objectives of this thesis were to study mechanisms conferring cefuroxime resistance in clinical non-ESBL (extended-spectrum β-lactamase) producing isolates of *Escherichia coli* and *Klebsiella pneumoniae*, as well as the presence of co-resistance to other antibiotics in the *K. pneumoniae* isolates. Further, the objectives were to study findings of and resistance patterns of Gram-negative bacilli isolated from patients seeking medical advice in Stockholm after the tsunami catastrophe.

**Materials and methods**. In paper I 30 clinical non-ESBL blood culture isolates of *E. coli* with various degrees of cefuroxime susceptibility were examined. Cefuroxime susceptibility was determined in the presence of the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN). Organic solvent tolerance (OST) was determined, as a phenotypic method for detecting efflux. In paper II 14 clinical non-ESBL isolates of *E. coli* with various degrees of cefuroxime susceptibility were examined. Transcription levels of *acrA* (encoding AcrA, part of the AcrAB-TolC efflux pump) and *ompF* (encoding the porin OmpF) were determined with quantitative RT-PCR. AmpC activity was determined with spectrophotometry. In paper III an epidemiological survey was made of findings of and resistance patterns of Gram-negative bacilli from patients seeking medical advice in Stockholm after the tsunami catastrophe. In paper IV ten blood culture isolates of *K. pneumoniae*, all cefuroxime resistant, but cefotaxime susceptible and a multiply antibiotic resistant (MAR) laboratory strain (selected by chloramphenicol) were examined. Transcription levels of *acrA*, *ompK35* (the homologue of *ompF*), and of the regulatory genes *ramA*, *marA* and *soxS* were determined with quantitative RT-PCR. Antibiotic susceptibility was also determined in the presence of PAβN.

**Results**. In paper I was shown that the organic solvent tolerant isolates had significantly decreased susceptibility to cefuroxime compared to the non-tolerant isolates. Further, the susceptibility to cefuroxime was increased by PAβN for the cyclohexane tolerant isolates. In paper II, increased *acrA* transcription was seen in seven of the eleven cefuroxime resistant isolates. Very low *ompF* transcription levels were seen in three and increased AmpC activity in two of the cefuroxime resistant isolates. Paper III showed that findings of various Gram-negative bacilli were common in secretion cultures from the tsunami victims and that resistant isolates of *E. coli*, *K. pneumoniae*, *Proteus mirabilis* and *Acinetobacter* spp. were more common in cultures from tsunami victims compared to the Swedish reference material, as was not the case for *Pseudomonas aeruginosa*. In paper IV all examined isolates and the laboratory strain showed similar antibiograms with decreased susceptibility to cefuroxime, chloramphenicol, nalidixic acid and tigecycline. All strains also had increased *acrA* transcription and decreased *ompK35* transcription. The laboratory strain and all the clinical isolates except one displayed increased *ramA* transcription. PAβN increased susceptibility to chloramphenicol, nalidixic acid and tigecycline, but not to cefuroxime.

**Conclusions**. In clinical non-ESBL cefuroxime resistant isolates of *E. coli* several resistance mechanisms (efflux of the AcrAB-TolC complex, lack of OmpF and increased AmpC activity) seem to contribute to cefuroxime resistance. In *K. pneumoniae* a multidrug resistant (MDR) phenotype was observed in all the examined isolates, with decreased susceptibility to cefuroxime, chloramphenicol, nalidixic acid and tigecycline. Further, the same resistance mechanisms were detected in almost all clinical isolates. The findings of various Gram-negative bacteria from tsunami victims affect which empirical antibiotic treatment should be given when these kinds of traumatic infections are treated.

# LIST OF PUBLICATIONS

I. **Källman O**, Fendukly F, Karlsson I and Kronvall G.
**Contribution of efflux to cefuroxime resistance in clinical isolates of *Escherichia coli*.**
Scandinavian Journal of Infectious Diseases. 2003; **35**: 464-470.

II. **Källman O**, Giske CG, Samuelsen Ø, Wretlind B, Kalin M and Olsson-Liljequist B.
**Interplay of efflux, impermeability and AmpC activity contributes to cefuroxime resistance in clinical, non-ESBL isolates of *Escherichia coli*.**
Submitted for publication in Microbial Drug Resistance.

III. **Källman O**, Lundberg C, Wretlind B and Örtqvist Å.
**Gram-negative bacteria from patients seeking medical advice in Stockholm after the tsunami catastrophe.**
Scandinavian Journal of Infectious Diseases. 2006; **38**: 448-450.

IV. **Källman O**, Motakefi A, Wretlind B, Kalin M, Olsson-Liljequist B and Giske CG.
**Cefuroxime non-susceptibility in multidrug-resistant *Klebsiella pneumoniae* overexpressing *ramA* and *acrA* and expressing *ompK35* at reduced levels.**
Journal of Antimicrobial Chemotherapy. 2008; doi: 10.1093/jac/dkn296. Epub ahead of print.

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ATCC | American Type Culture Collection |
| *bla* | β-lactamase (used for β-lactamase genes) |
| EARSS | European Antimicrobial Resistance Surveillance System |
| ESBL | Extended-spectrum β-lactamase |
| EUCAST | The European Committee on Antimicrobial Susceptibility Testing |
| ISA | Iso-Sensitest Agar |
| MDR | Multidrug resistant |
| MFP | Membrane fusion protein |
| MIC | Minimum inhibitory concentration |
| MNE | Mean normalised expression (transcription) |
| MYSTIC | Meropenem Yearly Susceptibility Test Information Collection |
| NE | Normalised expression (transcription) |
| OMF | Outer membrane factor |
| OMP | Outer membrane protein |
| OST | Organic solvent tolerance |
| PAβN | Phenylalanine arginine β-naphthylamide |
| PBP | Penicillin-binding protein |
| PhP | PhenePlate |
| RND | Resistance-nodulation-division |
| SRGA | The Swedish Reference Group for Antibiotics |

# 1 INTRODUCTION

## 1.1 FEATURES OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE*

*Escherichia coli* and *Klebsiella pneumoniae* are Gram-negative bacilli, belonging to the family Enterobacteriaceae. They are part of the normal human faecal flora. However, they are also common human pathogens, able to cause various infections, such as cystitis, pyelonephritis and bloodstream infection. *E. coli* is the most common cause of urinary tract infection and the most common Gram-negative bacterium isolated from blood cultures. Certain strains of *E. coli* can also cause enteric infections. *Klebsiella* spp. are the second most common Gram-negative bacteria isolated from blood cultures. *K. pneumoniae* can also cause pneumonia, especially in immunocompromised patients [1, 2].

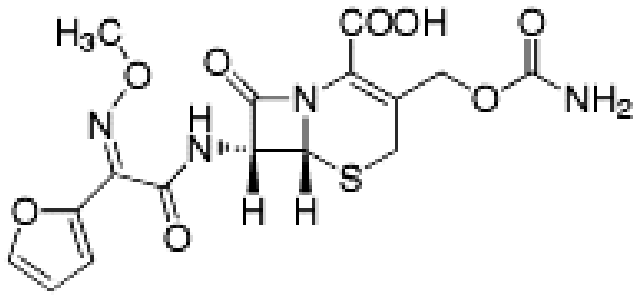
*E. coli* and *K. pneumoniae* are facultatively aerobic organisms and negative in the oxidase test. *E. coli* strains usually produce indole, but cannot hydrolyse urea and are negative in the Voges-Proskauer (VP) test, while *K. pneumoniae* strains usually are indole-negative, urease-positive and VP-positive [1, 2].

Infections with *E. coli* and *K. pneumoniae* often need to be treated with antibiotics, orally or parenterally, depending on the severity of infection. It is therefore of great importance to have access to several chemically unrelated non-toxic antibiotics, to which these bacteria are susceptible. Antibiotic resistance to different antibiotics is common in these bacteria, sometimes they are multidrug resistant. Therefore, there is also a need for development of new antibiotics [3].

## 1.2 CEPHALOSPORINS

Cephalosporins are broad-spectrum antibiotics which belong to the β-lactam antibiotic group. This group also includes penicillins, carbapenems and monobactams [4]. They all contain the characteristic β-lactam ring and their mechanism of action is by binding to penicillin-binding proteins, PBPs and interfering with the peptidoglycan synthesis in the cell wall synthesis of the bacterium [4, 5]. Commonly used cephalosporins for parenteral treatment in Sweden are the second generation cephalosporin cefuroxime (Fig 1) and the third generation cephalosporins cefotaxime and ceftazidime (www.apoteket.se). They all belong to the oxyimino cephalosporins [6]. Cefuroxime (and cefotaxime) has been used extensively in Sweden for the treatment of severe infections caused by *E. coli* and *K. pneumoniae*.

**Fig 1.** Cefuroxime




Wild type minimum inhibitory concentration (MIC) distributions of *E. coli* isolates are shown in Fig 2 for cefuroxime, in Fig 3 for cefotaxime and in Fig 4 for ceftazidime (www.eucast.org). MIC values are lower for cefotaxime and ceftazidime than for cefuroxime. Similar wild type distributions are seen for *K. pneumoniae* isolates (www.eucast.org).

**Fig 2**. Wild type distribution of MICs of cefuroxime, *E. coli*

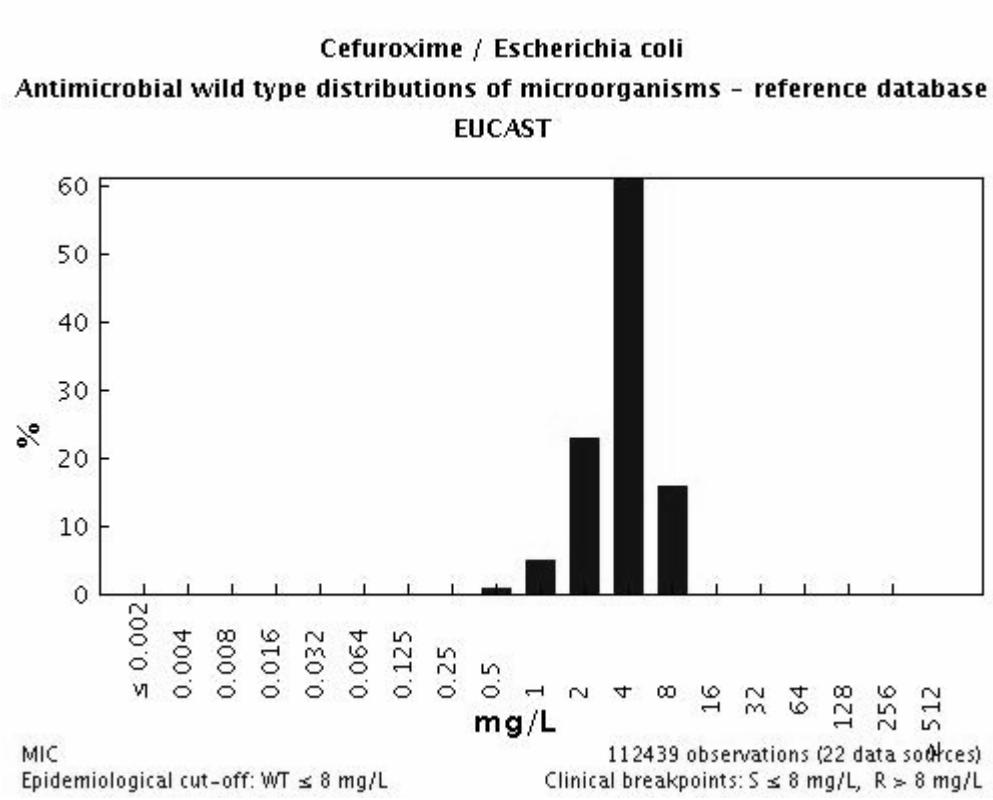



**Fig 3**. Wild type distribution of MICs of cefotaxime, *E. coli*

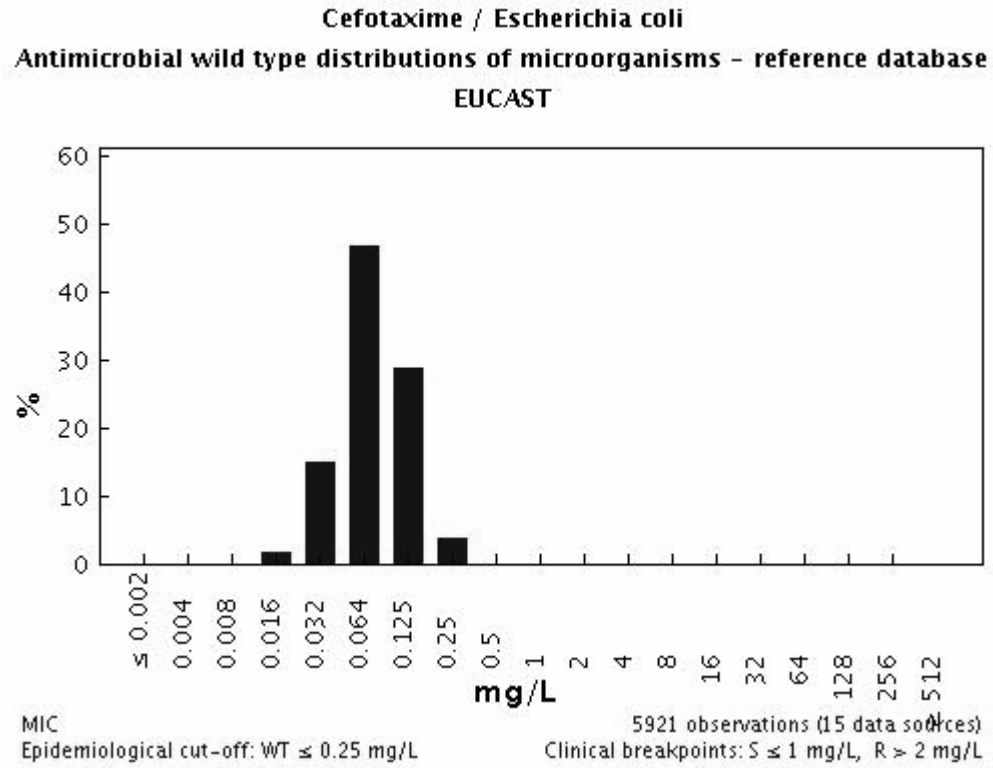
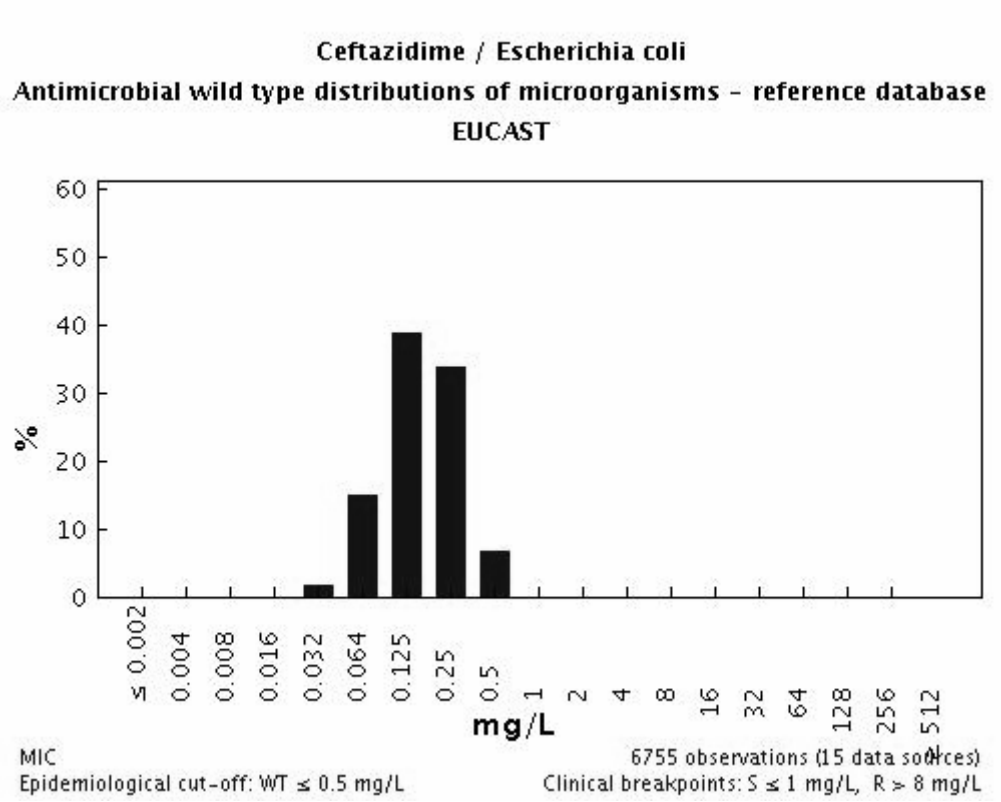


**Fig 4**. Wild type distribution of MICs of ceftazidime, *E. coli*

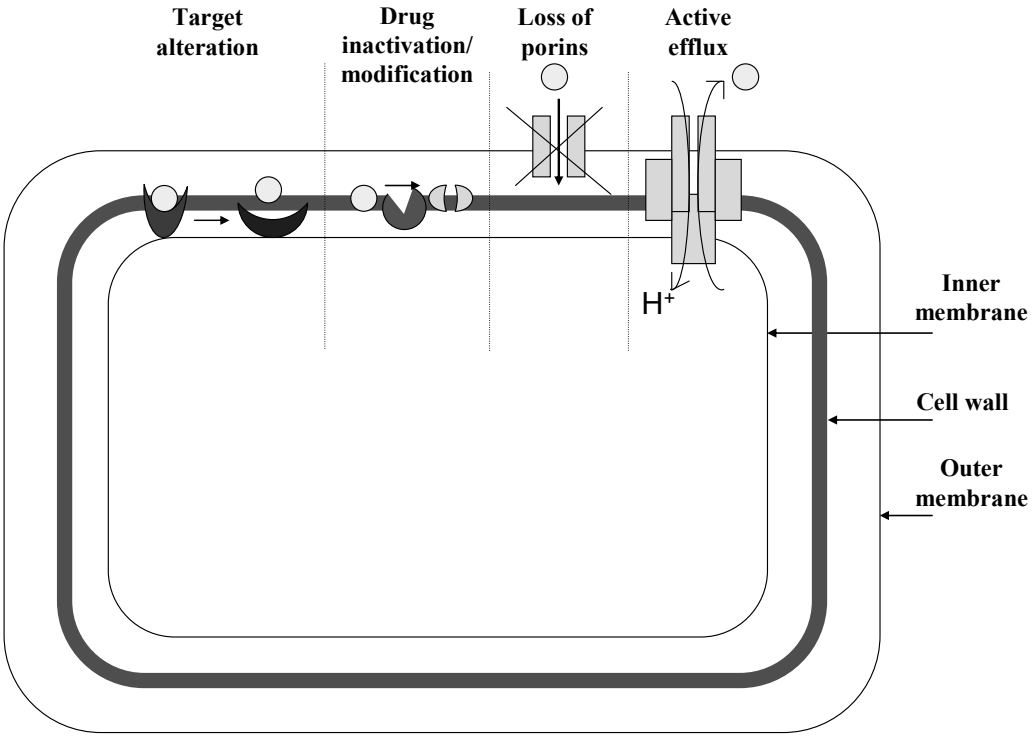


## 1.3 ANTIBIOTIC RESISTANCE MECHANISMS

Certain bacterial species are naturally resistant (intrinsically resistant) to certain antibiotics (e.g. intrinsic resistance to cephalosporins in enterococci) [4]. Frequently occurring in many bacterial species is acquired resistance, i.e. the occurrence of strains resistant to certain antibiotics, to which the wild type of that species is susceptible (e.g. penicillin resistance in *Staphylococcus aureus*) [4]. Acquired resistance is a substantial problem in clinical practice when treating bacterial infections.

The major mechanisms of acquired antibiotic resistance are drug inactivation/ modification, target alterations, reduced accumulation due to decreased permeability and reduced accumulation due to increased efflux (Fig 5) [7]. Three of these four mechanisms will be discussed more thoroughly in the following section, being the most important mechanisms for cephalosporin resistance in *E. coli* and *K. pneumoniae*.

**Fig 5.** Schematic picture of potential antibiotic resistance mechanisms in Gram-negative bacteria.



### 1.3.1 Beta-lactamase production

The production of β-lactamases, hydrolytic enzymes that can disrupt the β-lactam ring and thereby inactivate the antibiotic is a major mechanism of β-lactam resistance, especially in Gram-negative bacteria. The β-lactamase genes can be located both chromosomally and be plasmid borne. They are structurally related to PBPs and may have evolved from these [4]. They can be classified into four molecular classes, A-D, with different characteristics (Table 1) [4]. There is also a functional classification, the Bush-Jacoby-Medeiros classification, group 1-4 [8].

Two major groups of β-lactamases are of importance for cephalosporin resistance in *E. coli* and *K. pneumoniae*. AmpC enzymes render resistance to penicillins, cephalosporins and monobactams and can be inhibited by boronic acid or cloxacillin [4, 9, 10]. In *E. coli* the $bla_{\text{AmpC}}$ gene is generally poorly expressed in wild type isolates [11-13]. Cephalosporin resistance in *E. coli* can be caused by increased expression of the chromosomally encoded AmpC, typically causing resistance to cefuroxime, but with only moderately decreased susceptibility to cefotaxime and ceftazidime [14]. Increased $bla_{\text{AmpC}}$ expression in *E. coli* can be caused by mutations in the $bla_{\text{AmpC}}$ gene promoter region or by the existence of more than one copy of the $bla_{\text{AmpC}}$ gene [15]. There have also been reports of chromosomal *E. coli* AmpC enzymes with extended spectrum, ESAC, due to mutations in the $bla_{\text{AmpC}}$ gene [16, 17]. In *E. coli* ESAC enzymes in combination with deficiency of the porins OmpC and OmpF has been showed to confer decreased susceptibility also to carbapenems [18]. Plasmid borne $bla_{\text{AmpC}}$ genes are also increasingly observed in *E. coli* and *K. pneumoniae* [19]. Important examples of plasmid borne AmpC enzymes are CMY-enzymes, probably originating from chromosomal enzymes of *Citrobacter freundii* and *Aeromonas* spp. [19]. When present in *K. pneumoniae* they are always plasmid borne, since there is no $bla_{\text{AmpC}}$ gene in the *K. pneumoniae* genome [20].

The second group, extended-spectrum β-lactamases, ESBLs are plasmid borne and able to hydrolyse penicillins, cephalosporins and monobactams [4, 8]. They are inhibited by clavulanic acid [4, 8]. During the 1990s ESBLs caused by mutations in the genes encoding narrow-spectrum TEM- and SHV-penicillinases were globally dominant [21]. Today, CTX-M ESBLs have become the most frequently encountered [21].

Other β-lactamases of importance for cephalosporin resistance but with the ability to also hydrolyse carbapenems are metallo-β-lactamases, which have been reported in both *E. coli* and *K. pneumoniae* and KPC enzymes reported in *K. pneumoniae* [4, 22-24].

**Table 1**. Examples of β-lactamases in Gram-negative bacilli [4, 6, 8-10, 19]

| Ambler class | Type of enzyme | Preferred substrates | Inhibited by | Examples of chromo-somal enzymes | Examples of plasmid borne enzymes |
|---|---|---|---|---|---|
| A | Restricted spectrum β-lactamase | Penicillins, narrow-spectrum cephalosporins | CA | SHV-1[a], K1[b] | TEM-1, TEM-2 |
| | Extended-spectrum β-lactamase (ESBL) | Penicillins, cephalosporins, monobactams | CA | | Numerous TEM, SHV and CTX-M variants |
| | Inhibitor resistant β-lactamase | Penicillins, cephalosporins | - | | Several TEM variants |
| | Carba-penemase | Most β-lactams, including carbapenems | CA | | KPC variants |
| B | Metallo-β-lactamase | Most β-lactams, including carbapenems (except ATM) | EDTA | L1[c] | IMP, VIM variants |
| C | Extended-spectrum cephalo-sporinase | Penicillins, cephalosporins, cephamycins, monobactams | BOR, CL | Chromo-somal AmpC enzymes[d], ESAC | CMY, MOX, FOX variants |
| D | Penicillinase and ESBL | Penicillins, cloxacillin, cephalosporins | - | Some OXA variants[e] | OXA variants |
| | Carba-penemase | Penicillins, oxacillin, carbapenems | CA | OXA variant[f] | Some OXA variants |

ATM, aztreonam; BOR, boronic acid; CA, clavulanic acid; CL, cloxacillin
[a]in *K. pneumoniae*
[b]in *K. oxytoca*
[c]in *Stenotrophomonas maltophilia*
[d]e.g. in *E. coli*, *Shigella* spp, *Enterobacter* spp, *Citrobacter freundii*
[e]e.g. in *P. aeruginosa* and *Aeromonas* spp.
[f]in *Acinetobacter* spp.

### 1.3.2 Decreased permeability

Hydrophilic antimicrobials (e.g. β-lactams) enter the Gram-negative bacterium through channels in the outer membrane formed by porins. Porin loss has been related to resistance to β-lactams [4, 25]. In *E. coli* there are two major non-specific porins, OmpC and OmpF [25]. The functional pore of OmpF is slightly larger than that of OmpC [25]. They are both of importance for the entrance of β-lactams, but their role in β-lactam resistance is still somewhat unclear [25-27]. Alteration of outer membrane proteins has been proposed as a mechanism of cefuroxime resistance in *E. coli* [28]. Expression of OmpC and OmpF is controlled at the transcriptional level by the EnvZ-OmpR system and at the translational level by MicF and MicC [25, 29, 30]. Medium osmolarity affects OmpC and OmpF transcription, through the EnvZ-OmpR system. OmpF is preferentially produced at low osmolarity. The global regulatory protein MarA has been shown to decrease OmpF levels through increased production of the sRNA MicF [31-33]. In *K. pneumoniae* two major porins have also been described, OmpK35 (a homologue of OmpF) and OmpK36 (a homologue of OmpC). [25]. Decreased expression of OmpK35 and/or OmpK36 has been associated with resistance to cephalosporins and carbapenems [34-37]. OmpK35 deficiency has been associated with cefuroxime resistance [38]. In *K. pneumoniae* the regulatory protein RamA has been shown to decrease the amount of an outer membrane protein [39]. Minor porins (of unclear significance for antibiotic resistance) have also been identified, which are not expressed under standard laboratory conditions, e.g. NmpC and OmpN in *E. coli* and OmpK37 in *K. pneumoniae* [25, 40]. Other examples of porins are Omp35 in *Enterobacter aerogenes* and OprD in *Pseudomonas aeruginosa* and loss of porins has been associated with carbapenem resistance in *E. aerogenes* and *P. aeruginosa* [4, 41, 42].

### 1.3.3 Efflux

Active efflux of an antibiotic is a well-known resistance mechanism since almost 30 years, first described as a cause of tetracycline resistance in *E. coli* [43, 44]. Since then many different efflux pump systems have been described, in both Gram-negative and Gram-positive bacteria [7, 45, 46]. In *E. coli* genes encoding at least 37 efflux transporters, putative or proven, have been identified [45, 47]. Proposed natural functions of efflux pumps has been to help the bacteria to export potentially toxic substances, e.g. bile salts in its ordinary environment, or to mediate cell-cell communication in response to cell density [48, 49]. In *P. aeruginosa* it has been

suggested that the MexAB-OprM system is able to export virulence determinants, contributing to bacterial virulence [50]. Efflux pumps are also present in eukaryotes [45]. Bacterial drug efflux pump systems can be grouped into five families: the major facilitator superfamily (MFS), the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance-nodulation-division (RND) family [7, 45]. They are all energy-dependent; the ABC superfamily uses ATP as an energy source, while all the others are drug-proton antiporters [46]. Some efflux pumps are substrate specific and then typically occur on mobile genetic elements [7]. Others have a broad substrate range and are chromosomally encoded, e.g. the RND type efflux pumps [7, 46]. The RND type pumps are of importance for antibiotic resistance in many Gram-negative bacteria, especially *P. aeruginosa* and species belonging to the Enterobacteriaceae family [7, 45]. They typically operate as a tripartite system including the RND pump itself (located in the cytoplasmic membrane), a periplasmic membrane fusion protein (MFP) and an outer membrane factor (OMF) located in the outer membrane [7, 46]. Important examples are MexAB-OprM in *P. aeruginosa* and AcrAB-TolC in *E. coli* [45, 46].

The efflux pump AcrAB is of importance for efflux mediated resistance in several species belonging to the Enterobacteriaceae family [45]. AcrB is the RND pump itself and AcrA is the MFP [45]. In *E. coli* TolC is the OMF [45]. In *E. coli* AcrAB-TolC has been shown to cause efflux of organic solvents and different antibiotics, e.g. chloramphenicol, fluoroquinolones, nalidixic acid, β-lactams and tigecycline [45, 51-53]. It also (at least partly) explains the intrinsic resistance of *E. coli* to various antibiotics, such as erythromycin, fucidic acid and oxazolidinones [54]. Laboratory *E. coli* strains lacking AcrB or AcrAB showed increased susceptibility to cefuroxime, but not to cefotaxime and ceftazidime [55, 56]. Over-expression of *acrA* has been associated with organic solvent tolerance [57]. The ability of bacteria to grow in the presence of different organic solvents can be used as a phenotypic method to determine efflux, especially efflux mediated by AcrAB [57-59]. Wild type *E. coli* isolates grow in the presence of hexane, but not in the presence of cyclohexane or mixtures hexane-cyclohexane and growth in the presence of cyclohexane can suggest increased *acrAB* expression in *E. coli* [59]. Organic solvent tolerance has been associated with resistance to several antibiotics in *E. coli* [60]. Several genes are of importance for the regulation of *acrAB* expression in *E. coli*, e.g. the local repressor gene *acrR* and the global

regulator genes *marA, soxS* and *robA* [31, 48, 51, 52, 57]. Increased expression of *acrAB* during slow growth of *E. coli* has been reported [61]. More examples of *E. coli* efflux pumps are displayed in Table 2.

**Table 2.** Examples of efflux pumps in *E. coli*

| Name | Type of pump | Substrates | References |
|---|---|---|---|
| AcrAB-TolC | RND | AC, β-lactams, bile salts, CHL, ERY, FUS, FQ, LIN, NAL, OS, TET, TGC etc. | [45, 51, 53, 62] |
| AcrEF-TolC | RND | β-lactams, FQ, TET etc. | [45, 51] |
| AcrAD-TolC | RND | AG etc. | [45, 51] |
| TetA | MFS | TET | [62] |
| EmrAB-TolC | MFS | NAL etc. | [45, 51, 62] |
| EmrE | SMR | AC, EB | [45, 51, 62] |
| YdhE | MATE | AC, FQ | [45, 51] |
| MacAB-TolC | ABC | ML | [45] |

AC, acriflavin; AG, aminoglycosides; CHL, chloramphenicol; EB, ethidium bromide; ERY, erythromycin; FUS, fusidic acid; FQ, fluoroquinolones; LIN, linezolid; ML, macrolides; NAL, nalidixic acid; OS, organic solvents; TET, tetracycline; TGC, tigecycline

In *K. pneumoniae* the AcrAB efflux pump system has not been as well characterised as in *E. coli.* It has been associated with decreased susceptibility to fluoroquinolones, nalidixic acid, chloramphenicol, trimethoprim and tigecycline [63-66]. The OMF working together with AcrAB in *K. pneumoniae* had until recently not been identified [51]. However, a gene named *kocC*, encoding an ortholog of *E. coli* TolC, was recently identified from chromosomal DNA of *K. pneumoniae* [67]. Increased *acrA* expression has been associated with increased expression of the global regulatory gene *ramA* and with mutations in the local regulator gene *acrR*, but not with *marA* and *soxS* over-expression [64, 66].

An efflux pump inhibitor named phenylalanine arginine β-naphthylamide (PAβN, also called MC-207,110) has been used experimentally to inhibit efflux pumps [63, 68-71]. Its exact mechanism of action is unknown [71].

## 1.4 ANTIBIOTIC RESISTANCE EPIDEMIOLOGY

### 1.4.1 Global antibiotic resistance in *E. coli*, *K. pneumoniae* and *P. aeruginosa*

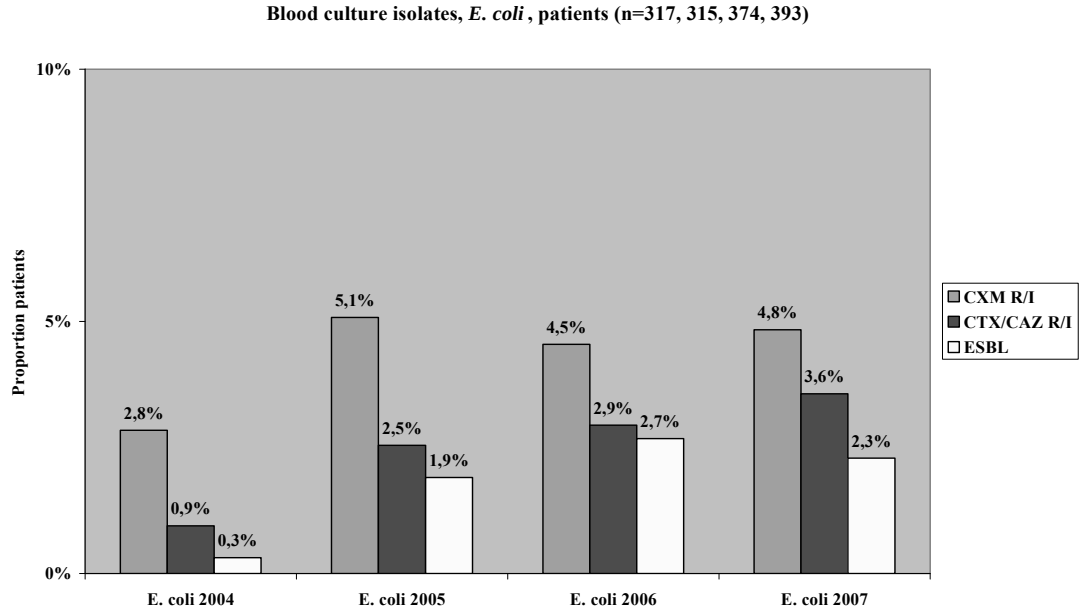
Antibiotic resistance is an increasing problem throughout the world (>95 000 hits in a PubMed search). Several surveillance programmes (e.g. MYSTIC, SENTRY and EARSS) monitor antibiotic resistance [72-74]. In USA, extended-spectrum cephalosporin resistance is increasing in *E. coli* and *Klebsiella* spp., as is also resistance to fluoroquinolones and aminoglycosides [72]. In *Klebsiella* spp. there is also increasing carbapenem resistance [72]. In Europe the trend is similar, with increasing resistance in *E. coli* and *K. pneumoniae* to extended-spectrum cephalosporins, fluoroquinolones and aminoglycosides. However, there are great regional differences. Generally, the resistance rates are lower in the northern of Europe. In Greece, Italy, Turkey and Israel increasing carbapenem resistance in *K. pneumoniae* is observed (http://www.rivm.nl/earss, EARSS annual report, 2006). This is very alarming, since carbapenems are often the last choice of treatment in isolates of Gram-negative bacteria resistant to other antibiotics.

For *P. aeruginosa* antibiotic resistance is common (>10%) to carbapenems, aminoglycosides, ceftazidime and/or fluoroquinolones in many European countries (http://www.rivm.nl/earss/database/, data from 2006).

### 1.4.2 Local resistance epidemiology

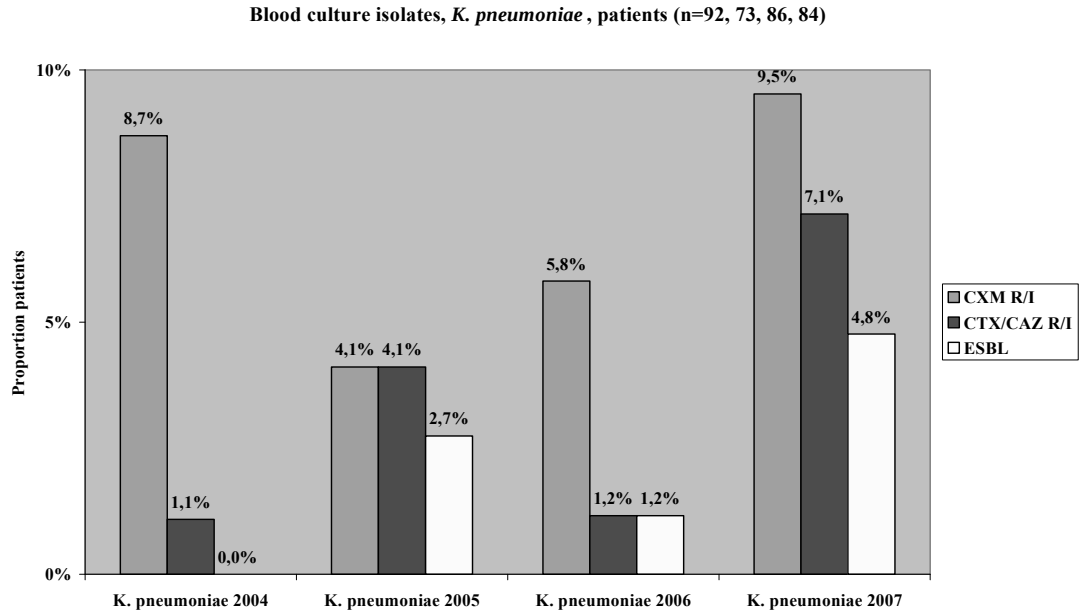
Resistance epidemiology of *E. coli* isolates at Karolinska hospital during 1988-1999 has been reported. No significant increase in cefuroxime resistance was seen [75, 76]. In Fig 6 cephalosporin resistance levels of blood culture isolates of *E. coli* from Clinical Microbiology, Karolinska University Laboratory, Solna, Sweden, during the years 2004-2007 is reported (unpublished laboratory statistics). Only one isolate per patient is included. There is a clear trend of increase in ESBL-producing isolates, causing resistance to cefuroxime, cefotaxime and ceftazidime. There is also a proportion each year (1.2-2.6%) of isolates which are non-susceptible to cefuroxime, but susceptible to cefotaxime and ceftazidime. Cefuroxime susceptibility is not always reported in global surveillance studies, but in a SENTRY report, of European *E. coli* isolates from 1997-98, 95% of the isolates were cefuroxime susceptible, while >98% were susceptible to ceftazidime and ceftriaxone (comparable to cefotaxime) [73].

**Fig 6**. Cephalosporin resistance in *E. coli* from blood cultures, Karolinska University Laboratory, Solna




For *K. pneumoniae*, the corresponding local cephalosporin resistance levels of blood culture isolates 2004-2007 are shown in Fig 7 (unpublished laboratory statistics). The material is smaller than for *E. coli*, but a trend of an increasing number of ESBL-producing isolates is observed (also supported by culture findings from other sample types, unpublished laboratory statistics). Also for *K. pneumoniae*, there are isolates which are cefuroxime non-susceptible but cefotaxime and ceftazidime susceptible.

**Fig 7**. Cephalosporin resistance in *K. pneumoniae* from blood cultures, Karolinska University Laboratory, Solna



### 1.4.3 Clinical impact of antibiotic resistance

Inadequate initial antimicrobial therapy for *E. coli* bacteraemia is associated with increased mortality [77]. Correct empirical therapy is therefore of great importance. This choice of empirical antibiotic for a bacterial infection is obviously complicated by the emergence of acquired antibiotic resistance. Infection with multidrug resistant ESBL producing isolates of *E. coli* or *Klebsiella* spp. is a risk factor for inadequate initial antimicrobial therapy [78]. Fluoroquinolone resistance has been shown as an independent risk factor for mortality in patients with health care acquired infections with *E. coli* and *K. pneumoniae* [79]. Another report (including patients with bacteraemia or pneumonia caused by Enterobacteriaceae) has not been able to show ESBL production in *E. coli* and *Klebsiella* spp. as an independent risk factor for treatment failure [80]. In a meta-analysis (based on 16 studies) of mortality and ESBL-bacteraemia, an association was found between ESBL production and mortality. Also found was an association between ESBL production and delay in effective therapy. However, only one study (of 16) reported relative risk controlled for confounding; making the results a bit hard to interpret [81].

## 1.5 CEFUROXIME RESISTANCE IN ISOLATES SUSCEPTIBLE TO EXTENDED-SPECTRUM CEPHALOSPORINS

Clinical isolates of *E. coli* and *K. pneumoniae* which are non-susceptible to cefuroxime, but susceptible to cefotaxime and ceftazidime occur (see above). The reason for this resistance pattern has not been entirely elucidated. Proposed mechanisms in *E. coli* have been efflux, hyperproduction of chromosomally encoded β-lactamases and changes in outer membrane permeability [28, 55, 56]. In *K. pneumoniae* OmpK35 deficiency has been associated with cefuroxime resistance [38]. Three of the four papers of this thesis (I, II and IV) focus primarily on examining resistance mechanisms possibly causing this resistance phenotype.

## 1.6 THE TSUNAMI CATASTROPHE

On 26th Dec 2004, the earthquake causing the tsunami (flood wave) in the Indian Ocean caused a catastrophe of great magnitude. Approximately 230000 people (from 55 nations) died. Around 227000 of them were from 14 countries bordering the Indian Ocean, of which 167000 were from Indonesia. Approximately 2000 tourists were killed, of which 543 were from Sweden (16 missing) (www.ne.se, Flodvågskatastrofen, Arne Järtelius). Many people were wounded and many Swedes sought medical advice in Sweden after returning from the catastrophe area.

Up to now, quite a few case reports have been published of different microbiological findings from tsunami victims. Uncommon fungi were reported e.g *Cladophialophora bantiana* in soft tissue infections (Sweden) [82], cutaneous mucormycosis (Australia) [83] and *Scedosporium apiospermum* as a cause of spondylodiscitis and intracerebral abscess (Switzerland) [84]. Further there were reports of nontuberculous mycobacteria, e.g. *Mycobacterium abscessus* causing soft tissue infections (Sweden) [82] and *Mycobacterium chelonae* causing wound infection (Switzerland) [84]. There were also several reports of wound infections due to various Gram-negative bacilli, e.g. from Australia [83], from Switzerland [84] and Germany [85]. Many species of Gram-negative bacilli were reported, e.g. *Acinetobacter* spp., *P. aeruginosa*, *Aeromonas* spp., *Achromobacter* spp., *E. coli*, *Enterobacter* spp, *Proteus* spp. and *Klebsiella* spp. [83-85]. ESBL-producing strains of *E. coli* and multidrug resistant *Acinetobacter* spp. were found [83-85]. Gram-negative bacilli were also reported from respiratory tract infections, e.g. from Germany [85], Thailand [86, 87], even as a case of "tsunami sinusitis" with culture findings from a maxillary sinuses of *Aeromonas* spp., *K. pneumoniae*, *E. coli* and *Proteus mirabilis* [88]. Reports came also of findings of *Burkholderia pseudomallei* (causing melioidosis) in respiratory tract infections in Thailand [86, 87] and Indonesia [89]. From Sweden a case of cutaneous melioidosis was reported [90]. There were fewer reports of Gram-positive bacteria, but *Nocardia* spp. in respiratory tract infection was reported from Indonesia [89] and in abscess from Switzerland [84]. Methicillin-resistant *Staphylococcus aureus* (MRSA) was also reported from wounds and respiratory tract [83, 85].

The Clinical Microbiology Laboratory of Karolinska University Laboratory (Huddinge and Solna) received many bacteriological culture samples (mostly wound cultures)

from patients wounded in the tsunami catastrophe. When analysing the wound cultures, it was soon clear that the microbiological flora was different from the one normally seen in wound cultures, with more (common and uncommon) Gram-negative bacilli and more resistant isolates (author's own observations). Therefore, a retrospective survey was performed of the findings and antibiotic resistance patterns of Gram-negative bacilli isolated from tsunami victims seeking medical advice in Stockholm. It was decided that the study would also be a part of this thesis, since it adds an epidemiological perspective of antibiotic resistance in Gram-negative bacilli, especially reflecting the situation in south-east Asia.

# 2 AIMS OF THE THESIS

The major aims of this thesis were:

- to examine possible mechanisms causing cefuroxime resistance in clinical non-ESBL isolates of *E. coli*, with focus on efflux, loss of porins and increased AmpC activity

- to examine possible mechanisms causing cefuroxime resistance in blood culture isolates of *K. pneumoniae*, resistant to cefuroxime, but susceptible to cefotaxime, with focus on efflux and loss of porins

- to examine the occurrence of resistance to other unrelated antibiotics in the same *K. pneumoniae* isolates and mechanisms behind that co-resistance

- to examine the occurrence and resistance patterns of Gram-negative bacilli in cultures from patients seeking medical advice in Stockholm after the tsunami catastrophe

# 3 MATERIALS AND METHODS

## 3.1 BACTERIAL STRAINS

### 3.1.1 Control strains

Control strains were *Escherichia coli* ATCC 25922 (papers I and II) and *Klebsiella pneumoniae* ATCC 25955 (paper IV).

### 3.1.2 Clinical isolates

Paper I: Thirty clinical isolates of *E. coli* with various degrees of cefuroxime susceptibility were examined. They were all isolated from blood cultures at the Department of Clinical Microbiology, Karolinska hospital, during 2001-2002.
Paper II: Fourteen clinical isolates of *E. coli* were examined. Thirteen of them were selected from the isolates used in paper I and the last isolate was derived from an abdominal secretion culture, isolated at Clinical Microbiology, Karolinska University Laboratory, Karolinska University Hospital, Solna in 2005.
Paper IV: All blood culture isolates of *K. pneumoniae* (at Clinical Microbiology, Karolinska University Laboratory, Karolinska University Hospital, Solna) during 2004-2006 which were cefuroxime non-susceptible but cefotaxime susceptible (n=10) were examined.

### 3.1.3 Multiply antibiotic resistant (MAR) laboratory strain

The multiply antibiotic resistant (MAR) laboratory strain was used in paper IV. It was derived by letting *K. pneumoniae* ATCC 25955 grow on Iso-Sensitest Agar (ISA) (Oxoid, Basingstoke, UK) containing chloramphenicol in increasing concentrations (3-4 cycles). A strain with chloramphenicol MIC >256 mg/L was selected. When grown on media not containing chloramphenicol, the strain quite easily reverted phenotypically to the original strain. It was therefore grown in media containing chloramphenicol 64 mg/L.

## 3.2 PHENOTYPIC METHODS

### 3.2.1 Antimicrobial susceptibility testing

The susceptibility of the examined strains to different antibiotics was determined with the disc-diffusion method (Oxoid, Basingstoke, UK) and/or with MIC determination using Etest (AB BIODISK, Solna, Sweden) according to SRGA (www.srga.org) [91]. ISA medium was used as recommended (www.srga.org).

### 3.2.2 ESBL-screening

For ESBL screening of the clinical isolates, the Oxoid combination disc range test was used in papers I and II. In paper IV the Etest ESBL for confirmation of extended-spectrum β-lactamase (cefotaxime/ cefotaxime+clavulanic acid and ceftazidime/ ceftazidime+clavulanic acid) (AB BIODISK) was used (www.srga.org).

### 3.2.3 Determination of cefuroxime susceptibility in the presence of clavulanic acid

In paper IV cefuroxime MICs were determined on ISA plates containing clavulanic acid (Sigma-Aldrich, St Louis, MO, USA) 2 mg/L, to detect the possible effect of hyperproduction of the chromosomal penicillinase SHV-1 on cefuroxime susceptibility. For each strain, the same inoculum was used for susceptibility testing without and with clavulanic acid.

### 3.2.4 Determination of antibiotic susceptibility in the presence of an efflux pump inhibitor

In papers I and IV MICs and/or inhibition zones were determined on ISA plates containing the efflux pump inhibitor, phenylalanine arginine β-naphthylamide (PAβN, also named MC-207,110) (Sigma-Aldrich) 20-40 mg/L [63, 68]. For each strain, the same inoculum was used for susceptibility testing without and with PAβN.

### 3.2.5 Determination of organic solvent tolerance

As an indicator of efflux, organic solvent tolerance (OST) was determined in paper I. The method is thoroughly described in paper I. Briefly, the ability of the bacterial strains to grow on LB Agar when overlaid with a layer of hexane (Sigma-Aldrich), cyclohexane (Sigma-Aldrich) or a mixture of hexane:cyclohexane 3:1 was determined. Strains which grew in the presence of pure cyclohexane or the mixture were classified

as organic solvent tolerant. Of these, strains which could also grow in the presence of pure cyclohexane were classified as cyclohexane tolerant.

### 3.2.6 Isoelectric focusing (IEF) for the detection of β-lactamases

Isoelectric focusing (IEF) was used in paper II. Analytical IEF of crude cell-free sonicates was performed according to Matthew *et al.* [92] on polyacrylamide gels (Ampholine PAGplate [pH 3.5 to 9.5]; Amersham Biosciences AB, Uppsala, Sweden) in a Multiphor II system (Pharmacia Biotech, Uppsala, Sweden). The pH gradient was measured with a surface pH electrode, and β-lactamase bands were detected by activity staining with nitrocefin. ATCC 25922 and three strains with defined β-lactamases were used as controls (NCTC 13351, NCTC 13352, NCTC 13353).

### 3.2.7 Spectrophotometric assay

The spectrophotometric assay was used to measure AmpC activity of crude cell lysates of the *E. coli* isolates in paper II. Cells from 20 mL overnight cultures were harvested by centrifugation, resuspended in 1 mL BugBuster (Novagen, Madison, WI, USA), and incubated at room temperature (r.t.) for 20 min. The cell lysates were obtained after centrifugation and stored on ice. Each cell lysate was diluted to a protein concentration of 0.5 mg/mL (resulting in a final concentration of 0.125 mg/mL in the assay) in 50 mM PIPES pH 7.0. AmpC activity of the crude cell lysate was determined spectrophotometrically at 486 nm using 100 μM nitrocefin (Oxoid) as substrate at 30°C by measuring the rate of hydrolysis (milliOD/min) in a SpectraMax (Molecular Devices, Sunnyvale, CA, USA). The hydrolytic activity of TEM-enzymes (present in some isolates) was inhibited by incubation of the crude cell lysate with clavulanic acid (SmithKline Beecham, Worthing, UK) to a final concentration of 5 mg/L for 15 min at r.t. before nitrocefin was added. Relative hydrolytic activity was calculated using ATCC 25922 as a wild type reference strain. Inhibition of AmpC using 3-aminophenylboronic acid (Sigma-Aldrich, Oslo, Norway) in a final concentration of 2.3 g/L was used to confirm that the increased hydrolytic activity was due to increased levels of AmpC. A clinical *E. coli* isolate producing TEM-1 (K27-41, TEM-1 verified with PCR and sequencing) was used as control for the inhibition of TEM-enzymes by clavulanic acid.

## 3.3 PCR AND SEQUENCING

### 3.3.1 Real-time RT-PCR for quantification of mRNA encoding various resistance genes

Real-time RT-PCR was used to analyse the mRNA (transcription) levels of the *E. coli* genes *acrA* and *ompF* in paper II and of the *K. pneumoniae* genes *acrA*, *ompK35*, *ramA*, *marA* and *soxS* in paper IV. As reference genes *rpoS* (paper II) and *rrsE* (paper IV) were used for normalising the transcription levels of target genes [64, 93]. This analysis was performed in three steps. The method has previously been described for *Pseudomonas aeruginosa* [94] and was used with some minor modifications.

1. Extraction of total RNA. One fresh colony of the bacterial strain was suspended in 10 mL LB broth (pH 7.5). Cells were grown over night at 37º C. The next day 0.5 mL was transferred to 9.5 mL fresh LB broth and incubated at 37º C for 60 min. OD600 was measured and 1-3 mL was used (depending on the OD value) for the extraction. Total RNA was extracted using an RNA extraction kit (High Pure RNA Isolation Kit, Roche, Mannheim, Germany) and stored in -69º C. The RNA concentration was measured (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, Delaware, USA) and the samples were diluted to a final concentration of 10 ng/µL.
2. cDNA synthesis. cDNA synthesis from the extracted RNA was performed using the 1$^{st}$ strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). Random primers were used to transcribe RNA into single-stranded cDNA. 20 ng (2 µL) of the RNA preparation was used for each reaction.
3. Real-time PCR for detection of cDNA-levels. With the Rotor-Gene 3000 real-time PCR instrument (Corbett research, Morlake, Australia) using SYBR Green (Qiagen, Hilden, Germany) for DNA detection and specific primers (Table 3, Cybergene, Huddinge, Sweden) for the genes examined, real-time PCR was performed to quantify cDNA. Each reaction contained 10 µL 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 6.8 µL RNase-free water (Qiagen), 1.2 µL primer mix (10 µM) and 2 µL of the cDNA preparation as template. The cycling programme comprised 15 min at 95º, followed by 40 cycles, 20 s each per cycle of 94-95º, 52º and 72º, followed by a hold for 30 s at 65º. The specificity of the generated product was then tested with melting-point analysis. A certain threshold value of fluorescence was set, and the number of cycles (CT

value) when reaching the threshold value was detected for each product. Every CT value of a target gene was directly compared to the CT value of the reference gene, rendering the normalised expression (transcription) value (NE value). The NE values were calculated with the Q-gene software [94, 95]. Amplification was performed in triplicate from three different cDNA preparations and the reference gene was always included as an internal control and to get the NE values for the further analyses. The mean of the normalised expression (transcription) values (MNE values) was calculated for each gene for each strain.

### 3.3.2 DNA sequencing

DNA sequencing was used to study possible mutations in the *acrR* regulatory gene in *K. pneumoniae* in paper IV. The gene was amplified using a forward primer located upstream of *acrR* and a reverse primer at the end of the gene (Table 3). DNA was extracted by boiling two colonies in 100 µL sterile water for two minutes. The lysate was then centrifuged 2 min at 13000 rpm and the supernatant used as DNA template. Amplification reactions contained 4 µL DNA template and 46 µL master mix. The master mix contained 4.6 µL PCR gold buffer (10x 10 mM Tris-HCl, 50 mM KCl), (Applied Biosystems, Stockholm, Sweden), 4.6 µL $MgCl_2$ (25 mM), (Applied Biosystems), 0.92 µL dNTPs (10 mM), (Amersham Biosciences, Uppsala, Sweden), 2.3 µL of each primer (10 µM), (Cybergene, Huddinge, Sweden), 0.23 µL of Ampli*Taq*Gold (5 U/µL), (Applied Biosystems) and 31 µL sterile filtered water. The cycling program comprised 10 min at 95ºC, followed by 30 cycles of 45 s at 94ºC, 45 s at 52ºC and 2 min at 72ºC, with a final extension for 10 min at 72ºC. PCR templates were purified with the Jetquick Spin Column Kit (Genomed; Saveen Werner AB, Malmö, Sweden) and diluted 1:2. DNA sequencing of PCR products was performed with the dideoxy chain-termination method [96]. The amplification primers were also used for the sequence reactions along with an additional internal forward primer (Table 3). Sequence reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing kit v.3.1 (Applied Biosystems) according to the manufacturer's instructions. Sequence analysis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) using Seqscape software v.2.5.0 (Applied Biosystems) for mutation profiling.

**Table 3.** Primers used for RT-PCR and sequencing

| Gene | Prod. size | Forward primer 5´-3´ (position in gene) | Reverse primer 5´-3´ (position in gene) | Use, paper | Source |
|---|---|---|---|---|---|
| *rpoS* | 288 | TGTCCAGCAA CGCTTTTTC (285-303)[a] | GCTTCTCAACA TACGCAACC (572-553)[a] | RT-PCR, II | This study |
| *acrA* | 265 | TTCGACTTACC AATGCGAC (632-650)[a] | CCTATCAGGC GACATACGAC (896-877)[a] | RT-PCR, II | This study |
| *ompF* | 134 | TGCGCAACTA ACAGAACGTC (239-258)[a] | CTGAAGTACG ACGCGAACAA (372-353)[a] | RT-PCR, II | This study |
| *rrsE* | 71 | TTGACGTTACC CGCAGAAGAA | GCTTGCACCCT CCGTATTACC | RT-PCR, IV | [64] |
| *acrA* | 101 | ATGTGACGAT AAACCGGCTC (72-91)[b] | CTGGCAGTTCG GTGGTTATT (172-153)[b] | RT-PCR, IV | This study |
| *ompK 35* | 81 | GAAGGTTCCC AGACCACAAA (265-284)[c] | ACGGCCATAG TCGAATGAAC (345-326)[c] | RT-PCR, IV | This study |
| *ramA* | 66 | GCATCAACCG CTGCGTATT | CGTTGCAGAT GCCATTTCG | RT-PCR, IV | [64] |
| *marA* | 84 | GAATGGCCGG TCTCTTTCTT (218-237)[d] | CCTGTCGCTGG AAAAAGTGT (301-282)[d] | RT-PCR, IV | This study |
| *soxS* | 101 | GCAGGCGGCG CTGGCGAATA (153-172)[d] | AGTCGCCAGA AAGTCAGGAT (253-234)[d] | RT-PCR, IV | This study |
| *acrR* | 673 | CGTAACCTCTG TAAAGTCAT (-26-(-7))[d] | GCTGACAAGC TCTCCGGGC (647-629)[d] | Amplification and sequencing, IV | This study |
| *acrR* | | AAGTGTGAGT TCGTCGGTGA (346-365)[d] | | Sequencing, IV | This study |

[a]Strain *E. coli* K12 MG1655 (GenBank accession no. U00096)
[b]Strain *K. pneumoniae* K23 (AJ318073)
[c]Strain *K. pneumoniae* 103624 (AJ303057)
[d]Strain *K. pneumoniae subsp. pneumoniae* MGH 78578 (NC_009648, CP000647)

## 3.4 EPIDEMIOLOGICAL TYPING

PhenePlate (PhP) typing was used in paper IV in order to confirm that the clinical isolates did not represent one clone of *K. pneumoniae* with the same antibiotic susceptibility pattern. A semi-automated system for biochemical fingerprinting (the PhenePlate or PhP system) was used for the ten clinical isolates (PhPlate AB, Stockholm Sweden). PhP-KL plates were used as recommended by the manufacturer (www.phplate.se). The method was used as previously described, except that the 23 most discriminatory reactions (of the 32 previously analysed) were included in the assay [97]. The PhP data for the isolates were compared pair wise and the correlation coefficient ($r$) between each pair of isolates was calculated. Isolates with a correlation coefficient above the identity level of $r \geq 0.97$ were considered to belong to the same PhP type, and hence to be potentially clonally related [97].

## 3.5 RETROSPECTIVE SURVEY OF LABORATORY DATA CONCERNING BACTERIOLOGICAL CULTURE FINDINGS AND RESISTANCE DATA

In paper I a survey of cephalosporin resistance data in blood culture isolates of *E. coli* from 1987, 1995 and 2002 was performed (analysed at the Department of Clinical Microbiology, Karolinska Laboratory, Karolinska hospital, Stockholm). The correlations between zone diameters of cefuroxime vs. cefotaxime, cefuroxime vs. ceftazidime and cefotaxime vs. ceftazidime were calculated. Exact analyses performed are more thoroughly described in paper I.

In paper III a survey was performed of clinical bacteriological culture findings from patients seeking medical advice after the tsunami catastrophe 26$^{th}$ Dec 2004 and from whom at least one bacteriological culture was taken from secretion, urine and/or lower respiratory tract (n=229) and received for analysis between 27 Dec 2004 and 28 Feb 2005 at Clinical Microbiology, Karolinska University Laboratory, Karolinska University Hospital, Huddinge or Solna, Sweden. Two major analyses of the material were performed. First, the findings of different species of Gram-negative bacilli in secretion cultures were analysed. Secondly, the antibiotic resistance pattern of five of the most commonly occurring and clinically important Gram-negative bacterial species was analysed (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *Acinetobacter* spp and *P. aeruginosa*). For the resistance analysis the whole material was included, i.e. findings

of cultures from secretion, urine and lower respiratory tract. Only one isolate per patient was included of each bacterial species. If different antibiograms were seen for different isolates of the same species in the same patient, the most resistant isolate was the one included. As a reference for the resistance analysis, the proportion of non-susceptible isolates (of the investigated antibiotics and species) in secretion cultures analysed at Clinical Microbiology, Karolinska University Laboratory, Solna from 1 Jan 2004 to 25 Dec 2004 was used.

## 3.6 STATISTICS

For paper I several non-parametric statistical tests were used. When the zone diameters for two antibiotics were correlated retrospectively the Spearman rank order correlation was calculated. The proportion of *E. coli* isolates resistant to cefuroxime, cefotaxime and ceftazidime (blood cultures from Karolinska Hospital, 2002) were compared with the $\chi^2$-test. When comparing the difference in cefuroxime MIC values between the organic solvent-tolerant isolates and the non-tolerant isolates the Mann-Whitney U-test was used. The same test was used for comparing the difference of zone diameters of different antibiotics between the two groups. To compare the antibiotic susceptibility measured with and without PAβN the Wilcoxon matched pairs test was used.

In papers II and IV the NE values for each strain was compared to the NE values of the ATCC strain using t-test for independent samples.

For all these calculations the Statistica software (StatSoft, Tulsa, OK, USA) was used.

# 4 RESULTS

## 4.1 PHENOTYPIC TESTS AND TRANSCRIPTION LEVELS OF RESISTANCE GENES

### 4.1.1 Paper I

Cefuroxime susceptibility (disc-diffusion and Etest), ceftazidime susceptibility (disc-diffusion), OST mean values and cefuroxime MICs in the presence of PAβN 20 mg/L are shown in Table 4. Also included in Table 4 are the corresponding names of the isolates when present in paper II.

Of the 30 clinical *E. coli* isolates, 14 were cefuroxime susceptible (MIC ≤8 mg/L). Four additional isolates would be interpreted as cefuroxime susceptible according to the disc-diffusion test. Three isolates were ceftazidime non-susceptible (disc-diffusion). All isolates were negative in the ESBL screening test. Eighteen of the isolates were classified as organic solvent tolerant (mean OST >2.5), seven of which were cyclohexane tolerant (mean OST >4.5).

The organic solvent tolerant isolates had significantly ($p<0.05$) increased cefuroxime MICs compared to the non-tolerant isolates (Fig 8). In the seven cyclohexane tolerant isolates, the cefuroxime MICs decreased significantly ($p<0.05$) in the presence of PAβN (Fig 9).

**Table 4**. Antibiotic susceptibility and OST (paper I)

| Strain no. | Strain no. paper II | CXM MIC S≤8, R>8 mg/L[a] | CXM MIC + PAβN 20 mg/L[b] | CXM 30 µg disc zone (mm)[c] | CAZ 30 µg disc zone (mm)[c] | OST[d] |
|---|---|---|---|---|---|---|
| ATCC 25922 | | 4 | 8 | 26 | 34 | 2 |
| 1 | | 8 | 8 | 20 | 35 | 1 |
| 2 | | 4 | 4 | 31 | 38 | 2 |
| 3 | | 2 | 4 | 29 | 37 | 2 |
| 4 | | 4 | 4 | 28 | 34 | 2 |
| 5 | *EC*-2 | 4 | 4 | 28 | 36 | 2 |
| 6 | | 4 | 8 | 26 | 35 | 2 |
| 7 | | 16[e] | 16 | 20 | 30 | 2 |
| 8 | | 32[e] | 32 | 17[e] | 32 | 2 |
| 9 | *EC*-9 | 32[e] | 64 | 15[e] | 30 | 2 |
| 10 | *EC*-3 | 4 | 8 | 27 | 36 | 2.3 |
| 11 | | 8 | 8 | 25 | 35 | 2.3 |
| 12 | | 4 | 4 | 28 | 35 | 2.5 |
| 13 | *EC*-1 | 4 | 4 | 29 | 36 | 2.7 |
| 14 | | 8 | 16 | 23 | 34 | 2.7 |
| 15 | | 4 | 4 | 30 | 35 | 2.8 |
| 16 | | 16[e] | 16 | 20 | 32 | 2.8 |
| 17 | | 4 | 4 | 27 | 35 | 3.3 |
| 18 | | 16[e] | 16 | 20 | 35 | 3.3 |
| 19 | | 8 | 8 | 23 | 23[e] | 4 |
| 20 | | 32[e] | 64 | 19 | 37 | 4 |
| 21 | *EC*-10 | 32[e] | 32 | 12[e] | 20[e] | 4 |
| 22 | *EC*-7 | 32[e] | 64 | 11[e] | 33 | 4 |
| 23 | *EC*-14 | >256[e] | >256 | 6[e] | 24[e] | 4.3 |
| 24 | *EC*-5 | 16[e] | 8 | 17[e] | 34 | 5 |
| 25 | *EC*-6 | 16[e] | 16 | 16[e] | 31 | 5 |
| 26 | | 32[e] | 16 | 16[e] | 32 | 5 |
| 27 | *EC*-8 | 32[e] | 16 | 15[e] | 34 | 5 |
| 28 | *EC*-12 | 64[e] | 32 | 11[e] | 31 | 5 |
| 29 | *EC*-11 | 64[e] | 32 | 11[e] | 32 | 5 |
| 30 | *EC*-13 | 64[e] | 32 | 8[e] | 28 | 5 |

CXM, cefuroxime; CAZ, ceftazidime; OST, organic solvent tolerance.

[a]Clinical breakpoints according to SRGA (www.srga.org) and EUCAST (www.eucast.org)

[b]CXM MIC determined in the presence of PAβN 20 mg/L

[c]Breakpoints at the time of paper I: for CXM: S≥19 mm,R≤15 mm, for CAZ: S≥27 mm, R≤23 mm

[d]A strain was considered organic solvent tolerant if the mean OST was >2.5 and also cyclohexane tolerant if the mean OST was >4.5

[e]Non-susceptible according to the used method and breakpoints

**Fig 8**. Cefuroxime MIC versus organic solvent tolerance

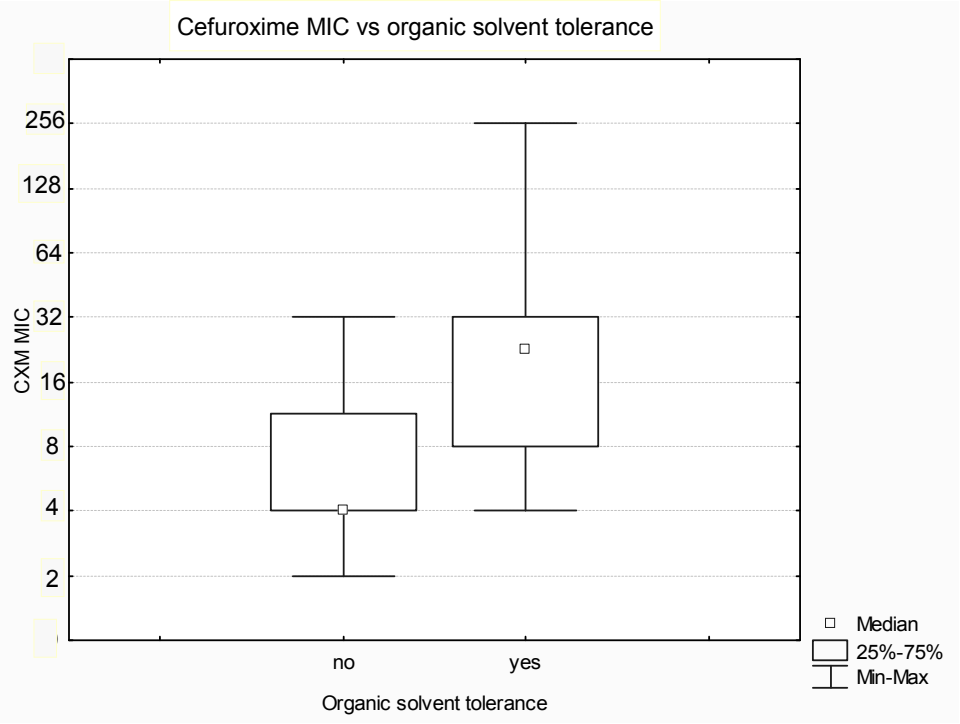
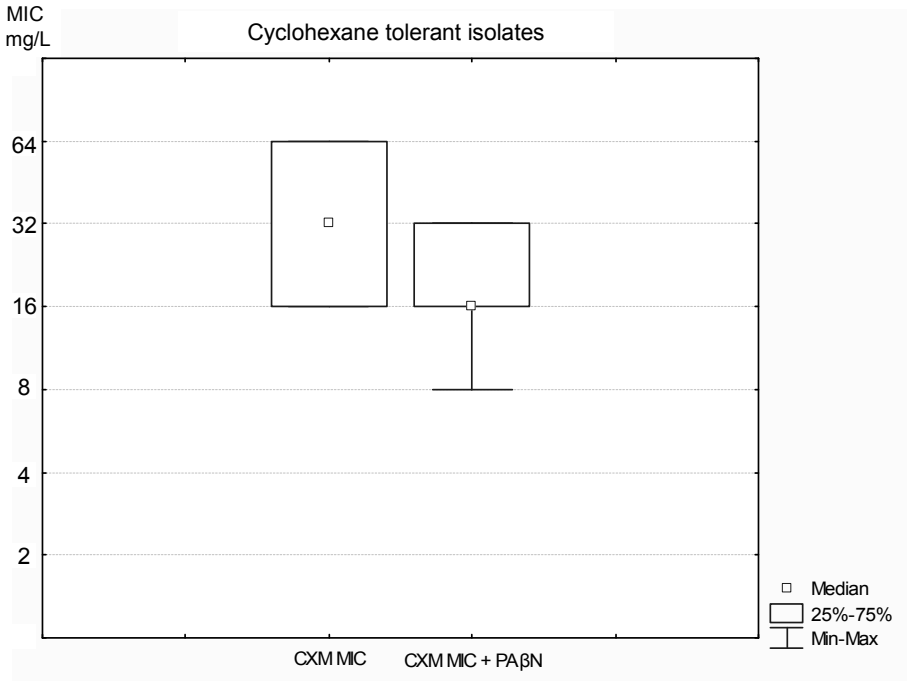



**Fig 9.** Cyclohexane tolerant isolates, cefuroxime MIC without and with PAβN 20 mg/L



### 4.1.2 Paper II

Antibiograms, relative transcription values of *acrA* and *ompF* (compared to the ATCC strain) and relative hydrolytic activity of AmpC (compared to the ATCC strain) are shown in Table 5. Three of the 14 examined clinical *E. coli* isolates were susceptible to all examined β-lactam antibiotics. The remaining eleven isolates were cefuroxime non-susceptible. Five of these were also ceftazidime non-susceptible, two of which were also cefotaxime non-susceptible. One isolate was imipenem non-susceptible. Ten isolates were cefoxitin resistant (defined in paper II as zone diameter ≤19 mm). All isolates were negative in the ESBL screening test.

Seven isolates had significantly increased *acrA* transcription, all of them cefuroxime resistant. All but one isolate had decreased *ompF* transcription, which could be confirmed statistically in seven of the isolates, two of which were cefuroxime susceptible. Three of the clinical isolates had very low *ompF* relative transcription levels (≤0.01). Two isolates (both cefuroxime resistant, *EC*-9 and *EC*-10) had increased AmpC activity (measured with the nitrocefin hydrolysis assay, reversible by boronic acid). The other isolates showed AmpC activity comparable to the ATCC strain. With IEF a β-lactamase with a high isoelectric point (pI >9), interpreted as AmpC, was detected in all clinical isolates and the ATCC strain (weak, moderate or strong bands). Also detected was a TEM-1 β-lactamase (pI 5.4) in four of the cefuroxime resistant isolates (*EC*-5, *E*C-10, *EC*-12 and *E*C-14) and in one of the susceptible isolates (*EC*-3).

**Table 5.** Antibiograms, relative transcription of *acrA* and *ompF* and nitrocefin hydrolytic activity (paper II)

| Strain no. | Minimum inhibitory concentrations, MICs mg/L[a] | | | | FOX 10 µg disc zone (mm)[b] | Relative transcription levels[c] | | Relative hydro-lytic activity + clav[d] |
|---|---|---|---|---|---|---|---|---|
| | CXM S≤8, R>8 | CTX S≤1, R>2 | CAZ S≤1, R>8 | IMI S≤2, R>8 | | *acrA* | *ompF* | |
| ATCC 25922 | 4 | 0.125 | 0.5 | 0.25 | 23 | 1 | 1 | 1 |
| *EC*-1 | 4 | 0.064 | 0.25 | 0.125 | 22 | 0.7 | 0.2[f] | 1.2 |
| *EC*-2 | 4 | 0.064 | 0.25 | 0.125 | 24 | 0.5 | 0.2 | 1.6 |
| *EC*-3 | 4 | 0.125 | 0.5 | 0.125 | 21 | 0.5 | 0.2[f] | 1.8 |
| *EC*-4 | 16 | 0.25 | 4 | 4 | 6 | 0.4 | 0.01[f] | 1.6 |
| *EC*-5 | 16 | 0.25 | 0.5 | 0.25 | 7 | 7.1[e] | 0.01[f] | 2.7 |
| *EC*-6 | 16 | 0.5 | 1 | 0.125 | 6 | 1.5 | <0.01[f] | 5.6 |
| *EC*-7 | 32 | 0.25 | 0.5 | 0.125 | 22 | 2.4[e] | 0.2 | 2.6 |
| *EC*-8 | 32 | 0.5 | 1 | 0.125 | 10 | 2.4[e] | 0.5 | 2.3 |
| *EC*-9 | 32 | 0.5 | 2 | 0.25 | 11 | 0.8 | 1.2 | 53[g] |
| *EC*-10 | 32 | 16 | 16 | 0.5 | 6 | 3.3[e] | 0.2[f] | 30[g] |
| *EC*-11 | 64 | 0.5 | 1 | 0.125 | 10 | 2.8[e] | 0.3[f] | 1.3 |
| *EC*-12 | 64 | 1 | 1 | 0.064 | 6 | 10[e] | 0.1 | 2.8 |
| *EC*-13 | 64 | 1 | 2 | 0.125 | 6 | 38[e] | 0.4 | 2.2 |
| *EC*-14 | >256 | 4 | 4 | 0.25 | 6 | 1.4 | 0.1 | 0.3 |

CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; IMI, imipenem; FOX, cefoxitin; clav, clavulanic acid 5 mg/L.

[a]Clinical breakpoints according to EUCAST (www.eucast.org)

[b]S ≥20 mm, R ≤19 mm

[c]Mean normalised expression (transcription) value (MNE value) for each strain / clinical isolate divided by the MNE value of the ATCC strain

[d]Nitrocefin hydrolysis value for each clinical isolate divided by the level of the ATCC strain (clavulanic acid was added to inhibit TEM-1 penicillinase)

[e]Statistically significantly increased *acrA* transcription levels compared to the ATCC strain ($p<0.05$)

[f]Statistically significantly decreased *ompF* transcription levels compared to the ATCC strain ($p<0.05$)

[g]Increased AmpC activity (reversible by addition of boronic acid)

### 4.1.3 Paper IV

Antibiograms for cephalosporins and chloramphenicol are shown in Table 6 and for other examined antibiotics in Table 7. The corresponding values when PAβN 40 mg/L was added to the medium are also shown (in parenthesis). All strains (the ten examined clinical isolates of *K. pneumoniae* and the MAR laboratory strain) showed similar antibiograms, with non-susceptibility to cefuroxime, chloramphenicol, tigecycline and nalidixic acid. They were all cefotaxime susceptible (which was part of the inclusion criteria), but cefotaxime MICs were increased compared to the wild type population. All clinical isolates were ESBL-negative. Clavulanic acid caused a two-fold decrease in cefuroxime MIC in the MAR laboratory strain, while the clinical isolates and the ATCC strain were unaffected (data not shown). This makes over-expression of SHV-1 as a cause of cefuroxime resistance unlikely. PAβN increased susceptibility significantly (≥ 4-fold MIC decrease or ≥ 5 mm increase in inhibition zone diameter) to chloramphenicol, nalidixic acid, tigecycline and trimethoprim-sulfamethoxazole in the MAR laboratory strain and in most clinical isolates, while the cephalosporin susceptibility was not affected significantly.

**Table 6.** MIC values of cephalosporins and chloramphenicol (in parenthesis ( ) when determined in the presence of PAβN 40 mg/L) (paper IV)

| | **Minimum inhibitory concentrations, MICs, mg/L[a]** | | | |
|---|---|---|---|---|
| | **CXM** | **CTX** | **CAZ** | **CHL** |
| **Strain no.** | **S≤8, R>8** | **S≤1, R>2** | **S≤1, R>8** | **S≤8, R>8** |
| *K. pneumoniae* ATCC 25955 | 2 (4) | 0.064 (0.125) | 0.25 (0.5) | 2 (1) |
| *K. pneumoniae* MAR laboratory strain 1 | 64 (64) | 1 (2) | 2 (2) | >256 (8) |
| *KP*-2 | 128 (256) | 0.5 (0.5) | 2 (2) | 32 (2) |
| *KP*-3 | 32 (32) | 1 (1) | 4 (1) | 128 (2) |
| *KP*-4 | 16 (16) | 0.5 (0.5) | 1 (1) | >256 (4) |
| *KP*-5 | 32 (32) | 0.5 (1) | 2 (2) | 64 (4) |
| *KP*-6 | 16 (32) | 0.5 (1) | 2 (2) | 64 (4) |
| *KP*-7 | 64 (128) | 0.5 (0.5) | 0.5 (0.5) | >256 (4) |
| *KP*-8 | 32 (64) | 1 (1) | 2 (2) | 64 (4) |
| *KP*-9 | 16 (32) | 0.5 (1) | 1 (1) | 64 (2) |
| *KP*-10 | 32 (64) | 0.5 (1) | 2 (1) | >256 (8) |
| *KP*-11 | 32 (32) | 1 (1) | 2 (1) | 64 (2) |

CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; CHL, chloramphenicol.
[a]Clinical breakpoints for MICs and inhibition zones according to EUCAST (www.eucast.org) and SRGA (www.srga.org)

**Table 7.** MIC values and inhibition zones of other antibiotics (in parenthesis ( ) when determined in the presence of PAβN 40 mg/L) (paper IV)

| | **Minimum inhibitory concentrations, MICs, mg/L** | | | | **NAL 30 µg disc** |
|---|---|---|---|---|---|
| **Strain no.** | **TGC S≤1, R>2[a]** | **TET[b] S≤2, R>2** | **CIP S≤0.5, R>1** | **SXT[c] S≤16, R>32** | **S≥20 mm, R≤19 mm** |
| *K. pneumoniae* ATCC 25955 | 0.25 (0.5) | 2 (4) | 0.064 (0.125) | 5 (2.5) | 30 (33) |
| *K. pneumoniae* MAR laboratory strain 1 | 8 (2) | 32 (32) | 0.125 (0.125) | 40 (5) | 12 (31) |
| *KP*-2 | 4 (0.5) | 8 (16) | 0.25 (0.125) | 10 (2.5) | 16 (35) |
| *KP*-3 | 16 (1) | 32 (16) | 0.5 (0.125) | 20 (2.5) | 12 (36) |
| *KP*-4 | 4 (0.5) | 8 (4) | 1 (0.25) | 40 (2.5) | 6 (33) |
| *KP*-5 | 4 (2) | 16 (16) | 0.5 (0.25) | 5 (2.5) | 14 (32) |
| *KP*-6 | 4 (2) | 16 (32) | 0.5 (0.5) | 10 (2.5) | 15 (32) |
| *KP*-7 | 4 (1) | 8 (8) | 2 (1) | 20 (5) | 6 (17) |
| *KP*-8 | 8 (1) | 16 (8) | 0.25 (0.125) | 10 (2.5) | 14 (36) |
| *KP*-9 | 2 (1) | 8 (8) | 0.25 (0.125) | 20 (5) | 17 (36) |
| *KP*-10 | 8 (2) | 32 (32) | 0.5 (0.25) | 40 (5) | 6 (30) |
| *KP*-11 | 4 (0.5) | 16 (8) | 0.5 (0.125) | 10 (1.25) | 12 (36) |

TGC, tigecycline; TET, tetracycline; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; NAL, nalidixic acid.
[a]Clinical breakpoints for MICs and inhibition zones according to EUCAST (www.eucast.org) and SRGA (www.srga.org)
[b]Tetracycline: general breakpoints, no specific breakpoints for Enterobacteriaceae, not recommended antibiotic for *K. pneumoniae*
[c]MIC read from the strip multiplied by 20, according to the manufacturer

The relative transcription (compared to the ATCC strain) of the examined strains for the genes *acrA*, *ompK35*, *ramA*, *marA* and *soxS* are shown in Table 8. All strains had increased *acrA* transcription levels and decreased *ompK35* transcription levels. Further, all strains except one (*KP*-4) had increased *ramA* transcription levels. Four of the clinical isolates and the MAR laboratory strain had significantly increased transcription levels of *marA*. For *soxS* only the MAR laboratory strain had increased transcription levels. Four of the isolates (*KP*-5, *KP*-6, *KP*-7 and *KP*-10) had a slightly lower melting point (1.7-1.8º C) for *soxS* than the rest. The length of the *soxS* PCR products (determined with gel electrophoresis) was the same for all strains, ~100 bp, as expected (Table 3).

**Table 8.** Relative transcription of the genes *acrA*, *ompK35*, *ramA*, *marA* and *soxS*

| | Relative transcription levels[a] | | | | |
|---|---|---|---|---|---|
| **Strain no.** | ***acrA*** | ***ompK35*** | ***ramA*** | ***marA*** | ***soxS*** |
| *K. pneumoniae* ATCC 25955 | 1 | 1 | 1 | 1 | 1 |
| MAR laboratory strain 1 | 4.9[b] | 0.05 | 60[b] | 8.1[b] | 2.3[b] |
| *KP*-2 | 2.0[b] | 0.1[c] | 13[b] | 0.8 | 0.5 |
| *KP*-3 | 6.9[b] | 0.03[c] | 57[b] | 1.4 | 0.3 |
| *KP*-4 | 1.8[b] | 0.06[c] | 0.6 | 1.7[b] | 1.0 |
| *KP*-5 | 6.3[b] | <0.01[c] | 64[b] | 2.2[b] | 0.4 |
| *KP*-6 | 3.1[b] | <0.01[c] | 26[b] | 1.3 | 0.2 |
| *KP*-7 | 3.7[b] | <0.01[c] | 69[b] | 3.5[b] | 0.6 |
| *KP*-8 | 4.1[b] | 0.1[c] | 74[b] | 1.5 | 0.4 |
| *KP*-9 | 2.8[b] | 0.3[c] | 16[b] | 1.1 | 0.9 |
| *KP*-10 | 4.2[b] | <0.01[c] | 74[b] | 2.6[b] | 0.3 |
| *KP*-11 | 4.2[b] | 0.08[c] | 74[b] | 1.8 | 0.7 |

[a]Mean normalised expression (transcription) value (MNE value) for each strain / clinical isolate divided by the MNE value of the ATCC strain
[b]Statistically significantly increased transcription levels compared to the ATCC strain ($p<0.05$)
[c]Statistically significantly decreased transcription levels compared to the ATCC strain ($p<0.05$)

## 4.2 DNA SEQUENCING

In paper IV the *acrR* gene was sequenced in the examined *K. pneumoniae* strains. Except for some silent mutations, the sequence analysis revealed one single amino acid change in one of the isolates, *KP*-3, in position Glu190Lys. The sequences were submitted to GenBank and the accession numbers are displayed in Table 2 of paper IV.

## 4.3 EPIDEMIOLOGICAL TYPING

The PhP typing of the clinical isolates of *K. pneumoniae* in paper IV revealed that two of the clinical isolates; *KP*-7 and *KP*-10 were found to have the same PhP-type ($r>0.97$). All the other isolates belonged to different PhP types.

## 4.4 RETROSPECTIVE SURVEY

### 4.4.1 Paper I

Correlations between zone diameters of cefuroxime vs. cefotaxime, cefuroxime vs. ceftazidime and cefotaxime vs. ceftazidime were compared for blood culture findings of *E. coli* from 1987, 1995 and 2002. The correlation between cefotaxime and

ceftazidime zone diameters was higher than the correlation of cefuroxime zone diameters with any of these agents. Also, the proportion of isolates that was non-susceptible to cefuroxime was larger than the proportion of cefotaxime non-susceptible isolates (2002).

### 4.4.2 Paper III

The findings of Gram-negative bacilli in secretion cultures from tsunami victims are shown in Table 9. From 209 patients at least one culture of secretion (mostly wound secretion cultures) was taken. From 98 of these 209 patients, Gram-negative bacilli were isolated. Both common human pathogens (e.g. *P. aeruginosa*, *E. coli* and *Klebsiella* spp.) and for wounds rather uncommon species (e.g. *Shewanella putrefaciens*, *Vibrio* spp., *Sphingomonas paucimobilis*, *Myroides odoratus* and *Bergeyella zoohelcum*) were isolated.

**Table 9.** Number of patients with findings of the following Gram-negative bacilli in cultures of secretion (paper III)

| **Bacterial species** | **Number of patients** |
|---|---|
| *Pseudomonas aeruginosa* | 46 |
| *Proteus* spp. | 34 |
| *Escherichia coli* | 24 |
| *Klebsiella* spp. | 21 |
| *Aeromonas* spp. | 18 |
| *Enterobacter* spp. | 16 |
| *Anaerobic Gram-negative bacilli* | 14 |
| *Acinetobacter* spp. | 11 |
| *Citrobacter* spp. | 8 |
| *Alcaligenes* spp. | 7 |
| *Serratia marcescens* | 7 |
| *Stenotrophomonas maltophilia* | 7 |
| *Morganella morganii* | 6 |
| *Shewanella putrefaciens* | 5 |
| *Providencia* spp. | 4 |
| *Vibrio* spp. | 3 |
| *Burkholderia cepacia* | 2 |
| *Sphingomonas paucimobilis* | 2 |
| *Chryseobacterium meningosepticum* | 2 |

Isolated from one patient each were the following species: *Pseudomonas* spp., *Bordetella trematum, Pantoea agglomerans, Myroides odoratus, Bergeyella zoohelcum and Haemophilus influenzae*

The resistance data was analysed for the whole material (secretion cultures, urine cultures and lower respiratory tract cultures, 229 patients) and is shown in Table 10. The proportion of resistant isolates was higher from the tsunami patients´ material, than for the reference material for *E. coli*, *K. pneumoniae*, *P. mirabilis* and *Acinetobacter* spp., but not for *P. aeruginosa*. The ceftazidime resistant isolates of *E. coli* and *K. pneumoniae* (in total eight isolates) were all ESBL-producers. From two patients multi-resistant isolates of *Acinetobacter* spp. were isolated (resistant to gentamicin, ciprofloxacin, trimethoprim-sulfamethoxazole and imipenem).

**Table 10.** The proportion of tsunami patients with non-susceptible resistance patterns (R/I) for chosen antibiotics for the clinically most relevant species, compared to (in parenthesis) the proportion of isolates with resistance patterns R/I from the ref. material. For the reference material, there were between 152 and 851 isolates of each species.

| **Species** | **Number of patients[a]** | **CAZ R/I (%)** | **IMI R/I (%)** | **GEN R/I (%)** | **TSU R/I (%)** | **CIP R/I (%)** |
|---|---|---|---|---|---|---|
| *E. coli* | 26-30 | 23 (4) | 0 (0) | 22 (3) | 62 (17) | 50 (8) |
| *K. pneumoniae* | 17 | 6 (1) | 0 (0) | 18 (0) | 29 (5) | 35 (5) |
| *P. mirabilis* | 20-26 | 5 (0) | 0 (0)[b] | 8 (1) | 35 (12) | 23 (6) |
| *Acinetobacter* spp. | 10-11 | [c] | 20 (3) | 45 (5) | 45 (5) | 45 (10) |
| *P. aeruginosa* | 47 | 6 (4) | 4 (14) | 6 (2) | [c] | 9 (13) |

CAZ, ceftazidime; IMI, imipenem; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin.
[a]the susceptibility for each antibiotic has not been tested for all isolates, therefore there is a varying denominator for each antibiotic.
[b]*P. mirabilis* is now considered a poor target for imipenem, and there are no current clinical breakpoints (www.eucast.org)
[c]not recommended antibiotic for that species

# 5 DISCUSSION

## 5.1 CEFUROXIME RESISTANCE IN *E. COLI* (PAPERS I AND II)

The principal purpose of papers I and II was to examine resistance mechanisms behind cefuroxime resistance in clinical, non-ESBL isolates of *E. coli*, which are susceptible to cefotaxime and ceftazidime or (in some isolates) have moderately decreased susceptibility to these agents. Therefore non-ESBL clinical isolates of *E. coli* with various degrees of decreased susceptibility to cefuroxime were selected and as a control material a number of susceptible isolates were selected.

Previous studies have proposed efflux, hyperproduction of chromosomally encoded β-lactamases and changes in outer membrane permeability as potential mechanisms of cefuroxime resistance in *E. coli* [28, 55, 56]. A pilot study of clinical isolates of *E. coli* performed in 2001 (Källman O *et al*, unpublished data) supported the hypothesis of efflux as a cefuroxime resistance mechanism. In paper I the possibility of efflux as a cefuroxime resistance mechanism was examined with phenotypic methods. The organic solvent tolerant isolates showed decreased cefuroxime susceptibility compared to the non-tolerant isolates. The cyclohexane tolerant isolates showed increased cefuroxime susceptibility when the efflux pump inhibitor PAβN was added to the medium. Both these findings support efflux as a cause of cefuroxime resistance. To look further into the exact mechanisms paper II was performed.

In paper II a smaller number of isolates were selected for further analyses. Transcription levels of *acrA* and *ompF* were determined, as was AmpC activity. Other β-lactamases were looked for with IEF. The results of paper II further confirm efflux due to the AcrAB-TolC efflux pump system as a cause of cefuroxime resistance, with seven of eleven cefuroxime resistant isolates having increased *acrA* transcription. A possible explanation of cefuroxime being more prone to efflux by the AcrAB-TolC efflux pump could be that cefuroxime has higher side-chain lipophilicity than cefotaxime [55, 98].

However, efflux did not seem to be the only mechanism. Seven isolates had significantly decreased *ompF* transcription, two of which were cefuroxime susceptible,

making the contribution of decreased *ompF* transcription a bit hard to interpret. Three isolates (all cefuroxime resistant) had very low *ompF* transcription (≤0.01 times the ATCC strain) and in those isolates lack of OmpF may have contributed to the cefuroxime resistance. Expression of *ompF* also depends on post-transcriptional modification, by the sRNA MicF. Effects of this might not be detected when transcription levels are analysed [31-33].

Increased AmpC activity (measured with spectrophotometry) was seen in two isolates (*EC*-9 and *EC*-10) in paper II. The increased AmpC activity observed in the two isolates is likely to have contributed to cefuroxime resistance and also to the decreased susceptibility to ceftazidime (observed in both isolates) and cefotaxime (observed in one of the isolates). Both *EC*-9 and *EC*-10 were cefoxitin resistant (Table 5), further supporting increased AmpC activity [99]. However, eight more isolates (all of them cefuroxime resistant) were cefoxitin resistant. The reason for cefoxitin resistance in these isolates is unknown. Loss of porins and efflux have been other proposed mechanisms of cefoxitin resistance [26, 55, 100].

In *P. aeruginosa*, which is intrinsically resistant to cefuroxime, a mutant deficient in AmpC and MexAB-OprM (an important efflux pump complex in *P. aeruginosa*) was susceptible to cefuroxime [101]. No conclusions can be drawn from this concerning other bacteria with different AmpC-enzymes and different efflux pumps. However, the observation that AmpC and efflux seem to be of importance for intrinsic cefuroxime resistance in *P. aeruginosa* is an interesting finding, which can be compared with the current results for *E. coli* (paper II).

One isolate (paper II, *EC*-4) was also imipenem non-susceptible. Imipenem resistance is still uncommon in *E. coli*. The isolate had very low *ompF* transcription, which could partly explain the imipenem resistance. No β-lactamases were detected. Other resistance mechanisms might be present, not detected in the current study. Loss of porins (OmpC and OmpF) in combination with production of plasmid-mediated β-lactamases (CMY-2) has been suggested as probable mechanisms of imipenem resistance in *E. coli* [102]. Metallo-β-lactamases have also been reported in *E. coli* [22].

In conclusion, current findings suggest that both efflux due to the AcrAB-TolC system, down-regulation of OmpF and increased AmpC activity all contribute to cefuroxime

resistance in clinical non-ESBL isolates of *E. coli*. However, in one of the cefuroxime resistant strains (*EC*-14), none of these resistance mechanisms was found. Other potential mechanisms of resistance remain to be examined, e.g. down-regulation of other porins (e.g. OmpC) and efflux due to other efflux pump systems.

## 5.2 CEFUROXIME RESISTANCE AND ASSOCIATED RESISTANCE TO OTHER ANTIBIOTICS IN *K. PNEUMONIAE* (PAPER IV)

Paper IV was performed to examine if efflux due to the AcrAB efflux pump and down-regulation of OmpK35 (a homologue of OmpF) contribute to cefuroxime resistance in clinical blood culture non-ESBL isolates of *K. pneumoniae*. Therefore blood culture isolates which were cefuroxime resistant but cefotaxime susceptible were selected. A laboratory strain, selected by chloramphenicol, was also examined. Since efflux can cause decreased susceptibility to chloramphenicol, nalidixic acid and tigecycline [63, 64], susceptibility to these agents were also examined.

The reason for cefuroxime resistance in non-ESBL isolates of *K. pneumoniae* has not been fully elucidated. Loss of OmpK35 has been one proposed cause [38]. In a study of cefuroxime resistance in clinical isolates of *K. pneumoniae*, some alterations of OMP patterns were seen in some isolates, though of unclear relevance [103]. Strains of *K. pneumoniae* with cross-resistance to nalidixic acid and chloramphenicol (in the first and third reports also to trimethoprim) have been described [64, 104, 105]. However, in those studies, cefuroxime susceptibility was not reported.

In paper IV, ten clinical blood culture isolates were selected because they were cefuroxime non-susceptible but cefotaxime susceptible. Interestingly, they all had similar antibiograms, with decreased susceptibility to unrelated antibiotics as chloramphenicol, tigecycline and nalidixic acid. Although they were cefotaxime susceptible, they showed decreased susceptibility to both cefotaxime and ceftazidime compared to the wild type population (www.eucast.org). Only two of the clinical isolates belonged to the same PhP type and could therefore possibly be part of the same clone. However, more than two years passed between the isolation of the two isolates, and they were isolated from patients seeking medical care at two different hospitals, which makes clonal spread unlikely. Also, the MAR laboratory strain, selected by chloramphenicol, showed a similar antibiotic susceptibility pattern. However, the

laboratory strain was not stable, suggesting that it was phenotypically adapted to high antibiotic concentrations.

Similar resistance mechanisms were seen in all strains, with increased *acrA* transcription and decreased *ompK35* transcription in all strains. There was also increased transcription of the regulatory gene *ramA* in the laboratory strain and all but one of the clinical isolates. Transcription levels of *marA* were increased in four of the clinical isolates and in the laboratory strain and *soxS* levels were increased only in the laboratory strain. Four of the clinical isolates had a slightly different melting point for *soxS*, probably reflecting the presence of mutations (of uncertain relevance). Thus, *ramA* seems to be the most important regulatory gene involved in the MDR phenotype of the examined strains, though *marA* and *soxS* may be of importance in the laboratory strain. This is also in agreement with earlier findings, where over-expression of *ramA* (but not of *marA* and *soxS*) was shown to be related to increased *acrAB* expression in clinical isolates of *K. pneumoniae* [66].

Only one mutation leading to an amino acid change was seen in the *acrR* gene. This amino acid change has to our knowledge not been described before and has an uncertain relevance. It should however be noted that the shift of an acidic to a basic residue could change the configuration of this regulatory protein. The previously described *acrR* mutations associated with increased expression of *acrA* was not found in any of the examined strains [66]. In that paper also seven point mutations in the *acrR* gene were described in seven of the examined strains, compared to the reference sequence (from *K. pneumoniae* K23 (AJ318073)), leading to amino acid shifts of uncertain relevance. However, one of these strains was a susceptible wild type isolate and another strain was also ciprofloxacin susceptible. The authors concluded that these changes represented genetic variation and were not linked to the OST phenotype. Current data confirms this, as another *acrR* sequence was used as reference in paper IV, from strain *K. pneumoniae subsp. pneumoniae* MGH 78578 (NC_009648), which has the described mutations compared to *K. pneumoniae* K23. MGH 78578 is indeed multiply antibiotic resistant [106]. However, the fully susceptible reference strain *K. pneumoniae* ATCC 25955, used in paper IV, has an identical *acrR* sequence (EU411049) as MGH 78578, further confirming that the differences compared to the reference strain earlier used (*K. pneumoniae* K23 (AJ318073)) [66] are not of importance for the multidrug resistance observed.

The PAβN assay results, with increased antibiotic susceptibility for the non-β-lactam-antibiotics chloramphenicol, tigecycline, nalidixic acid and trimethoprim-sulfamethoxazole, suggest that efflux due to increased expression of *acrAB* (or possibly other efflux pumps) was responsible for the decreased susceptibility to these antibiotics, while the reduction of *ompK35* expression probably was the most important factor for the decreased susceptibility to cephalosporins. In a Danish study of antibiotic cross-resistance in *K. pneumoniae*, 91% of isolates with decreased susceptibility to cefuroxime were also chloramphenicol non-susceptible, compared to 7% of the cefuroxime susceptible isolates [107]. This provides further support to the assumption of a common resistance mechanism for these chemically unrelated antimicrobial agents.

Tigecycline is a quite new broad-spectrum glycylcycline used for treatment of severe infections, particularly those caused by multi-resistant Enterobacteriaceae. Most species in this family are naturally susceptible to tigecycline, with the important exception Proteae [108]. In *Proteus mirabilis* the AcrAB system has been shown to be associated with this intrinsic tigecycline resistance [109]. There have also been reports on tigecycline non-susceptible isolates of *K. pneumoniae* [64, 110]. Of ten selected clinical blood culture isolates of *K. pneumoniae*, selected only because of the combination of cefuroxime non-susceptibility and cefotaxime susceptibility, all were tigecycline non-susceptible. Further, current data support earlier findings that efflux due to increased *acrA* expression (secondary to increased *ramA* expression) contributes to tigecycline resistance in *K. pneumoniae* [64].

In conclusion a multiply antibiotic resistant phenotype of *K. pneumoniae* was found in multiple blood culture isolates, with decreased susceptibility to unrelated antibiotics as cefuroxime, chloramphenicol, nalidixic acid and tigecycline. Resistance mechanisms contributing to this phenotype were increased expression of the regulatory gene *ramA*, leading to increased *acrA* expression and decreased *ompK35* expression. Cefuroxime susceptibility was not increased in the presence of PAβN, implying loss of OmpK35 as the probable cause of cefuroxime resistance, while increased efflux of the AcrAB pump explains the decreased susceptibility to chloramphenicol, nalidixic acid and tigecycline.

## 5.3 COMPARISON BETWEEN *E. COLI* AND *K. PNEUMONIAE*

*E. coli* and *K. pneumoniae* both belong to the Enterobacteriaceae family and have many genes in common. Common resistance mechanisms are observed, e.g. production of ESBLs. However, concerning cefuroxime resistance mechanisms, there are also some differences, obvious when the findings of papers I and II are compared with the findings of paper IV. For *E. coli* all examined resistance mechanisms (increased *acrA* transcription, decreased *ompF* transcription and increased AmpC activity, paper II) seemed to contribute to cefuroxime resistance in the examined isolates. However, the material was heterogeneous, with different resistance mechanisms observed in different isolates and also one isolate (*EC*-14) not displaying any of the examined mechanisms. The PAβN assay in paper I, in which the cyclohexane tolerant isolates showed increased cefuroxime susceptibility, can be argued against. It was only a one-step MIC decrease. However, the fact that there was a statistically significant change (though not big) led to the conclusion that efflux contributed to the cefuroxime resistance observed in the cyclohexane tolerant isolates.

For *K. pneumoniae*, the selected material turned out to be much more homogenous, with all clinical isolates displaying similar antibiograms and similar transcription levels of resistance genes (with some small exceptions). No $bla_{\text{AmpC}}$ gene is present in the *K. pneumoniae* genome [20], therefore increased production of chromosomal AmpC was not needed to be looked for. In the examined *K. pneumoniae* isolates PAβN did not affect cefuroxime susceptibility (or other cephalosporins) and therefore efflux did not seem to cause cefuroxime resistance (as it seems to do in *E. coli*), although cefuroxime resistance obviously was associated with increased *acrA* transcription levels. Instead, probably lack of OmpK35 (the homologue of OmpF) contributed to the cefuroxime resistance, in analogy with the findings of some of the *E. coli* isolates. Also, in *K. pneumoniae*, the gene *ramA* (not present in the *E. coli* genome) seems to be the most important of the examined regulatory genes in causing the observed phenotype.

Of course the selection criteria of the clinical isolates might have affected the results. In paper I, non-ESBL *E. coli* blood culture isolates with various degrees of cefuroxime susceptibility were selected. When designing the experiments of paper II, isolates were selected from paper I, but also included was an imipenem non-susceptible isolate from a secretion culture. When analysing which cefuroxime resistant isolates that were

selected from paper I to be part also in paper II (Table 4), it is clear that there was a certain overrepresentation of the cyclohexane tolerant isolates, which were more likely to display increased *acrA* transcription. If a more representative isolate collection had been selected, it is possible that there would have been more isolates with other resistance mechanisms than efflux, e.g. increased AmpC activity. Therefore, no conclusion can be drawn from paper II about the frequency of AcrAB-TolC efflux as a cause of cefuroxime resistance in non-ESBL isolates of *E. coli*, only that it seems to contribute in some isolates. In paper IV, the selection criteria were more defined; all blood culture isolates 2004-2006 of *K. pneumoniae*, which were cefuroxime resistant but cefotaxime susceptible, were selected. The only susceptible strain used as a reference was ATCC 25955. These more distinct selection criteria might partly explain why the *K. pneumoniae* isolates were more homogeneous than the *E. coli* isolates, but it is anyway surprising that they had almost identical antibiograms and very similar transcription levels for the examined resistance genes. In fact, they were so similar, that an epidemiological typing was performed, to make sure they were not part of a clonal spread. The observation of this MDR phenotype in clinical isolates of *K. pneumoniae*, with antibiotic resistance to the unrelated antibiotics cefuroxime, tigecycline, chloramphenicol and nalidixic acid, is indeed one of the most important findings of this thesis.

## 5.4 CEFUROXIME USE FOR THE TREATMENT OF INFECTIONS CAUSED BY *E. COLI* AND *K. PNEUMONIAE*

Cefuroxime was the most common antibiotic for parenteral use in Sweden 2007 (www.apoteket.se). It is used mainly for severe respiratory tract infections, upper urinary tract infections and septicaemia of unknown origin. It is so common, that it is called “the wine of the house” in many Swedish hospitals. For Enterobacteriaceae infections, it is in Sweden currently only recommended for infections originating from the urinary tract caused by *E. coli*, *Klebsiella* spp. or *P. mirabilis*. The Swedish Reference Group for Antibiotics (SRGA) has now recommended that the use of cefuroxime should be minimized, for several reasons (www.srga.org). The main reason is to avoid a routine use of a cephalosporin for different severe infections, as cephalosporin use might contribute to the selection of ESBL producing isolates, an emerging problem in Sweden at the moment [111]. MICs of cefotaxime and ceftazidime are lower than for cefuroxime for the wild type population of *E. coli* and *K.*

*pneumoniae* (Fig 2, 3 and 4, www.eucast.org). Apart from the lower activity of cefuroxime as compared to third generation cephalosporins against wild type *E. coli* and *K. pneumoniae*, levels of acquired resistance to cefuroxime are also higher in these species. In this thesis we have demonstrated that isolated resistance to cefuroxime is mainly due to chromosomal resistance mechanisms that do not affect third generation cephalosporins as much. It is not known whether this type of resistance can be selected during cefuroxime therapy or whether it is associated with an adverse outcome. However, the association to antibiotic efflux makes it likely that not only cefuroxime therapy, but also other unrelated antibiotics that are substrates for the same efflux pumps may select for cefuroxime resistance. In light of these observations it seems prudent to follow the current recommendations from SRGA to avoid cefuroxime for empirical therapy when Gram-negative aetiology is suspected.

## 5.5 FINDINGS OF GRAM-NEGATIVE BACILLI FROM TSUNAMI VICTIMS (PAPER III)

The findings of paper III, with a high frequency of Gram-negative bacilli in secretion cultures (mostly wound cultures) from the tsunami victims, were probably explained by the way the wounds were contracted. They were traumatic wounds, contaminated by water, sand, soil and in many cases sewage contaminated water. Common water borne bacteria as *P. aeruginosa*, *Aeromonas* spp. and *Vibrio* spp. were isolated, as were common faecal bacteria as *E. coli*, *Klebsiella* spp. etc. The clinical relevance of findings of Gram-negative bacteria in these wounds was uncertain in several cases. Generally Gram-negative bacteria in superficial wounds are rather considered as colonising than infecting agents. However, many of the tsunami victims had deep wounds and sometimes open fractures, where findings of Gram-negative bacteria may be of clinical importance. The findings are in analogy with case reports after the tsunami catastrophe from other countries [83-85]. Also, reports from other kinds of natural catastrophes and wound infections after marine trauma have shown similar findings [112, 113]. Paper III is, to the author's knowledge, the largest survey of findings of Gram-negative bacilli in secretion cultures after the tsunami catastrophe; other publications have mostly been case reports.

The high proportion of resistant isolates of *E. coli*, *K. pneumoniae*, *P. mirabilis* and *Acinetobacter* spp. probably reflects the antibiotic resistance situation of South-East

Asia. Previous reports of antimicrobial resistance from Asia reports high resistance levels in *E. coli* to fluoroquinolones and in some countries also to third generation cephalosporins [114, 115]. Interestingly, in the resistance analysis in paper III, the proportion of *P. aeruginosa* isolates resistant to common antibiotics was not higher than for the Swedish reference material. The reason for the discrepancy between the resistance rates of *P. aeruginosa* and the other examined species is probably that many of the *P. aeruginosa* isolates were wild type isolates from the water and soil, while the resistant isolates of the other bacteria were probably from contamination of the flood water by sewage containing "human" isolates. There were also some multi-resistant isolates, e.g. of *Acinetobacter* spp. and ESBL-producing *E. coli* and *K. pneumoniae*. They may have been acquired nosocomially before transport to Sweden. However, this remains to be examined, in an analysis where clinical information and microbiological findings are connected.

In conclusion, infections with Gram-negative bacilli are common in patients with traumatic wounds contaminated by sea-water, soil and sewage. This should be taken into consideration in empirical antibiotic treatment of such patients, as should the antibiotic resistance epidemiology of the country in which the trauma took place. Of importance is also that correct infection control measures are undertaken when treating the patient, to avoid clonal spread of MDR isolates. Suggestions of recommendation for control measures in those kinds of situations have been made [116].

## 5.6 FUTURE PROJECTS

In this thesis the author has examined resistance mechanisms behind cefuroxime resistance in clinical non-ESBL isolates of *E. coli* and *K. pneumoniae*, and also resistance to other antibiotics associated with cefuroxime resistance in *K. pneumoniae* (papers I, II and IV). A survey of findings and resistance patterns of Gram-negative bacilli from tsunami victims has also been performed (paper III). Several conclusions have been drawn. However, several issues remain to be addressed in future projects. Other resistance mechanisms remain to be examined, e.g. increased expression of other efflux pumps and decreased expression of other porins. Different methods could be used, methods in which the amount of protein is determined, instead of the amount of mRNA (papers II and IV), to also be able to detect post-transcriptional modification. The patient material of paper III could also be more thoroughly investigated, an

analysis in which clinical data were coupled to microbiological findings would be very interesting to perform.

# 6 CONCLUSIONS

The major conclusions of this thesis were:

- For *E. coli*:
    - In cefuroxime resistant clinical non-ESBL isolates of *E. coli* several mechanisms seem to contribute to the cefuroxime resistance, e.g. efflux by the AcrAB-TolC efflux pump system, loss of the porin OmpF and increased AmpC activity.
- For *K. pneumoniae*:
    - A multidrug resistant (MDR) phenotype was identified among clinical isolates of *K. pneumoniae*, with non-susceptibility to unrelated antibiotics as cefuroxime, chloramphenicol, nalidixic acid and tigecycline.
    - The resistance mechanisms observed were increased transcription of the regulatory gene *ramA*, increased *acrA* transcription (encoding AcrA, part of the efflux pump AcrAB) and decreased *ompK35* transcription (encoding the porin OmpK35).
    - Loss of OmpK35 seems to be the mechanism contributing to cefuroxime resistance, while efflux of the AcrAB efflux pump contributes to non-susceptibility to chloramphenicol, nalidixic acid and tigecycline.
- Thus, the mechanisms causing cefuroxime resistance in *E. coli* and *K. pneumoniae* seem to be (at least partly) different.
- Findings from tsunami victims:
    - From secretion cultures from tsunami victims seeking medical advice in Stockholm, findings of Gram-negative bacilli were common.
    - Antibiotic resistance was more common in cultures from the tsunami victims, compared to a Swedish reference material, for *E. coli*, *K. pneumoniae*, *P. mirabilis* and *Acinetobacter* spp., but not for *P. aeruginosa*.
    - Empirical antibiotic treatment of infections secondary to trauma in patients seeking medical advice after natural catastrophes requires special microbiological considerations.

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