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ANTIBIOTIC RESISTANCE AND ANTIBIOTIC CONSUMPTION IN SWEDEN WITH FOCUS ON *Escherichia coli* AND *Pseudomonas aeruginosa*

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ABSTRACT

Aims: The general aims of the present studies were to assess the levels of antibiotic resistance, in relation to antibiotic consumption at the Karolinska University Hospital, Solna (KS) and at 11 other Swedish hospitals, furthermore to assess the role of the membrane protein OprD and penicillin-binding proteins in *Pseudomonas aeruginosa* resistance to imipenem.

Methods: Resistance figures were retrieved from the microbiology service databases for the period 1989-99, at the 12 above mentioned hospitals, including their intensive care units (ICU). Antibiotic consumption figures were obtained from the National Corporation of Swedish Pharmacies database during the same period.
In order to study molecular mechanisms of carbapenem resistance, we produced transconjugants from clinical isolates of carbapenem resistant *P. aeruginosa* in a sensitive PAO18 after selection for a proline marker (*proB*). The active sites of penicillin-binding proteins PBP1b, PBP2, PBP3 and PBP6 were sequenced, and the expression of *oprD*, *pbp2* and *pbp3* genes was measured using quantitative real-time PCR.

Results: Resistance to ciprofloxacin increased in *Escherichia coli* and *P. aeruginosa* in parallel with an increased quinolone consumption in all included hospitals. The use of cephalosporins increased two and a half times, while the level of resistance in *E. coli* to cefuroxime and cefotaxime remained stable at KS. A third pattern was observed for co-trimoxazole resistance in *E. coli*, which increased at KS as well as the other 11 Swedish hospitals, while consumption of co-trimoxazole and trimethoprim decreased during the 12 year study period. Resistance rates at KS were still generally low, but there were increasing trends for some antibtiotic-microbe combinations. *E. coli* resistance to ciprofloxacin increased from 0% in 1991 to 11% in 1999 and co-trimoxazole resistance increased in *E. coli* from 7.5% to 14% during the study period. For *E. coli*, resistance to ciprofloxacin was higher at the hospital than at the ICUs. There were considerable fluctuations in resistance prevalence over time, especially at the ICU. Imipenem resistance in *P. aeruginosa* was particularly noticeable at the ICU, with resistance peaks of 15% and 28% in 1992 and 1999, respectively. These peaks were due to outbreaks. Sequencing of *P. aeruginosa* genes for PBP1b, PBP2, PBP3 and PBP6 showed no differences in amino acid sequence, but the gene for OprD porin was downregulated in all imipenem resistant clinical strains and their transconjugants.

Conclusions: The significant trend of increased resistance to antibiotics over time constitutes an important warning system. The relation between antibiotic consumption and antibiotic resistance was not always parallel. Three different patterns were observed which suggests that different mechanisms were operating. We also found in some cases, higher resistance rates at the hospital than at the ICUs emphasizing the importance of including all sectors of a hospital. Also, antibiotic resistance figures fluctuated substantially over time, illustrating the value of long surveillance periods. Finally, in imipenem resistant *P. aeruginosa*, a previously unknown gene for regulation of *oprD*, is most likely located close to the *proB* marker.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals I-IV:

I. Sörberg M, Farra A, Ransjö U, Gårdlund B, Rylander M, Wallen L, Kalin M, Kronvall G.
Long-term antibiotic resistance surveillance of gram-negative pathogens suggests that temporal trends can be used as a resistance warning system.
Scandinavian Journal of Infectious Diseases, 34: 372-378, 2002.

II. Sörberg M, Farra A, Ransjö U, Gårdlund B, Rylander M, Settergren B, Kalin M, Kronvall G.
Different trends in antibiotic resistance rates at a university teaching hospital.
Clinical microbiology and infection, 9: 388-396, 2003.

III. Farra A, Skoog G, Wallen L, Kahlmeter G, Kronvall G, Sörberg M.
Antibiotic use and *Escherichia coli* resistance trends for quinolones and co-trimoxazole in Sweden.
Scandinavian Journal of Infectious Diseases, 34: 449-455, 2002.

IV. Farra A, Strålfors A, Sörberg M, Wretlind B.
Role of outer membrane protein OprD and penicillin-binding proteins in *Pseudomonas aeruginosa* resistance to imipenem
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LIST OF ABBREVIATIONS

ACS	Apotekets Centrala Statistiksystem
ATC	Anatomical Therapeutic Chemical (ATC) classification
CDC	Center for Disease Control and Prevention
DDD	Defined Daily Dose
DHFR	Dihydrofolate reductase
EARSS	European Antimicrobial Resistance Surveillance System
ESAC	European Surveillance of Antimicrobial Consumption
ESBL	Extended spectrum ß-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GT	Glycosyltransferase
HMM	High molecular mass
ICU	Intensive care unit
KS	Karolinska University Hospital, Solna
LMM	Low molecular mass
MDR	Multi-drug resistant
MIC	Minimal inhibitory concentration
MRSA	Methicillin Resistant *Staphylococcus Aureus*
NNISS	National Nosocomial Infection Surveillance System
PABA	p-amino benzoic acid
PBP	Penicillin-binding protein
PFGE	Pulsed Field Gel Electrophoresis
PHLS	Public Health Laboratory Service
PMQR	Plasmid mediated quinolone resistance
PRP	Penicillin resistant *Streptococcus pneumoniae*
qRT-PCR	Quantitative reverse transcriptase PCR
RND	Resistance-nodulation-division
SRGA	Swedish Reference Group for Antibiotics
STRAMA	Swedish Strategic Programme for the Rational use of Antimicrobial Agents and Surveillance of Resistance
TP	Transpeptidation
VRE	Vancomycin Resistant Enterococci
WHO	World Health Organisation

1 INTRODUCTION

Antimicrobial resistance is a growing problem worldwide and has now become a major public health issue. The 58th World Health Assembly in 2005 has acknowledged the fact that the containment of antimicrobial resistance is now a priority (WHA resolution on antimicrobial resistance-WHA 58.27).

Antibiotics are among our most important clinical tools. Only a few years after the introduction of penicillin, resistance to this drug emerged. Bacteria, like all living creature adapt to their environment, following the survival of the fittest and developing resistance to antibiotics.

Very few new drugs are being developed. This places pressure on maintaining the effectiveness of currently available agents as long as possible until newer agents become available.

All antibiotic use whether appropriate or inappropriate exerts selective pressure for the emergence of resistant bacteria[1-3]. Our only means of handling the situation at the moment is through prudent use of antimicrobial agent, improved diagnostics, and infection control [4].

Surveillance of antimicrobial resistance is of great help for selection of empirical therapy, for detecting the emergence and spread of new resistances, and assessing the level of resistance and impact of infection control interventions. In 1999, when we started our work, very little was known about the resistance levels in Sweden, even less about antibiotic consumption. The Swedish Strategic Programme for the Rational use of Antimicrobial Agents and Surveillance of Resistance (STRAMA) was still new, and many of the big European networks we have today did not exist [5]. The microbiology database used at Karolinska University Hospital as well as at eleven other Swedish hospitals, had 12 years of results stored, unused and unsorted: a mine of information just waiting to be revealed and used. We therefore decided to analyze this material to detect resistance trends, and to put them in relation to antibiotic consumption.

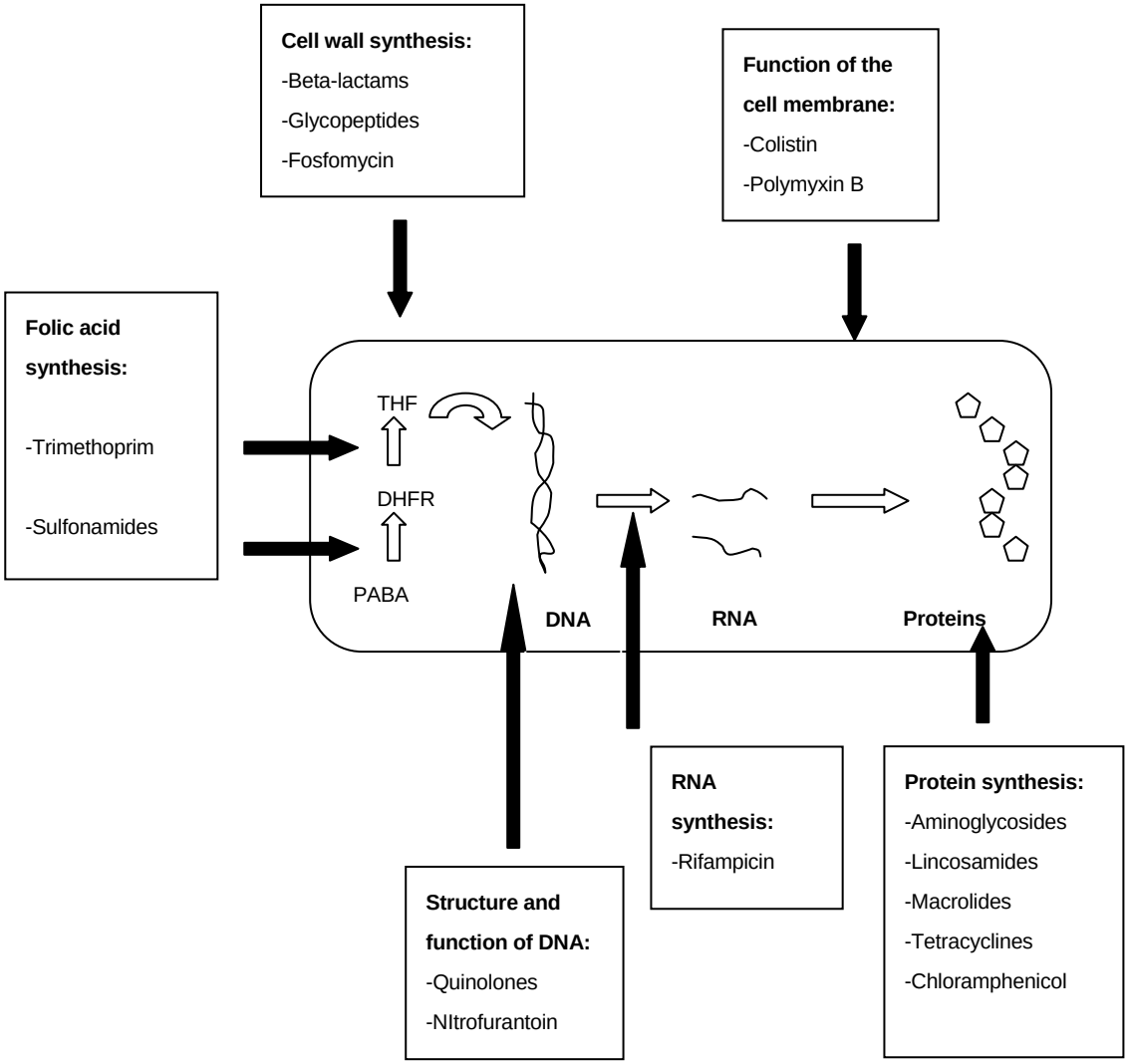
2 ANTIMICROBIAL AGENTS

The history of antimicrobial agents started at the beginning of the last century in 1907 with Paul Ehrlichs research and development of the “magic bullet”, Salvarsan, an arsenic derived drug [6]. Alexander Fleming in 1928 discovered the effect of penicillin, but it was not until the 1940s that penicillin could be produced as an effective drug [7]. New antibiotics came at a very quick pace until the late 1960s: sulfonamides, ß-lactams, aminoglycosides, chloramphenicol, tetracyclines, macrolides, glycopeptides, lincosamides, streptogramins, trimethoprim, and quinolones.

Between 1968 and 2000 no new class of antimicrobial drug was introduced. The oxazolidinones, lipopeptides and glycylcyclines [8, 9] were introduced in the early 2000. The first two mentioned are designed for gram-positive bacteria. Although a variety of agents targeting gram negative bacteria are being investigated, none has entered the clinical development phase, and it may take 10 to 15 years before any of them may be available for clinical use [10].

The targets for the antimicrobial substances are despite the large number of drugs surprisingly few [10]. The main targets are in gross: the cell wall synthesis and cell membrane, DNA construction and repair, RNA translation and transcription, protein synthesis and folic acid metabolism. The basic mechanisms for antimicrobial action on the bacterial cell are shown in Figure 1.

Figure 1. Mechanisms of action for the most important groups of antibiotics



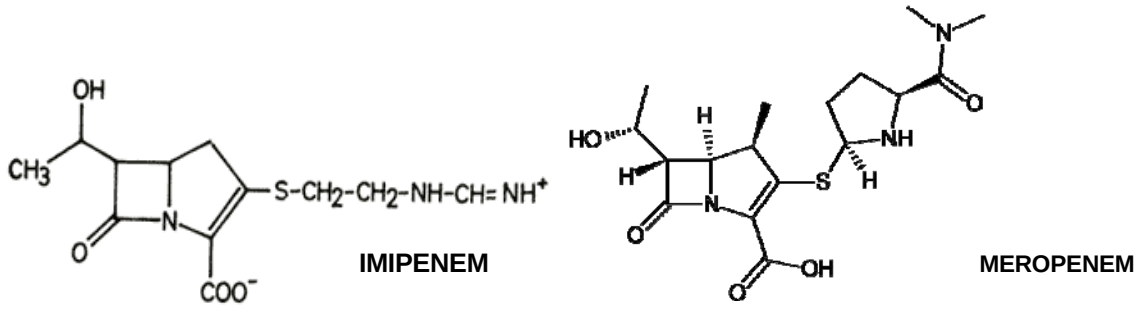
2.1 ß-LACTAM ANTIBIOTICS

The ß-lactam antibiotics are bactericidal cell-wall synthesis inhibitors (Figure 1). Beta lactam drugs are the most widely used group of antibiotics, owing to their high effectiveness, low cost, ease of delivery and minimal side effects [11]. They include penicillins (e.g. ampicillin), cephalosporins, monobactams, and penems. The characteristic of the ß-lactam antibiotic structure is the four member lactam ring [12]. Various chemical side chains have been synthetically linked to the ring structures producing antibiotics with different properties. The

cell wall of bacteria is a complex structure composed of a tightly cross-linked peptidoglycan net that protects the cell from the osmotic pressure. The ß-lactam antibiotics bind to and inhibit enzymes involved in the cross-linking of peptidoglycan: the penicillin-binding proteins (PBP) (described in paragraph 5.3). Once cell wall synthesis is inhibited, enzymatic autolysis of the cell wall can occur.

2.1.1 Carbapenems

Figure 2. Chemical structure of imipenem and meropenem



Carbapenems are ß-lactam antibiotics. They have the widest spectrum of antimicrobial activity, including gram-positive and negative bacteria as well as anaerobic bacteria. They have a side chain with a hydroxyethyl side chain in trans configuration at position 6, which confers stability toward most ß-lactamases, including the extended spectrum ß-lactamases (ESBL) [13]. Clinically available carbapenems in Sweden are imipenem, meropenem and ertapenem. Ertapenem has no effect on *Pseudomonas aeruginosa*. Carbapenems exert their action in *P. aeruginosa b*y binding to protein-binding protein 2 (PBP2) [14].

Carbapenems are indicated for severe infections in the lung, abdomen, central nervous system, septic arthritis and for initial treatment of fever of unknown origin in neutropenic patients [15]. Carbapenems are among the most used antibiotics in Swedish ICUs [16].

2.2 QUINOLONES

Quinolones are bactericidal and exhibit concentration-dependent killing. The targets of quinolone activity are the bacterial DNA gyrase and topoisomerase IV, enzymes essential for regulation of super coiling and decatenation of bacterial DNA [17]. Earlier compounds with similar activity, such as nalidixic acid, had poor systemic distribution and limited activity and were used primarily for gram-negative urinary tract infections. The next generation of agents acting on DNA topoisomerases, the fluoroquinolones (i.e., ofloxacin, norfloxacin and ciprofloxacin,), were more readily absorbed and displayed increased activity against gram-negative bacteria. Newer fluoroquinolones (i.e., levofloxacin, moxifloxacin) have enhanced activity against many gram-negative and gram-positive organisms (www.cdc.org).

Ciprofloxacin is widely used both in hospital and ambulatory settings mainly for upper tract urinary infections and exacerbations of chronic bronchitis [15]. Levofloxacin and moxifloxacin are used for treatment of atypical pneumonia, since they are effective against Mycoplasma, Chlamydia and Legionella.

2.3 CO-TRIMOXAZOLE

Co-trimoxazole is a combination of two antibiotics, both of them antifolates: trimethoprim and sulfamethoxazole. The two drugs separately are bacteriostatic while a combination in certain concentration relationships may have a synergistic bactericidal effect. Both trimethoprim and sulfamethoxazole target enzymes in the folic acid pathway. Folic acid and folate are necessary for DNA replication. Dihydrofolate reductase (DHFR) is an essential enzyme in this pathway. Trimethroprim is a structural analogue to this enzyme and thus a competitive inhibitor. The human DHFR is endogenously resistant to trimethoprim, which is the basis for its selectivity and clinical use. Sulfonamides also act as a competitor inhibitor of another enzyme in the pathway: dihydropteroate synthase, as they are structural analogues of this enzyme substrate p-amino benzoic acid (PABA) (Figure 1) [18]. This enzyme only exists in bacteria and some eukaryotic

cells such as *Pneumocystis jiroveci* (formerly *carinii*), *Toxoplasma gondii* and *Plasmodium falciparum* [19]. In Sweden, trimethoprim is used on its own mainly for lower urinary tract infections, while co-trimoxazole is used for upper urinary tract infections, and in paediatric care. Co-trimoxazole is also used for treatment and prophylaxis of other infections such as *Pneumocystis jiroveci* pneumonia and toxoplasmosis in HIV patients [20, 21]. Co-trimoxazole use has been and still is very common worldwide as the drug is accessible and inexpensive.

3 RESISTANCE MECHANISMS

Resistance to antimicrobial drugs in bacteria can be intrinsic, or acquired. Acquired resistance is caused by genetic alterations leading to protection of the bacteria from the action of an antibiotic drug. The mechanisms of acquired resistance are multiple and varied but can be divided into four main principles [22]:

- Inactivation of the antimicrobial drug (i.e. ß-lactamases)
- Change in target for antibiotic action: mutations in the target (i.e. PBP mutations), production of alternative targets or protection of the target
- Changed access (i.e. down regulation of porins)
- Extrusion of the antibacterial agent (i.e. efflux pumps)

3.1 RESISTANCE TO ß-LACTAM ANTIBIOTICS

There are three major ways bacteria avoid the effect of ß-lactam drugs:

3.1.1 Production of ß-lactamases

ß-lactamases are enzymes that hydrolyze the amide bond of the ß-lactam ring of the antibiotic, they thereby render the drug inactive before it reaches the PBP target [23]. They constitute the most common mechanism of resistance in gram-negative bacteria. The ß-lactamase genes are often integrated within mobile genetic elements, such as transposons or plasmids, and can therefore easily be transferred between bacteria. Their expression is often induced by ß-lactam antibiotic [11]. There is an immense and increasing number of different ß-lactamases that can hydrolyze different ß-lactam antibiotics. There are two different classification systems for ß-lactamases: based on amino acid sequence (Ambler classes A to C) or substrate inhibitor profile (Bush-Jacoby-Medeiros group 1 to 4). Of particular concern, are the class C cephalosporinase (AmpC), the extended spectrum beta lactamases (ESBL) and the carbapenemases as they are able to target most beta lactams [23].

AmpC (Ambler class C) is a class of chromosomal ß-lactamases found in several gram-negative bacteria (like Pseudomonas). AmpC can be induced by ß-lactam antibiotics in Enterobacter, Serratia, Citrobacter and Pseudomonas and degrade cephalosporins. Ceftazidime and other cephems are stable against AmpC enzyme hydrolysis[24]. AmpC can also be derepressed by mutations in regulator genes resulting in selection of derepressed mutants over expressing AmpC [25]. Examples of *ampC* genes being mobilized on plasmids, and spreading to species normally not carrying the *ampC* gene has been observed [26]. High levels of AmpC exert resistance to penicillins, beta-lactamase inhibitors, cefoxitin and ceftazidime [23].

Most ESBL are the result of genetic mutation from other beta-lactamases (i.e. TEM-1, TEM-2 and SHV-1), resulting in a "novel" beta-lactamase able to hydrolyze cefotaxime, ceftazidime and aztreonam. CTX-M ESBLs arose by plasmid acquisition of preexisting chromosomal ESBL genes from the *Kluyvera* spp [23]. ESBL can be plasmid mediated and thus capable of spread. ESBL-producing isolates remain susceptible to carbapenems [27].

The carbapenemases are further described in paragraph 5.2.3.

3.1.2 Control of ß-lactam intracellular concentration

Some ß-lactams enter the bacteria via porins in the outer membrane. If the amount of porins is significantly decreased or the porins have a structural change, the antibiotic may no longer be able enter the bacteria and resistance develops.

Another way bacteria can keep the intracellular concentration of the drug low is to transport the antibiotic out of the cell by the effect of efflux pumps. Five families of efflux systems have been described. They are the ATP-binding cassette family, the resistance-nodulation-division family (RND), the multidrug and toxic compound extrusion family and the small multidrug resistance family. RND is thought to be the most involved in ß-lactam resistance [11].

3.1.3 Altered target for ß-lactam action

The target of the beta lactam antibiotic, the penicillin binding proteins (PBP, further described in paragraph 5.3), can by several mechanisms acquire induced resistance: acquisition of a "new" less sensitive enzyme, mutation of an endogenous PBP so that it exerts less affinity for the antibiotic drug or up regulation of expression of PBP [11].

3.2 RESISTANCE TO QUINOLONES

Resistance to quinolones occurs through mutations in the genes encoding DNA gyrase (topoisomerase II) and topoisomerase IV subunits *gyrA* and *gyrB,* or *parC* and *parE* respectively [17]. Resistance mediated by these mutations is enhanced by porin structure changes and efflux pump activity [28]. Another more recently discovered mechanism, is a plasmid mediated quinolone resistance (PMQR) gene encoding a Qnr protein capable of protecting DNA gyrase from quinolones [29]. These resistance mechanisms are additive, and can be combined. In gram-negative bacteria, DNA-gyrase tends to be the primary target for fluoroquinolones. Mutations in *gyrA* are found in isolates with low-level resistance, whereas higher minimal inhibitory concentrations (MIC) are associated with additional mutations in *parC, gyrB, parE* and expression of efflux pumps [30]. The PMQR confers low level resistance to quinolones in itself, but increases resistance levels if combined with another resistance mechanism. Another aspect of plasmid borne resistance is co transmission of PMQR, amino glycoside-modifying enzymes, broad spectrum ß-lactamases and even carbapenemases [31].

3.3 RESISTANCE TO CO-TRIMOXAZOLE

Resistance to co-trimoxazole is in fact resistance to trimethoprim, to sulfamethoxazole or both combined.

3.3.1 Trimethoprim resistance

There are three types of chromosomally conferred resistance to trimethoprim: loss of thymidylate syntase activity making the dihydrofolate reductases redundant for the bacteria since

it will depend on external supply of thymine, changes in the structure or expression of porins (seen in Gram negative species as strains of Klebsiella, Enterobacter and Serratia) and through mutations in the *folA* gene encoding the bacterial dihydrofolate reductase, DHFR, which lead to a lower affinity of the drug to the enzyme and thus to a lower level of enzyme inhibition [32].

The clinically most important trimethoprim resistance in Gram negative bacteria is conferred by alternative resistant dihydrofolate reductases which are encoded by a number of different *dfr*-genes. Most of these genes are recognised as integron-borne gene cassettes, where the mechanisms for recruitment are still unknown, and are thus very horizontally mobile [19, 32, 33].

3.3.2 Resistance to sulfonamides

Mutational changes in the chromosomal gene (*folP*) for dihydropteroate synthase, results in lowered affinity for the inhibiting sulphonamide in the expressed enzyme [32].

The horizontally transferred sulfonamide resistance genes are in several studies responsible for the majority of sulfonamide resistance studied in Gram negative isolates. Only three *sul*-genes are known at present. Still, these genes seem to be efficiently spread. The first two genes, *sul1* and *sul2* have been known to be plasmid-borne since the 1960s and were for a long time described to be equally distributed [34] among resistant isolates while the more recently described *sul3* has now been seen in clinical isolates [35].

4 ANTIMICROBIAL RESISTANCE

4.1 ANTIMICROBIAL RESISTANCE RATES

Antibiotic resistance rates for *E. coli* and *P. aeruginosa* are shown in tables 1 and 2.

Table 1- Resistance rates of *E. coli* in various parts of the world

Place	Year	Setting	Quinolones C=ciprofloxacin	Co-trimoxazole	Reference
Sweden	2005	Various isolates	9.3%		[36]
Greece	2006	Various isolates	13%		EARSS
Canada	2003-2004	Outpatient urine	1.2% (C)	17.3%	[37, 38]
USA	2003-2004	Outpatient urine	6.9% (C)	22.6%	[37, 38]
UK and Ireland	2001-2002	Hospital Blood	11.1% (C)		[38, 39]
Spain	2001-2003	Hospital blood/CSF	19.9% (C)	32.8%	[38, 40]
USA	2002	Hospital Blood	13.3% (C)	25.2%	[41]
USA	2002	ICU Blood	14.3% (C)		[41]

Table 2- Resistance rates of *P. aeruginosa* in various parts of the world

Place	Year	Setting	Ciprofloxacin	Imipenem	Meropenem	Ref.
KS	2005	ICU		13%	5%	[42]
Sweden	2005	ICU, dialysis, haematology Various isolates		17.5%	12.7%	[43]
USA	2001-2002	ICU Nosocomial	29.2%	17.4%		[44]
USA	2001	ICU	28%	19.3%	19.9%	[45]
USA	2001	Hospital	30.2%	14.5%	14.5%	[45]
Europe	2002-2004	Hospital	36%	30%	24%	[46]
North Am.	2002-2004	Hospital	29%	15%	11%	[46]
South Am	2002-2004	Hospital	55%	48%	43%	[46]
USA	2001-2003	Various isolates	33.5-31.2%	15.6-21.2%	14.2%(2003)	[47]
Asia-pacific	2001-2004	Various isolates	18.8%	17.8%	14.3%	[48]
Europe	2001-2004	Various isolates	29.9%	21.9%	19.4%	[48]
Latin America	2001-2004	Various isolates	57.3%	33.9%	32%	[48]
North Am	2001-2004	Various isolates	25.1%	13.1%	10.6%	[48]

4.2 DEVELOPMENT AND SPREAD OF ANTIMICROBIAL RESISTANCE

Antibiotic use is the primus motor of antibacterial emergence. There is a close association between the use of antibiotics and the emergence of subsequent antibiotic resistance in both gram negative and gram positive bacteria [49-52], even though this relationship is complex [53]. Prolonged regimens of antimicrobial agents are one factor promoting the emergence of antibiotic resistance [54]. Another more frustrating reason is the unnecessary and inappropriate use of antibiotics that creates pressure for the selection of resistant strains. In the United States, several studies have suggested that a big part of antimicrobial use might be unnecessary or inappropriate [55, 56]. Decreasing the use of a certain antibiotic does not necessarily mean a decrease in resistance [57-60]. Indeed concordant to our findings in paper III, Sundquist et al. [61] found that a 85% decrease of trimethoprim use during a period of two years did not result in a decrease in resistance in *E. coli*.

Strategies aiming at limiting or modifying the administration of antimicrobial agents have the greatest likelihood of preventing the emergence, but not always the prevalence, of resistance to these agents, a resistance that might be difficult to revert. Good antimicrobial stewardship involves selecting the most appropriate drug at its optimal dosage and duration of therapy to eradicate an infection while minimizing side effects and pressures for the selection of resistant strains [4].

Other factors promoting antimicrobial resistance include long hospitalization stays, the presence of invasive devices such as catheters, endotracheal tubes and inadequate infection control practices [62]. Preventing horizontal transmission of antibiotic resistant bacteria is important in reducing antibiotic resistance rates. In health care facilities, person-to person transmission of multidrug-resistant organisms by indirect and direct contact constitutes the major route of transmission and dissemination. Hand hygiene is considered the most important and effective measure to prevent health care associated infections and the spread of resistant pathogens [63].

The use of surveillance cultures to identify patients colonized with antibiotic-resistant bacteria, allowing them to be placed in isolation in an efficient manner, may help to reduce the spread of resistant bacteria [64]. This method demands a lot of resources and an aggressive policy to be successful. However, the threat and consequences of horizontal spread of antibiotic resistant bacteria probably outweighs the potential risks of isolation practices.

4.3 THE ICU SETTING

The intensive care units are considered to be the place with the highest risk of resistance development and spread. Critically ill patients that are treated there and antimicrobial therapy is common: up to 74% of Swedish ICU patients were treated with antibiotics [16]. Many of the patients are admitted because of infections leading to organ failure. Moreover, many ICU patients acquire defects in host defence mechanisms from the immuno-suppressive effect of underlying diseases (i.e. diabetes, immunosuppression, and trauma). Thus patients are at high risk for nosocomial infection, further increased by the exposure to several invasive devices such as mechanical ventilation and insertion of diverse catheters.

Data from the Centre for Disease Controls (CDC) National Nosocomial Infection Surveillance System (NNIS) have documented the magnitude of the resistance problem in ICUs. In this setting, Methicillin Resistant *Staphylococcus Aureus* (MRSA) accounted for almost 60% of staphylococcal infections, Vancomycin Resistant Enterococci (VRE) for 28% of enterococcal infections and 31% of Enterobacter infections were caused by enterobacter species resistant to 3rd generation cephalosporins [65, 66]

Even though the antibiotic consumption is high at Swedish ICUs (74% of ICU patients were treated with antibiotics), and most treatment decisions (70%) were made without microbiological data, the empirical treatment was adequate in vitro in 95% of the cases, reflecting the fortunate ecological situation in Swedish hospitals [16].

4.4 MEASURING ANTIMICROBIAL RESISTANCE

Surveillance has been described as: "the ongoing and systematic collection, analysis, and interpretation of health data in the process of describing and monitoring a health event" [67].

In Sweden, statutory notification of certain communicable diseases is regulated in the Communicable Disease Act (SFS 1988:1472). Both the clinician caring for the patient and the laboratory diagnosing the pathogen causing the disease are obliged to notify. This double notification enhances the sensitivity of the surveillance system. There are four antibiotic resistant pathogens that are included in the list of diseases to notify: penicillin resistant *Streptococcus pneumoniae* (PRP) (since 1996), MRSA (since 2000) and vancomycin resistant *Enterococcus faecalis* and *Enterococcus faecium* (VRE) (since 2000).

Sweden has a resistance surveillance program since 1994 (www.strama.se) where resistance data is collected and presented yearly. 30 microbiology laboratories send in quantitative resistance data (zone diameters) for defined antibiotics for 100 consecutive clinical isolates of a selected number of bacterial species. *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Haemophilus influenzae* have been included all years, while *E. coli* and *P. aeruginosa* have not been included all years but on several occasions [36]. These figures give a good estimate of the resistance rates in the country, but give no information about local data, like single hospitals or wards.

Several European antimicrobial resistance networks have been created or implemented since 1999, among them European Antimicrobial Resistance Surveillance System (EARSS-1999) [5], European Surveillance of Antimicrobial Consumption (ESAC-2001), European Committee on Antimicrobial Susceptibility Testing (EUCAST-1996, restructured in 2002), and last ReAct (Action on antibiotic resistance).

EARSS is based on methods used routinely in clinical laboratories all over Europe, and the value of the results is thus dependent on comparable methods being used by participating laboratories

[5]. EUCAST has the objective of harmonizing the results of susceptibility tests in different countries or cross correlate the results of various national methods [68].

All resistance studies have limitations, but a lot of effort and resources are now put on resistance control surveillance, enhancing the attention and change in attitudes in patient healthcare.

4.5 ANTIBIOTIC CONSUMPTION

Antibiotic use significantly contributes to increasing rates of resistant pathogens [69]. However, antibiotic consumption is not easy to measure. In Scandinavia we have used the ATC/DDD system since the eighties. The DDD (Defined daily dose) is the assumed average maintenance dose per day for a drug in its main indication for adults. It is a measurement unit to be used during drug use studies and does not necessarily reflect the recommended or prescribed daily dose for individual patients or specific patient groups (http://www.whocc.no/atcddd/atcsystem.html), [70]. Unfortunately, different "DDD" were used in the USA and in Europe, until the early 2000, as not all used the WHO-assigned international measurement unit for each antimicrobial. There is now a free tool for calculating antimicrobial use available at http://www.escmid.org/esgap.

The ATC/DDD system has its limitations, such as the measurements of antimicrobial use in paediatric wards when patient-level data are not available [71], or to use the proper denominator for measurements in hospitals [72, 73].

Data on antibiotic use is crucial for control of antibiotic use, and thus the European Surveillance on Antimicrobial consumption (ESAC) was established in 2001 with support of the European Commission. In 2002, the median national hospital antibiotic consumption in Europe was 2.1DDD/1000inhabitants/day, ranging from 3.9 in Finland to 1.3 in Sweden [74].

During the 2000-2005 period, antibiotic use in Sweden in hospital settings increased by 13% DDD/1000inhabitants, or 42% (1994-2004) DDD/1000 patient care days. These numbers might be overestimated due to fact that both number of beds as well as length of stay has decreased in

Swedish hospitals [36]. The total use of antibiotics in Sweden has been stable since year 2000 (around 15DDD/1000inhabitants/day).

4.6 RESISTANCE CONTROL STRATEGIES

There are guidelines for resistance control, both in Europe and North America. Most studies to measure the impact of these measures are done on MRSA or VRE, and it is difficult to evaluate every precaution on its own [75]. All categories of health care workers should be aware and active in implementing the local guidelines.

The CDC (Center for Disease Control and Prevention) in USA has at the moment a campaign to prevent antimicrobial resistance in health care settings, divided in 4 steps that I chose to use for presenting this issue:

4.6.1 Preventing infection

Vaccination for influenza and pneumococcus for at risk patients does minimize the overall infection load, and its complications [76].

All invasive devises are a risk for infection and colonization. Catheters should therefore be used only when needed, correctly used and inserted and removed as soon as they are not needed any longer [77].

4.6.2 Diagnose and treat infection effectively

Culturing the patient is a simple but often forgotten measure. It is the key, not only to know the infections aetiology, but also a way to know the local pathogens and their susceptibility profile. This information is needed for choosing the correct empiric therapy and thus grossly increasing the infected patients chances of recovery [78]. Targeting empiric therapy to likely pathogens and local antibiogram is crucial for survival of severely ill patients, and thus consulting the infectious diseases experts is strongly recommended.

4.6.3 Use antimicrobials wisely

Local data is of great importance: knowing the patient population and antibiogram is important as mentioned previously, and thus engaging in local antimicrobial control efforts is a good way to minimize unnecessary antibiotic use.

Contamination or colonization should not be treated. A way to avoid contamination is to use proper methods to obtain and process all cultures. Colonization is unavoidable, and culture results should always be read with this in mind.

Minimizing unnecessary antibiotic use is also to narrow down the antibacterial spectra once culture results are retrieved, and to stop antibiotic treatment when infection is cured or unlikely [4]. Consulting the expert is always encouraged [79].

4.6.4 Prevent transmission

Hygiene play a big role in transmission prevention: hand wash and disinfection prior to patient care [80], correct handling of needles, correct disinfection of all invasive material (ventilators, endoscopes equipment, dialysis apparatus etc.) just to name some [81]. Proper hygiene routines should be followed at all times in all patient health care aspects. In some cases, special measures have to be taken as patient isolation or screening cultures [64].

4.7 IMPACT OF RESISTANT BACTERIA

Treatment factors may contribute to adverse outcomes in patients infected with a resistant pathogen. These factors include decreased effectiveness, increased toxicity, and/or improper dosing of antimicrobial agents available for treatment; a delay in treatment with or the absence of microbiologically effective antimicrobials; and an increased need for surgery and other procedures as a result of these infections [82].

As the bacteria become resistant, we are forced to use older and more toxic drugs, like for instance colistin for *Pseudomonas aeruginosa*. Colistin is both nephro and neurotoxic [83].

Second-line therapy is often more expensive than first-line a heavy burden especially for developing countries [84]. We are now faced for instance with tuberculosis strains so resistant that almost no chemotherapy is available (XDR tuberculosis) [85].

In a milestone study by Kollef [78], it was demonstrated that inadequate initial treatment of infections among patients requiring ICU admission was the most important determinant of hospital mortality. Antimicrobial resistance increases the risk of choosing the "wrong" antibiotic, and thus increases the mortality risk.

Increased length of hospital stay and higher costs of care for patients infected with a resistant organism may also result from an increased frequency of surgical interventions required to control infection [86].

In conclusion, there is an association between the development of resistance and increases in mortality, length of hospitalization, and costs of healthcare [82].

5 PSEUDOMONAS AND CARBAPENEM RESISTANCE

5.1 PSEUDOMONAS AERUGINOSA

Pseudomonas aeruginosa is an aerobic gram-negative rod. It is widely distributed in nature and can adapt to many ecological niches, from water and soil to plant and animal tissues, and can thus be isolated from nearly any conceivable source within hospitals [87]. *P. aeruginosa* is an important cause of both community acquired and hospital acquired infections. Community acquired infections include keratitis, otitis externa and skin and soft tissue infections (especially in immunocompromised patients such as those with diabetes mellitus). *P. aeruginosa* infection is especially problematic in patients suffering from the genetic disease cystic fibrosis, where it colonizes the airways and causes recurrent infections. Nosocomial infections caused by *P. aeruginosa* include pneumonia, urinary tract infections, bloodstream infections, surgical site infections and skin infections in the setting of burn injuries [88]. Infections with *P. aeruginosa* have been associated with high morbidity and mortality when compared with other bacterial pathogens [89].

P. aeruginosa infections are difficult to treat because of high intrinsic resistant to many antibiotics and a high risk of emergence of resistance during therapy. The carbapenems, including meropenem and imipenem, are among the few therapeutic options still available for treating infections caused by *P. aeruginosa.*

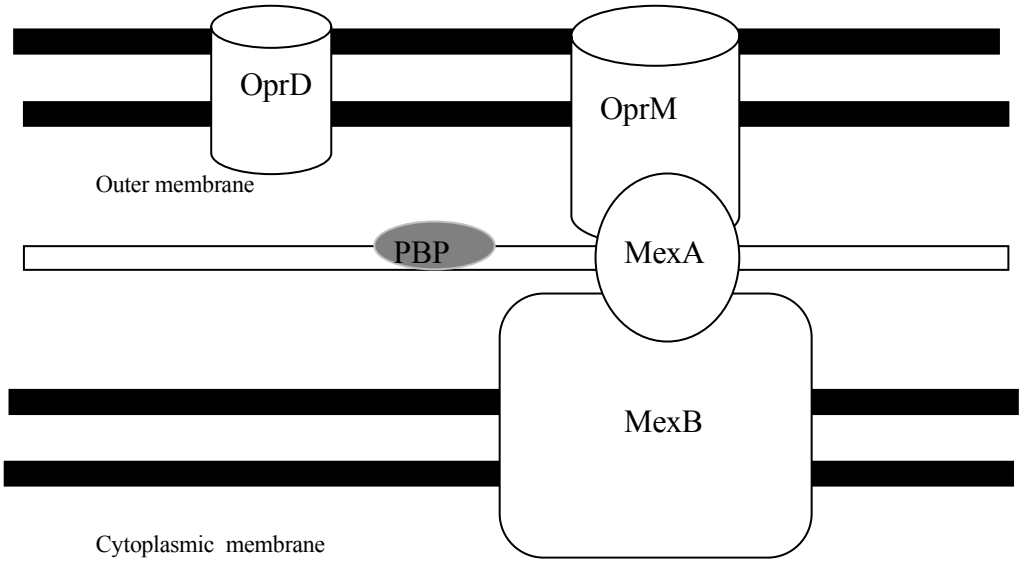
5.2 CARBAPENEM RESISTANCE MECHANISMS

The main known mechanisms of resistance to carbapenems in *P. aeruginosa* are through control of intracellular concentration of the antibiotic: alterations in or decreased production of outer membrane porin OprD [90], multi-drug efflux pumps [91] or hydrolysis by metallo ß-lactamases (MBL) [92, 93].

A study done by El Amin et al [94] indicated that also other mechanisms were involved in imipenem resistance, perhaps through mutations in genes for penicillin-binding proteins (PBP).

5.2.1 Outer- membrane proteins

Figure 3. Cell wall of *Pseudomonas aeruginosa*



As Pseudomonas has an outer membrane with low permeability, many substrates necessary for growth have to utilize specialized pathways: thus the variety of gated channels; the porins. *P. aeruginosa* has 3 large families of porins; the OprD family of specific porins, the OprM family of efflux porins, and the TonB-interacting family of gated porins [95]. OprD porin plays a major role in imipenem resistance [93]. Mutations in loops 2, 3 of the OprD protein [96], lead to imipenem resistance. Changes in loop 5, 7 or 8 have been found to expand the channel, thus leading to hyper susceptibility. The most important mechanism of resistance to imipenem in clinical strains is down-regulation of OprD [90]. Loss of the porin OprD raises the imipenem minimal inhibitory concentration (MIC) from 1-2mg/L to 8-32mg/L [93], but does not affect meropenem susceptibility much. OprD is regulated by multiple systems; it is repressed by salicylates and catabolite repression, and activated by arginine and a variety of other amino acids

[95]. MexT (PA2492) is a transcriptional repressor that down-regulates *oprD* and up-regulates genes for the efflux pump MexEF-OprN (so called NfxC class mutants). Mex EF-OprN efflux pump mediates resistance to several antibiotics, including quinolones. MexS (PA2491) [97] and mvaT (PA4315) [98] have similar effects.

5.2.2 Multi-drug efflux pumps

These efflux systems are composed of three proteins physically linked (Figure 3). The systems include a pump located in the cytoplasmic membrane (e.g. MexB), an outer membrane porin (e.g. OprM) and third protein (e.g. MexA) that physically link the two other components.

Efflux pumps, all belonging to the resistance nodulation family (RND) are also involved in carbapenem resistance: MexAB-OprM, MexCD-OprJ and MexXY [91]. MexAB-OprM is the one that functions primarily and effectively in the extrusion of penems (mainly meropenem) while MexCD –OprJ holds a compensatory mechanism, and MexXY has a small impact [99].

5.2.3 ß-lactamases

Carbapenems are stable to almost all clinically relevant ß-lactamases, but there are exceptions: the class A carbapenemases and the metallo ß-lactamases (MBL).

The class A carbapenemases include chromosomal, integron or plasmid encoded enzymes. Over the past years the most notable expansion group has been the plasmid encoded carbapenemases. The most important of the plasmid serine carbapenemases are the *Klebsiella pneumoniae* carbapenemase and the OXA-type carbapenemase [23].

Metallo ß-lactamases (Ambler class B) are enzymes that use one of two zinc atoms for inactivating penicillins and cephalosporins. In bacteria, MBL confers resistance to carbapenems, cephalosporins and penicillins. MBL are found on a variety of genetic elements (chromosome, plasmid and integrons). Bacteria possessing MBLs are among the most resistant phenotypes

encountered by clinicians. MBL are the carbapenemases found in *P. aeruginosa*, and little is known about their dissemination and spread.

5.3 PENICILLIN BINDING PROTEINS

The bacterial peptidoglycan is a three-dimensional netlike mesh that lines the exterior of the cell membrane. It protects the bacteria from osmotic shock, determines the cellular shape, and serves as attachment sites for virulence factors and adhesins. Synthesis in an untimely manner or erroneously will lead to fragility or instability of the bacterial cell. PBP catalyse the final stages of the peptidoglycan synthesis within the periplasm [100].

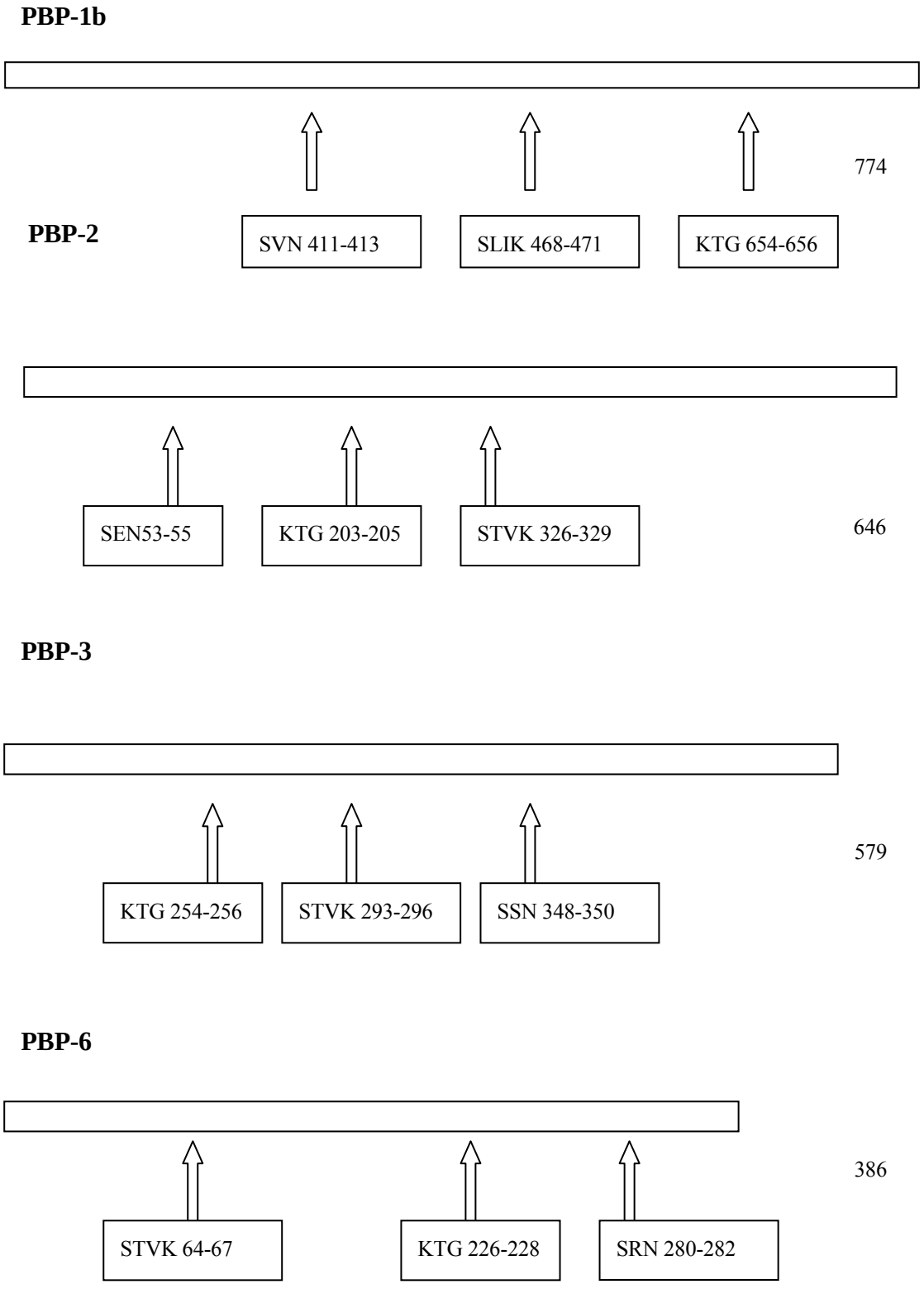
Bacteria have multiple PBPs with different roles during cell division. There are high molecular mass (HMM) and low molecular mass (LMM) PBPs. HMM PBPs are divided into class A and B. Class A are bifunctional enzymes that catalyze both the polymerization of the GlcNAc-MurNAC chains (Glycosyltransferase, GT) and the cross linking of adjacent stem peptide (Transpeptidation, TP) reactions. Class B are monofunctional and present only TP activity [101]. LMM-PBPs catalyze a carboxypeptidation reaction that prevents further cross linking of the peptidoglycans and are thus involved in the regulation of peptidoglycan reticulation [102].

There are not many studies on *P. aeruginosa* PBPs. Up to date, there seem to be 8 different PBPs, PBP-1a, -1b, -2, -3, -3a, -4, -6 (or 5 depending on the nomenclature), -7 that are homologues of *E. coli* PBPs -1a,-1b, -2, -3, -4, -5 and -7 [103, 104] [105, 106] (Table 3).

Table 3- The penicillin binding proteins in *P. aeruginosa*

	Gene names	**Locus ID**	**Location**	**Type**	**Corresponding PBP in *E.coli***	**References**
PBP-1a	*ponA*	PA5054	5681kb	HMW(A)	PBP-1a	[106]
PBP-1b	*ponB*	PA4700	5280kb	HMW(A)	PBP-1b	[107]
PBP-2	*pbpA*	PA4003	4485kb	HMW(B)	PBP-2	[108]
PBP-3	*pbpB*	PA4418	4954kb	HMW	PBP-3	[109]
PBP-3a	*pbpC*	PA2272	2501kb	HMW	PBP-3homolog	[103]
PBP-4	*dacB*	PA3047	3410kb	LMW	PBP-4	[110]
PBP-6	*dacC*	PA3999	4480kb	LMW	PBP-5	[111]
PBP-7	*pbpG*	PA0869	950kb	LMW	PBP-7	[105]

Figure 4 – Schematic view of the conserved sites of PBP-1b, -2, -3 and -6



5.4 CLINICAL IMPACT OF PSEUDOMONAS RESISTANCE

Carmeli et al. published several studies addressing outcomes associated with antimicrobial resistance in gram-negative pathogens. There were no differences in mortality or length of hospital stay between patients infected with a resistant isolate at baseline and those infected with a susceptible isolate at baseline. In contrast, the emergence of resistance was associated with a greater risk of death and a longer duration of hospital stay. The emergence of resistance was also associated with an increased risk of secondary bacteraemia [112]. Infection or colonization with multi-drug resistant (MDR) *P. aeruginosa* was associated with increased mortality, increased length of hospital stay and the need for more surgery and other procedures. Also, the functional capacity of the MDR *P. aeruginosa* carrying patients at discharge was poorer than that of the controls [113]. In patients with cystic fibrosis, infection with MDR *P. aeruginosa* was associated with accelerated progression of cystic fibrosis and increased likelihood of undergoing lung transplantation [114]. Imipenem-resistant *P. aeruginosa* has been found to be associated with increased in hospital mortality rates, increase in hospitalization duration and hospital charges [115].

6 AIMS OF THE THESIS

I- Estimate resistance trends by use of routine clinical microbiology results over a defined longer period of time.

II- Study long term trends in antibiotic resistance of common bacterial species isolated at a university hospital and in its intensive care units (ICUs).

III- Analyze long term trends in antibiotic resistance of *E. coli* to quinolones and co-trimoxazole at 12 Swedish hospitals in relation to antibiotic consumption.

IV- Investigate the role of outer membrane protein OprD and penicillin-binding proteins in resistance to imipenem in *Pseudomonas aeruginosa.*

7 MATERIAL AND METHODS

7.1 DATA COLLECTION

7.1.1 Susceptibility figures selection

The susceptibility test results of clinical isolates were obtained from the respective hospital microbiology service computer database, ADBakt data system (Autonik AB, Sweden). This is an M-technology based, post-relational database system for the microbiology laboratory with registration of requests and results and it also allows queries with file output for further analysis in Microsoft Excel. Our analysis included pathogens collected from all types of patient specimens, from the hospitals in and out-patients. All isolates marked as R were included. Only one isolate of the same species and type of specimen was included from each patient during each year. This selection was used to avoid duplicate isolates and to include all blood isolates.

In article I and II figures were taken from Karolinska University Hospital, Solna, Sweden. In article III, figures were taken in the same way from 12 hospitals in Sweden. The clinical microbiology laboratories included in the present studies were all providing full diagnostic bacteriology service to their respective hospital and its surrounding city and province. The diagnostic methods followed established procedures in clinical bacteriology with required quality control programs passed. The studies included isolates over a 12 year period ranging from 1988 to 1999.

7.1.2 Antibiotic sales

Antibiotic sales data were obtained from National Corporation of Swedish Pharmacies in the "Apotekets centrala statistiksystem" (ACS) database. Pharmacies are organized in a government owned company, which has monopoly on selling pharmaceutical preparations in Sweden. Figures are presented as defined daily doses according to the Anatomic Therapeutic Chemical classification system defined by WHO Collaborating Centre for Drug Statistics Methodology

(http://www.whocc.nmd.no/). Defined daily dose (DDD) is a unit based on the average daily maintenance dose used for the main indication of the drug in adults (http://www.whocc.nmd.no/). Even though the figures did not show the actual consumption, they still give a good estimate of increase or decrease of antibiotic use within each hospital.

7.1.3 Selection of isolates

In a study done by El Amin et al. [94] , clinical strains of *P. aeruginosa* resistant to different carbapenems were selected during 2001-2003 at Karolinska Hospital and were examined for mutations in and expression of the genes coding for the outer membrane protein OprD and the efflux pump-protein MexB. The clinical strains that were resistant to imipenem and not ß-lactamase producing were conjugated with a well known PAO strain and selected for auxotrophic markers (see conjugation). The imipenem resistant conjugates were then chosen for further analysis.

7.1.4 The Hospitals

The Karolinska University Hospital, Solna (KS) is a highly specialized university hospital. There are altogether six ICUs within the hospital. For comparisons of resistance rates, only the non-paediatric ICUs were included, i.e. the general surgery ICU (eight beds, increased to 12 beds in 1998), the burns unit ICU (six beds, reduced to four beds in 1991), the thoracic surgery ICU (eight beds), and the neurosurgery ICU (opened in 1996, 11 beds).

The total number of beds in the hospital was reduced from 1325 in 1989 to 1132 in 1998, and the number of inpatient bed-days from 339 547 in 1989 to 261 508 in 1998. The number of admissions, however, increased from 42 801 in 1989 to 55 753 in 1998. Outpatient departments deal mainly with referred patients.

In study III, twelve hospitals were included, designated here as hospitals A (KS) to M. Hospitals A and B were university teaching hospitals with 1132 and 1048 beds (1998 figures throughout

for hospital beds). Hospitals C and D had 802 and 978 beds. The other hospitals had between 300 and 500 beds, except hospital L which had 215. As for the ICUs, the children ICUs and cardiac intensive care units were not included. The hospitals were located from the very south of Sweden up to 300 km north of Stockholm, covering most of the populated parts of the country.

7.2 SUSCEPTIBILITY TESTING

The antibiotic susceptibility of clinical isolates was determined using the disk diffusion method standardized according to SRGA, the Swedish Reference Group for Antibiotics, (http://www.srga.org) [116] with interpretations adjusted for species groups [117]. The clinical strains were inoculated onto Ovoid Iso-Sensitest Agar (Oxoid, Basingstoke, Hampshire, UK) or onto PDM agar (AB Bio disk, Sweden). Antibiotic disks were purchased from Oxoid or from AB Biodisk. According to SRGA there is practically no difference between results using one medium or the other (http://www.srga.org). Antibiotic disks were placed on the inoculated surface followed by pre incubation at room temperature for 30 minutes and then by overnight incubation at 36°C ±1°C. Inhibition zone diameter values were read by a pair of callipers in millimetres. All laboratories participated in quality control assessments by SRGA, under the Swedish Medical Association, and all laboratories included performed excellently according to these external control parameters.

The susceptibility tests of study 4 were performed by determining MIC using Etest (AB Biodisk). The Etest is a plastic strip containing a predefined gradient of antibiotic concentrations. MICs were read where the ellipse intersected the plastic strip according to the manufacturer's description. MICs were performed on Oxoid Iso-Sensitest Agar (Oxoid). Interpretative susceptibility breakpoints were derived from SRGA.

7.3 PULSED-FIELD GEL ELECTROPHORESIS

Under normal electrophoresis, large nucleic acid particles (above 30-50 kb) migrate at similar rates, regardless of size. By changing the direction of the electric field at a certain frequency (thus

the name Pulsed-field gel electrophoresis), much greater size resolution can be obtained. The smaller nucleic acid pieces are able to re-orient to the new field more quickly than are larger ones. This delay in re-orientation means larger pieces end up migrating down the gel slower than smaller ones. This method is used as a molecular typing method: genomic DNA is cut by restriction enzymes and then run through a PFGE gel giving a specific profile for a specific strain [118] . The *Spe1* restriction enzyme was used [119]. DNA fragments were electophoresed in 1% Speakem Gold Agarose in 0.5x Tris-borate-EDTA buffer at 6V/cm for 14h with pulses ranging from 5 to 40 s. Gel pictures were compared PFGE patterns scanned.

7.4 CONJUGATION

Conjugation is a method to pass over genetic material from one bacterial strain to another by mating using a conjugative plasmid. In *P. aeruginosa* only small parts of the chromosome (less than 10% of the genome) are transferred and recombined in conjugation between clinical Pseudomonas strains and strain PAO because of strong restriction systems in PAO (B. Wretlind, unpublished data). We have used conjugation with a genetically well characterized strain (PAO) to elucidate genes contributing to carbapenem resistance. We selected for auxotrophic markers because of high frequency of spontaneous mutations to imipenem resistance.

The conjugative plasmid R68.45 was transferred from *P. aeruginosa* PAO25 (R68.45) to the clinical strains by selection for kanamycin resistance. PAO18SR (proB64, pur-66, strR rifR), and PAO236 (*ilv-226, his-4, lys-12, met-28, trp-6, proA, nalA*) were used as recipient strains [120]. Conjugation was performed as described previously except that the recipient strain was grown at 42^{o}C to overcome restriction [121] with the clinical strains containing plasmid R68.45 as donors. Selection was done for the two markers of PAO18 on minimal agar plates containing streptomycin (1 g/L) and rifampicin (80mg/L). Only conjugants that required either proline or adenine for growth and were resistant to imipenem (2mg/L) were selected for further studies. We

used serotyping to verify the conjugants using a kit from Bio-Rad, Marnes-La-Coquette, France. All the conjugants had the same serotype as PAO18.

7.5 STATISTICS

Statistical analysis of the resistance data in article I and II was calculated using the Spearman's rank order correlation. The qRT-PCR results of article IV used a 2 sided t-test and a conventional significance level ($p<0.05$). A clinically significant alteration of gene-transcription levels was considered as corresponding ratios of >2.5 or <0.4. All analyses were performed using Statistica (Statsoft, Tulsa, OK, USA).

In study III, logistic regression analysis was used to analyze the trends of *E. coli* resistance to different antibiotics.

7.6 PCR METHODS

Chromosomal DNA was extracted using a DNA extraction kit (QIAamp DNA mini kit, Qiagen, Valencia, CA, USA). The genes of interest were amplified using PCR. Primers for sequencing (Table 2) were designed, based on the active site of the PBPs in *Escherichia coli* and the nucleotide sequence information was obtained from the Pseudomonas genome project (http://v2.pseudomonas.com). Primers were from Thermo Electron, Ulm, Germany. All reactions were run in a total volume of 50 µl with 50 ng of genomic DNA, 20 pmol of each amplification primer, 10 nmol of each deoxynucleotide triphosphate (Sigma), 75 nmol $MgCl_2$, and 1.25 U TaqGold (Applied Biosystems, Foster City, CA). Reactions were heated at 95°C for 10 minutes and subjected to 35 cycles of amplification (1 min of denaturation at 94°C followed by annealing for 30 sec at 58 or 61°C, 2 min of extension at 72°C) before a final extension of 10 minutes at 72°C. The sizes of the PCR products were analyzed on a 1.5% agarose gel and the products were purified using PCR purification kit (PCR Clean Up System, Viogene, Sunnyvale, CA, USA). For sequencing of PBP-3, PCR was performed using a modified mastermix containing 4% of pure dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA).

Table 4. Sequences of primers used for qRT-PCR, PCR and sequencing

Primer	Sequence	Position in gene	Reference	Annealing temperature
Sequencing				
pbp-1b-f	5' -TCG TGA CCA ATC CGG AAA C- 3'	1298-1317	Study IV	61°C
pbp-1b-r	5' -GCG GTG GAC AGG TTG TAG GAG- 3'	1581-1602	Study IV	61°C
pbp-3-f	5' -TAC CTG GCT CAT CGC GAACTG- 3'	865-886	Study IV	61°C
pbp-3-r	5' -GGA TGC CGG TGA GAT CGA G- 3'	1018-1037	Study IV	61°C
Pbp3-seq1	5' -CCT GAA GGT GCC CGG CGT CTA- 3'		Study IV	
Pbp3-seq2	5' -ACC CTG CAG ATC GGC CGC TAC- 3'		Study IV	
pbp-2-a-f	5' -GCC GAG CTA CGA CCC CAA CCT- 3'	858-879	Study IV	61°C
pbp-2-a-r	5' -CCA GCA GCG CGG TCA T- 3'	1393-1409	Study IV	61°C
pbp-2-b-f	5' -ATG CCC GAC ATC GTG CTG- 3'	1483-1501	Study IV	61°C
pbp-2-b-r	5' -AGC AGC CAG GCG TCC ATC- 3'	1823-1841	Study IV	61°C
pbp-6-a-f	5' -ACA GCA TCC GCG TGG C- 3'	33-49	Study IV	63°C
pbp-6-a-r	5' -ATC GCG TAG TGG CTC GGC TCA- 3'	717-738	Study IV	63°C
oprD-a-f	5' -ATG AAA GTG ATG AAG TGG AGC- 3'	1-21	[94]	58°C
oprD-a-r	5' -AGG GAG GCG CTG AGG TT- 3'	671-655	[94]	58°C
oprD-b-f	5' -AAC CTC AGC GCC TCC CT- 3'	655-671	[94]	58°C
oprD-b-r	5' -ATA CTG ACC TCT CCT GTT CG- 3'	1329-1310	[94]	58°C
RT-PCR				
Rpsl-f	5' –GCT GCA AAA CTG CCC GCA ACG- 3'	69-89	[94]	60 or 62°C
Rpsl-r	5' –ACC GCA GGT GTC CAG CGA ACC- 3'	318-298	[94]	60 or 62°C
oprD1-f	5' -CGA CCT GCT GCT CCG CAA CTA- 3'	125-149	[94]	60°C
oprD1-r	5' -TTG CAT CTC GCC CCA CTT CAG- 3'	426-406	[94]	60°C
mexB-f	5' -CAA GGG CGT CGG TGA CTT CCA G -3'	507-528	[94]	62°C
mexB-r	5' –ACC TGG CAA CCG TCG GGA TTG A- 3'	779-758	[94]	62°C
pbp-2 –f	5' -GCC CAA CTA CGA CCA CAA G- 3'	1071-1089	[42]	62°C
pbp-2 -r	5' -CGC GAG GTC GTA GAA ATA G- 3'	1179-1161	[42]	62°C
Pbp3-f	5' –TGA TCA AGT CGA GCA ACG TC-3'	1037-1056	[42]	62°C
Pbp3-r	5' –TGC ATG ACC GAG TAG ATG GA-3'	1112-1093	[42]	62°C

7.7 DNA SEQUENCING

Sequencing of the PCR product was preformed with the dideoxy-chain termination method. Cycle sequencing was preformed with the Big Dye Terminator Ready Reaction Kit (Applied Biosystems, Forster City, CA, USA). The temperature profile of the PCR was initial heating at 96ºC 30sek, 25 cycles of 96ºC for 10sek, 50ºC for 5sek and 60ºC for 4 minutes and a final extension at 60ºC for 1 min. The extension products were purified by ethanol/sodium acetate precipitation and the samples were analyzed by electrophoresis in an ABI PRISM 310™ Genetic Analyzer Applied Biosystems, Forster City, CA, USA). All results were confirmed by sequencing of both strands of the gene or part of the gene. The nucleotide sequences were

transcribed into the amino acid sequence using a DNA translation tool from http://www.expasy.org/tools/dna/html. Both the nucleotide sequences and the amino acid sequences were compared between the clinical isolates, conjugants, PAO18SR and the reference gene (from pseudomonas genome project, http://v2.pseudomonas.com/) using the sequence alignment program clustalW (http://www.ebi.ac.uk/clustalw/index.html). Sequencing of *oprD,* and the active sites for the penicillin binding proteins *ponB* (PBP-1a), *pbpA* (PBP-2), *pbpB* (PBP-3) and *dacC* (PBP-6) (Figure 4) was preformed for the clinical isolates, conjugants and PAO18SR. Primers were designed to include the conserved region SXXK of the active site, containing the catalytic nucleophile serine. For PBP2, SXXK and KTG regions were included, and for PBP-3 the whole gene was sequenced.

7.8 QUANTITATIVE REVERSE TRANSCRIPTASE PCR

To evaluate gene expression, we measured the mRNA by real-time PCR. mRNA must first be copied into cDNA by reverse transcription. Real time PCR is a method where the PCR products, during the amplification reaction are continuously measured by use of a fluorescent dye or probe. We used SYBR Green which is a fluorescent dye when bound to double-stranded DNA. The fluorescence is measured at every cycle. When the fluorescence is strong enough, depicting a certain amount of PCR amplicons, the number of cycles is registered as the CT (cycle threshold). The more cycles are used to reach the threshold, the lesser is the amount of amplicons produced at every cycle. The specificity of the amplified products is analysed by use of a melting curve [122].

Total RNA was extracted using a RNA extraction kit (high pure RNA Isolation Kit, Roche). Strains were grown at 37°C over night in LB medium. Next morning the cells were diluted in a fresh culture (1:100) and were gown to a mid exponential phase (OD_{595} 0.5). The bacteria were collected by centrifugation and the cell pellets were resuspended in 10mM tris, pH 8.0. The cells (approximately 10^8) were disrupted with lysozyme (1mg/ml). RNA extraction and purification were performed as described in the manual. RNA concentration was measured using a

spectrophotometer. RNA was stored at –67ºC until used. Total RNA (500ng) from all strains was reverse transcribed into cDNA using 1st strand cDNA Synthesis Kit for RT-PCR (Roche). Until used, the cDNA was stored at -20°C.

Transcription levels of *oprD, pbpA, pbpB* and *mexB* were analyzed using real-time PCR. Gene specific primers were used (Table 4). cDNA for the ribosomal protein S12, Rpsl was used as a reference. All amplifications were done in triplicate using different cDNA preparations except mexB. Analysis of the transcription product was done using an Excel sheet following the Pfaffl equation [123].

8 RESULTS

8.1 RESISTANCE RATES

8.1.1 Resistance rates at Karolinska Hospital

The isolation of various pathogenic bacterial species at the Karolinska Hospital during the 12-year period (1988 to 1999) was analyzed, with regard to both absolute numbers and percentage of the total number of bacterial isolates per year. The general pattern observed was a slight shift from Gram-positive organisms towards Gram-negative species. There was a significant increase over time in the bacterial species *P. aeruginosa*, *Stenotrophomonas maltophilia*, *E. coli*, *Citrobacter freundii*, *Serratia marcescens*, and *Acinetobacter species* ($P < 0.04$ to $P < 0.000002$, Spearman rank order correlation). *Proteus vulgaris* showed a slight but significant decrease ($P < 0.01$). In the ICUs, there were no significant changes over time for percentage occurrence of the common bacterial species. The most common bacterial species registered in 1999 are shown in Table 3 for both the Karolinska Hospital and for its ICUs separately. The increased resistance to ciprofloxacin among *E. coli* and *P. aeruginosa* was particularly evident (Table 5 and 6) at KS. The same increasing resistance trend for *E. coli* and quinolones was also seen in the 12 hospitals (Article III) studied.

Table 5. The most common bacterial species isolated in 1999 at the Karolinska Hospital as a whole and in its ICUs

Organism	KS			ICUs		
	No.	%	Rank	No.	%	Rank
Escherichia coli	3484	21.01	1	103	7.62	4
Staphylococcus aureus	2625	15.83	2	169	12.51	3
Coagulase-negative staphylococci	2174	13.11	3	213	15.77	1
Enterococcus spp.	1796	10.8	4	115	8.51	2
Streptococcus group B	751	4.53	5	23	1.7	14
Haemophilus influenzae	703	4.24	6	73	5.4	5
Klebsiella pneumoniae	678	4.09	7	63	4.66	7
Pseudomonas aeruginosa	643	3.88	8	63	4.66	8
Streptococcus pneumoniae	597	3.60	9	33	2.44	9
Moraxella catarrhalis	514	3.10	10	24	1.78	13
Streptococcus group A	474	2.86	11	6	0.44	25
Enterobacter cloacae	362	2.18	12	66	4.89	6
Proteus mirabilis	285	1.72	13	18	1.33	17
Streptococcus group G	268	1.62	14	15	1.11	18
Klebsiella oxytoca	250	1.51	15	25	1.85	11
Acinetobacter spp.	162	0.98	16	25	1.85	12
Citrobacter freundii	126	0.76	17	10	0.74	19
Bacteroides fragilis	100	0.60	18	7	0.52	23
Stenotrophomonas maltophilia	84	0.51	19	20	1.48	15
Bacillus cereus	77	0.46	20	29	2.15	10
Morganella morganii	68	0.41	21	9	0.67	21
Proteus vulgaris	65	0.39	22	5	0.37	26
Bacteroides spp.	65	0.39	23	3	0.22	27
Serratia marcescens	62	0.37	24	20	1.48	16
Streptococcus group C	59	0.36	25	7	0.52	24
Enterobacter aerogenes	59	0.36	26	10	0.74	20
Bacillus spp.	54	0.33	27	8	0.59	22

8.1.2 Resistance rates for *E. coli*

A marked increase of resistance was seen in *E. coli* for ciprofloxacin, from 0% to 10.5% at the Karolinska Hospital as a whole ($P < 0.000001$, Spearman rank order correlation), and from 0% to 4.8% in the ICUs (Table 6). The lower levels in the ICUs, compared to the whole hospital,

were explained by relatively higher levels in some wards, particularly urology, surgery, rehabilitation and oncology.

Table 6. Proportions of resistance levels for *Escherichia coli* during 1988–99 shown as percentages

Year	Ampicillin S ≥ 30/R ≤ 11		Piperacillin S ≥ 21/R ≤ 17		Cefuroxime S ≥ 19/R ≤ 15		Gentamicin S ≥ 21/R ≤ 17		Ciprofloxacin S ≥ 26/R ≤ 19		Co-trimoxazole S ≥ 17/R ≤ 13	
	KS	ICU	KS	ICU	KS	ICU	KS	ICU	KS	ICU	KS	ICU
1988	19.6	22.6	13.6	5.1	3.4	3.6	1.0	0.0	–	–	7.5	6.0
1989	19.0	19.3	10.4	2.0	3.6	10	0.8	2.0	–	–	7.9	2.9
1990	22.5	23.0	11.9	1.7	5.8	6.8	0.6	0.0	–	–	7.6	3.6
1991	19.5	29.9	7.9	16.0	4.5	6.6	0.0	0.0	0.0	–	4.5	6.7
1992	18.5	25.8	14.3	-[a]	3.4	0.0	0.6	0.0	1.3	0.0	5.9	6.3
1993	21.5	19.2	10.8	–	3.0	1.6	0.5	0.0	2.5	0.0	7.9	1.7
1994	20.9	25.0	15.9	–	4.1	3.3	0.9	1.7	3.8	0.0	9.4	6.8
1995	22.1	21.2	6.7	2.6	4.3	4.3	1.5	0.0	5.1	4.6	11.0	7.6
1996	22.1	17.0	9.1	5.6	4.3	2.8	1.5	0.0	4.8	0.0	11.6	3.0
1997	23.7	24.5	9.5	2.4	3.9	1.2	1.2	1.2	7.0	1.3	11.8	7.8
1998	24.7	20.3	8.2	6.0	5.0	3.5	0.5	0.0	7.2	2.3	13.3	10.6
1999	26.5	28.3	23.7	15.1	5.3	3.5	2.4	2.3	10.8	4.8	14.4	6.2
N[b]	2102	58	343		669	70	439	66			609	68

[a]Number of isolates equal to or below 20.

[b]*N* = mean number of isolates per year.

8.1.3 Resistance rates for *P. aeruginosa*

For *P. aeruginosa* there was an increasing trend for ciprofloxacin resistance in the entire Karolinska Hospital from 2.5% in 1991 to 12.5% in 1999 ($P < 0.01$, Spearman rank order correlation) (Table 7). In the ICUs, there was also an increase, but generally lower than that for the whole hospital, and with fluctuations. An even more marked resistance increase was seen for imipenem (Table 7); this was particularly noticeable in the ICUs, where it fluctuated, but with peaks in 1991–92 and 1998–99, reaching 25% and 28% during the last 2 years. In the medical/surgical ICU, 14 patients in 1998 and 10 patients in 1999 were infected with bacteria that were shown by PFGE analysis to belong to one single clone representing nosocomial outbreaks of imipenem-resistant isolates. It is probable that the rise in resistance in 1991–92 was also due to an outbreak, but data are not available on the clonality of these isolates.

Table 7. Proportions of resistance levels for *Pseudomonas aeruginosa* during 1988–99 shown as percentages

	Piperacillin S≥21/R≤17		Gentamicin S≥21/R≤17		Ciprofloxacin S≥32/R≤24		Ceftazidime S≥23/R≤19		Imipenem S≥23/R≤17	
Year	KS	ICU	KS	ICU	KS	ICU	KS	ICU	KS	ICU
1988	0.6	0.0	0.6	4.4	-[a]	-	1.2	0.0	0.0	-
1989	0.8	2.6	0.4	0.0	-	-	0.4	2.6	-	-
1990	4.4	16	5.9	16	-	-	0.5	0.0	4.6	-
1991	1.7	4.9	2.0	4.9	2.5	0.0	0.4	0.0	2.5	10
1992	1.5	4.1	1.5	1.3	3.6	1.5	0.6	1.4	5.7	14
1993	0.5	0.0	1.8	1.3	8.8	2.9	1.0	1.3	4.3	6.6
1994	0.8	1.5	1.7	1.5	9.7	10	0.6	1.5	6.3	6.2
1995	0.6	1.9	0.9	3.8	7.8	6.4	0.3	0.0	3.7	3.8
1996	0.8	1.7	1.3	1.7	14	2.0	0.5	3.5	1.6	0.0
1997	0.9	2.3	1.6	4.4	8.9	6.2	0.2	0.0	5.0	13
1998	1.2	0.9	1.1	2.8	12	8.9	0.2	0.0	9.0	28
1999	2.2	3.5	1.0	0.0	13	3.9	1.5	2.3	9.9	25
n[b]	377	65	378	65			376	65		

[a] - number of isolates equal to or below 20

[b]- n = mean number of isolates per year

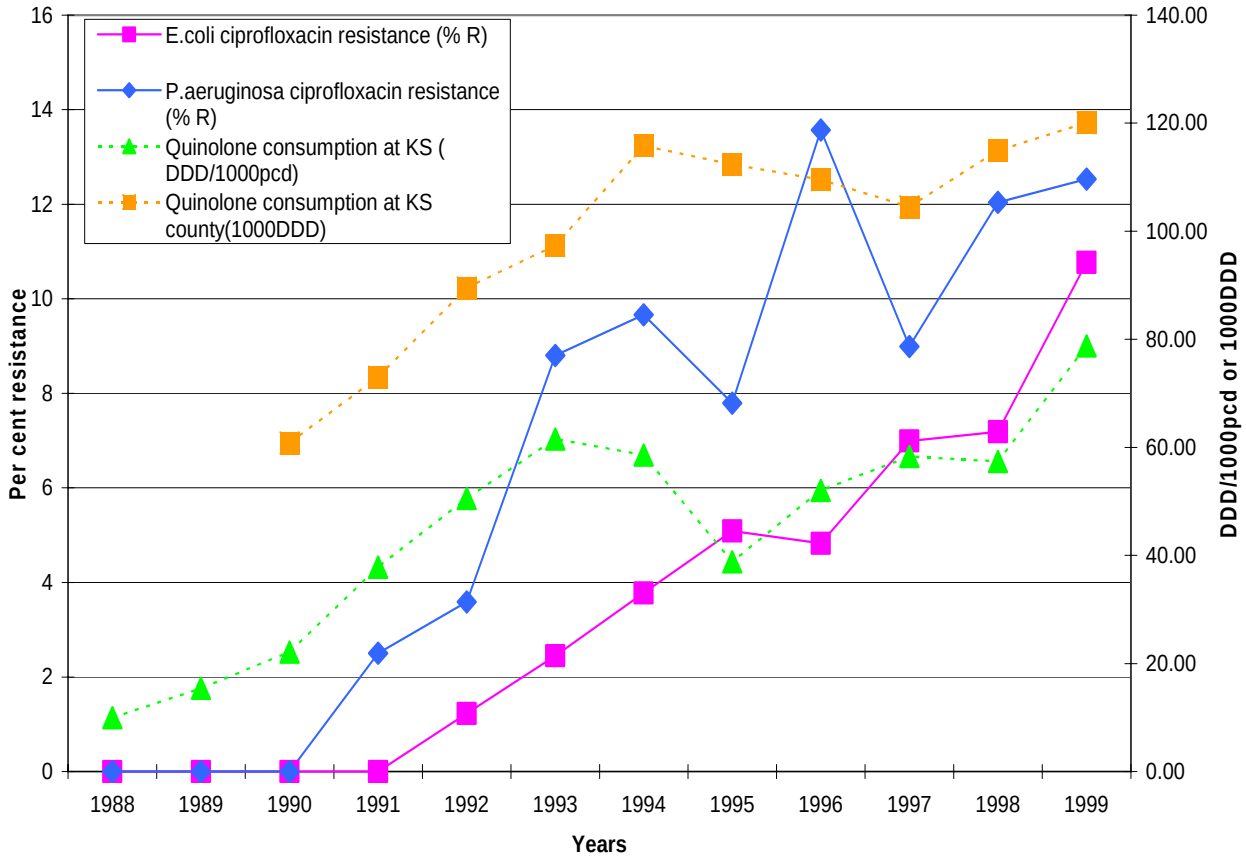
8.2 RESISTANCE RATES IN RELATION TO ANTIBIOTIC USE

In Study I antibiotic consumption was put in relation to antibiotic resistance. We found 3 different patterns:

1. Increased consumption paralleled with increasing resistance rates as in quinolone consumption and resistance (Figure 5).
2. Increasing consumption but stable resistance (Figure 6).
3. Decreased consumption with increasing resistance figures (Figure 7).

8.2.1 Quinolone consumption and resistance

Figure 5. Quinolone consumption and ciprofloxacin resistance in *E. coli* and *P. aeruginosa* at KS



For ciprofloxacin and *E. coli* a marked increase of resistance was seen from zero to 11% ($p<0.000001$, Spearman rank order correlation) (Figure 5). This significant trend was also evident from an analysis of resistance levels during the early years 1991 – 1995. It is apparent that trends in antibiotic resistance might be more important than the actual levels themselves, which might be influenced by the inherent bias of the material. The highest rate of *E. coli* isolates resistant to ciprofloxacin was found at the urology ward. Ciprofloxacin resistant *E. coli* originated from 202 patients in 21 departments during the study period: 50 in urology, 21 in

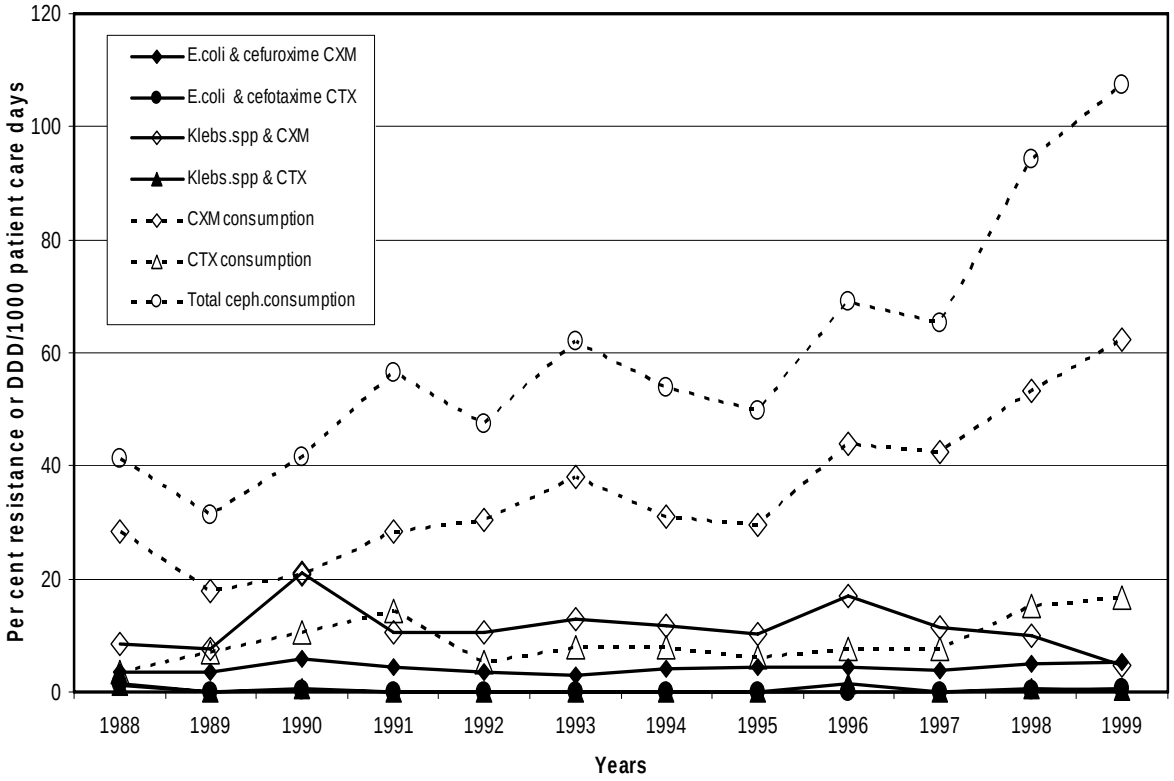
surgery, 23 in rehabilitation and 17 in oncology. During 1999 the consumption of quinolones was high in all these clinics.

Among *P. aeruginosa*, there was an increasing trend for ciprofloxacin resistance at the entire KS, from 2.5% in 1991 to 12.5% in 1999 ($p<0.01$ Spearman rank order correlation) (Figure 5). Ciprofloxacin resistant *P. aeruginosa* were isolated in 242 patients originating from 21 departments during the study period: 43 in urology, 29 in rehabilitation, 21 in dermatology, 21 in surgery and 24 in intensive care units.

Ciprofloxacin resistance rates in *Klebsiella spp* increased from 0.7 to 4.0%, and for *P. mirabilis* resistance to ciprofloxacin increased from zero to 5.6%. Resistance levels for *Stenotrophomonas maltophilia* fluctuated for ciprofloxacin between 10 and 20% (1992-1999). Ciprofloxacin resistance was uncommon among Enterobacter spp except during occasional years, but never exceeding 4.4%. For *H. influenzae* ciprofloxacin resistance was rare and below 1% at KS during the study period.

8.2.2 Consumption of ß-lactam and resistance

Figure 6. Total cephalosporin consumption and resistance in *E. coli* and *K. pneumoniae* at KS



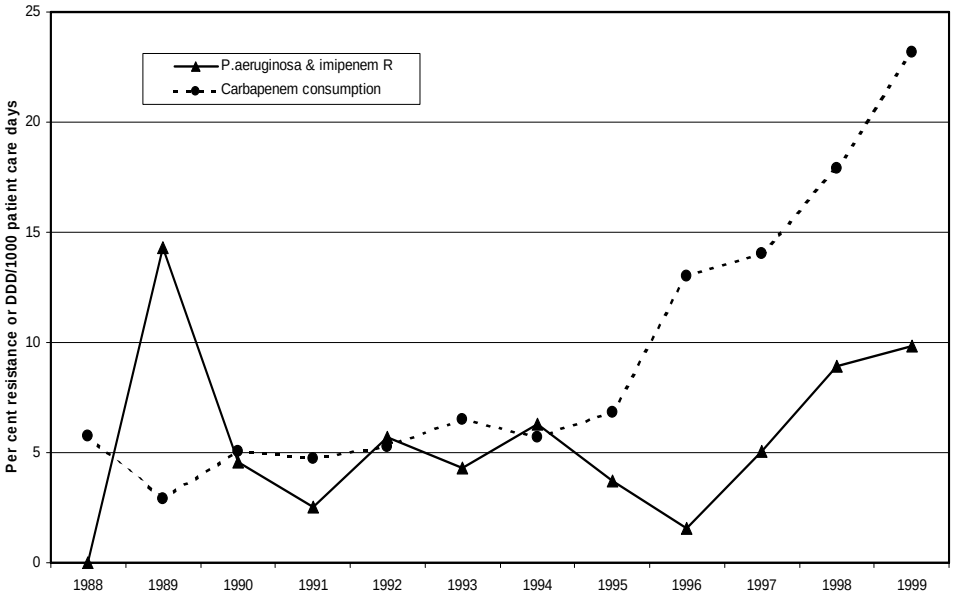
Increasing volumes of cephalosporins were prescribed during the study period, from 40 DDD/1000pcd to above 100 DDD/1000pcd (Figure 6). About 60 per cent of the cephalosporin consumption was due to cefuroxime which is the preferred prophylactic drug in abdominal surgery and is often used in the treatment of pneumonia (Figure 6). The second most often prescribed cephalosporin was cefotaxime but it constituted only 15% of the total cephalosporin amount used (Figure 6). The use of piperacillin with or without tazobactam also increased several fold over the study period but was still relatively sparingly prescribed (9 DDD/1000pcd in 1999).

For *E. coli*, ampicillin, cefuroxime and gentamicin resistance rates were essentially fluctuating at around 22, 4.0, and 1.5% respectively. Piperacillin resistance rates were varying between different years, but with an increase in 1999. Cefotaxime and ceftazidime resistance rates at KS never exceeded 1.3%. Klebsiella spp. resistance rates to cefuroxime at KS varied between 5.0%

and 16%. For ceftazidime and cefotaxime, the number of isolates was too low to permit analysis**.** No resistance was seen to cefotaxime for *P. mirabilis* at KS. Among *P. aeruginosa*, the resistance to ceftazidime never exceeded 1.5% at KS during the study period. No resistance was found among S. maltophilia for ceftazidime most of the years, except for occasional resistant strains giving per cent figures up to 5.6. Resistance to cefotaxime for *Enterobacter spp.* at KS reached 25% some years with a fluctuation between 5.8 to 25% during the study period. Ceftazidime resistance for *Enterobacter spp.* at KS rose from around 10% the first three years to around 20% the last four years. Ampicillin resistance among *H. influenzae* varied between 10 and 19% the last ten years. Resistance to cefuroxime was generally low, below one per cent except for 1996 (3.2%) and 1997 (1.4%) at KS.

8.2.3 Imipenem consumption and resistance

Figure 7. Carbapenem consumption and resistance at KS



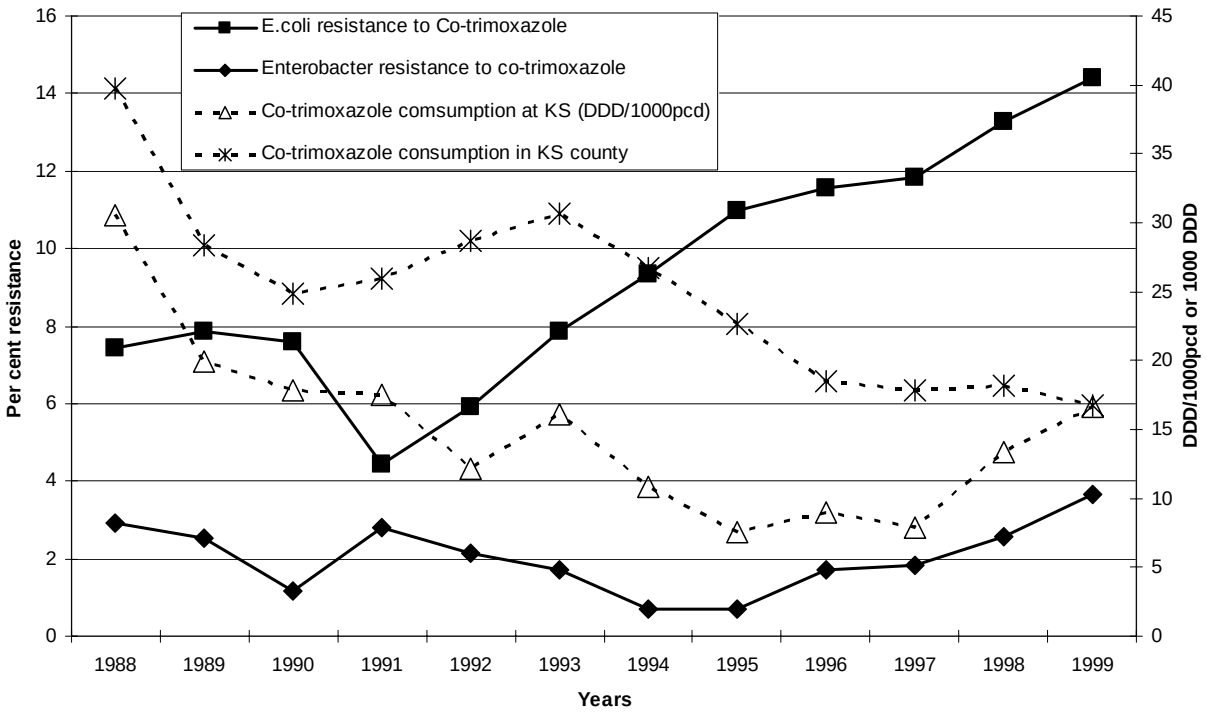
Carbapenem (imipenem and meropenem) use was quite stable until 1995 and then increased fourfold to about 23 DDD/1000pcd in 1999 (Figure 7). Imipenem resistance levels for *P. aeruginosa* were variable and around 5% in 1990-1994 and then rose from 2% in 1996 to 10% in 1999 (Figure 7). Imipenem-resistant *P. aeruginosa* strains were isolated in 196 patients originating from 24 departments: 17 in neurosurgery and 85 in intensive care units. In the medical/surgical ICUs, 14 patients in 1998 and 10 patients in 1999 were infected with bacterial strains that were shown with PFGE analysis to belong to one single clone and constituted one single nosocomial outbreak. No resistance to imipenem was seen among *E. coli*, *Klebsiella sp*p, or *P. mirabilis* isolates.

8.2.4 Co-trimoxazole consumption and resistance

The use of co-trimoxazole has been decreasing from 30 to below 10 DDD/1000pcd but increased since 1997 to over 15 DDD/1000pcd (Figure 8). The use of co-trimoxazole in the Karolinska county has also decreased from 42,000 DDD in 1988 to 17,000 DDD in 1999 (Figure 8). Co-

trimoxazole resistance levels increased for *Escherichia coli* from 7.5 to 14% (Figure 8). For co-trimoxazole and *Klebsiella spp* resistance levels showed a fluctuation independent of time between zero and 7.3% at KS. For co-trimoxazole and *P. mirabilis* resistance levels fluctuated between 1.6 and 11%. Few *Stenotrophomonas maltophilia* isolates were tested with co-trimoxazole before 1996. Between 1997 and 1999, co-trimoxazole resistance was below 10%. For co-trimoxazole and Enterobacter spp there was a downward trend from 1989 to 1994 (Figure 8). After that time, there was an increasing trend up to 3.7% (Figure 8). Co-trimoxazole resistance among *H. influenzae* was quite stable at the 10% level at KS.

Figure 8. Co-trimoxazole consumption and resistance in *E. coli* and *Enterobacter spp*



In study III, where 12 hospitals were included, the same patterns could be observed for *E. coli*, trimethoprim and co-trimoxazole, with increasing resistance in spite of decreasing consumption, as well as for *E. coli* and quinolones, with resistance increasing in parallel with consumption.

8.3 PSEUDOMONAS AND PBP

8.3.1 Conjugation

True conjugants, auxotrophic for at least one of the markers of PAO18SR or PAO236, had the same serotype as PAO and grew on imipenem containing plates were selected, and their MICs for imipenem, meropenem, ciprofloxacin and ceftazidime were tested (Table 8). Of the 13 selected clinical strains only 3 produced imipenem resistant conjugants after selection for *proB* in PAO18SR: CG1, CG2 and CG13. The MICs of imipenem of the conjugants ranged between intermediate (4 mg/L) to highly resistant (24 mg/L). The purine marker of PAO18 or the *proA* marker of PAO236 gave no imipenem resistant recombinants.

Table 8. MICs of clinical strains of *P. aeruginosa* and their conjugants

Strain	Sero-type	MIC imipenem (mg/L)	MIC meropenem (mg/L)	MIC Ceftazidime (m/L)	MIC Ciprofloxacin (mg/L)
PAO18	PME	0.75	0.38	1	0.094
CG 1	PMA	32	4	1,5	0.125
1a	PME	24	2	0.5	0.64
1b	PME	24	1.5	0.5	0.64
1c	PME	32	2	0.5	0.64
CG2	PMA	16	2	1	0.125
2a	PME	>32	4		
2c	PME	24	4		
2e	PME	32	6		
2f	PME	32	4		
2g	PME	24	2		
2h	PME	4	1		
CG 13	NT	32	1	2	0.016
13 a	PME	16	2	1	0.064
13 c	PME	24	2	1.5	0.64
13 e	PME	24	1.5	1	0.064
13 f	PME	24	2	1	0.064

NT, non-typable

MIC breakpoints according to EUCAST (European Committee on Antimicrobial Susceptibility Testing): IP(S≤4,R>8), MP(S≤2,R>8), TZ(S≤8,R>8), CI(S≤0,5, R>1)

8.3.2 Sequencing

The active sites of the penicillin-binding proteins located close to both markers were sequenced. We focused on the SXXK box holding the active site serine. The penicillin-binding proteins that were sequenced were *ponB* and *pbpB,* located close to *proB.* We also sequenced *pbpA* and *dacC*. No significant mutations were seen, neither in the clinical strains, in the conjugants nor in the reference strain, although some of the clinical strains had variations in the third codon (Table 9). In order not to miss mutations outside the conserved region that could have an impact on the active site, the complete *pbpB* genes of strains PAO18SR, CG13 and conjugant 13c were sequenced. Only silent mutations in CG13 were found, resulting in no amino-acid changes.

Table 9. Sequencing of penicillin-binding proteins in clinical strains and conjugants

	Imipenem MIC mg/L	**PBP-1b**	**PBP-2**	**PBP-3**	**PBP-6**
CG1	32	**	**	**	**
1b	24		*		
1c	32	*	*	**	*
CG2	16		**		**
2c	24		*		
2e	32	*			
2f	32	*	*		
CG13	32	**	**	**	*
13c	24	*	*	*	*

* = same nucleotide sequence as PAO18
** = same amino acid sequence as PAO18

Imipenem resistance has been shown to correlate with alterations in the OprD porin [124]. Therefore the *oprD* gene was sequenced for the different strains. All of the clinical isolates had mutations that could explain some of their resistance patterns. However, none of the conjugants had mutations indicating that this part of the bacterial genome was not transferred during the conjugation.

8.3.3 RT-PCR

The expression of *oprD, pbpA, pbpB* and *mexB* was measured using real-time PCR (Table 6). *oprD* was down regulated in all of the clinical strains and in all conjugants. The clinical strain CG1 had upregulated *pbpA* and *pbpB,* however, all other strains had normal levels of the corresponding mRNAs. *mexB* was slightly down regulated in two conjugants.

9 DISCUSSION

9.1 CAN ROUTINE RESISTANCE RESULTS BE USED AS A RESISTANCE WARNING SYSTEM?

In paper I, II and III we used routine clinical microbiology resistance results of tested unselected clinical isolates. The isolates used came from different types of specimens. To circumvent this, we compared our results with blood isolate figures where the sample material is homogenous. We could see that the resistance trends in blood isolates were quite similar to our initial material (Paper III). In paper III, data was retrieved from several hospitals: different laboratories could have used different measuring routines, have different laboratory traditions in test performance, and changes might have happened over time within the same laboratory. To be able to compare results over time, a rigorous quality control is required, and even more so if comparisons are being made between laboratories. In Sweden, very strict guidelines exist (SRGA) and they are also followed nationwide. There is quality assessment schemes followed on a regular basis, and laboratory results are therefore comparable. Despite these drawbacks, routine data was relatively easy to retrieve, and even more so when all hospitals have been using the same data software and similar registration codes for several years. The number of isolates included is therefore much larger than it would be in a prospective study.

Retrospective studies of the present kind are subject to bias in terms of the material studied. This was apparent in paper I, from the differences in absolute levels of resistance to ciprofloxacin and norfloxacin. This difference was even more marked because the interpretative breakpoints used for the two quinolones would favour interpretations of resistance to norfloxacin. In the present material ciprofloxacin was tested in fewer, more complicated cases and resistance levels were therefore exaggerated. However, the built-in bias of the material was stable over the study period and the results were therefore comparable on a temporal basis. The important conclusion to be drawn from our results is that, regardless of the absolute levels of resistance, changes over time

are highly significant. We observed a significant trend in ciprofloxacin resistance among *E. coli* isolates and this trend was apparent also for the period 1991–95 when the resistance level rose to a mere 5%. Routine data however, can not be used as a basis for absolute resistance figure calculations, but rather to study temporal profiles in individual hospitals. Different trends can be seen over time that could be used as indicators of further analysis and as a warning system for upcoming resistance. Similar conclusions have been drawn by Livermore et al. [125], where they compared trends in resistance among *E. coli* for trimethoprim and ciprofloxacin in routine data reported to the Public Health Laboratory Service (PHLS) with the ones performed at the PHLS, and found them to be comparable.

We conclude that significant changes with time should be recognized regardless of the level of resistance. Routine susceptibility results obtained in a clinical microbiology laboratory constitute an important source of information for the detection of an emerging increase in resistance. This kind of information should therefore be collected regularly for use as a warning system for the detection of antibiotic resistance.

9.2 ARE ICUS ALWAYS THE HOT-SPOT FOR RESISTANCE EMERGENCE AND SPREAD?

From an international perspective, the prevalence of infections is usually higher in ICUs than in other wards, and nosocomial outbreaks are also more frequent in ICUs [51].

The most important result in study II was the demonstration of differences in resistance levels between the whole hospital and the ICUs. Antibiotic consumption is consequently relatively high in ICUs [126]. Because of these factors, selection of resistant strains is expected, and hence many antibiotic resistance surveillance studies have been carried out in ICUs only [43]. Only a few studies have compared antibiotic resistance in ICUs and that in other wards [127, 128]. Therefore, longitudinal studies, comparing ICUs with the whole hospital, are of value.

For some antibiotic and bacterial species, the resistance levels were high in the ICUs, but even higher in other parts of the hospital. Antibiotic resistance surveillance in the hospital should therefore cover all wards. In article III, there were too few isolates from the ICUs to permit analysis. A recent study done by Erlandsson et al. encountered the same problem, the number of cultures taken was too low to provide data on species distribution and susceptibility [16].

The findings of less antimicrobial resistance for some antibiotic and bacterial combinations in the ICUs compared to the whole hospital might have several explanations. Our ICUs are characterized by short stays, and a majority of the cases are hospitalized directly after emergency admittance. Isolation care in general is essential to prevent nosocomial transmission of infection and colonization with resistant bacteria, and detailed guidelines are available [129]. For the nursing of potentially contagious cases, the importance of good-quality barrier nursing[130], preferably in single rooms [131], has been well documented as a means to avoid the spread of resistant organisms. Cross-transmission of multi-resistant micro organisms is common, particularly in ICUs [132]. If transmission occurs, this is an indicator of poor quality of nursing care, nursing overload and crowding being most important [133]. At the Karolinska Hospital, the reasons for lower resistance rates may be sought among these circumstances. Antimicrobial regimens were available and fairly strictly followed, and most therapy was supervised daily by an infectious disease consultant. The ICU patients also seem to be well protected from transmission of infection/colonization by good barrier nursing, except during periods of overcrowding and understaffing.

Many published studies on antibiotic resistance cover only shorter time periods [43, 128, 134-136]. We found large fluctuations over time in our study, indicating that it is important to perform antibiotic resistance surveillance studies over longer time periods. This is especially important for ICUs, where the fluctuations seem to be more pronounced. These fluctuations might be explained partly as a result of outbreaks of nosocomial infections. The two outbreaks

of imipenem-resistant *P. aeruginosa* in the medical/surgical ICU occurred during periods of overcrowding and understaffing at the Karolinska Hospital. Moreover, previous publications have given the results of multi center studies, with the data pooled from different centres [43, 125, 134-136]. Other studies have indicated that a detailed analysis of the source of the data might provide a more differentiated view of resistance emergence [128]: registered data of MDRO from units outside ICUs have been found at rates as high as ICUs [137, 138]. Our data concerning ICU in relation to the whole hospital support this view. Further analysis of antibiotic resistance in ICUs should be performed at the Karolinska Hospital in order to answer these questions.

P. aeruginosa is a leading cause of nosocomial infections [65]. Nearly half of the imipenem-resistant *P. aeruginosa* strains were isolated from the ICUs, which contained only 6% of the hospital beds. The high prevalence of imipenem-resistant *P. aeruginosa* among isolates from the ICUs has been reported by other investigators [65]. The high ICU resistance rates of 28% in 1998 and 25% in 1999 in our studies were found to be due to an accumulation of isolates from nosocomial outbreaks in the medical/surgical ICUs.

9.3 ANTIBIOTIC CONSUMPTION AND RESISTANCE: IS THE RELATION ALWAYS PARALLEL?

The increased ciprofloxacin resistance in *E. coli* and *P. aeruginosa* during the study period in paper I was paralleled by an increased consumption of quinolones (Figure 5). The parallel increase by time between consumption and resistance was evident also from another point of view. Wards with the highest consumption had the highest levels of resistance and wards with the lowest consumption had the lowest levels of resistance. There are only a few studies on the relationship between antibiotic consumption and antibiotic resistance over longer time periods and a causal relation between antibiotic consumption and antibiotic resistance is often difficult to

prove [2, 139, 140]. The association between the use of antibiotics and antimicrobial resistance levels in the community is supported by other investigators [1-3, 141, 142]. Despite the paucity of studies on the relationship between antibiotic consumption and antibiotic resistance there is a belief that reduction of antibiotic consumption would favour a decrease in antibiotic resistance prevalence. Our data in studies I and III show a more complicated situation with different patterns and associations between antibiotic use and antibiotic resistance. In paper I, the total use of cephalosporins increased two and a half times, while the resistance levels of *E. coli* resistant to cefuroxime and cefotaxime remained stable (Figure 6). In contrast increasing co-trimoxazole resistance in *E. coli* was paralleled by decreasing use of co-trimoxazole in both studies I and III. There could be several explanations to the different patterns seen in our studies. Co-trimoxazole is largely used in children, where the dose "per head" is lower. Furthermore most of the antibiotics are used in the community and not in the hospitals [136]. However, in the case of co-trimoxazole the decreased consumption in the hospital was paralleled by a similar decrease in the surrounding county. Explanations for the findings could also be sought in the fact that there is linkage between several genes for resistance to different antibiotics[143, 144] .Therefore, an increased use of one antibiotic could cause an increase in antibiotic resistance to another antibiotic.

Antibiotic consumption was measured in DDD per patient day in paper I and only DDD in paper III. There are obvious limitations to the accuracy of the DDD per patient day figures; some antibiotics delivered to the wards may not have been used, other dosages than those defined by DDD may have been practiced, especially on the paediatric wards and several patients were certainly treated with two or more antibiotics concurrently. Nevertheless, the use of antibiotics is massive and ecological effects on the hospital micro flora, including emergence of antibiotic resistance and a shift to increased prevalence of more resistant bacterial species can be

anticipated and was also noted in our as well as in many other studies. It has been emphasized that a strict antibiotic policy is necessary for the control of antibiotic resistance [4].

9.4 IMIPENEM RESISTANCE IN PSEUDOMONAS

9.4.1 PBP

The penicillin-binding proteins PBP-2 and PBP-3 have been mentioned to be involved in carbapenem resistance in *E. coli* [145]. We sequenced the active sites of *ponA* (corresponding to PBP-1a), *pbpA* (PBP-2), *dacC* (PBP-6) and the whole *pbpB* gene (PBP-3), however no mutations were found in the clinical strains or in the conjugants that could explain imipenem resistance. In a study by Legaree [108], it was shown that mecillinam in concentrations between 200 and 400 mg/L as well as mutations in *pbpA* causes spherical cells. In our study absence of morphological changes in the recombinants growing in the imipenem restriction zone in Etest (data not shown) indicates lack of alterations in PBP-2.

Expression of PBP-2 and PBP-3, was decreased in all strains compared to PAO18SR, except for the clinical strain CG1 were there was a clear increase in both PBPs. The resistance pattern of CG1 and its conjugant 1c were very similar (Table 8), so the increased expression of the genes for PBP-2 and PBP-3 is probably not important for resistance to carbapenems.

Over expression of genes for the MexAB-OprM efflux pump contributes multidrug resistance including meropenem. Since the clinical strains and conjugants had increased MICs of meropenem, we determined the expression of *mexB* which was down regulated in all analyzed conjugants and thus not involved in meropenem resistance seen in our conjugants.

9.4.2 OprD

The clinical strains included here had alterations in the porin OprD that might have an impact on imipenem susceptibility, however the conjugants had the same sequence as PAO18SR, demonstrating that the *oprD* gene was not transferred during the conjugation.

The most important mechanism of resistance to imipenem in clinical strains is decreased production of OprD, and loss of OprD raises the imipenem MICs from 1-2 mg/L to 8-32 mg/L [93]. Decreased transcription of *oprD* was found in both clinical strains and conjugants explaining imipenem resistance and probably also the increase in MIC seen for meropenem. This indicates that down regulation of the porin gene alone is enough to induce high level of imipenem resistance. OprD is regulated by multiple systems and is repressed by salicylates, subject to catabolite repression, and activated by arginine and a variety of other amino acids [95]. MexT (PA2492) is a transcriptional repressor that down-regulates *oprD* and up-regulates genes for efflux pump MexEF-OprN (so called nfxC class mutants). Mex EF-OprN efflux pump mediates resistance to several antibiotics, including quinolones. *mexS* (PA2491) and *mvtA* have similar effects [97, 98], but none of them are close to the *proB* marker. None of our recombinants was resistant to ciprofloxacin indicating that these regulator genes were not affected; also, none of the clinical strains had any significant increase of *mexF* [94]. We propose that one or more regulatory genes for *oprD* are located close to the *proB* gene (PA4565 at 5113 kb). Further experiments to identify the responsible gene(s) are in progress.

10 CONCLUSIONS

We demonstrated that routine microbiology data analysis is an easy and reliable way to note and estimate resistance trends. Regardless of the absolute level of resistance, changes over time are highly significant. Routine susceptibility results obtained in a clinical microbiology laboratory constitute an important source of information for the detection of an increase in resistance, working as an early warning system.

We found that relationships between antibiotic use and antibiotic susceptibility showed different patterns at KS. Ciprofloxacin resistance of *E. coli* and *P. aeruginosa* increased during the study period paralleled by an increased consumption of quinolones. The total use of cephalosporins increased 2.5-fold, while the levels of *E. coli* resistance to cefuroxime and cefotaxime remained stable. A third pattern was seen with co-trimoxazole, namely increasing resistance of *E. coli* as the use of co-trimoxazole declined. The analysis of resistance levels and antibiotic consumption in the present study indicates that their relation is not parallel, and that more complex mechanisms are involved.

We also found, that resistance rates at the Karolinska Hospital are still generally low, but increasing resistance rates were seen for some antibiotic–microbe combinations in recent years. For some antibiotic and bacterial species, the resistance levels were high in the ICUs but even higher in other parts of the hospital, emphasizing the importance of including all sectors of a hospital in resistance surveillance studies. There were considerable fluctuations in resistance prevalence during the study period, especially in the ICUs, illustrating the value of long surveillance periods.

Furthermore, in order to determine if the results found at KS with increasing resistance levels in *E. coli* for quinolones and co-trimoxazole were just a local phenomenon or a part of a general trend in Sweden, we extended our surveillance study to include 12 Swedish hospitals. We found that increase in norfloxacin and ciprofloxacin resistance in *E. coli* started relatively

simultaneously in all hospitals, in parallel with an increase in quinolone consumption. Co-trimoxazole resistance has been increasing significantly in most hospitals, in spite of decreasing sales of co-trimoxazole and trimethoprim. These two findings were in accordance to the findings at KS. We can also conclude that clinical laboratory data are subject to bias but surveillance over longer periods of time provides a useful method for detecting trends in increasing resistance and therefore functions as a resistance warning system.

Finally, high resistance rates were seen for imipenem in *Pseudomonas aeruginosa* at the ICU at KS. In order to assess the role of the membrane protein OprD and penicillin-binding proteins in *P. aeruginosa* resistance to imipenem, we produced transconjugants from clinical isolates of carbapenem resistant P. aeruginosa in a sensitive PAO18 after selection for a proline marker (*proB*). Sequencing of P. aeruginosa genes for PBP1b, PBP2, PBP3 and PBP6 showed no differences in amino acid sequence, but the OprD porin was down regulated in all imipenem resistant clinical strains and their transconjugants. We believe a previously unknown gene for regulation of *oprD*, is most likely located close to the *proB* marker.

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