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Antibiotic Resistance and Population Dynamics of *Escherichia coli* in Relation to a Large Scale Antibiotic Consumption Intervention

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Abstract
Sundqvist, M. 2010. Antibiotic Resistance and Population Dynamics of *Escherichia coli* in Relation to a Large Scale Antibiotic Consumption Intervention. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 512. 78 pp. Uppsala. ISBN 978-91-554-7700-4.

Antibiotic resistance challenges the practice and development of modern medicine. The aim of this thesis was to test the hypothesis that antibiotic resistance is reversible once the selection pressure of an antibiotic is removed. A decisive reduction (85%) in trimethoprim and trimethoprim-sulfamethoxazole over 24 months in Kronoberg County, Sweden, is described. The resistance baseline prior to the intervention and the effects of the intervention on resistance levels, trimethoprim resistance genes (*dfr*-genes) and population structure in *Escherichia coli* were studied.

The effects of different algorithms for excluding patient duplicate isolates were small but systematic. An identical algorithm was used throughout.

The drastic decrease in the use of trimethoprim containing drugs did not result in a corresponding decrease in trimethoprim resistance. This was true both for total trimethoprim resistance and for trimethoprim mono-resistance. The distributions of *E. coli* phenotypes, *dfr*-genes and *E. coli* sequence types were stable. The marginal effect on resistance rates was explained by a low fitness cost of trimethoprim resistance observed *in vitro* and the high levels of associated resistance in trimethoprim resistant isolates.

Trimethoprim resistance was, although widespread in the *E. coli* population, more common in certain *E. coli* sequence types. The distributions of *dfr*-genes were different in *E. coli* and *K. pneumoniae* and between different *E. coli* sequence types. These results indicate mechanisms related to the genetic back-bone of *E coli* to be important for the acquisition and persistence of antibiotic resistance.

The findings of this thesis indicates that, at least for some classes of antibiotics, we may have overestimated the usefulness of a strategy for reversing antimicrobial resistance based on the fitness cost of resistance. We have equally underestimated the conserving effects of associated resistance. The stability of the *dfr*-genes and *E. coli* sequence types underlines the importance of associated resistance and successful lineages in the spread and maintenance of antibiotic resistance in *E. coli*.

Keywords: reversibility, trimethoprim, dfr, associated resistance, MLST

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Sundqvist M**, Kahlmeter G. (2007) Effect of excluding duplicate isolates of *Escherichia coli* and *Staphylococcus aureus* in a 14 year consecutive database. *Journal of Antimicrobial Chemotherapy* **59:** 913-18

II **Sundqvist M**, Geli P, Andersson DI, Sjölund-Karlsson M, Runehagen A, Cars H, Abelson-Storby K, Cars O, Kahlmeter G. (2009) Little evidence for reversibility of trimethoprim resistance after a drastic reduction in trimethoprim use. *Journal of Antimicrobial Chemotherapy* Nov 8 (e-pub ahead of print)

III Brolund A, **Sundqvist M**, Kahlmeter G, Grape M. Molecular Characterisation of Trimethoprim Resistance in *Escherichia coli* and *Klebsiella pneumoniae* during a 2 year intervention on Trimethoprim use. (Submitted)

IV **Sundqvist M**, Granlund S, Naseer U, Brolund A, Sundsfjord A, Kahlmeter G, Johansson A. Persistent Endemicity of Trimethoprim Resistant Uropathogenic *Escherichia coli* despite a large scale antibiotic Intervention. (In manuscript)

Contents

Abbreviations

ATCC	American Type Culture Collection
CDC	Center for Disease Control
CGA	Clonal Group A
CLSI	Clinical Laboratory Standards Institute, (former NCCLS)
DDD	Defined Daily Doses
dfr	Gene encoding for trimethoprim resistance
ECDC	European Centers for Disease prevention and Control
ECOFF	Epidemiological Cut-off
ESBL	Extended Spectrum β-Lactamase
EUCAST	European Committee on Antibiotic Susceptibility Testing
IDSA	Infectious Diseases Society of America
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PHC	Primary Health Care
PNSP	Penicillin Non-Susceptible Pneumococci
SNP	Single Nucleotide Polymorphism
SRGA	Swedish reference Group on Antibiotics
strama	The Swedish strategic programme against antibiotic resistance
TIND	Thousand Inhabitants and Day
UTI	Urinary Tract Infection
Susceptibility categories	
S	Susceptible
I	Intermediate
R	Resistant
Antibiotics	
AMP	Ampicillin
CFR	Cefadroxil
FQX	Fluoroquinolone
MEC	Mecillinam
NAL	Nalidixic acid

NIT	Nitrofurantoin
SXT	Trimethoprim-Sulphamethoxazole
TMP	Trimethoprim

Introduction

Antibiotic resistance challenges the current practice and further development of modern medicine. [1] The relation between antibiotic use and increasing antibiotic resistance is well established. [2-4] The direct link between the use of a specific antibiotic and resistance has been shown in some cases [5-8] but not in all. [7-10] Studies on antibiotic resistance in bacteria from bloodstream infections, [11] respiratory tract infections [2] and from uncomplicated UTI [12, 13] have shown pronounced variation in antibiotic resistance in Europe with the highest figures in the Mediterranean countries and the lowest in Scandinavia. The Sentry program collecting clinical isolates from all over the globe has shown high resistance rates in *Escherichia coli* from South America and Asia while levels are lower in Europe and North America [14, 15] The prevalence of antibiotic resistance in a community seems to be determined by the volume of antibiotic use and factors affecting the spread of bacteria like crowding [16] and low economical standards [17, 18] together with bacterial characteristics. [19-21]

Several national and international organisations are developing strategies where the prudent use of antibiotics in both humans and animals and the importance of hygiene measures in health care facilities are promoted (WHO, EU, CDC, ECDC, IDSA). The adopted measures have been reported successful to varying extent in keeping resistance levels under control. [7, 22] There are however so far conflicting evidence on whether antibiotic resistance is reversible in any practical sense. [23-29]

This thesis deals with the background and practice of reversibility of antibiotic resistance in a community where the use of an antibiotic was drastically reduced. All four papers are related to a large scale intervention on the use of an entire class of antibiotics, drugs interfering with folic acid synthesis in bacteria (trimethoprim and the combination with sulphamethoxazole). The baseline, i.e. antimicrobial resistance prior to the intervention, and factors influencing the baseline was thoroughly characterized and the effect of the intervention on resistance levels, genes and population structure in *E. coli* was studied.

Antibiotic resistance – basic concepts of development, spread and reversibility

Bacteria can acquire antibiotic resistance through mutations, [30] or through incorporation of DNA from other bacteria or occasionally from the milieu. [31] Long before the introduction of antibiotics for treatment of infections environmental bacteria had developed antibiotic resistance mechanism in response to the presence of antibacterial substances produced by other organisms in their environment or by themselves. [32] In clinical isolates from the pre-antibiotic era (the Murray collection) plasmids able to transfer genetic elements by conjugation were present [33, 34] but antibiotic resistance was not expressed. [32] The introduction of resistance mechanisms in bacteria of direct interest to man, the development of new resistance mechanisms and the spread of these mechanisms thus seems to have occurred during the last 70 years. [32]

Several antibiotics will, through their action, increase the probability of an uptake of (resistance) genes in a bacterium. This may occur by transformation, [35] the induction of plasmid and transposon transfer [36] or by stimulating the stress response system (SOS-response), leading to an increased exchange of antibiotic resistance genes. [37] Mutational resistance is usually developed through a series of point mutations, enhanced by the presence of antibiotics, leading to different levels of reduced susceptibility. [38, 39] This may occur shortly after the introduction of new antibiotics. [40]

The development of antibiotic resistance in bacteria will be driven by antibiotic use but several biological mechanisms contribute to this development. The basics of these mechanism will be reviewed and then further discussed in relation to the results of the thesis in the general discussion.

A bacterium will acquire antibiotic resistance as a response to the environment. Although *mutations* will cause antibiotic resistance in some cases [30] the most important mechanism for the acquisition of antibiotic resistance is *horizontal gene transfer (HGT)*, in Enterobacteriacae mainly mediated by conjugation. [31] The conjugative plasmids responsible for the spread of antibiotic resistance usually contains an integron structure flanked by a transposon. [41] These *integrons* always consists of an *Int*l-gene encoding the integrase that catalyzes the integration and excision of the *gene cassettes* encoding antibiotic resistance situated downstream the *Int*l-gene. A continuous exchange of gene cassettes both within and between integrons occurs. [42] The acquisition of the mutation/resistance gene might impose a *fitness cost*, usually measured as a decreased growth rate, on the bacterium. [43-49] The fitness cost will be deleterious to the newly resistant strain unless exposed to a continuous positive *selection* [46] leading to a non-competitive close environment and/or a rapid development of compensatory mutations. [50, 51] The positive selection pressure will additionally contribute to the dissemination of the resistance gene both through clonal expansion and through spread to new bacteria

of the same or other species. [52] The clonal expansion within the host will increase the probability of further successful *spread* to other hosts. [52] To become a clinically significant and successful resistance mechanism, it should concern an important antibiotic and ideally be introduced into a pathogen with high epidemic potential. [53-55] The newly incorporated resistance mechanism is unlikely to be the only resistance mechanism in the bacterium as an isolate resistant to one antibiotic is prone to be resistant to one or more other antibiotics. [56] This phenomenon is called *associated resistance* [56] and has huge implications for the clinical impact of antibiotic resistance. The observed fitness cost of resistance genes/mutations [43-49] is a prerequisite for the *reversibility* of antibiotic resistance by reduced antibiotic use. Mathematical models have predicted a faster reversibility in the hospital than in the community due to the dynamics of individuals moving in and out of the system studied. [57] So far the clinical evidence for reversibility is limited. [23, 24]

Interventions on antibiotic use

The use of antibiotics changes over time. Only rarely do we manage to design drugs with novel targets in bacteria. More often our efforts result in a "variation on a theme" and since bacteria cannot read the package insert they end up not understanding our ingenuity. There are instances where resistance development has necessitated drastic changes in antibiotic use. In other cases the discovery of serious side effects have caused pronounced shifts. Planned interventions on antibiotic use to reduce antibiotic resistance have been performed mainly in hospital settings but there are a few examples of interventions in communities usually in response to emerging resistance problems.

A number of shifts in antibiotic policy in hospitals in response to the emergence of resistance problems have been reported over the last years. [6, 58-60] Two of these studies can be mentioned. The first showed that a decisive reduction in the use of third generation cephalosporines was followed by a significant reduction in the acquisition of ESBL-producing *K. pneumoniae*. [60] In the second a new antibiotic guideline, excluding the use of cephalosporines resulted in a reduction in cephalosporin use. This led to a significant reduction in the isolation rate of ceftazidime resistant *K. pneumoniae*. [61] The concomitant increase in imipenem resistance in *Pseudomonas aeruginosa* was attributed to the increased carbapenem use. [61]

Outside hospitals and institutions existing studies are few and show conflicting results. Two studies, one on *S. pyogenes* [23] and the other on *S. pneumoniae.* [24] show decreased resistance after antibiotic intervention whereas in two studies from Great Britain there was no effect on resistance rates in *E. coli* despite the fact that the use of respective antibiotic ceased almost completely. [25-27] These and more recent studies will be thoroughly discussed in the General discussion.

Escherichia coli - acquisition, persistence and population structure

Escherichia coli is a facultative pathogen residing as the most common facultative aerobic organism in the intestines of birds and mammals. [62, 63] It is one of the most important human pathogens responsible for up to 90 % of all UTIs in outpatient care. In addition it is an important cause of both community- and hospital acquired septicaemia, leading to considerable morbidity and death. [64, 65] Gastroenteritis caused by specific groups of *E. coli* are also common infections worldwide and neonatal meningitis and wound infections including necrotising fasciitis are serious, although more uncommon, infections. *Escherichia coli* are indigested through food and water and will then have the opportunity to colonize or infect the intestines. [66] To understand the development, stability and potential for reversibility of antibiotic resistance in *E. coli* we have to consider some underlying features of *E. coli* transmission, colonization and population structure.

Acquisition and Persistence of *Escherichia coli*

The establishment of *E. coli* in the human intestines starts early in life and recent studies have shown that in Sweden 45% of the infants are colonized with a mean of 2.1 strains at day 3 in life. [67] Interestingly this flora was found stable and the corresponding carriage rate was only 61% at 6 months of age indicating an environment in the modern society almost free of contaminating faecal bacteria. [67] This should be compared to the rapid turnover of strains in developing countries where 8.5 *E. coli* strains were found as the mean carriage rate in a comparable group of infants. [68]

Later in life a newly introduced *E. coli* strain will not necessarily replace and out compete already existing strains in the intestines.[69-71] In all humans there are some strains that will reside for many weeks and months (resident strains) while other will persist only for days or weeks (transient strains). [19, 69] Resident *E. coli* strains have an increased capacity to adhere to colonic epithelial cells, [72] present uropathogenic virulence characteristics [72] and are more likely to be of phylogenetic group B2 origin. [73] *Escherichia coli* able to infect extra intestinal sites (ExPEC) usually have sets of virulence factors including adhesion molecules and iron acquisition

systems [74] Not all isolates found in urinary cultures have the ExPEC virulence profile so there is an association of these factors with urinary tract virulence rather than a prerequisite. [75, 76] In fact virulence in *E. coli* isolates belonging to the phylogenetic group B2 has been found to be a "coincidental by-product of commensalism". [77]

Population structure of *Escherichia coli*

The population structure of *E. coli* is considered basically clonal. [78] However, already in the 1980:s the studies utilizing Multi Locus Enzyme Electrophoresis (MLEE) [79, 80] showed that recombination was important in the evolution of *E. coli*. In 1997 Roger Milkman reviewed this field concluding that broadly favourable mutations, although rare, will carry their respective genomes to high frequency (clonal sweeps). These new clones will then be speckled with recombination, thus no longer being clones, but still have common ancestral DNA (clonal frames). [81] This framework of *E. coli* evolution has later been verified in the studies using Multi Locus Sequence Typing (MLST, see below) [82] showing that recombination have an even more important role in the evolution of *E. coli* than earlier anticipated. [83] The evolutionary relationships are now more and more studied through the comparative studies of the entire *E. coli* genome. [84] Although detailed, these latter studies are so far based on a maximum of 20 genomes from widely different strains and have to be interpreted with caution on their overall relevance of phylogeny facing the huge diversity in *E. coli*. [83]

Regarding antibiotic resistance some investigators have proposed the presence of lineages [85] or even clones [86] within the *E. coli* population carrying most of the widely spread resistance determinants. For trimethoprim resistance the horizontal dissemination is so far considered to be main route of spread. [87]

Urinary Tract Infections (UTI)

Bacterial colonisation and infection in the urinary tract is very common in women, with the highest incidence in the elderly. [88] The lifetime incidence of UTI in women are 53-60% [89] and as many as 27% of all women >18 years are reporting symptoms of UTI (dysuria and/or pollakisuria) during one year. [90] Risk factors for UTI are sexual activity, mother with UTI, diabetes and incontinence. [91]

Uncomplicated UTI constitutes 75% of all UTI diagnosed in Swedish PHC leaving recurrent UTI, complicated UTI (including UTI in men) and febrile UTI (pyelonephritis) as less common causes. [92] Urinary tract infections are the second most common reason in Swedish PHC for receiving an antibiotic prescription [93] and 9.1% of the women >18 years old were reported to receive an "UTI-antibiotic" prescription for a UTI in 2005-2006. [94] Roughly 20% of these women got another prescription during the coming year and 9 and 4% received 2 and 3 more prescriptions, respectively.[94] The most common treatment for uncomplicated UTI in Sweden is in rank order pivmecillinam, trimethoprim, nitrofurantoin, ciprofloxacin and cephadroxil. [93]

Virtually any bacteria can occasionally cause an UTI but irrespective of sex, age and geography *E. coli* is the dominating pathogen [13, 95-97] and is the causative agent in 90% of the cases in younger patients with uncomplicated UTI. [12, 65, 98] In older women the etiological spectrum is more complex. [99, 100] The *E. coli* strains responsible for UTIs are characterized by faecal abundance, a higher virulence factor score and to be of phylogroup B2 origin in comparison with those strains found in faecal samples only. [75].

Antibiotics used in the treatment of UTI-substances and corresponding resistance mechanisms

This section will review the antibiotics most used in the treatment of UTI in Sweden. Some basics about the indications for use and briefly about their present use in Sweden are commented for all antibiotics. Specific information on the antibiotic resistance and associated resistance in *E. coli* of these antibiotics are described.

Trimethoprim and trimethoprim-sulphamethoxazole

Sulphonamides interferes with the first step of folinate synthesis in bacteria and was launched already in the 1930ies. [101] When combined with trimethoprim, a dihydrofolate-antagonist interfering with the purine synthesis through the inhibition of dihydrofolate reductase, (Figure 1) a bactericidal effect was achieved resulting in a better treatment for severe infections. [102] The combination (co-trimoxazole or trimethoprim-sulphamethoxazole) has since the introduction in the late 1960:s been widely used around the globe because of its chemical stability and low cost. [101] Sulphonamides are however toxic, at a level not tolerable in uncomplicated infections and TMP treatment alone was shown in the 1970:s to be equally effective as in combination with sulphamethoxazole in the treatment of uncomplicated UTI. [103] Trimethoprim had excellent activity against most organisms involved in UTI in both women and men and there was a hope that TMP resistance would not appear in isolates resistant to sulphonamides [104] However, increasing resistance to TMP are now limiting its potential as first line treatment. [105]

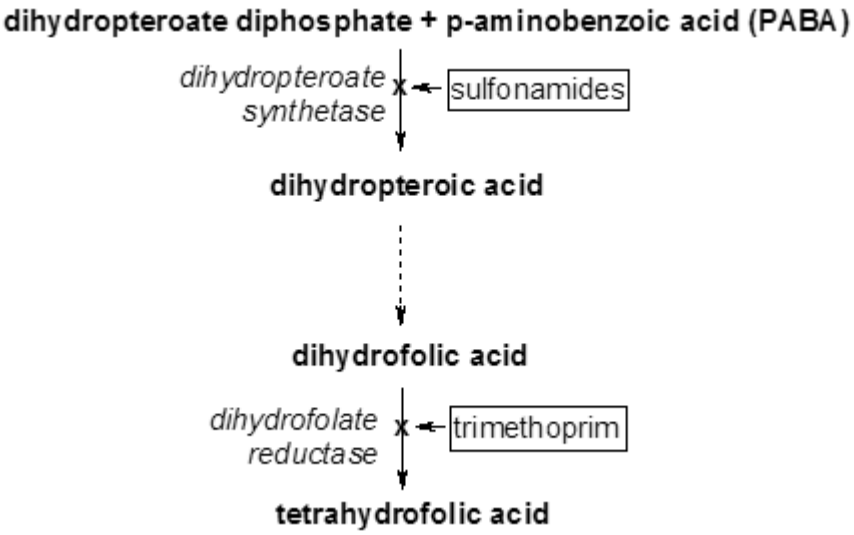

Figure 1. The bacterial folate synthesis showing where sulfonamides and trimethoprim exerts their effect.

Trimethoprim resistance

Trimethoprim resistance was first described in 1968 [102] and in 1971 1% of *E. coli* isolates from urinary specimens were reported resistant. [106] Since then there has been a constant increase in TMP resistance world wide and in some regions only a minor part of the *E. coli* population is still susceptible to TMP. [107] Trimethoprim resistance in Enterobacteriaceae is mediated by at least 30 different *dfr*-genes. [108] The *dfr*-genes encode modified dihydrofolate reductases whose configuration will escape the action of TMP. [101] The localization of these genes has been reported to be chromosomal [109, 110] but the most recent studies indicate that a plasmid location within integron structures is dominating. [87]

Although epidemics of UTI caused by SXT resistant *E. coli* have been suggested [111, 112] the *dfr*-genes are mainly transferred horizontally. [87] UTIs caused by TMP-resistant *E. coli* have been associated with worse clinical outcome and an increased workload in general practice. [17, 113] The early studies on TMP resistance development [114-116] did not observe any increase in resistant bacteria in the faecal flora due to TMP treatment. However, the last recent antibiotic treatment, [113, 117, 118] TMP treatment within the last six months, [117, 119] travel abroad, male sex [120] and recent hospitalization [120] have been shown to increase the risk of being infected with a TMP resistant *E. coli*.

β-lactam antibiotics

The most widely used group of antibiotics are the β-lactams. They exhibit a rapid bactericidal effect and are well tolerated. [121] In the treatment of uncomplicated UTI ampicillin (with or without clavulanic acid), oral cephalosporines and, in the Scandinavian countries, pivmecillinam has been widely used. [92, 122] In pyelonephritis and septicaemia intravenous formulations of cefotaxime, ceftriaxone and lately ceftibuten has been used. [122, 123]

Resistance to β-lactams in Enterobacteriaceae is mainly exerted by different enzymes, β-lactamases, encoded by chromosomally or plasmid encoded enzymes [38] The recent emergence of ESBL-producing *E. coli* and *K. pneumoniae* has led to therapeutic failures and death when treating septicaemia with standard agents, like cefotaxime. [124, 125]

Ampicillin and amoxicillin

Ampicillin and amoxicillin are widely used in respiratory tract infections (amoxicillin) and in severe infections (ampicillin). [121, 126] Traditionally amoxicillin also has been used in the treatment of uncomplicated UTI. The usefulness in UTI treatment has however decreased as a consequence of increasing resistance, mainly due to β-lactamases like TEM and SHV. [38] The action of these enzymes can in most cases be overcome with the addition of a β-lactamase inhibitor like clavulanic acid. [127] In Sweden amoxicillin is not recommended for empirical treatment of UTI.

Cephalosporines

Cephalosporines was developed as a response to the increase in penicillinase producing strains of *S. aureus* in the 1940:s. The first generation cephalosporines have been widely used in the treatment of all kinds of uncomplicated infections and CFR is in Sweden still recommended as second line treatment in uncomplicated UTI. [94]

The third generation cephalosporines, like cefotaxime [128] have been very valuable agents in the treatment of septicaemia of unknown origin and uro-sepsis [129], their efficiency is now threatened by the increase in ESBL-producing pathogens.

Ceftibuten is an oral cephalosporin shown to be comparable to SXT in the treatment of pyelonephritis in children. [123] This agent also seems to be more stable against ESBL:s of SHV type *in vitro* [130] but there is so far no clinical evidence supporting their use in these infections.

Pivmecillinam

Pivmecillinam is increasingly common in the treatment of uncomplicated UTI in Swedish women. [131] It is a pro-drug hydrolysed to mecillinam and approximately 60% is excreted in the urine reaching concentrations exceeding 1g/L. [121] Pivmecillinam has a limited effect on the colonic- and the vaginal flora [132, 133] and is moderately stable against most β-lactamases (including ESBLs). [134] Resistance to MEC is so far uncommon. [12, 135] and isolates with ESBL production rends susceptible (MIC≤8 mg/L) [136]

The exact mechanism of MEC resistance has not been described in clinical isolates but in studies on the elongation process of *E. coli* MEC resistance has been associated with a distorted action of PBP2. [137-141]

Quinolones

Fluoroquinolones are broad spectrum antibiotics exhibiting a bactericidal effect against a wide range of bacteria. [142] The first quinolone was nalidixic acid in 1962 [142] followed by the fluoroquinolones (FQX) with ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin with good bioavailability and spectrum. [142] Ciprofloxacin is the FQX recommended in the treatment of complicated UTI and pyelonephritis in Sweden. Fluoroquinolones affects both aerobic and anaerobic bacteria in the faecal flora and the vaginal flora. [143]

Resistance to FQX increases dramatically in Enterobacteriaceae. [39] This is mainly due to a series of mutations in the *gyr*A and *par*C genes. The first mutations in *gyr*A will result in resistance to NAL [39] and after additional mutations to ciprofloxacin and other FQX. [39] This acquisition has been shown to be associated with an initial fitness cost [46] later restored by additional resistance mutations. [51] Low level resistance to FQX has been shown to be encoded by plasmid mediated resistance genes. [144]

Nitrofurantoin

Nitrofurantoin has been used in the treatment of UTI for a long time. [121] The action is considered to be a combination of inhibition of the protein synthesis and a direct effect on the DNA synthesis. [121] The main part of NIT is excreted unchanged in the urine and a concentration exceeding 100 mg/L is reached. [145] More than 95% of NIT is absorbed and seems to have limited effects on the faecal and vaginal flora. [146] Resistance to NIT in *E. coli* is continuously low and due to mutations in *nsfA* and *nfsB* genes encoding oxygen-insensitive nitroreductases shown to impose a fitness cost. [49]

Fosfomycin

The use of fosfomycin has during the last years increased in many countries mainly in infections with multi resistant *E. coli*. Fosfomycin inhibits the cell wall synthesis by irreversibly inhibiting enolpyruvyl transferase catalyzing the first step in the biosynthesis of peptidoglycan. It is considered particularly active against *E. coli.* Fosfomycin resistance has been shown to impose a fitness cost [147]and is still uncommon (0-1.5%). [13] Single dose treatment with fosfomycin has been shown to be effective in the treatment of uncomplicated UTI.

Antibiotic susceptibility testing

Antibiotic susceptibility tests are performed by microbiological laboratories to determine the likelihood of therapeutic success and failure with antibiotic therapy. Tests can be phenotypic, i.e. quantify the level of susceptibility as a biological phenomenon or genotypic, i.e. detect specific resistance and predict, but never guarantee, susceptibility.

The basic concept of phenotypic test systems is to allow the bacteria optimal growth in the presence of a series of antibiotic concentrations. The lowest concentration, in a two-fold dilution series, which macroscopically inhibits the growth of the organism, is designated the MIC, the Minimum Inhibitory Concentration. All phenotypic test systems are calibrated against the MIC. The reference system for non-fastidious bacteria is Micro Broth Dilution (ISO 20776-1).

The most commonly used routine method for susceptibility testing is the disk diffusion method where a disk, containing a defined amount of antibiotic, is placed on the agar surface. A concentration is rapidly established around the disk. The growth of the organism is inhibited by the presence of the antibiotic (Figure 2). Depending on the level of susceptibility of the organism it may grow closer to or farther from the disk. The ensuing inhibition zone diameter is correlated to the MIC of the isolate – the higher the MIC, the smaller the zone. Inhibition zones can be read manually with callipers or electronically with a variety of devices. When stored in a database they can be re-analysed at any point in time. MIC-breakpoints can be given zone diameter correlates, thus allowing the susceptibility categorization to S (susceptible), I (intermediate) and R (resistant).

Zone diameter and MIC distributions (Figure 3) can be compared over time, both for epidemiological purposes and for purposes of internal quality control and external quality assessment. Furthermore, distributions can be analysed with both clinical breakpoints and epidemiological cut-off values.

According to EUCAST (www.eucast.org) a micro-organism is categorized as S, I or R by applying the appropriate breakpoint in a defined phenotypic test system. This breakpoint takes into account a number of parameters (see below) and importantly may be altered with legitimate changes in circumstances (e.g. changes in commonly used dosages, emergence of new resistance mechanisms etc.).

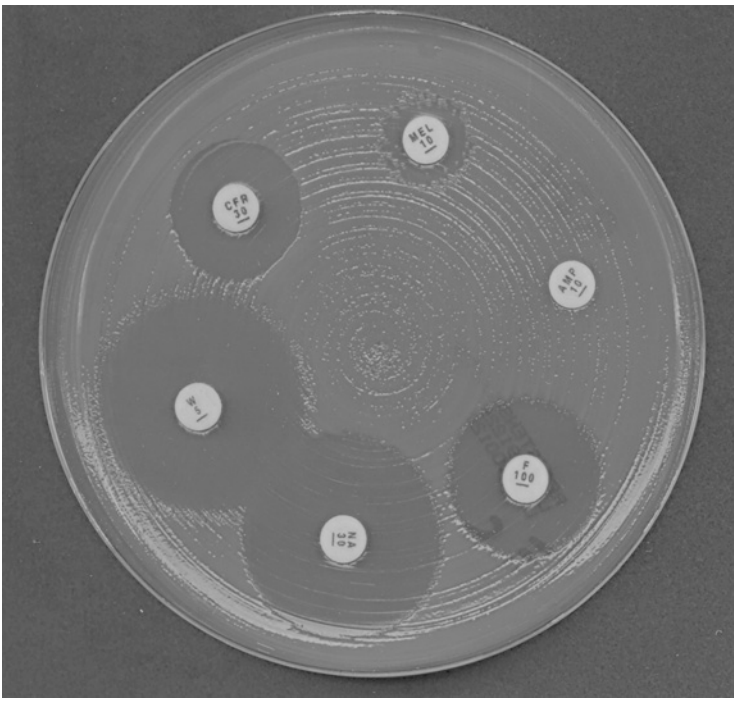

Figure 2. An example of susceptibility testing using disc diffusion. In this case there is growth of *E. coli* close to the ampicillin and mecillinam disc. This isolate is thus resistant to ampicillin and mecillinam.

The definitions agreed through EUCAST (www.eucast.org) of S, I and R are listed below:

S- a micro-organism is defined as susceptible by a level of antimicrobial activity associated with a high likelihood of therapeutic success

I- a micro-organism is defined as intermediate by a level of antimicrobial agent activity associated with uncertain therapeutic effect. It implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

R- a micro-organism is defined as resistant by a level of antimicrobial activity associated with a high likelihood of therapeutic failure.

Clinical Breakpoints

Clinical breakpoints are determined by international or national breakpoint committees, either as part of the process of approval of a new antibiotic or because there were reasons to review previously determined breakpoints. The setting of breakpoints takes into account target species, target indications, MIC-distributions of relevant micro-organisms, resistance mechanisms, clinical success and failure in relation to MIC, normal and maximum dosing, pharmacokinetic and pharmacodynamic properties of the drug. To allow reproducibility in susceptibility testing, the breakpoint should not divide the distributions of wild type bacteria. (www.eucast.org)

Breakpoints need to be reviewed at intervals - dosing schedules may change, indications extended or withdrawn, new resistance mechanisms appear, etc. - and breakpoints are thus prone to change over time. Seen over a period of 40 years almost all revisions have lowered existing breakpoints. [148]

Isolates categorized as susceptible to a drug, may still have resistance mechanisms to the drug in question, whereas others are characterized as resistant despite a complete lack of resistance mechanisms (eg. intrinsic resistance).

Zone diameter breakpoints for disk diffusion are either calibrated against clinical MIC breakpoints or (for screening purposes) against the presence or absence of a specific resistance mechanism (cefoxitin breakpoints for detection of MRSA, oxacillin breakpoints for detection of penicillin non-susceptibility in Streptococcus pneumoniae etc)

The Epidemiological Cut-off

For each species and antibiotic it is possible to define the MIC-concentration and the zone diameter that separates isolates without ("wild type organisms") and with resistance mechanisms. [149-151] EUCAST has named this concentration the epidemiological cut-off (ECOFF) (www.eucast.org). The typical MIC-distribution for wild type organisms of a species covers 3 – 5 two-fold dilution steps which correspond to 10 – 14 mm in the zone diameter histogram. (www.srga.org & www.eucast.org)

The wild type distribution and thus the ECOFF for a defined species and antibiotic will be unaffected by time, differences in geographic origin or the host species (man, animal etc). [152, 153] The advantage of the ECOFF is that it describes susceptibility and resistance as a biological phenomenon, it is a more sensitive and stable measurement of resistance than CBPs since it does not change over time as a result of decisions by breakpoint committees. In this thesis, all results are based on ECOFFs and an organism is categorised as "resistant" when outside the defined wild type distribution (Figure 3). With

modern breakpoints the difference between using CBPs and ECOFFs is not great for almost all antibiotics used in II-IV.

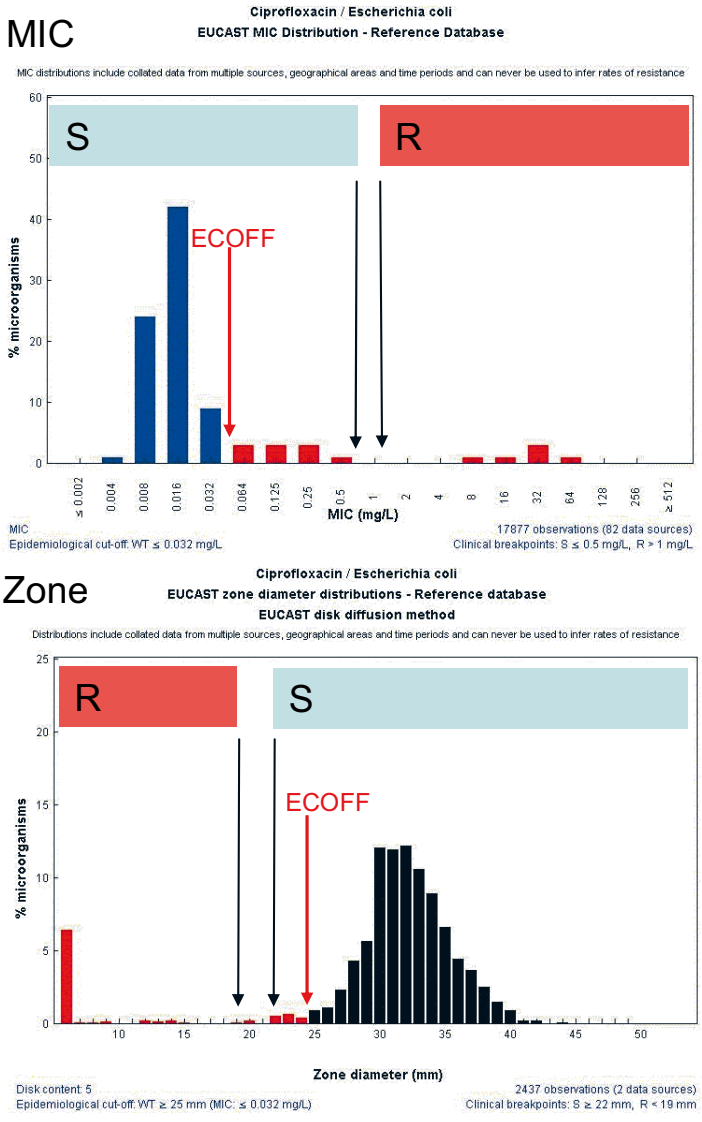

Figure 3. The *E. coli* ciprofloxacin MIC (mg/L) distribution and a corresponding distribution of inhibition zone diameters (mm), both from the EUCAST website (www.eucast.org). Current EUCAST clinical breakpoints (for S- and R-categories) are marked in blue and red. For each distribution the ECOFFs have been indicated by red arrows. The red bars in both graphs indicate non-wild type organisms, i.e. organisms with resistance mechanisms, irrespective of whether these are categorized as clinically resistant or not.

The epidemiological use of antibiotic susceptibility test data

The most common way of presenting antibiotic resistance data is probably through a graph or a table showing resistance or susceptibility rates over time for a defined species and a defined antibiotic (as exemplified with yearly resistance rates in Figure 4).

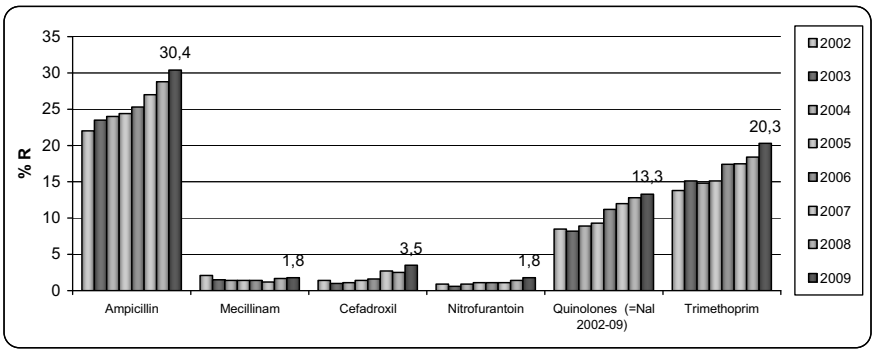

Figure 4. Resistance rates in Swedish urinary tract isolates of *E. coli*. (Data from ResNet (www.srga.org), where each Swedish county (n=26 – 28 laboratories) supplies quantitative results on at least 100 consecutive isolates per year (courtesy of Barbro Olsson Liljequist, Smittskyddsinstitutet, Stockholm, Sweden).

These graphs are straight forward and easy to understand but tend to hide important information for our understanding of antibiotic resistance development. This is indicated by the fact that in Kronoberg County the proportion of *E. coli* isolates fully susceptible to all 6 antibiotics in Figure 4 decreased by only 2.3% during the period 1993 to 2004 while the increase in resistance for the individual antibiotics was clearly higher. [154] Resistance development thus seems not to affect all bacteria within a species. This has further been illustrated by the associated resistance, i.e. the fact that an isolate being resistant to antibiotic X will be at a higher risk/chance of being resistant to antibiotic Y than an isolate not resistant to antibiotic X. [56, 154] The impact of associated resistance on resistance rates is illustrated in Figure 5 showing the

resistance development expressed as specific antibiogram patterns with three or more resistances.

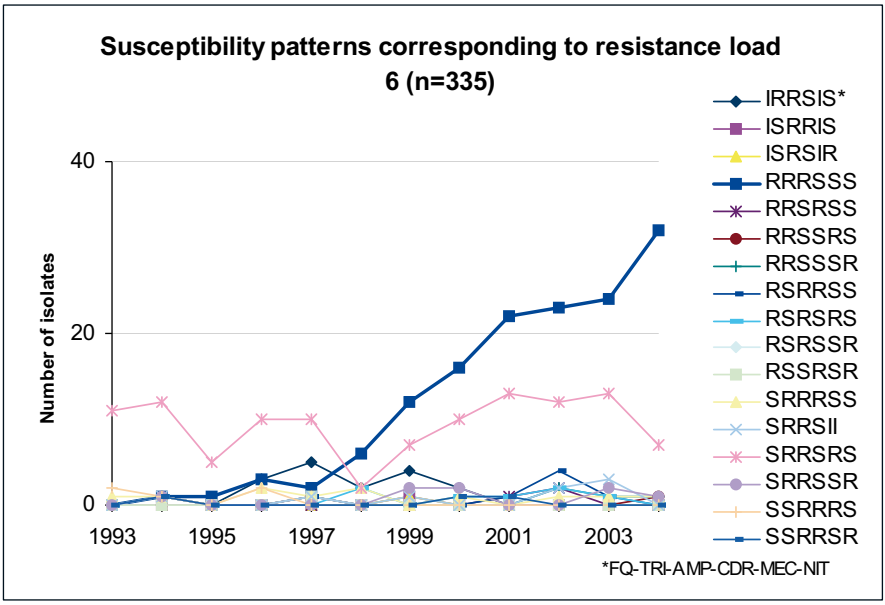

Figure 5. Proportion of *E.coli* isolates with three resistances or two resistances plus two intermediate results (resistance load 6), Kronoberg county 1993-2004. The blue line with squares shows the phenotype resistant to a quinolone, ampicillin and trimethoprim. Published with kind permission of A. Wimmerstedt.

Antibiotic susceptibility test data from clinical isolates are usually stored in local databases and used for local and national surveillance of antibiotic resistance. It is preferable to store quantitative data, either MICs or inhibition zone diameters, but in many laboratories only the result of the interpretation, the S, I or R, are stored. Quantitative data can be retrieved and re-interpreted following changes in breakpoints. The data is used to guide empirical therapy[155] and to warn against local resistance development or outbreaks of resistant bacteria and emerging new resistance. [54]

To ensure the quality of the surveillance data the following points should be considered:

a) Patients with resistant pathogens will fail on treatment more often than patient with susceptible pathogens. Pathogens that cause treatment failures will reappear in the data base and may cause “falsely” high resistance rates. This is addressed in I.

b) From 2010 there will hopefully be only two CBP-systems used in the world, the CLSI and the EUCAST. Importantly and unfortunately only few breakpoints are identical in the two systems. The EUCAST system has just systematically revised all breakpoints whereas the CLSI is just now beginning a similar process. It is important to state both which system was used and to list the actual breakpoints when presenting resistance data.
c) Data from routinely collected isolates is accompanied by a sampling bias since in most cases only specific groups of patients were cultured. [4] Awareness of this is necessary to interpret the surveillance data correctly.
d) Laboratories use different algorithms when setting up their susceptibility testing panels. Most laboratories have a first-line panel limited to 5 – 8 antibiotics. It is extended to cover another 4 – 6 antibiotics if the organism was resistant to many or most of the first-line drugs. This is perfectly reasonable and safe and cost-effective. However, when resistance rates are based on follow-up-antibiotics resistance will be seriously overcalled. [154, 156]

Ideally a baseline for an intervention study consists of quantitative susceptibility data obtained with a standardized methodology stable over a very long period. The random fluctuations in resistance rates should be characterized. The effect of including and excluding duplicate samples should be mapped. [157] Only resistance rates for antibiotics which were included in the primary susceptibility test may be used and the denominator of both cultures and positive isolates should be known.

Typing methods

Typing of bacteria is used to study both evolutionary and epidemiological associations between isolates. [158] Ideally a typing method has a high resolution with a solid relation to long term evolution and species. [158] This is however not always possible to obtain and the choice of typing method also involves considerations on typeability, practicality and cost. [159] In this thesis one phenotypic (PhenePlate^{TM}-system) and one genotypic method (MLST) was used. To understand the relation of these methods to other commonly used systems the following section will give an introduction to these techniques with a focus on their utility in typing of *E. coli*.

Genotyping of *E. coli*

Pulsed Field Gel Electrophoresis (PFGE) has been used extensively in the typing of *E. coli*. It utilizes restriction enzymes and the fragments are separated by a pulsed electrophoresis. It is considered the gold standard typing technique and has excellent discriminating capacity and typeability. [158] It is however labour intensive and problems in the comparison of data between different laboratories has been hard to overcome. [158]

More simple methods are those based on Polymerase Chain Reaction (PCR) like Rapid Arbitrary Polymorfic DNA [160] and the Enterobacterial Repetitive Intergenic Consensus PCR (ERIC). [86] These are widely used to define local outbreaks [161, 162] and the ERIC PCR has also been used to analyse large collections of *E. coli.* [86, 112, 163] Although practical these methods deals with some problems with inter laboratory variation. [159]

Multi Locus Sequence Typing (MLST)

To overcome the methodological difficulties with the above mentioned techniques sequenced based methods are now preferred in the typing of *E. coli*.[159] These methods utilizes housekeeping genes (MLST) or variable tandem repeats (MLVA). They have a good typeability, excellent reproducibility, good discriminatory power and have the advantage that data can be stored and easily utilized in open databases. [158]

Multi Locus Sequence Typing (MLST) is based on the sequence variation of a variable number (usually six to eight) of housekeeping genes in the bac-

teria. The housekeeping genes are amplified and the PCR fragments are sequenced on both strands. The extensive amount of sequence data are stored in a database and analyzed using specially designed software. In *E. coli* two MLST schemes are available: "The French scheme" [164] and the more widely used "German Scheme" available at http://web.mpiib-berlin.mpg.de/. [83] The sequence data is used to define Sequence Types (ST) that are specific for the present study or compared with other studies using the same MLST scheme. MLST has been used to describe the *E. coli* population in relation to environmental niches [165, 166] and virulence factors [167, 168] and correlates with the phylogenetic branches A, B1, B2 and D. [168, 169]

The association of antibiotic resistance to specific ST:s in *E. coli* have so far been based on limited numbers of clinical isolates of *E. coli* [20, 170-172] or the association of successful lineages to ST. [168, 173] The knowledge on the Sequence type distribution in *E. coli* from urinary tract infections is so far limited. [20]

Phenotyping of *E. coli*

All phenotyping is based on the phenotypic appearance of the bacterium under standardized conditions. This is utilized in Serotyping, antibiogram based typing, PhenePlate^{TM}-system and in Multi Locus Enzyme Electrophoresis (MLEE).

Serotyping

Serotyping has in *E. coli* traditionally been a widely used method to describe lineages and population composition. [174] It is based on antibodies directed against 173 O antigens, 80 K-antigens and 56 H antigens. The association of these antigens to specific virulence or hosts varies and some O types are associated with UPEC, [74, 175] The flagella (giving the H antigens) have a defined role in the pathogenesis of UTI [176] and some K antigens are associated with virulence and avoidance of phagocytosis. [74] Serotyping is today used in the description of diarrhoeal strains. [177] Serotyping is time consuming and has not enough discriminatory power to be considered efficient in epidemiological studies. [158]

Multi Locus Enzyme Electrophoresis (MLEE)

A great deal of our knowledge of the population structure in *E. coli* is based on the electrophoretic pattern of cellular enzymes (Multi Locus Enzyme Electrophoresis). [178] This method detects the allele variation within the genome by measuring the expressed protein pattern and will thus only identify genetic substitutions that results in the substitution of at least one amino

acid. Despite this limitation the results of a MLEE protocol based on 15-25 enzymes representing structural genes did provide a lot of information on the diversity and relatedness between strains within several bacterial populations. [78, 178]

PhenePlate™-system

The PhenePlate™system [179] utilizes the dynamics of 11 or 23 biochemical reactions and has been reported suited for epidemiological typing. [180, 181] The reactants (sugars and amino acids) are fixed in special 96-well plates where one loop-ful (1µl) of bacteria is incubated during 48 hours in 350µl PhP suspending medium. Reactions are measured at 8, 24 and 48 hours using a scanner. Every isolate then gets a digital row of 11/23 figures ranging from 0-24 referring to both the speed and type of reaction for each reactant. These isolate specific rows then form the basis for calculating the diversity of the studied population, for pair wise comparisons presented as Phenotypes and for clustering in dendrograms.

The PhenePlate™system has been shown to be a useful and comprehensive tool in analysing large numbers of isolates both in *E. coli*[180] and *Enterococcus* spp. [182] The reproducibility is high[180] and its discriminatory power has been shown to be comparable with PFGE although not necessarily resulting in matching clusters. [183]

Aims

The primary aim of this thesis was to test the hypothesis that a drastic change in the use of antibiotics in a community would affect the corresponding levels of antibiotic resistance. Additional aims were to study the effect of the intervention on the distribution of trimethoprim encoding resistance genes and *Escherichia coli* genotypes.

The following specific questions were asked:

- What is the impact on resistance rates and trends of including or excluding patient duplicate isolates? (I)

- Will a drastic reduction in the use of trimethoprim and trimethoprim-sulphamethoxazole in a defined geographic area (Kronoberg County) affect:
  - corresponding antibiotic resistance rates? (II)
  - the *dfr*-gene distribution in *E. coli*? (II and III)
  - the population structure in *E. coli*? (II and IV)

- Will resistance to trimethoprim confer a fitness cost in clinical isolates of *E. coli* as measured with growth rate? (II)

- Is the *dfr*-gene distribution in *K. pneumoniae* similar to that reported in *E. coli*? (III)

- Do certain Sequence Types of *E. coli* harbour antibiotic resistance determinants to a larger extent than other Sequence Types? (IV)

Materials and methods

General description and demographics

Kronoberg county is a rural forested part of Sweden, approximately 150 by 120 km with a population of 178 000 (mean age 41.3 years). The health care system is funded at the county level and includes two hospitals and 25 primary health care centres. The population structure and population density has been virtually unchanged during the last twenty years. No major changes regarding the recommendations on when and how to perform a urinary culture was made during the studied period. The number of yearly urinary specimens for culture varied from 1991-2009 between 16 777 and 19 635. The corresponding percentage of *E. coli* isolated were 22-24% without a trend.

All studies in this thesis were based on resistance rates in clinical isolates of bacteria isolated at the Dept. of Clin. Microbiology, Växjö, Sweden through the years 1991-2008. Resistance rates are based on quantitative data (inhibition zone diameters in the SRGA standardised disk test) from primary susceptibility testing. In a study from 2001 (Österlund & Kahlmeter, unpublished) where women in Kronoberg county with uncomplicated UTI were cultured (results not disclosed to physician) prior to receiving empiric antibiotic therapy the resistance rates corresponded to within +/-1 % of resistance rates based on all clinical samples. Papers II, III and IV were based on isolates from urinary specimens only.

All Enterobacteriaceae isolated from urine specimens from June 2004 (i.e. 4 months prior to the intervention) – October 2008 (n=24 672) were stored at -70˚C and used for the studies in II-IV.

Bacterial isolates (I-IV)

In I, susceptibility testing results of all consecutive *E. coli* and *S. aureus* identified in the period 1990 to 2003 at the Dept. of Clin. Microbiology, Central Hospital, Växjö, Sweden, were analysed. The exclusion of repeat samples using several different algorithms was performed by the laboratory based computer system (ADBakt™). This was to determine the possible variability in the baseline data due to the presence of duplicate isolates.

In II, all susceptibility results on consecutive *E. coli* isolated from urinary specimens at the Depts. of Clin. Microbiology, Central Hospital, Växjö and Kalmar County Hospital, Kalmar, Sweden, since 1991 were included in the analysis (n= 71 290 and 89 837, respectively). The resistance rates in *E. coli* for TMP were retrieved as monthly figures from both counties. Trimethoprim resistance rates in other species than *E. coli* were retrieved from Kronoberg County only. Monthly resistance rates in *E. coli* to AMP, NAL, NIT, MEC and CFR were retrieved from Kronoberg County only.

In III, consecutive TMP resistant *E. coli* isolates from three time periods were analysed; prior to intervention (n=106) , mid-intervention (n=105) and at end of intervention (n=109). In addition all TMP resistant *Klebsiella pneumoniae* from urinary samples from June 2004 to September 2007 were analyzed.

In IV, 348 consecutive TMP-susceptible and 200 TMP-resistant *E. coli* with known *dfr*- and integron contents were sequence typed. Half of the isolates from each cohort were from before the intervention.

Susceptibility testing (I-IV)

Since 1986 all bacterial findings and susceptibility data produced at the department of clinical microbiology, Växjö, Sweden, is stored in the computer based laboratory system (ADBakt™). During the studied period (1991-2008) susceptibility testing and quality control were performed in strict accordance with the recommendations of the Swedish Reference Group on Antibiotics (SRGA) using Iso-sensitest agar and antibiotic discs from Oxoid Ltd (Basingstoke, UK). [184] Inhibition zone diameters were stored together with all patient data in the database. The methodology for culturing and susceptibility testing was stable throughout the period except for the testing of fluoroquinolone susceptibility in *E. coli* where the test substance used was changed over time (norfloxacin 10 µg during 1990-1993, ciprofloxacin 5 µg 1994-2000 and nalidixic acid 30µg 2001-2003). In I the CBPs set by the SRGA were used. In II-IV the epidemiological cut-offs were used calculated for each antibiotic using the Normalized Resistance Interpretation methodology. [151]

Removal of duplicate isolates (I)

Duplicate isolates were identified and excluded by the laboratory computer system (ADBact™) on the basis of bacterial species and the unique personal identification number given to all Swedes at birth. In I, the following algorithms were investigated: 7, 14, 30, 45, 90, 180, 270 and 365 days interval between sampling dates for the first and following isolates of *E. coli* vs.

AMP and TMP and for *S. aureus* vs. fusidic acid and clindamycin. For other antibiotics cut-off values of 30 and 365 days were analysed. Methicillin-resistance was not included in the analysis since less than 20 unique isolates were available in any of the 14 years. The following antibiotic susceptibility test results were analysed for *E. coli* - AMP, MEC, CFR, TMP, NIT and FQX and for *S. aureus* - clindamycin and fusidic acid. Antibiotics were chosen to represent different classes of antibiotics and because they belonged to the standard routine panel of antibiotics used over the whole period. In II, the time for exclusion of duplicates were 30 days, as decided to be the most suitable time in I, and in III and IV the principle of one isolate per patient and study period was used.

Associated resistance (II-IV)

To permit analysis of the influence of co-selection during the intervention (II), associated resistance frequencies [154] (the proportion of isolates resistant to an antibiotic in the presence of resistance to TMP) were calculated. Associated resistance rates to TMP were also calculated for the materials in III for the analysis of association with integrons and or specific *dfr*-genes with multi resistance. In IV the resistance rates were calculated in TMP susceptible population and TMP resistant population to give a measure of the associated resistance in relation to population structure.

Growth rate measurements (II)

A subset of *E. coli* isolates resistant to TMP only (n=50) and *E. coli* susceptible to all tested antibiotic agents (n=50) were analyzed. To estimate the fitness, the growth rate was determined using BioScreenC, (Oy instruments, Helsinki, Finland) a turbidometric reader which continuously monitors bacterial growth. Each isolate was inoculated to a final density of 10^5 cfu/mL in a well with 0,4 mL LB. Growth was recorded during 8h at 37˚C as an increase in optical density at 540 nm as previously described. [147] Isolates were run in triplicate in the same run. *E. coli* ATCC 25922 was run in all experiments as control.

Phenotyping (II)

To detect changes in the distribution of *E. coli* phenotypes during the intervention(II), consecutive *E. coli* isolates from the 4 months preceding the intervention (n=1369), the 4 months immediately following the start of the intervention (n=1445) and from the last 4 months of the intervention

(n=1461), were analysed using the PhenePlate™ (PhP) System. [180] Pair wise comparison and cluster analysis was performed using PhPWin4 software. A phenotype (categorized A-Z) was defined as at least 1% of the isolates presenting the same (97.5% identical) biochemical pattern.

Analysis of *dfr*-genes and integrons (III and IV)

In TMP resistant *E. coli* (n=320) and *K. pneumoniae* (n=54), the presence of the five most common *dfr*-genes described in human infections, *dfr*A1, A5, A7, A12 and A17[87] [185] were analyzed using a multiplex real time PCR.[108] The isolates negative for these genes were analysed further for the presence of *dfr*-genes 2d, A3, A8, A14 and A24 using specific PCRs. In addition all isolates were analysed for the presence of Integrons class I and class II by specific primers targeting the Integrase genes (Primer sequences and detailed methodology are described in III).

Multi Locus Sequence Typing (MLST) (IV)

The MLST scheme described by Wirth et al. [83] (http://mlst.ucc.ie/mlst/dbs/Ecoli), utilizing seven housekeeping genes: adenylate cyclase (*adk*), fumarate hydratase (*fum*C), DNA gyrase (*gyr*B), isocitrate/isopropylmalate dehydrogenase (*icd*), malate dehydrogenase (*mdh*), adenylosuccinate dehydrogenase (*pur*A) and ATP/GTP binding motif (*rec*A) was used. The PCR reactions were performed using a T Gradient Thermocycler (Biometra GmbH, Goettinger, Germany) and are described in IV. For sequencing reactions Big Dye® Terminator v3.1 cycle sequencing kit (Applied biosystem, Foster City, CA, USA) was used and analyzed in a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) according to manufacturers' instructions. All sequence traces were imported, aligned, trimmed and quality controlled aided by functions of the software Bionumerics v.6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium). Re-sequencing was performed if required and MLST allele designations were determined via the electronic MLST database. Novel ST designations were provided by the curator of the database.

Analysis of MLST data

Minimum Spanning Trees were constructed in Bionumerics v.6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium) using the option for categorical data with single and double locus variant priority rules. In addition, the software ClonalFrame v1.1 was run to determine phylogenetic relationships between the individual sequence types. This software is based on a model of

genetic diversification that accounts for recombination that occurs in bacterial populations. This enables the inference of phylogenetic relationships even if sequence data from different genes are partly incongruent. The software was run at default values for all options using 100 000 iteration for burn-in and 200 000 additional iterations that were retained for the analysis.

The location of *dfr*A1, *dfr*A5, *dfr*A17 and bla_{TEM} (IV)

To investigate the location of the *dfr*-gene and the bla_{TEM} gene eighteen isolates of three different ST-*dfr* combinations (ST73-*dfrA1* (n=6), ST69-*dfrA17* (n=6) and ST58-*dfrA5* (n=6)) were analyzed. The presence of *dfr*A1, *dfr*A5 and *dfr*A17 was determined using specific PCRs as described in III. The bla_{TEM} were amplified using a specific PCR.[186]

PFGE and Southern blot hybridisation

S1-nuclease-digested (Promega, Madison, WI, USA) linearized plasmid DNA from all isolates were examined by PFGE, run in a Chef-DRs III System (BioRad, Oslo, Norway). Each generated band was considered a unit length linear plasmid. The plasmids DNA was further blotted onto positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany) using the VacuGene^{TM} XL Vacuum Blotting System (Amersham and Bioscience, Oslo, Norway) for transfer, and hybridized with PCR generated probes of the *dfr*A1, *dfr*A5, *dfr*A17 and bla_{TEM} genes. The PCR DIG Probe Synthesis Kit (Roche Diagnostics) was used for probe labeling, and detection was carried out using the DIG Luminescent Detection Kit (Roche Diagnostics). [187]

Analysis of uropathogenic linages in *E. coli* - Clonal group A and O15:K52:H1 (IV)

Two PCR:s [188, 189] has been reported to be highly sensitive and specific for the detection of CGA and of O15:K52:H1. As both these are based on the presence of a specific SNP in the *fum*C gene, included in the used MLST scheme, the *fum*C-sequences from all isolates were screened for the presence of these specific SNPs (C288T (CGA) and G594A (O15:K52:H1).

Antibiotic use (II)

Antibiotics are in Sweden available only via prescription and were at the time of the study sold through the National Corporation of Pharmacies (Apoteket AB) only. Sulphonamides in other forms than co-trimoxazole have not been used in Sweden since the 1970-ies. No changes in the national or local recommendations for the treatment of UTI were made during the study time. Monthly sales data based on the patients residency, were retrieved for the period 1991 and onwards from Apoteket AB for Kronoberg and Kalmar County. From 1991 to 1995 these data were based on random samples (every 25:th prescription); from 1996 all data were actual sales data.

Intervention (II)

The intervention was 24 months starting October 1st 2004. All 464 physicians in Kronoberg County were during September 2004 asked to substitute TMP and SXT with other antibiotics. All received written information and a short pamphlet describing existing alternatives for the treatment of UTIs. Immediately prior to the intervention, all PHC and those Hospital clinics prescribing the main part of TMP and SXT were visited by members of the study group. Monthly e-mails were sent to the physicians employed by the county during the first year of the intervention. These e-mails contained some cheering but also a coded graph where all PHCs and clinics could monitor their own prescribing of TMP containing drugs. Susceptibility testing for TMP and SXT was performed but the results were made available only on demand.

With two exceptions, surgical prophylaxis and prophylaxis in immunocompromised patients, all indications for TMP and SXT were included in the intervention. Since SXT was standard therapy for upper UTI in children at the time of the intervention all children with clinical signs of upper UTI were referred to the paediatric department during the intervention and the suggested therapy for these children were orally administered ceftibuten.

The Swedish medical board, The Swedish Medical Product Agency and the pharmaceutical industry was informed about the study by the study group. Citizens in Kronoberg County were informed via local and national media. Physicians in Kalmar County were not specifically informed of the intervention. The study was approved by the regional ethics committee of Linköping University, Sweden (Dnr 03-04).

Statistics (I-IV)

In I the exact two-sided test of proportions based on the binomial distribution was used, $p<0.001$ was considered significant.

To evaluate the intervention effect segmented regression analysis [190] and a three parameter mathematical model, thoroughly described in II, was used. Student's t-test was used when comparing growth rate for resistant and susceptible isolates. ($p<0.001$ was considered significant)

Chi square test was used in III to evaluate differences between *E. coli* and *K. pneumoniae* and to test the association of resistance to *dfr*-genes ($p<0.001$ were considered significant)

Chi square test was used in IV for analysing the differences in antibiotic resistance and clinical parameters between TMP-resistant and TMP-susceptible isolates. The distribution of ST:s in relation to age group, sex, origin of specimen (i.e. Hospital or Community) and type of infection (where stated (n=346)) were analysed for the ten most common ST:s (all having $\geq$10 isolates) using Pearson Chi square test ($p<0.05$) (Statistica, StatSoft, Tulsa, OK, USA). The relation between specific ST:s and phenotypic resistance was analysed using Logistic regression (SPSS version 14.0 software (SPSS Inc., Chicago, IL, USA)) with a following analyses of the identified associations using Fischer's exact test on all ST-resistance combinations displaying a resistance higher or lower than the resistance rate given by the stratification of the material. Odds Ratios (OR) were calculated on the combinations with a significant ($p<0.05$) relationship.

Results and comments

Duplicate isolates - implication for re-infections with resistant bacteria

The effect on resistance rates of excluding duplicate isolates was in general small but consistent throughout the years except for the combination of *S. aureus* and fusidic acid. The pattern was different for the two pathogens studied (*E. coli* and *S. aureus*) indicating differences in carriage and/or infection patterns of these bacteria.

A continuous decrease in resistance rates in *S. aureus* when excluding duplicates were seen indicating a long carriage time (>one year) of the same strain. An interesting exception was seen in the years of a monoclonal epidemic of a fusidic acid resistant *S. aureus* strain. [54, 191] In these years an U-formed pattern was observed, especially prominent in the younger age-groups affected by the epidemic strain. [54, 191] This change was not due to an increased number of cultures. The pattern indicated that children infected with the fusidic acid resistant strain were re-infected and re-cultured (maybe because of the epidemic) and then with a fusidic acid susceptible isolate.

In *E. coli* a U-shaped curve was formed for all antibiotics except for FQX. (Figure 6) This pattern would suggest that after the first infection the risk of being re infected with the same, or another similarly resistant strain, is greatest up to 90 days after the index culture. After that the likelihood of being infected with a susceptible strain increases continuously (Figure 6). This finding is in line with previous reports an increased risk of re infection with a TMP resistant strain up till 180 days after the last TMP treatment. [119] For *E. coli* resistant to FQX a pattern of decreased resistance rates independent of algorithm was seen. This was not because of a relation of FQX resistance to urinary catheters in our material but indicates a longer carriage time of FQX resistant isolates or could be due to factors associated with complicated UTI, as the same pattern of continuous decrease was seen for all analysed resistance determinants in *K. pneumoniae*, *Proteus mirabilis* and *Enterococcus faecalis* (data not shown) more commonly found in association with complicated UTI. [192, 193]

Although the effect of excluding duplicate samples was limited it was consistent and stable over time for all antibiotics tested and clearly suggested that the same exclusion algorithm should be employed throughout the whole

intervention study (II). That algorithm should preferably be the same as the time between each data point (in the trimethoprim intervention 30 days (II)).

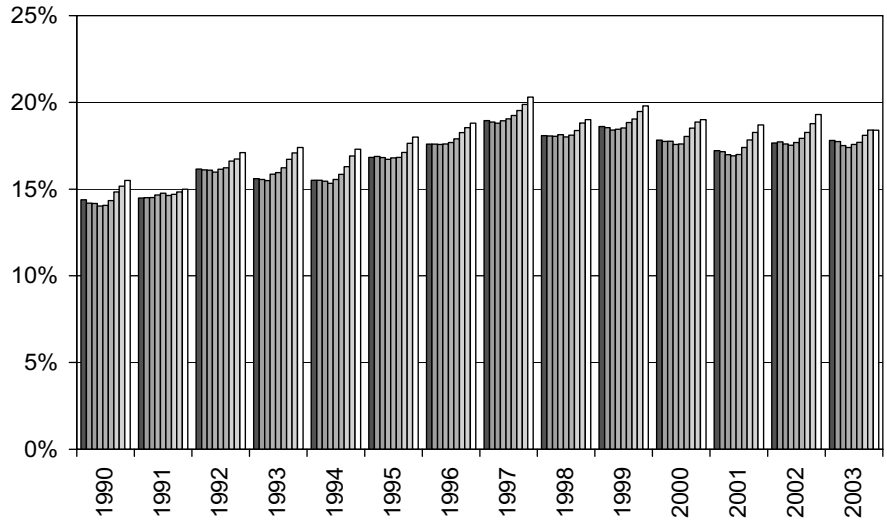

Figure 6. The effect on ampicillin resistance in *E. coli* of excluding duplicate isolates with the time algorithm 7, 14, 30, 45, 90, 180, 270 and 365 days from first positive *E. coli*. The first bar represents no exclusion. The exclusion rendered no clinically important difference in reported resistance rates or trend, but the pattern was consistent over time. (I)

The trimethoprim intervention (II)

So far this is the only controlled prospective intervention on antibiotic use with the purpose to test the hypothesis if reduced TMP and SXT use would result in a corresponding decrease in TMP resistance in a community.

The 2-year intervention was initiated the 1st of October 2004. An immediate 85% decrease in the use of TMP was achieved and this was sustained throughout the study period. The fact that physicians were allowed to replace the TMP containing drugs rather than avoid therapy altogether ensured good compliance (Figure 7).The decrease in the use of TMP was (as suggested by the study group) primarily replaced by MEC, NIT and to some extent FQX.

The effect on TMP resistance was limited (Figure 7). Segmented regression analysis could not detect any effect of the intervention on TMP resistance. This method does however not take into account the possible lag be-

tween the intervention and the effect on resistance rates. Because of this the three dimensional model was built to analyze the data. The model is described in detail in II and describes the competition for nutrients (space) between susceptible and resistant *E. coli* within a theoretical common gastrointestinal tract of all individuals in the investigated county. The relation between the TMP use in the studied community and the level of TMP resistance in the "common intestine" are built up by the first months of prescribing and resistance data available. With this model a significant decrease in trimethoprim resistance would be expressed as a fitness cost of TMP resistance.

The best fit of the model was associated with a small yet statistically significant fitness cost of TMP resistance. It predicted that during a continued intervention the TMP resistance would reach the 1991 level (i.e. 7-8%) after 12 years. The predictions of the model are intriguing as we could detect no effects on any of the experimentally assessed biological parameters. The distribution of phenotypes (II), Sequence Types (IV) and the distribution of specific TMP resistance encoding *dfr*-genes (Table 1) were all stable. The fraction of TMP-resistant isolates which were resistant to TMP only, was not affected, no *in vitro* fitness cost could be detected in TMP-resistant isolates and as expected [56, 154] high levels of associated resistance in TMP-resistant *E. coli* were seen throughout the study. At the same time the model predictions were mathematically accurate.

This conflict has some possible explanations: i) an influx in the intervention county of one or several successful TMP-resistant strains could mask a greater effect than that observed. This explanation is unlikely as only small and apparently random changes in strain distribution were observed and the intervention would be expected to undermine the positive selection of these strains specifically. ii) The population sampled might not have been fully representative for the intervention population. This is probably true to some extent as the recommendation in Sweden is not to culture uncomplicated UTI. TMP resistance rates reported from the Dept of Clin Microbiology, Växjö, are however historically at the same level as in uncomplicated UTI (Österlund A and Kahlmeter G, unpublished data) so this seems a rather unlikely cause for the discrepancy. iii) A third explanation could be that the treatment of infections caused by TMP resistant *E. coli* would have been cured more often during the intervention, leading to lower sampling of TMP resistant *E. coli*. This would result in the observed difference but would also implicate a trend towards lower sampling rates during the intervention, which could not be observed.

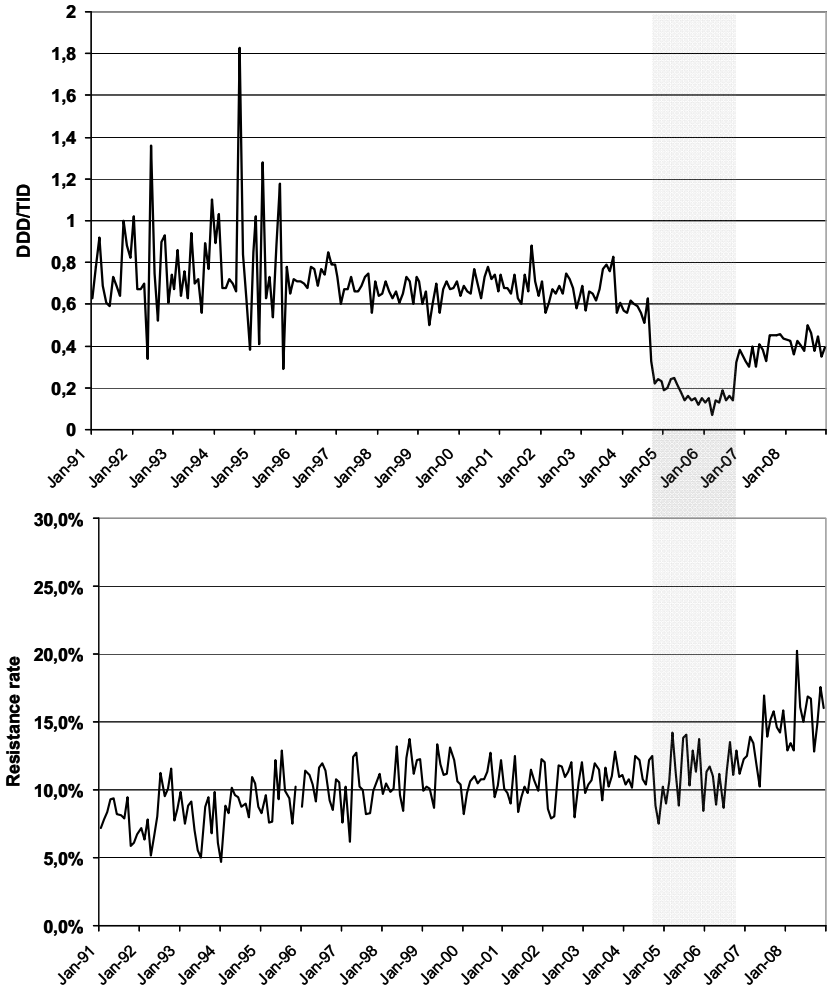

Figure 7 Consumption of trimethoprim and trimethoprim/sulphamethoxazole and corresponding resistance to trimethoprim in *E. coli*. Monthly data for Kronoberg county, Sweden, 1991-2008.

Over the last years, TMP resistance in *E. coli* has increased in all Swedish counties, including Kronoberg. (ResNet 2009, www.srga.org) In 2007 new Swedish guidelines concerning the treatment of uncomplicated UTI were adopted nationally and for the first time in more than 25 years, TMP was no longer among the first-line drugs. The use of TMP has since 2007 slowly decreased all over Sweden. Despite this TMP resistance increases all over Sweden (www.srga.org/ResNet_sok.htm) and the last figure for Kronoberg (2009) shows a 19% TMP resistance in *E. coli*.

The result in II through the model suggests that the *dfr*-genes provide *E. coli* with a small (>1%) fitness cost. The in vitro data measuring fitness cost

as growth reduction do not indicate a fitness cost among TMP resistant clinical isolates. (II) [194] Even if there is a fitness cost imposed by *dfr*-genes it would be less than 6% and seems to be epidemiologically compensated by the increased or unchanged use of antibiotics not directly involved in the intervention. The mechanism for this is co-selection of TMP resistant organisms due to the very high levels of associated resistance to these antibiotics.

dfr-genes and integrons in relation to species (III and IV)

Horizontal gene transfer is central in the dissemination of resistance genes both within [87, 195] and between species. [196] The rapid dissemination of $bla_{\text{CTX-M}}$ in several species is only one example. [197] As many resistance genes (among these the *dfr*-genes) are carried on conjugative plasmids[87, 198] the spread of these resistance mechanisms will be partly limited by the host range of the plasmids carrying these genes. [198] However, as the resistance genes are usually situated as gene cassettes within an integron structure transfer between different plasmids residing in the same bacterium will give a specific resistance gene the possibility of a much broader host range. Plasmids carrying *dfr*-genes (often together with several other resistance genes) have been shown to be easily transferred from an *E. coli* donor to *K. pneumoniae* in a mouse model. [199] As both *E. coli* and *K. pneumoniae* reside in the human intestine you would expect that the *dfr*-genes would be reasonably equally distributed in *E. coli* and *K. pneumoniae.* On the contrary the results in III showed a striking difference in the presence and distribution of the 13 investigated *dfr*-genes in *E. coli* and *K. pneumoniae* ($p<0.001$). In *K. pneumoniae* 69.0% of the isolates carried any of the *dfr*-genes studied whereas in *E. coli* 97.5% of the TMP resistance was explained by the same genes. The *dfr*A17 (being the second most frequent *dfr*-gene in *E. coli*) was only found in one single isolate of *K. pneumoniae* and *dfr*A1 was significantly more common in *E. coli* ($p=0.003$). *dfr*A5 was found equally common in both species (Table 1). Thus, the exchange of *dfr*-genes between *K. pneumoniae* and *E. coli* is not necessarily a common event. This has implications for the importance of the combination of core genome properties (IV) and plasmid encoded features [200] for the spread of antibiotic resistance.

Within *E. coli* the earlier reported [87, 185] relative importance of *dfr*-genes A1, A5, A7, A12 and A17 were confirmed in III showing the stability of these genes within the *E. coli* population. The stability of these genes both throughout the intervention, over time [87] and geography [87, 185] further underlines that the epidemiological fitness cost of these *dfr*-genes probably is low.

Table 1. No. of isolates positive for the investigated *dfr*-genes and integrons in *E. coli* collected consecutively at different time periods of the trimethoprim intervention and *K. pneumoniae* collected consecutively during the intervention.

	Escherichia coli				***Klebsiella pneumoniae***
	Pre intervention n=106	Mid intervention n=105	Post intervention n=109	Total n=320 (%)	Total n=55 (%)
dfrA1	36	41	33	110(34)	8 (15)
dfr2d	1	0	0	1(0.3)	0
dfrA3	0	0	0	0	1 (2)
dfrA5	17	17	18	52(16)	7 (13)
dfrA7	3	7	5	15(5)	1 (2)
dfrA8	3	3	8	14(4)	7 (13)
dfrA9	0	0	0	0	1 (2)
dfrA10	0	0	0	0	0
dfrA12	6	2	6	14 (4)	7 (13)
dfrA14	9	4	6	19 (6)	5 (9)
dfrA17	26	29	27	82 (26)	1 (2)
dfrA24	1	0	0	1 (0.3)	0
dfrA26	0	0	0	0	0
Integron 1	75	74	82	230 (72)	29 (53)
Integron 2	10	19	14	43 (13)	2 (4)

In contrast to the other *dfr*-genes, being equally associated with several other resistance determinants investigated, *dfr*A8 was associated to AMP resistance only and only one out of these ten isolates were additionally positive for any integron (Integron class I). Whether this indicates a more recent evolution, a different location of this *dfr*-gene or is just a coincidence due to low number of isolates remains to be investigated. Integron class I and/or II was identified in 85 % of TMP resistant *E. coli* and multi-resistance was associated with high prevalence of Integron class I as earlier reported.[18] Isolates with TMP mono-resistance was associated with no integron found or presence of integron class II ($p<0.001$). Integron class II are usually associated with a conserved sequence of gene-cassettes (*dfr*A1, *sat*1 and *aad*A1). [109] None of the corresponding phenotypic resistances were investigated in this study and the level of TMP mono-resistance could thus have been overestimated. Further limitations of III were that our analysis did not reveal the genetic position of the *dfr*-genes studied to integrons or other resistance determinants. Except for the few isolates analysed with Semi-Random PCR, the associations reported was purely based on the presence of genes/phenotypic expression in the same isolate.

Antibiotic resistance and population structure in *Escherichia coli* (IV)

As shown in III the distribution of antibiotic resistance encoding genes are not necessarily equally distributed between species within the Enterobacteriaceae. This disequilibrium has also been suggested within a species as the resistance development to a large part seems to take place within the "already resistant" population. [154] In IV the analysis of the *E. coli* population structure in TMP resistant (n=200) and TMP susceptible (n=348) isolates using MLST was performed. The sequence type (ST) distribution was not equal in TMP resistant isolates and TMP susceptible isolates. (Figure 8B) Six ST:s showed significantly higher TMP resistance than the stratified TMP resistance rate (36.5%): ST10 ($p=0.008$), ST58 ($p=0.001$), ST69 ($p<0.001$), ST131 ($p=0.003$), ST393 ($p=0.01$) and ST405 ($p=0.02$). ST69 (CgA), [53, 86, 171] ST393 (O15:K52:H1) [173] and ST131 have all, although in the latter in association with fluoroquinolone resistance[85] and ESBL-production,[20] been described as successful lineages with high levels of TMP resistance.[170] The TMP resistant isolates of these six lineages constituted 37% of the TMP resistant group.

The *dfr*-genes were additionally unequally distributed between ST:s. Twenty one out of a total of 24 TMP resistant ST73 isolates carried *dfr*A1 while in ST69 and in ST393 20/26 and 5/5 TMP resistant isolates were positive for *dfr*A17 respectively. All TMP resistant isolates of ST58 were positive for *dfr*A5. These associations were not considered due to small epidemics of these ST-*dfr*-gene combinations as the isolates were from patients of different age and residency. The localization of the *dfr*A1, *dfr*A5 and *dfr*A17 in the hybridization assay were heterogeneous. The ST69 isolates contained 2-5 plasmids and the *dfr*A17-gene was located on four of these plasmids indicating multiple acquisitions of this gene [163] or a single acquisition [63] followed by a continuous exchange between plasmids within this ST. In ST73, the isolates carried only one or two plasmids and the *dfr*A1-gene was located either on the same 145 kb plasmid or on the chromosome. ST58 showed a 1-2 plasmid carriage with the *dfr*A5 situated on a 145kb plasmid in 5 out of six isolates. These results are consistent with the genetic back-bone, as measured by MLST, to affect the effective host range of plasmids and resistance genes.

The host range of plasmids have been considered to be determined by plasmid encoded features like the coping with restriction enzymes, [201] plasmid addiction systems (toxin-antitoxin systems) [202] and incompatibility and plasmid partition systems. [203] In addition the driving forces for integration of plasmid borne genes into the chromosome [204] and the effect of the continuous rearrangements of the gene cassettes within and between integrons might be involved. [205] Some studies have suggested chromosomal genes to regulate these processes as the expression of virulence factors

has been shown to be dependent on phylogenetic group [167, 175] and the stability of broad host range plasmids, encoding antibiotic resistance within (*Pseudomonas putida*) or between species [166, 206].

In conclusion, the distribution of ST:s among TMP resistant and TMP susceptible isolates differed and the *dfr*-genes were additionally unevenly distributed. These differences may be explained by a control of plasmid stability and compatibility encoded by chromosomally located genes reflected by the ST.

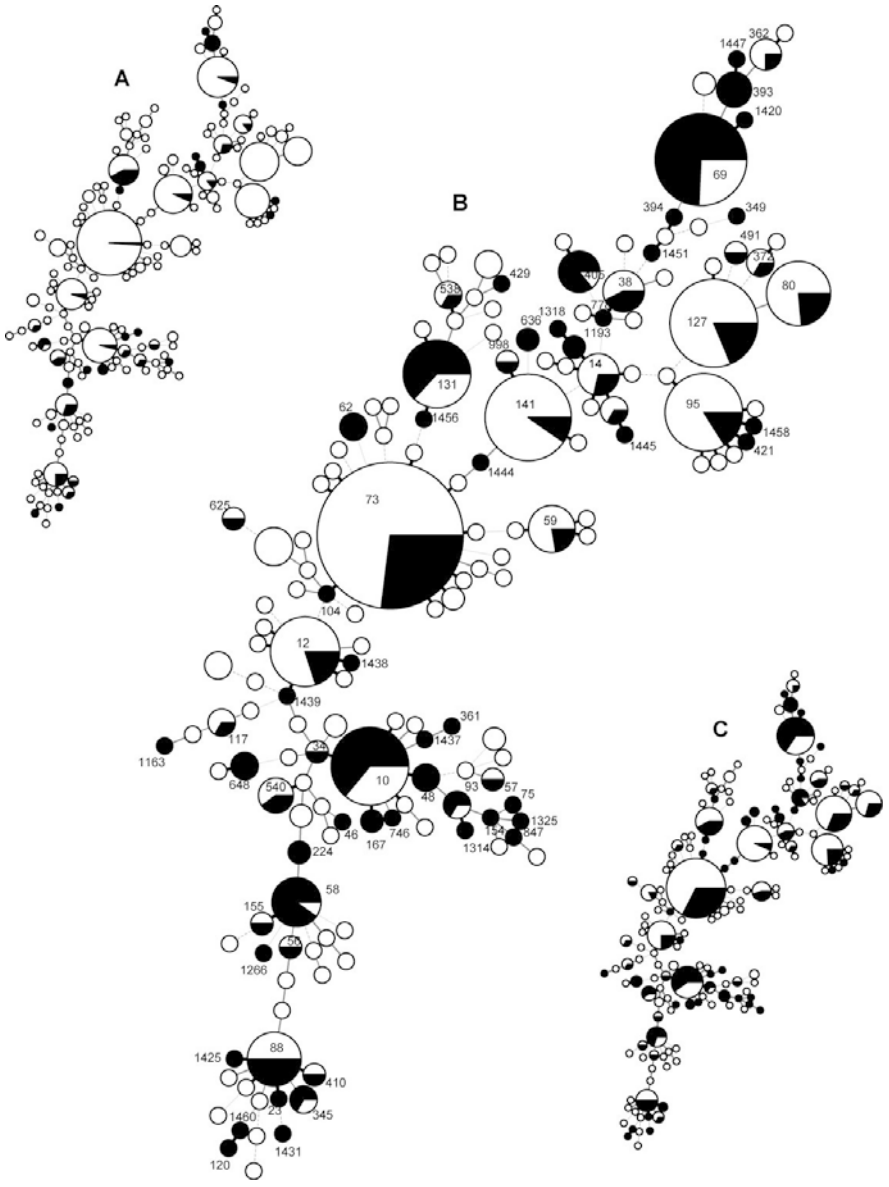

Figure 8. The distribution of antibiotic resistance in relation to Sequence Type (ST) presented as minimum spanning trees. Each panel represents 548 *E. coli* isolates (200 TMP-R and 348 TMP-S) from urinary specimens. The trees are based on the degree of allele sharing as determined by MLST analysis. Clonal complexes composed of at least three ST members are indicated by dots proportional in size to the number of ST:s within them. Lines connecting pairs of ST:s indicate that they share six (thick lines) or five (thin lines) alleles. Dotted connecting lines represent less allele sharing. Antibiotic resistant isolates are black: A) Ampicillin resistance, B) Trimethoprim resistance and C) Nalidixic acid resistance. Note that the material was stratified according to trimethoprim resistance.

General discussion

The inherent fitness cost of antibiotic resistance will not always impose an epidemiological fitness cost.

It has been shown that some antibiotic resistance confers a measurable *in vitro* fitness cost. [46, 49, 147] The postulated fitness cost have influenced the construction of mathematical models on antibiotic resistance development [57, 207, 208] leading to the idea that a reduction in antibiotic use would counteract the selection of resistant organisms and allow expansion of more fit susceptible strains and/or loss of genes encoding antibiotic resistance. This would result in a drop of resistance rates as measured in bacteria isolated in clinical samples. These *in vitro* studies have typically measured the fitness cost as the difference in growth rate between strains with and without resistance to defined agents. [46, 49, 147] Experimentally, resistance has been introduced in well defined susceptible strains through HGT [209] or by induction of resistance mutations. [210] The constructed iso-genic pair is allowed to compete in a common culture. The strain dominating the culture after X generations is considered the more fit strain. For clinical isolates, the growth rate is determined by using a spectrophotometer where continuous reading allows accurate determination of the maximum growth rate. [147]

The growth rate data in II was obtained in *E. coli* isolated from urine, mostly from patients with UTI. Clinical isolates will have a natural variation in growth rate and the sensitivity of such assay will therefore be reduced in comparison with competitions of constructed strains. On the other hand, the isolates from urinary specimens tested were considered relevant in relation to the intervention.

The reversibility of TMP resistance in the clinical setting studied in II is either extremely slow or non-existent. The fraction of TMP mono-resistant *E. coli* was not affected (II figure 2) and we could not demonstrate any decrease in growth rate *in vitro* in *E. coli* isolates mono-resistant to TMP or in combination with AMP and/or NAL resistance. (II) [194] This finding might be due to compensatory mutations[211, 212] and was in line with previous studies on resistance mechanism mainly spread by HGT. [209, 213, 214] Only occasionally have horizontally transferred resistance mechanisms been

shown to confer a substantial fitness cost. [213] More often the acquisition of resistance has been neutral [209, 213, 214] or even beneficial for the studied strain. [215-219]

Growth rate is only one way of measuring fitness. Several other factors will help determine the survival and establishment of resistant strains in bacterial populations. The incorporation of an *E. coli* strain in the intestinal flora will depend on the growth rate but also on features affecting colonization and virulence of the resistant strain (i.e. adherence factors etc). [220] Additionally the flora composition at challenge [71] and the selective pressure by antibiotics [221] will affect the survival of the strain.

All these factors taken together will affect the epidemiological fitness [207, 222-224] of a resistance mechanism - growth rate, transmission capacity, [224] transmission dynamics in relation to selection [222] and persistence despite a lack of selective pressure. [223] Based on the results of this thesis, it would seem appropriate to appoint co selection a more significant role in future modelling.

Stable distribution of *dfr*-genes in a stable *E. coli* population

Resistance rates in *E. coli* to AMP, TMP and SXT are now so high that empirical treatment will fail increasingly often. [17, 124] In addition increasing resistance to FQX, third generation cephalosporines and aminoglycosides is seen. [12, 13, 15] All these resistances are highly associated [56, 154, 156] sabotaging many proposed strategies for reducing or reverting resistance. Associated resistance in TMP resistant isolates is considerable. More than 75% of the TMP resistant *E. coli* isolated during the intervention was resistant to at least one of the other antibiotics used. (II)

The fact that associated resistance is so pronounced has many implications. Firstly, isolates resistant to several drugs are effectively selected for by all of them – which mean that these strains are more favoured than those resistant to fewer drugs. Consequently, the resulting increase in resistance will hit two or more antibiotics rather than only the “culprit-drug”. Secondly, in a patient infected with an isolate resistant to one drug the chance of finding another drug which is active (either blindly as empirical therapy or prospectively after susceptibility testing) is considerably lessened. [126]

The stability of the distributions of *dfr*-genes and ST:s was striking throughout the intervention. This could be explained by co selection due to the high degree of associated resistance and by the association of TMP resistance to well adapted lineages of *E. coli* prone to cause UTI. (IV) Since most *dfr*-genes are located on plasmids(IV) in association with integrons [225] the recent findings that the activity of the integrase is closely dependent on the SOS-response [37] is interesting as several antibiotics are potent activators of this stress response in bacteria. [37] A high level of SOS-response has been shown to increase the exchange of antibiotic resistance

genes [37] and we can thus expect a reduction in antibiotic use to result in a lower level of SOS-response indirectly stabilising the organisation of the antibiotic resistance genes within the integron. This possibly limits the effect of an intervention on antibiotic use as the resistance genes will be stabilized in the integron. In agriculture the ban on avoparcin caused an initial dramatic decrease in the levels of glycopeptide resistance in enterococci isolated from pigs and pig farm environments. This was of course facilitated by the normal fate of most pigs but interestingly low levels of glycopeptide resistance still remains on the investigated farms and is predicted to remain for a long time. [226] The stability in the distribution of both resistant and susceptible sequence types and in the *dfr*-genes further supports the stability of antibiotic resistance in *E. coli* once established.

In summary, antibiotic resistance in *E. coli* once established appears to be very stable. This is supported by the finding that the distributions of both resistant and susceptible sequence types and of the *dfr*-genes were stable throughout the intervention.

The *E. coli* genetic backbone and the control of acquisition and stability of antibiotic resistance

The associated resistance phenomenon would predict that some lineages within a species are more prone or better equipped to acquire resistance genes than others. In *E. coli* this implies that some lineages would be able to both control the acquisition and the stabilization of plasmids and resistance mutations.

A species will have an evolutionary advantage by upholding certain diversity, as the susceptibility to evolutionary bottlenecks will be reduced. [81, 227] In Enterobacteriaceae the conjugative plasmids are able to spread resistance genes [228, 229] and/or virulence genes. [167, 206] Plasmids are able to pass into a varying number of species – those with only few hosts are called narrow host range plasmids [230] as opposed to broad host range plasmids. [200, 231] The host range is considered to be determined by plasmid encoded features. [201-203]

Both *E. coli* and *K. pneumoniae* are normal inhabitants of the human intestinal tract and in a mouse model the transfer of plasmids between these two species was common. [199] The difference between the two species in the overall prevalence of *dfr*-genes observed in III was thus unexpected. Successful transfer of *dfr*-genes between the two species seems to be an uncommon event. Thus, the effective host-range of plasmids carrying *dfr*-genes might be narrower than one would expect.

The results in IV showed an uneven distribution of ST:s in the TMP susceptible and TMP resistant group. The diversity was comparable in both groups. This discrepancy indicates the genetic backbone, as measured with

MLST, to in part determine the host range of plasmids carrying resistance mechanisms. This hypothesis is further supported by similar findings in recent studies. [231-233] The association seen between specific *dfr*-genes and certain ST:s indicates that *dfr*-genes might be carried by plasmids with extremely narrow host range. The position of the *dfr*-genes were analysed in some strains giving a more complex result. It seemed as if ST69 was capable of carrying more plasmids than ST73 with the *dfr*A17 situated on several plasmids indicating either multiple acquisitions or a continuous recombination between different plasmids within ST69. Interestingly chromosomally encoded resistance mechanisms too were associated with some ST:s(IV). These observations show the complexity of the acquisition and stability of antibiotic resistance and furnish a biological fundament for the understanding of associated resistance.

Several studies have shown an association between resistance determinants and phylogenetic groups. [234-236] According to the results in IV this association will be imprecise since there are ST:s with both very low (ST127, ST141) and very high (ST131) resistance rates to TMP and other antibiotics as within each phylogenetic group (in this case group B2). Three ST:s, not previously reported to be associated with TMP resistance, were found in IV, indicating that screening large materials for one clonal group [86] will not add very much to our understanding of TMP resistance epidemiology.

The ST:s displaying exceptionally low resistance rates would appear to be "protected" from the acquisition of antibiotic resistance. Recent data on the Clustered, Regularly Interspaced, Short Palindromic Repeats (CRISPR) in the protection against foreign DNA acquisition in bacteria [237] might be of importance in our understanding of this phenomenon.

TMP resistance is mainly disseminated horizontally but the endemicity of some lineages more likely to cause UTI and to carry TMP and other resistance determinants seems to play an important role in the increasing resistance.

What seems easy in the hospital is hard to achieve in the community

The hospital and the community have traditionally been seen as two separate compartments with regard to antibiotic resistance. Hospitals have reported higher resistance rates than the community. [105, 193] Although this may often be true, the opposite is increasingly common. High levels of ESBL producing *E.* coli are seen in community acquired UTI. [238] MRSA were for a long period mainly associated with hospital outbreaks but over the last decade community acquired skin and soft tissue infections caused by MRSA have become commonplace. [239] These are examples of how certain lineages are better suited to one environment whereas others thrive primarily in the opposite milieu. [170, 240]

Hospital

Resistance rates often decrease in hospitals when decisive shifts in the use of antibiotics [6, 58-61] and/or improvements to infection control are instituted. [241] However, whether in burn units, intensive care units or in neonatal care facilities, these interventions are performed in "shoebox" compartments. Dynamics are rapid and effects of changes in antibiotic use are enhanced by the rapid turnover of patients. In most cases, the intervention was performed in response to a clinical problem with high rates of a specific resistant bacterium, mostly as a result of an epidemic outbreak with a resistant bacterium, such as ESBL-producing *K. pneumoniae*. Under these premises a shift in antibiotic policy has been shown to be effective as the isolation rate of the studied resistant bacteria has declined. [6, 58-61] Sometimes the intervention has coincided with enforced hospital infection control measures. [58]

Increasingly often, patients are colonized with one or several multi-resistant bacteria already at admission. [124, 242-244] The impact of sudden shifts in the use of two antibiotics to reduce the spread of a specific multi-resistant organism in hospitals will in this situation eventually diminish. Increasing multi-resistance will render any antibiotic an efficient selective force for keeping almost any resistance .[245] The logical conclusion is that to be successful the overall use of antibiotics must be reduced[59] and this must be coupled with stringent enforcement of infection control measures. These measures must be instituted and rigorously upheld in every institution involved in health care.

Community

Generalizing from the results in II, reducing the consumption of one antibiotic might delay the development of antibiotic resistance but not reverse it. This finding was in line with the earlier studies in *E. coli* from Great Britain where SXT was used until 1991 when its popularity gradually declined because of its adverse effects. Formal prescribing restrictions were implemented in 1995 and in the period up to 1999 there was a 97% decrease in the consumption of SXT. Despite this, the resistance level to sulphamethoxazole was virtually unchanged in 2004. [25, 27] Partly using the same set of isolates, it was shown that streptomycin resistance remained stable although the use in Great Britain had been very low for 30 years. [26, 27] These studies were retrospective and performed in a before/after fashion based on short collection periods of bacteria with a large proportion coming from inpatient specimens. In the study on sulphonamide resistance the concomitant use of trimethoprim as single agent was reported to increase, but the level of associated resistance to trimethoprim in sulphamethoxazole resistant isolates was not reported. [25]

At the time of the intervention study (II) two community interventions on antibiotic use, both resulting in apparent decrease in target antibiotic resis-

tance, had been performed. [23, 24] Both studies were performed in response to a sudden dramatic increase in resistance. The first study was conducted in Finland in response to a nationwide increase in erythromycin resistance in *S. pyogenes*. National recommendations advocating a decreased use of macrolide antibiotics were launched at the end of 1991. [23] During 1992 the use of macrolides decreased from 2.40 to 1.38 DDD/TIND and remained at this level during the study period. The increase in erythromycin resistance in *S. pyogenes* seen in 1992-1993 was followed by a significant decrease in 1994 and 1995. When examining the tables and figures presented in the article, the use of macrolides significantly decreased already in 1989. The reduction in macrolide resistance was thus seen 5 years after the initial decrease in macrolide sales indicating that other factors than the decreased use might have contributed to the decrease in resistance seen in 1994-1995. *S. pyogenes* are always susceptible to β-lactam antibiotics and associated resistance was uncommon in erythromycin resistant *S. pyogenes* at the time of the intervention. [21]

Table 2 Studies evaluating the effect on resistance rates of large scale reductions in antibiotic use in the community.

Country (ref)	Species	Antibiotic (s)	Intervention/ evaluation	Study design	Resistance rates
Finland [23]	*S. pyogenes*	macrolides	nationwide/ nationwide	prospective	decrease
Island [24]	*S. pneumoniae*	β-lactams and more	nationwide/ nationwide	prospective	decrease
Great Britain [25, 27]	*E. coli*	SXT	nationwide/ local	retrospective	increase
Great Britain [26]	*E. coli*	Streptomycin	nationwide/ local	retrospective	no effect
Sweden (II)	*E. coli*	TMP, SXT	county/ county	prospective	marginal effect
Great Britain [28]	*E. coli*	AMP, TMP and more	PHC/ PHC	retrospective	decrease
Israel [29]	*E. coli*	FQX	county/ county	retrospective	decrease

In Iceland, resistance to penicillin in *S. pneumoniae* increased from 1989 to 1993. This initiated a nationwide campaign to reduce the prescriptions of antibiotics. The reductions and changes in consumption together with restrictions on day-care centre attendance for children colonized with non-susceptible *S. pneumoniae* resulted in a decrease in the rates of PNSP.[24] However, in 2006 it was shown that this decrease was short-lived as a continued conservative use of antibiotics was not accompanied by sustained low level of PNSP. This was attributed to a clonal spread of successful PNSP serotypes. [246, 247]

Two recent studies, performed after the intervention in II, show that a reduction in antibiotic use seems to result in decreased resistance rates in *E. coli*. [28, 29] Butler and co-workers followed the antibiotic prescriptions and resistance rates in a large number of PHC:s in Wales and could show that the PHC:s having the largest reduction in AMP and TMP prescriptions during seven years also experienced a decrease in the corresponding resistance in *E. coli* in their communities. [28] The decrease was 58.7 to 53.5% for AMP and 29.1 to 25.7% for TMP resistance. No data were presented giving the reader a chance to evaluate whether the decrease was continuous or extrapolated from two measurements. Importantly, the decrease was from a very high level of resistance and the change could not possibly affect the empirical utility of these drugs.

Gottesman and co-workers presented data on ciprofloxacin resistance in *E. coli* in relation to a nationwide restriction on ciprofloxacin use in Israel (due to a threat of anthrax attacks). Interrupted time series analysis showed a significant decrease in ciprofloxacin resistance from 12 to 9% in the course of a six month intervention gradually reaching a reduction from 7000 DDD/month to 4500 DDD/month.[29] The study has several limitations. There was little information on methodological or interpretative aspects of susceptibility testing. Associated resistance rates in FQX resistant *E. coli* was not reported or discussed, nor were the possibility of FQX-resistant clones. The rapid effect on resistance rates is theoretically unlikely as recent data indicates the initial fitness cost of FQX resistance in *E. coli* to be rapidly compensated. [51]

The reversibility of resistance seems to be related to the fitness cost of the resistance mechanism studied, the epidemic potential and the transmission route of the species investigated. Both *S. pyogenes* and *S. pneumoniae* have a well known epidemic potential [248-251] and are easily spread in daycare centers [252] and in military camps. [250] The transmission routes for *E. coli* and other Enterobacteriacae are more indirect and will be dependent on living standards, water and food supply. [253, 254] These differences are probably of great importance. In two of the studies where reversibility of resistance was shown, the intervention coincided with a decline in an epidemic dissemination of resistant isolates. [21, 247]

In view of the results of the seven studies performed so far on the effects of reduced antibiotic use in the community (table 2) together with the pronounced associated resistance rates and seemingly low epidemiological fitness cost of resistance determinants, we should probably not expect that even major antibiotic interventions will dramatically affect resistance rates in the community. [226]

Conclusions

The effect of excluding duplicate isolates on resistance rates was influenced by the species, the antibiotic and on whether or not there was an outbreak of a specific resistant strain. The effects on resistance rates were systematic but small. The choice of exclusion algorithm did not affect the resistance trend over time. It was decided to use identical algorithms for excluding duplicate isolates during baseline and intervention evaluation of resistance. The algorithm used for exclusion of duplicate isolates should be stated when publishing or reporting on antimicrobial resistance surveillance data.

A drastic decrease in the use of trimethoprim containing drugs did not result in a corresponding decrease in trimethoprim resistance. This was true both for total trimethoprim resistance and for trimethoprim mono-resistance

The composition of the *dfr*-gene pool in *E. coli* remained unaffected by the intervention. The distribution of *dfr*-genes differed in *E. coli* and *K. pneumoniae*.

The population structure of trimethoprim susceptible and trimethoprim resistant *E. coli* were unaffected by the intervention. Six sequence types, as determined by MLST, were associated with high trimethoprim resistance rates, and some also to nalidixic acid and nitrofurantoin. A close association was seen between bla_{TEM} and *dfr*-genes giving the molecular basis for co selection.

No fitness cost of trimethoprim resistance could be detected *in vitro*.

The findings of this thesis indicates that, at least for some classes of antibiotics, we may have overestimated the usefulness of a strategy for reversing antimicrobial resistance based on the fitness cost of resistance. We have additionally underestimated the conserving effects of associated resistance. The stability of the *dfr*-genes and *E. coli* sequence types underlines the importance of associated resistance and successful endemic lineages in the spread and maintenance of antibiotic resistance in *E. coli*.

Future strategies to combat antibiotic resistant bacteria – the decision is ours

Bacteria adapt rapidly and there are no new antibiotic classes in the pipeline. Antibiotic resistant *E. coli* have been found in almost every environment exemplified by the finding of resistant *E. coli* in juvenile birds in Siberia, [255] swine, [256, 257] poultry, [66, 258] and people living in very remote areas. [256] We can expect every molecule of antibiotics wherever spent to select for the persistence of antibiotic resistant strains. The animal reservoir of antibiotic resistance [209, 259-261] will often end up in retail meat [209, 259] and might thus contribute to the resistance levels seen in human infections. The combination of indigestion of resistant strains with extra intestinal pathogenic capacity being selected in human intestines and the acquisition of antibiotic resistance by resident strains [77, 262] through HGT and *de novo* mutations [195, 263]are both favoured by antibiotic use. [199, 264] The normal faecal flora is an important reservoir for the development and selection of resistant bacteria. [265, 266] The carriage of ESBL producing *E. coli* has increased dramatically during the 1990s [242] underlining the importance of the continuous selection of subpopulations of already resistant bacteria in the faecal flora.

Globalisation, the rapid and frequent travelling and the increasing international market exchange of foods and feeds, and modern healthcare will increase the spread of resistant bacteria and new immuno suppressive drugs will increase the need of effective bactericidal antibiotics. With no new antibiotics in sight we must start choosing carefully who, when and how to treat with antibiotics. Antibiotics have a limited effects in uncomplicated infections[267] and we probably need to find areas where antibiotics can be restricted. The prudent use of antibiotics should always be promoted. Prudent means: always appropriate, less in many cases but occasionally some patients will need more and broader treatment. Normally one should advocate that narrow spectrum antibiotics with low impact on the normal flora are used. In the treatment of UTI three antibacterial agents have fared better than others in relation to resistance development. Resistance to fosfomycin, nitrofurantoin and pivmecillinam is still very low (1-5%) [12, 13] and this is in spite of quite intense use in some countries. [93] These agents have in common that they are all excreted mainly in urine and the impact on the normal gut flora is limited. [145] and they are almost only used for UTI. In addition

resistance to these agents has been shown to confer a fitness cost to the bacterium. [49, 147, 194]

Multi-resistance causes deaths [124, 268] and clinical microbiologists and infectious disease physicians have to take the initiative in the decisions on antibiotic use and infection control. We have to help our colleagues in a wait and see approach in uncomplicated infections and refrain from using broader agents unnecessarily. Such strategies have been proven useful and will reduce antibiotic use in the hospitals. [269] We should always promote infection control bundles to keep the transmission of micro organisms (susceptible or resistant) as low as possible.

The new situation calls for a more active participation in everyday clinical care and choice of antibiotics. Both clinical microbiologists and infectious disease specialists will need to master the intricacies of new and combined resistance mechanisms to be able to correctly help other clinical colleagues in their choice of therapy. Rapid transport systems for both samples and results together with new diagnostic tools, enhanced culturing, [270] rapid susceptibility testing and continuous resistance surveillance of good quality to guide empirical treatment must be priorities for clinical microbiology laboratories.

All these measures will help to reduce the speed of antibiotic resistance development, but not lead to decreased levels of antibiotic resistance. As these measures ideally will buy us some time, the development of new antibiotics has to be prioritized among pharmaceutical companies and governments.

Future research

This thesis provides a basis for further research in the field of antibiotic resistance.

The hypothesis that the genetic-backbone restricts the host range of plasmids should be confirmed or refuted. The importance of CRISPRs is interesting in this context and should be further studied.

The increasing knowledge on mechanisms steering dissemination of antibiotic resistance between and within bacterial species, such as the SOS-response, needs more attention and could eventually result in new strategies for containing antibiotic resistance. This could be as important as developing new antibiotics.

It appears that some antibiotic resistance is coupled with a persistent fitness cost and this may prevent the dissemination of resistance. If so, it is important to elucidate why and what the differences are between these types of resistance mechanisms and those where compensatory mechanisms seem to rapidly nullify fitness cost.

It is doubtful if more studies on the effect of large scale community single-drug interventions on antibiotic use are useful. They are costly and cum-

bersome and will most probably not add more information regarding the potential of this concept. However, should someone have the courage and means to study the effect of a decisive and dramatic general reduction in the use of antibiotics in a community of some size, this would add to our knowledge.

The fact that associated resistance is a general phenomenon with all species and all drugs is not generally known or appreciated. It has been shown for Enterobacteriaceae, *Pseudomonas aeruginosa*, *S. aureus*, *S. pneumonia*e and *S. pyogenes*. [154] The genetic background, the importance for resistance development and the clinical implications of this phenomenon should be studied.

Further studies screening large *E. coli* materials for only one [86] to three [85] lineages already known to be associated with TMP resistance will not provide more information regarding the increase in TMP resistance seen worldwide. Instead more focus should be put on how to counteract the spread of multi-resistant bacteria in the community and in hospitals.

Sammanfattning på svenska

Andelen infektioner som orsakas av antibiotikaresistenta bakterier ökar över hela världen. För att det ska vara medicinskt försvarbart att genomföra transplantationer, protesingrepp mm så krävs möjlighet till effektiv antibiotikabehandling. Den långsamma utvecklingen av nya antibiotika innebär därför att vi måste hitta nya strategier för att hushålla med de antibiotika vi har idag.

Resistens hos bakterier utvecklas genom mutationer och/eller inkorporering av genetiskt material från andra bakterier via plasmider eller direkt från omgivningen. Vissa resistensmekanismer har visats vara en belastning för bakterien i avsaknad av antibiotika. Det vanligaste är att denna biologiska kostnad ses som en minskad tillväxthastighet hos den resistenta bakterien men kan också uttryckas genom minskad förmåga till kolonisation eller minskad möjlighet till spridning mellan individer. Om den resistenta bakterien exponeras för antibiotika så får den ökad chans till överlevnad genom minskad konkurrens från andra (känsliga) bakterier och genom att kompensera den initiala kostnaden genom ytterligare mutationer. Denna relation mellan antibiotika och kostnad har lett fram till matematiska modeller och ett fåtal kliniska studier som visat att man kan vända antibiotikaresistensutvecklingen hos bakterier genom minskad antibiotikaanvändning.

Plasmider som bär antibiotikaresistensgener innehåller oftast integroner som reglerar integrering och uttryck av de genkassetter som kodar för resistens mot t.ex trimetoprim. Trimetoprim är ett antibiotikum som hämmar tillväxten hos bakterierna genom att inverka negativt på folsyrasyntesen. Trimetoprim är ett effektivt antibiotika och används i Sverige nästan uteslutande i behandlingen av urinvägsinfektioner (UVI).

Okomplicerad UVI orsakas i närmare 90% av fallen av *Escherichia coli* som normalt finns i tarmfloran hos djur och människor. *Escherichia coli* kan delas in i phylogenetiska grupper där vissa har egenskaper som gör att de har högre sannolikhet att kolonisera tarmen under lång tid och även orsaka urinvägsinfektion (UVI). Den *E. coli* stam som man finner i urinen vid UVI dominerar oftast tarmfloran vid samma tillfälle. Trimetoprimresistens orsakas av minst 30 olika *dfr*-gener som kodar för ett modifierat enzym vilket förhindrar trimetoprims verkan. En stadigt ökande resistens hos *E. coli* har noterats under de senaste åren och resistens mot trimetoprim förekommer i 15-65% av *E. coli* stammarna beroende på var i världen man befinner sig.

Syftet med den aktuella avhandlingen var att testa hypotesen att en drastiskt förändrad antibiotikaanvändning i samhället skulle påverka relaterad

resistens. Vidare undersöktes effekten av denna intervention på fördelningen av *dfr*-gener och populationsstrukturen hos *E. coli*.

Antibiotikaresistensutvecklingen följs vanligen genom studier på bakterier framodlade från patientprover. Dessa data innehåller olika nivåer av upprepade bakteriefynd från samma patient vilket gör att jämförelser mellan olika studier blir osäkra. I den första studien undersöktes betydelsen av olika tidsgränser för uteslutning av dessa upprepade isolat i de data som sedan används som baslinje för interventionen i II. Hos *E. coli och Staphylococcus aureus* gav de olika tidsgränserna små men konsekventa effekter på resistensnivåerna, vilket gav ökad förståelse för risken att återinfekteras med resistenta bakterier. Slutsatsen var att den algoritm som används för uteslutning av upprepade isolat ska anpassas till den mätperiod man har och alltid anges i samband med publicering av antibiotikaresistensövervakning.

I studie två beskrivs effekten på trimetoprimresistens av en drastiskt minskad användning av trimetoprim och trim-sulfa. Alla läkare i Kronobergs län uppmanades att inte använda trimetoprim och trim-sulfa från oktober 2004 till september 2006. Användningen minskade omedelbart med 85% och ersattes av nitrofurantoin, mecillinam och till viss del av ciprofloxacin. En matematisk modell utvecklades för att analysera interventionseffekten. Trimetoprimresistens hos *E. coli* minskade inte men modellens bästa passning mot observerade data visade en utplanad resistensutveckling uttryckt i modellen som en liten biologisk kostnad för trimetoprimresistens hos *E. coli*. Denna kostnad kunde inte verifieras i tillväxtförsök *in vitro*. Fördelningen av fenotyper hos 4275 *E. coli* påverkades inte av interventionen och inte heller andelen av de trimetoprimresistenta *E. coli* som var resistenta enbart mot trimetoprim. Den begränsade effekten av interventionen bedömdes bero på en liten eller icke existerande kostnad av trimetoprimresistens samt coselektion genom användningen av andra antibiotika som trimetoprimresistenta *E. coli* också var resistenta mot i hög utsträckning.

För att öka förståelsen av den begränsade effekten av interventionen analyserades i studie 3 fördelningen av *dfr*-gener och integroner hos *E. coli* och *Klebsiella pneumoniae.* Fördelningen av dessa gener var stabil i båda arterna genom hela interventionen och de *dfr*-gener som tidigare rapporterats vanliga hos *E. coli* dominerade även det aktuella materialet. Fördelning av integroner och *dfr*-gener i *K. pneumoniae* skiljde sig förvånande nog från den i *E. coli* vilket antyder att utbytet av resistensgener mellan dessa båda arter är begränsat.

Under senare år har man noterat att större delen av antibiotikaresistensutvecklingen verkar ske i en begränsad del av *E. coli* populationen. I den fjärde studien användes Multi Locus Sequence Typing, MLST, för att studera detta fenomen hos sammantaget 548 *E. coli* (200 trimetoprimresistenta och 348 trimetoprimkänsliga) i relation till interventionsstudien. De trimetoprimresistenta isolaten hade tidigare analyserats i studie 3 och var därför välkarakteriserade avseende *dfr*-gener och integroner. Fördelning av sekvenstyper var

stabil i relation till interventionen. Trimetoprimresistens var spridd i en stor del av *E. coli* populationen vilket tidigare har antagits, men sex sekvenstyper visade signifikant högre trimetoprimresistens än bakgrundsnivån i materialet. Några av dessa var dessutom kopplade till resistens mot andra antibiotika. Detta antyder att mekanismer relaterade till bakteriernas genetiska bakgrund har betydelse för integrering och stabilisering av antibiotikaresistens hos *E. coli*. Tre *dfr*-gener var starkt associerade med vissa sekvenstyper vilket inte kunde förklaras av mindre epidemier utan sannolikt beror på ytterligare detaljerad styrning av vilka genetiska element som kan inkorporeras och samexistera i *E. coli*

Sammanfattningsvis visar avhandlingen att det är osannolikt att man kan vända antibiotikaresistensutvecklingen genom minskad användning av en antibiotikaklass på samhällsnivå. Olika delar av *E. coli* populationen verkar ha olika förutsättningar att ta upp och stabilisera antibiotikaresistens vilket kommer att få konsekvenser för framtida strategier för behandlingen av antibiotikaresistenta bakterier.

Ännu större vikt måste läggas vid att försöka minska utvecklingstakten av antibiotikaresistens. Detta kan åstadkommas med en kombination av rationell antibiotikaanvändning, aktivitet för att öka förståelsen för betydelsen av basal vårdhygien och en ökad aktivitet hos mikrobiologiska laboratorier för att ta fram snabba metoder för resistensbestämning. Infektionsläkare och kliniska mikrobiologer måste ta initiativet och fortlöpande blanda sig i enskilda antibiotikaval, inte bara de mest komplicerade. Det finns idag mycket evidens som betonar den begränsade nyttan av antibiotika vid okomplicerade infektioner och att korta behandlingstider kan användas. Våra kollegor behöver stöd för att använda denna kunskap.

All onödig användning av antibiotika såväl inom jordbruk, olika industriella processer och i human medicin måste upphöra för att kommande generationers infektioner effektivt ska kunna behandlas.

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