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INTRODUCTION

Antibiotics are compounds that kill or inhibit the growth of bacteria while causing no or only limited harm to the human body. This phenomenon, termed "selective toxicity" by the immunochemist Paul Ehrlich (1854-1915), is based on differences in structure and metabolism between microorganisms and host cells. Antibiotics exert their action on target sites in bacteria that are absent in host cells.

The first antibiotics described and introduced into clinical practice were compounds synthesized by microorganisms, such as penicillins and aminoglycosides. Today, an increasing number of antibiotics are synthetically produced, e.g. sulfonamides and quinolones. Many antibiotics are semisynthetic, i.e. natural substances chemically modified in the laboratory [7].

ANTIBIOTIC MECHANISMS

Antibiotics may be classified according to whether they are bactericidal, i.e. kills the bacteria, or bacteriostatic, i.e. inhibits their growth. The most common classification of antibiotics is by their mechanism of action on the bacteria.

1. Inhibition of cell wall synthesis
2. Inhibition of protein synthesis
3. Inhibition of folic acid synthesis
4. Inhibition of DNA replication
5. Inhibition of RNA transcription

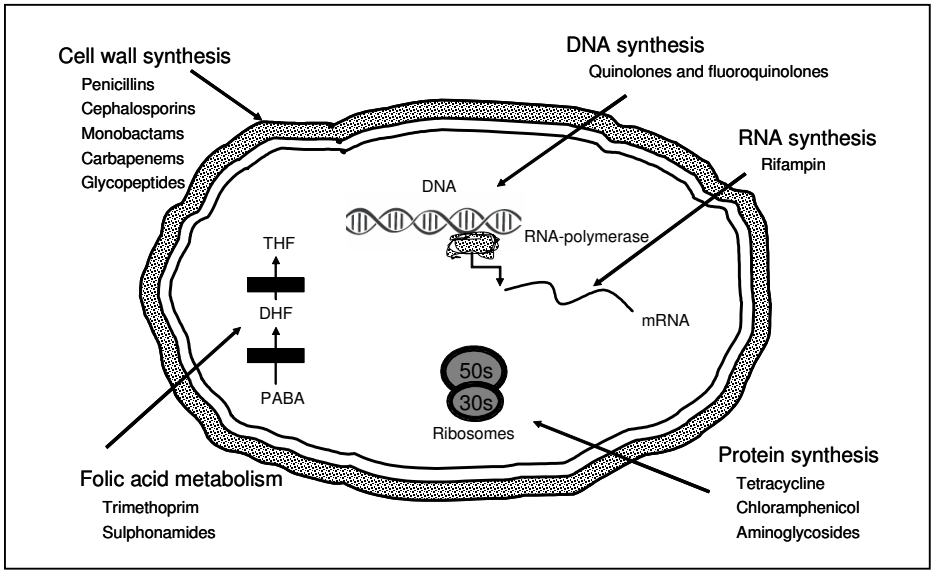


Figure 1. Mechanisms of action for the most important groups of antibiotics

1. Inhibition of cell wall synthesis

Bacteria are covered by a rigid cell wall composed mainly of peptidoglycan, which is unique to bacteria and therefore an excellent target for selective toxicity. Loss of peptidoglycan causes lysis and death of bacteria. Examples of antibiotics, which interfere with peptidoglycan synthesis, are penicillins, cephalosporins and glycopeptides [12].

Peptidoglycan consists of a linear backbone composed of sugars (glycan) cross-linked with chains of amino acids (peptides)(Fig. 2).

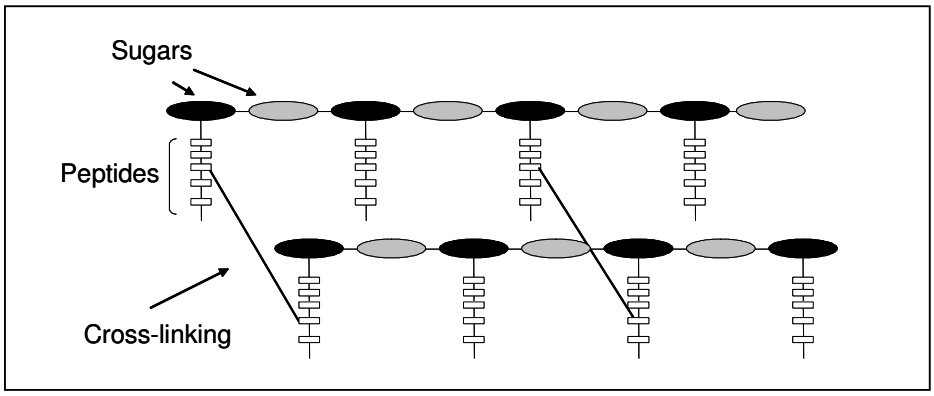


Figure 2. The structure of peptidoglycan.

Peptidoglycan synthesis starts in the cytoplasm by binding the peptide chains to one of the sugar molecules. In the cytoplasmic membrane, the other sugar molecule joins creating disaccharide subunits, which are then released on the outside of the cytoplasmic membrane and linked together to form long peptidoglycan strands. Finally, the strands are linked together by cross-linking of the peptide chains. This reaction is catalyzed by specific transpeptidases located in the cytoplasmic membrane (Fig. 3).

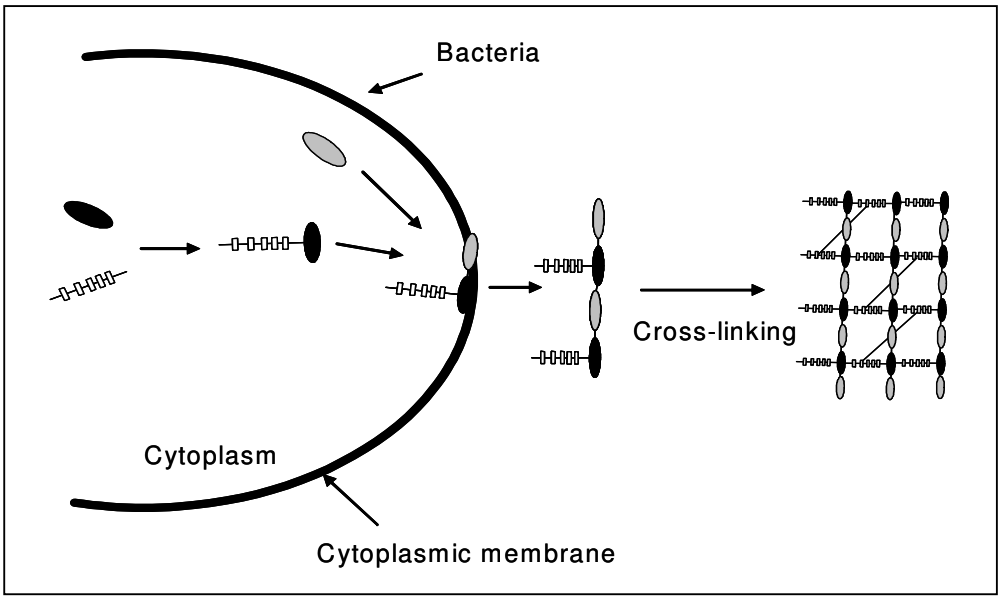


Figure 3. Synthesis of peptidoglycan in bacteria.

The cell walls differ between gram-positive and gram-negative bacteria. Peptidoglycan is present in both gram-positive and gram-negative bacteria, but the peptidoglycan layer is much thicker in the former group. In gram-negative bacteria, the peptidoglycan layer is covered by an outer membrane. There are channels (porins) in the outer membrane that allow small molecules to diffuse into the space between the outer membrane and the cytoplasmic membrane.

Beta-lactams

The common compound in all beta-lactam antibiotics is a four-membered ring that consists of three carbon atoms and one nitrogen atom (Fig. 4). This ring is the active part of all beta-lactam antibiotics. It binds to enzymes responsible for cross-linking the peptidoglycan

chains, these enzymes are also called penicillin binding proteins (PBPs). Binding of the beta-lactam results in an accumulation of unlinked cell wall units and cell lysis.

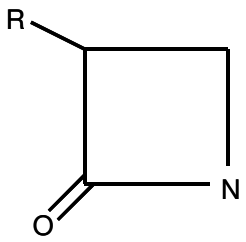


Figure 4. Beta-lactam ring structure.

The beta-lactam antibiotics comprises of penicillins, cephalosporins, carbapenems and monobactams. The different groups of beta-lactam antibiotics are distinguished by the structure attached to the beta-lactam ring [7].

Penicillins

Penicillin was discovered by Alexander Fleming in 1929. He was interested in finding an effective treatment for wound infections caused by *Staphylococcus aureus* which was of importance in war wound infections. The substance described by Fleming was a natural antibiotic produced by the mould *Penicillum notatum* [1]. In 1940, Chain and Florey turned penicillin into an effective antibiotic and started to produce it in large scale. In penicillin a five-membered ring is attached to the beta-lactam ring (Fig. 5).

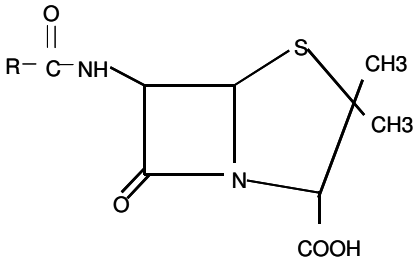


Figure. 5. Structure of penicillins .

The penicillin discovered by Fleming was named benzylpenicillin or penicillin G (PcG). PcG must be given parenterally because it is unstable in acidic medium and thus

destroyed in the stomach. Phenoxymethylpenicillin or penicillin V (PcV) has been modified to permit oral administration. PcG and PcV are both narrow spectrum antibiotics, acting on most gram-positive and gram-negative cocci. They are not active on gram-negative bacteria such as *Enterobacteriaceae*, because their outer membrane is impermeable to PcV and PcG.

A derivate of penicillin, ampicillin, was developed by attaching an amino group to the beta-lactam ring that increased their activity against gram-negative bacteria. Ampicillin exhibits acceptable stability in an acidic medium, allowing transit through the gastrointestinal tract, but it is poorly absorbed. Amoxicillin is a derivate of ampicillin, which is well absorbed after oral administration. Amoxicillin can be used in combination with clavulanic acid. Clavulanic acid is a very weak beta-lactam antibiotic and binds covalently to bacterial proteins, which causes resistance to amoxicillin. The binding destroys these proteins and makes it possible for amoxicillin to be extremely active against gram-positive and gram-negative bacteria.

Cephalosporins

Cephalosporin was first isolated from a mold named *Cephalosporium*. Cephalosporins have a six-membered ring attach to beta-lactam ring (Fig 6). The low activity of natural cephalosporins could be increased by various substitutions to their six-membered ring.

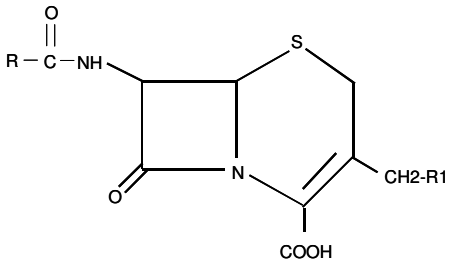


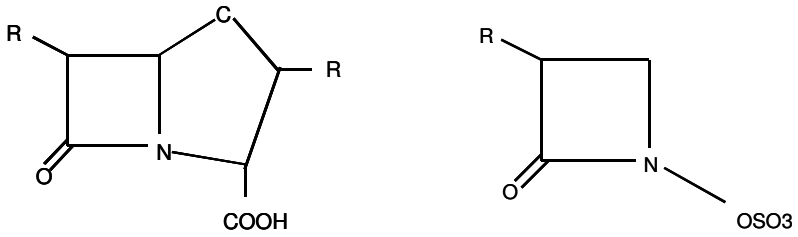
Figure. 6. Structure of cephalosporins

Cephalosporins have a broader spectrum of antibiotic activity than penicillins. Several classes of cephalosporins have been developed, often referred to as first, second, third and fourth generation of cephalosporins. The first generation has the most narrow spectrum with good activity against gram-positive and poor activity against gram-negative bacteria. Cefadroxil and cephalotin are examples of this generation. The second generation

cephalosporins exemplified by cefaclor, cefuroxime and cefoxitin, have increased activity against gram-negative compared to the first generation. The third generation of cephalosporins, e.g. cefotaxime, cefibuten, ceftriaxone and ceftazidime are much more active against *Enterobacteriaceae* than the first generation of cephalosporins due to their ability to pass through the outer cell membrane. The fourth generation of cephalosporins represented by cefepime and cefpirome have good activity against *Enterobacteriaceae*, and no activity against enterococci or anaerobes.

Carbapenems and monobactams

Carbapenems and monobactams (Fig. 7) were developed to counteract the increasing prevalence of bacterial resistance to penicillins and cephalosporins. The carbapenems, i.e. meropenem and imipenem have activity against most gram-positive as well as gram-negative bacteria. Monobactams, i.e. aztreonam, is limited to aerobic gram-negative bacteria.



Carbapenems monobactams

Figure 7. Structure of carbapenem and monobactam

Glycopeptide antibiotics

The glycopeptide antibiotics vancomycin and teicoplanin were discovered in the 1960s. Vancomycin and teicoplanin are big molecules that cannot pass through the pores of the outer membrane of gram-negative bacteria, which is why they are effective only against gram-positive bacteria. These antibiotics inhibit bacterial cell wall synthesis. They act by binding to the peptide chains of peptidoglycan and prevent the binding transpeptidase and cross linking of the peptidoglycan chains (Fig. 8).

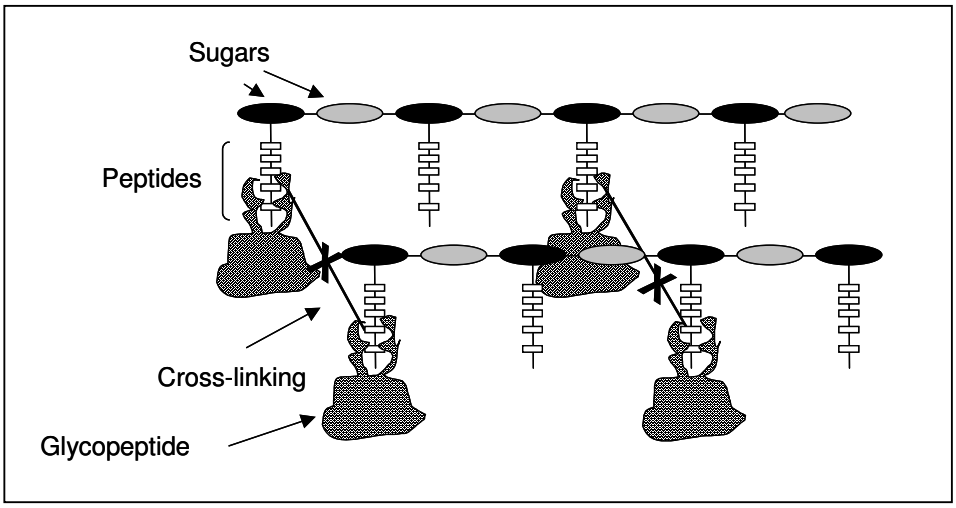


Figure 8. The inhibition of peptidoglycan synthesis by glycopeptides.

2. Inhibition of protein synthesis

Protein synthesis takes place on bacterial ribosomes where mRNA translates into proteins. Bacterial ribosomes consist of two subunits, 30S and 50S each consisting of one or two strands of RNA and several proteins. They differ structurally from the ribosomal of mammals that are composed of 40S and 60S subunits. Antibiotics can bind to one or both of these subunits and inhibit the protein synthesis by interfering with one of several stages i.e. initiation, elongation, translocation or termination.

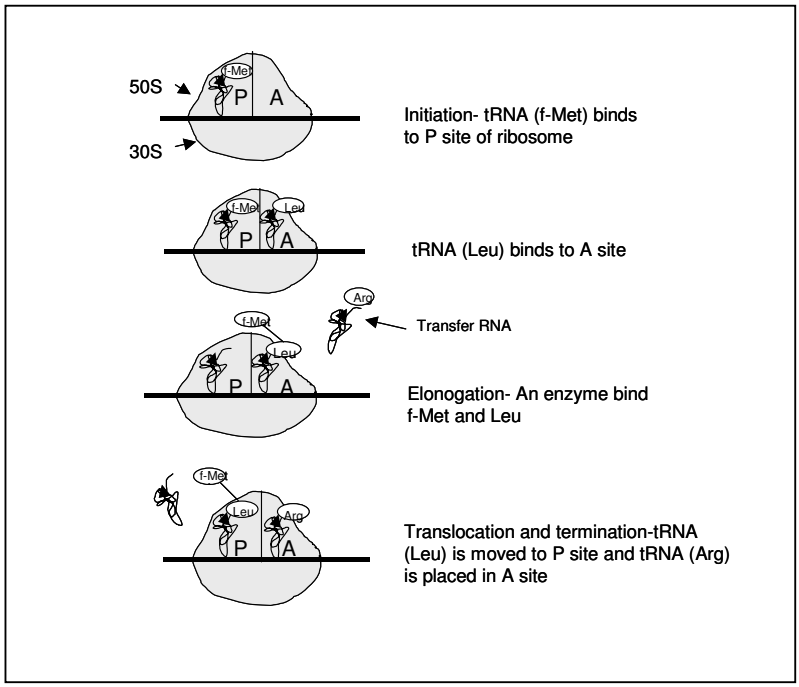


Figure 9. Protein synthesis in bacteria. A = acceptor site, P = peptide site

Tetracyclines

Tetracyclines bind to the 30S subunit of the bacterial ribosomes. This binding alters the A site of the ribosome and prevents the aminoacyl-tRNA from entering this site, hence no proteins are produced [2, 3]. Tetracyclines are broad-spectrum agents active against both gram-positive and gram-negative bacteria, and atypical organisms such as chlamydia, mycoplasmas, and rickettsia. Tetracycline penetrates bacterial cells by passive diffusion and are bacteriostatic in their action [4]. Tetracyclines are inexpensive, have low toxicity and can be given orally. These traits have led to an overuse of tetracyclines in clinical practice, and also in veterinary medicine. Furthermore, tetracyclines have also been given as feed additives to promote growth of livestock. Tetracyclines are not given to children, since they incorporate readily into growing bones and teeth.

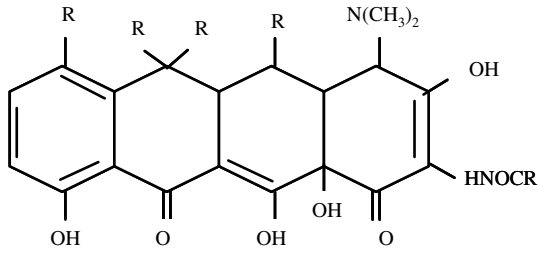


Figure 10. Structure of tetracycline

Chloramphenicol

Chloramphenicol binds to the 50S subunit of the ribosomes. It interferes with the binding of new amino acids to the growing peptide chain. Chloramphenicol is a broad spectrum antibiotic that acts against many gram-positive and gram-negative bacteria. Chloramphenicol has a toxic effect on liver and bone marrow stem cells which limits its use.

Aminoglycosides

Aminoglycosides bind to the 30S subunit of the bacterial ribosome, which prevents joining of the 50S subunit to the 30S to form the active ribosome and thereby protein synthesis [5]. Aminoglycosides, e.g. streptomycin, tobramycin, gentamicin and amikacin, are effective against many classes of bacteria. Their use is limited by toxic side effects on to the inner ear resulting in permanent loss of hearing and balance.

Macrolides

Macrolides, e.g. erythromycin, inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and inhibiting protein elongation. Macrolides have a relatively broad spectrum and affect gram-positive and some gram-negative bacteria e.g. *Haemophilus*, *Mycoplasma* and *Chlamydia*. Macrolides are bacteriostatic and have also been used on livestock, primarily to prevent shipping sickness.

Lincosamides

Lincosamides (clindamycin) have the same mechanism of action as the macrolides. They bind the large subunit of bacterial ribosomes and prevent elongation of peptide chains. Lincosamides have a broad spectrum against aerobic gram-positive cocci and anaerobes, whereas all *Enterobacteriaceae* are resistant.

3. Inhibition of folic acid synthesis

Sulfonamides and trimethoprim

Bacteria need folic acid to synthesize DNA and a number of amino acids. Bacteria lack a system to obtain folic acid from the environment and they must synthesize it by themselves. Sulfonamides and trimethoprim interfere with the production of the active form of folic acid, i.e. tetrahydrofolic acid (THF), by imitating the substrates of two different enzymes i.e. para-aminobenzoic acid (PABA) and dihydrofolate (DHF) [6]. The combination of sulfonamide and trimethoprim causes a double block of the folate system in the bacterial metabolism. Trimethoprim has a broad antibacterial spectrum including *Staphylococcus*, *Streptococcus* and *Enterobacteriaceae* and is a first line of treatment against uncomplicated urinary tract infection [7].

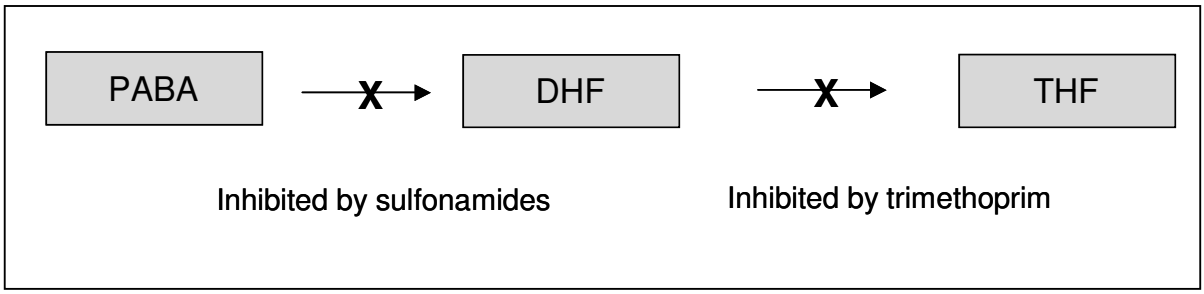


Figure 11. Mechanism of action of trimethoprim and sulfonamide.

4. Inhibition of DNA replication

Quinolones and fluoroquinolones

In the early 1960s, nalidixic acid was introduced as the first quinolone. Nalidixic acid had poor activity against gram-negative bacteria and no activity against gram-positive bacteria, still it was extensively used in laboratory experiments. Quinolones are modified types of nalidixic acid with good antibiotic activity. Fluoroquinolones are fluorinated quinolones and include norfloxacin and ciprofloxacin. All fluoroquinolones have good activity against gram-positive and gram-negative organisms. Quinolones and fluoroquinolones are synthetic antibiotics.

Opposed to eukaryotic DNA, bacterial chromosome is highly supercoiled. The bacterial chromosome DNA is very long and it is necessary to be coiled to fit inside the cell after the DNA replicated. This coiling regulates by four different types of enzymes, termed topoisomerase (I to IV), which are involved in returning replicated DNA to its supercoiled form. Quinolones and fluoroquinolones bind tightly to topoisomerases and prevent their action. Topoisomerase II (gyrase) is the target for quinolones in gram-negative bacteria, whereas topoisomerase IV is the target in gram-positive bacteria.

5. Inhibition of RNA synthesis

Rifampin

Rifampin binds to DNA-dependent RNA polymerase and inhibits the initiation of RNA synthesis. Rifampin is very active against *Mycobacterium tuberculosis* and aerobic gram-positive cocci.

USE OF ANTIBIOTICS IN SWEDEN

Antibiotic consumption is measured as defined daily doses (DDD), a unit based on the average daily dose used for the drug. Sales data from the National Corporation of Swedish Pharmacies were calculated as the number of grams of drugs, converted it into a number of defined daily doses and then adjusted for 1000 inhabitant per day [8] [9].

Penicillin V is the most prescribed antibiotic in Sweden. In 2005 PcV and PcG comprised 26% of the total defined daily doses (DDD/ 1000/day) in out-patient care, tetracycline 22%, amoxicillin with clavulanic acid 9% and trimethoprim 4% in 2005 [10]. Table 1 shows the historical consumption of drugs in Sweden in children 0-6 years of age [11]. Note that tetracycline is not prescribed to children.

Table 1. Use of antibiotics (DDD/1000/day) in out-patients 0-6 years of age in Sweden.

Antibiotic	1974	1985	1993	2000	2001	2003	2005
PcV/PcG	4.6	5.0	6.3	4.5	4.6	3.7	3.4
Ampicillin/amoxicillin	0.5	0.9	2.5	1.4	1.4	1.3	1.4
Tetracycline	0.03	0	0	0	0	0	0
Trimethoprim and sulfonamides	-	0.1	0.1	0.2	0.2	0.1	0.1

ANTIBIOTIC RESISTANCE

Antibiotic resistance can be divided into intrinsic and acquired resistance.

Intrinsic resistance describes a status of a genus- or species-specific insensitivity of bacteria to an antimicrobial agent. This can be due to lack of target structures for certain antibiotics, e.g. cell wall-free bacteria such as mycoplasma are intrinsictly resistant to beta-lactam antibiotics. It can also be due to impermeability of the bacterial cell to an antibiotic. In this way, gram-negative bacteria are intrinsictly resistance to glycopeptides, as the molecule is too large to permeabe their outer membrane. All members of the genus/ species have the same intrinsic resistance.

Acquired resistance results from changes in the bacterial genome, due to mutations, or to horizontal acquisition of genetic information from other bacteria [12]. Acquired resistance is a property of the individual bacterial strain within a species or genus.

Mutations

Mutations occur spontaneously and might affect any gene in the bacterial genome. The frequency of mutations may differ between genes. Mutations in the target gene, which leads to antibiotic resistance, are usually caused by multiple mutations [13]. The frequency of mutations that causes antibiotic resistance may vary depending on the antibiotic. For example, mutations by streptomycin, nalidixic acid or rifampin resistance are more common than mutations leading to resistance to vancomycin [14] [12].

Horizontal gene transfer

The most important mechanism of acquired resistance is horizontal acquisition of resistance genes. The same or closely related resistance genes are found in bacteria of different species or genera, suggesting the exchange of resistance genes by horizontal gene transfer [15, 16]. Acquisitions of genes occur through any one of the following processes:

1. Conjugation (direct cell-to-cell transfer of DNA)
2. Transformation (uptake of DNA from the environment)
3. Bacteriophage transduction (transfer by bacterial viruses)

Conjugation is supposed to be responsible for most of the transfer of resistance genes among bacteria [17]. This process is accomplished by mobile DNA elements such as plasmids and transposons. It is necessary to describe these elements before explaining conjugation process.

Mobile DNA elements

Mobile DNA elements refer to a DNA sequence that can move between cells or between DNA molecules within the bacterial cell.

Plasmids

Plasmids are extrachromosomal elements that replicate in an autonomous, self-controlled way and vary in size from 1500 bp to 400,000 bp. Small plasmids often exist in over 20 copies in a cell, while large plasmids often exist in a single copy. Plasmids contain genes that are not necessary for the survival but which are often beneficial for the bacteria, such as genes for antibiotic resistance, metabolic functions and virulence factors [18]. Plasmids may be divided according to their mobility as follows:

1) Conjugative plasmids, which carry genes for their own transfer from the donor bacteria to recipient cells.

2) Mobilisable plasmids, which can be transferred with the assistance of a conjugative plasmids.

3) Non-mobilisable plasmids, which are not able to transfer at all [17].

Plasmids can also be classified according to their replicon. The replicon contains the genes required for plasmid maintenance [19]. The conjugative plasmids have the necessary genes for their transfer in the *tra* region. *Tra* regions are large in size, therefore plasmids containing this certain region were found to be at least 30 kb [20].

Transposons

Transposons are mobile DNA sequences that can transfer within the genome, from chromosome to plasmid or from plasmid to chromosome. The most basic transposons are called insertion sequences (IS) and encode genes for their own transfer, termed transposases, flanked by inverted repeats in both ends (Fig. 12). The central region in transposon carries genes for e.g. antibiotic resistance. Transposas mediates the excision- integration reactions i.e. it enables the transposon to separate and incorporate to the new chromosome or plasmid. Transposons do not possess replication system and must therefore integrate into a chromosome or plasmids [17].

Only conjugative transposons are able to move from a cell to another [21]. This is done by first making themselves free from the DNA and create a circular double-strand form. Then only one of the strands moves to the other cell and integrates to the chromosome or plasmid [22].

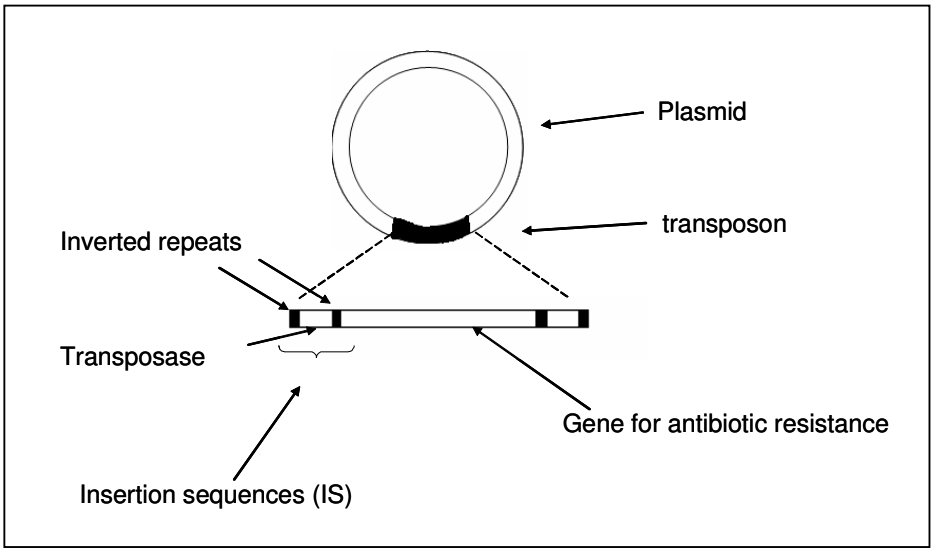


Figure 12. Structure of transposon

Conjugation

Conjugation is passage of DNA directly from one cell to another. Conjugation was discovered in the 1940s [23], when it was shown that genes could be transferred from one strain of *E. coli* to another. The cell that donated the DNA was called F+ for "fertility-proficient" and the recipient cell was called F-. The F+ strain was shown to posses a F plasmid that encodes genes for the F pilus, which establishes physical contact between donor and recipient, forming a channel where plasmid DNA is transferred. Conjugation always results in a one-way transfer, from the F+ strain to the F- strain. The F plasmid (also called conjugative plasmid) is relatively large (90 kb) and contains a *tra* region which encodes the F pilus that is essential to plasmid transfer function and a short specific nucleotide sequence, termed the *oriT* region, that serves as the origin of transfer [17]. Conjugation can be described as follows; the donor cell makes contact by F pilus, a channel forms between the cells, a nick at the *oriT* in the donor plasmid initiates the synthesis of a new copy of plasmid DNA, while the old copy is transferred to the recipient. The DNA is transferred as a single-stranded molecule, which is recircularized to synthesize its complementary strand (Fig 13) [24].

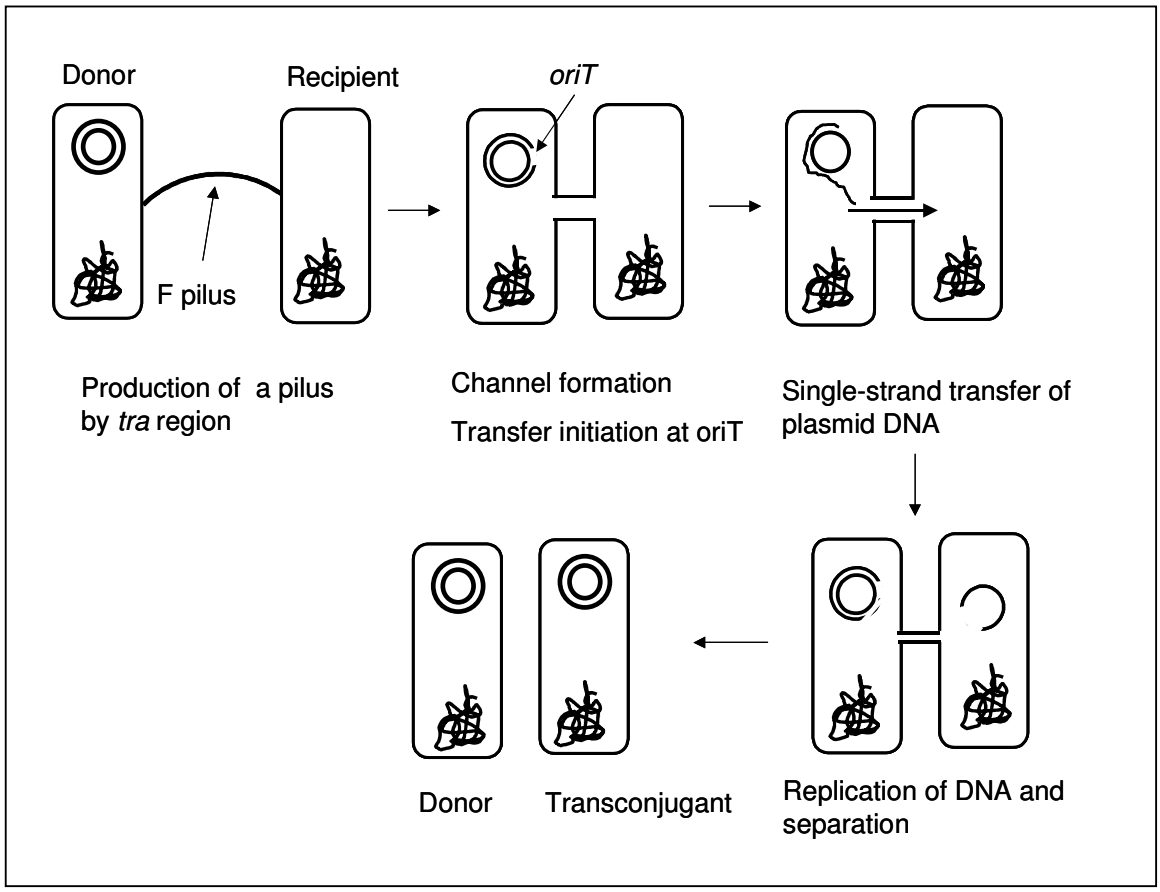


Figure 13. Mechanism of conjugation

Transformation

In transformation, free DNA is transferred to a recipient. In transformation, the donor cell generally lyses, releasing DNA into the medium, and some of this free DNA is taken up by recipient cells. This process may be used for introducing plasmids into host bacteria *in vitro*. *In vivo*, transformation seems to be of limited significance in the transfer of resistance genes, although pneumococci may acquire resistance by the uptake of free DNA from the environment [25].

Transduction

In transduction, bacterial DNA is incorporated into a phage and transferred to new bacteria by the virus. It is speculated that transduction are of importance in the acquisition of resistance in staphylococci [26].

DETERMINATION OF RESISTANCE

Antibiotic resistance testing may be performed by either broth dilution, disc diffusion test, or E test (Fig. 14) [27].

1) The broth dilution method is the golden standard for determination of the susceptibility of a bacterial strain to a certain antibiotic. The read-out is the minimum inhibitory concentration (MIC). In the broth dilution test, the antibiotics are prepared as twofold serial dilutions in tubes (macrodilution) or in a microplate (microdilution). A standardized concentration of bacteria is added to each tube/ well and the suspension is incubated overnight. The lowest concentration of antibiotic that inhibits the growth of the bacteria is the minimum inhibitory concentration (MIC), which expressed in μg/ml.

2) The disc diffusion test was developed as a simple and versatile means to determine whether a bacterial strain is resistant or susceptible to an antibiotic. A bacterial suspension is inoculated onto agar plates onto which paper discs containing various antibiotics in defined concentrations are applied. The agar plates are incubated overnight. During this incubation, the antibiotics in the discs diffuse through the agar creating a gradient in the agar with the highest concentration near the disc. Resistant bacteria can grow without being inhibited by the drugs, whereas susceptible ones cannot grow close to the disc and a clear zone is formed. The zone diameters are measured. Each bacterial isolate is judged to be susceptible (S), indeterminate (I) or resistant (R) to the drug tested. This is based on previous measurements of zone diameters in large population of bacteria. The Swedish Reference Group for Antibiotics (SRGA) provides species-specific zone break-point for all clinically relevant bacteria-antibiotic combination [11].

3) E test was developed as a more practical means to determine the MIC. In this method, a plastic strip, which is coated with an antibiotic gradient, is placed onto an agar plate inoculated with bacteria. During overnight incubation the antibiotic diffuses into the agar and creates a clear zone around the strip. The MIC can be read directly from a scale on the strip where the oval growth of bacteria intercepts the strip [27].

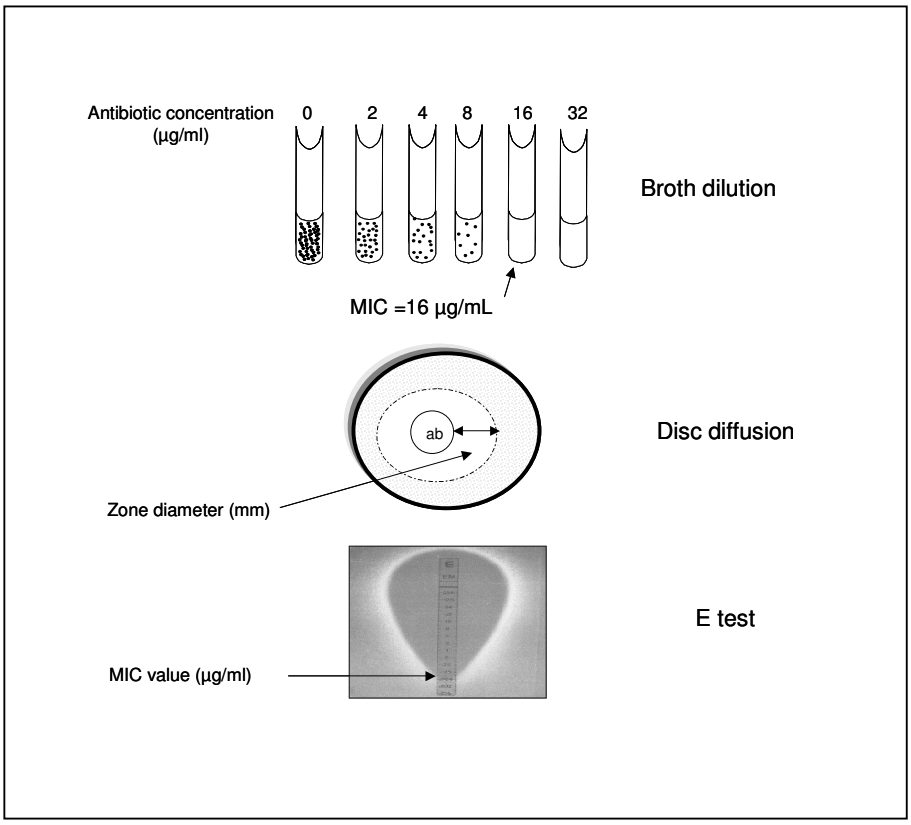


Figure 14. Methods for determining antibiotic resistance

MIC values obtained from broth dilution or E test can be correlated to the zone diameter of inhibition. The correlation can be shown as a regression lines between MICs and zone sizes. The MIC values may be plotted against matching zone diameters, obtained from a series of disc diffusion test against a number of certain species of bacteria. Fig 15 illustrates a regression line between zone diameter and MICs.

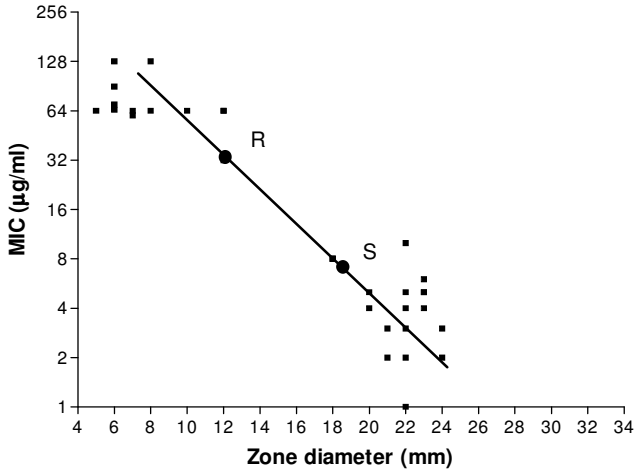


Figure 15. Correlation between zone diameters and MIC.

Bacteria may have high or low level of resistance to antibiotics, which can be determined from evaluation of their MIC values. Isolates with a MIC value slightly higher than the susceptible population are considered having low level resistance [28]. A high-level resistance is often mediated by acquired foreign DNA such as plasmid or transposons, while a low-level resistance is depends on mutations in housekeeping genes. Low level resistance may stepwise evolve to high-level resistance under antibiotic pressure [28]

MECHANISMS OF RESISTANCE

Bacteria have developed various mechanisms to confer antibiotic resistance [12]. The most common mechanisms are the following:

1. Enzymatic drug inactivation

Bacteria produce enzymes that alter or modify the antibiotic in a way that destroys its antibacterial activity.

2. Modification of the drug target

The target for the antibiotic can be modified so that the drugs no longer can bind to the target.

3. Drug efflux pumps

Active efflux is an energy-dependent mechanism used by bacteria to reduce the drug concentration in the cell. Some efflux pumps act on specific drugs, e.g. tetracycline, and generally confer high-level resistance. Other pumps are active on multiple drugs and generally confer low-level resistance.

4. Reduced drug uptake

Reduced uptake is an important mechanism of resistance in gram-negative bacteria, where drugs enter the bacterial cell through porins in the outer membrane. Mutations leading to loss or reduced size of porin channels usually confer low level resistance to their drugs.

5. Target protection

Resistance by protection of the drug target means that a protein is produced that binds to target structure and protects it from binding the antibiotic.

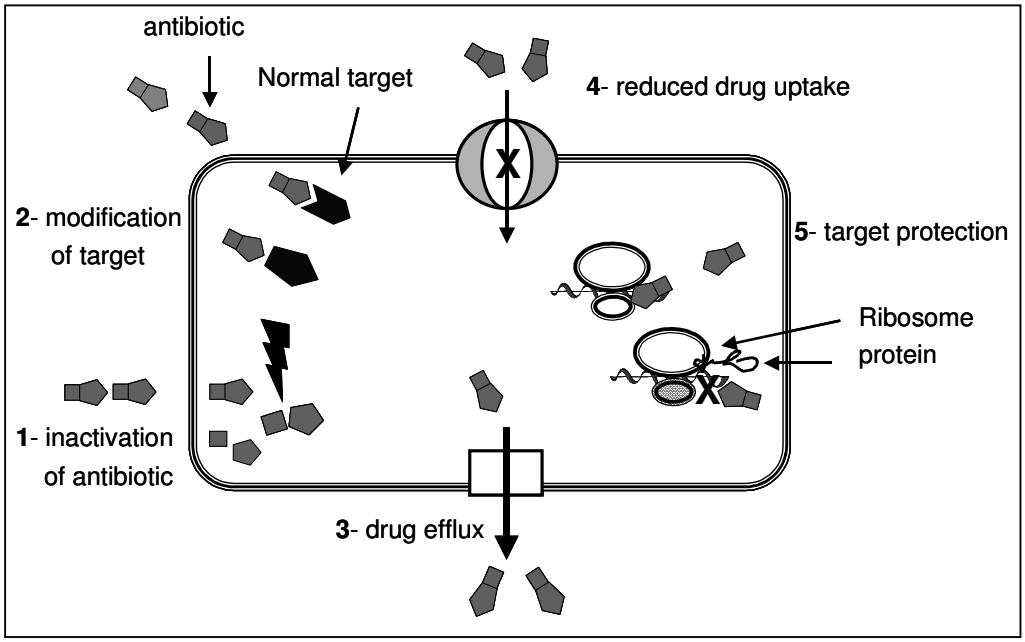


Figure 16. Mechanism of antibiotic resistance

Bacteria have acquired resistance to several different classes of antibiotics. The most important antibiotics and their mechanism of resistance are listed in Table 2. The resistance mechanisms of beta-lactam and tetracycline are presented in more detail in the following sections.

Table 2 Antibiotics and their mechanisms of bacterial resistance [5, 7].

Antibiotics	Resistance mechanism
Beta-lactam Penicillins (PcV/PcG, ampicillin, amoxicillin) Cephalosporins (cefotaxime, ceftazidime, cefadroxil) Monobactam (aztreonam) Carbapenems (imipenem)	- beta-lactamases - mutation in PBP - reduced uptake - efflux pump
Glycopeptides (vancomycin, teicoplanin)	- alteration in the target of antibiotic by presence of *van* gene
Tetracycline (doxycycline, lymecycline)	- efflux pump - protection of ribosomes - enzyme for inactivation of tetracycline
Chloramphenicol (chloramphenicol)	- enzyme for inactivation of chloramphenicol
Aminoglycosides (streptomycin, gentamicin, tobramycin)	- aminoglycoside-modifying enzymes
Macrolides (erythromycin)	- ribosomal metylation by *erm* genes - efflux pump - inactivating of antibiotics
Lincosamides (clindamycin)	- ribosomal metylation by *erm* genes - efflux pump - inactivating of antibiotics
Trimethoprim and sulfonamides (trimethoprim/ sulfamethoxazole)	- overproduction of DHFR - mutation in DHFR
Quinolones and fluoroquinolones (nalidixic acid, norfloxacin)	- mutation in *gyr(A)*
Rifampin (rifampin)	- mutation in RNA polymerase

The origin of antibiotic resistance genes

Antimicrobial resistance genes are thought to originate in naturally antimicrobial-producing bacteria and fungi that make them to protect themselves from the antibiotics they produce [29]. It is speculated that the microbes surrounding these organism have acquired resistance genes from the producers and thereby the ability to survive in their environment. Thus, a

gene found in tetracycline-producing strains of *Streptomyces* origin showed to be similar to a certain tetracycline resistance gene [30].

Another possibility is that antibiotic resistance genes have evolved from mutations in various housekeeping genes in bacteria. Many resistance genes are similar to housekeeping genes that are responsible for building the cell wall or synthesis of bacterial proteins. This is exemplified by a considerable homology between the DNA sequence of PBPs and beta-lactamases [31].

Resistance to beta-lactam antibiotics

Classification of beta-lactamases

Beta-lactamases may be classified into four classes based on their nucleotide sequence from class A through D, termed Ambler classification [32]. Class A, C, and D enzymes have a serine at their active site, while class B enzymes have four zinc atoms at their active site. More commonly classification of beta-lactamases is based on their substrate profiles and termed Bush-Jacoby-Medeiros groups [33].

Mechanism of resistance

Bacterial resistance to beta-lactam antibiotics can be mediated through four mechanisms:

1- Production of beta-lactamases, which are proteins that bind to and hydrolyze the beta-lactam ring. This mechanism is the most common mechanism for beta-lactam resistance in gram-negative enterobacteria.

2- The next common mechanism is by mutation in the penicillin-binding proteins, resulting in their loss of affinity for beta-lactam antibiotics. This mechanism is important in gram-positive cocci [34].

3-4 Active efflux of beta-lactamase or reduced impermeability by mutational loss in porin proteins confers resistance to the bacteria [35]. The combination of efflux and reduced impermeability is the second important mechanism in gram-negative bacteria [36]

Beta-lactamases

Plasmid-mediated beta-lactamases

Ampicillin was introduced in the 1960s and after a few years ampicillin mediated beta-lactamase started to appear [37]. Resistance to ampicillin is mediated by TEM, SHV and OXA genes. TEM-1 is the most common of the plasmid-mediated beta-lactamases found in *E. coli* [38] [39]. The TEM-1 β-lactamase gene is further subdivided into the *bla*$_{\text{TEM-1a}}$, *bla*$_{\text{TEM-1b}}$, *bla*$_{\text{TEM-1c}}$, *bla*$_{\text{TEM-1d}}$, *bla*$_{\text{TEM-1e}}$, and *bla*$_{\text{TEM-1f}}$ varieties, based on small differences in nucleotide sequences [40]. SHV-1 is most common in enterobacteria, especially in *Klebsiella species*. Strains that produce high level of these enzymes show resistance to first generation cephalosporins like as cephalotin.

Inhibitor resistant beta-lactamases

One group of TEM beta-lactamases in *E. coli* make the bacteria resistant to beta-lactam, as well as to the beta-lactamase inhibitor, clavulanic acid. These are designated "Inhibitor-Resistant TEMs" or IRTs. At least two major mechanisms may explain this resistance: either overproduction of the TEM beta-lactamase or a modification of the beta-lactamase structure gene [41] [42].

Chromosomal-mediated beta-lactamases

Many bacterial species, including *E. coli*, contain chromosomally encoded beta-lactamases, called AmpC enzymes. These genes are normally expressed at a low level in *E. coli*, which is not sufficient to give clinical resistance. Mutation in *ampC* genes can increase enzyme activity and render the bacteria resistant. AmpC also confer resistance to the second and third generation of cephalosporins [43]. In the recent years, plasmid mediated AmpC beta-lactamases are also reported which is of great concern [44, 45].

Plasmid-mediated extended spectrum beta-lactamases

The exposure of bacteria to an increasing number of beta-lactams with extended spectra has lead to the emergence of new beta- lactamases, called extended spectrum beta-lactamases (ESBL)[46]. These new enzymes have expanded activity and confer resistance to the third and fourth generation of cephalosporins such as ceftazidime, cefotaxime and cefepime, and

also aztreonam. These ESBLs enzymes are plasmid borne and have evolved from point mutations in the active site of the TEM-1, TEM-2, and SHV-1 beta- lactamases [47]. CTX-M is another example of an ESBL enzyme among *Enterobacteriaceae* which renders the bacteria resistance to cefotaxime and ceftazidime [45].

Promoters of beta-lactamase TEM genes

Promoters are DNA sequences to which RNA polymerase binds and transcription initiates. Promoters with high affinity to RNA polymerase are classified as "strong" promoters and those with a low affinity as "weak" promoters.

The expression of *bla*$_{TEM}$ genes is controlled by four different promoters, *P3*, *Pa*/*Pb*, *P4* and *P5*. Two regions in promoters, referred as –10 and -35 are critical in determining their strength. Mutations in these regions of promoters affect their affinity [48]. *Pa*/*Pb*, *P4* and *P5* differ from P3 by point mutations in –10 or –35 regions. The strength of promoters is: *P3* < *P4* < *Pa*/*Pb* < *P5* [49, 50] .

Resistance to tetracyclines

The emergence of tetracycline resistance in the last 50 years has accelerated by the frequent use of this agent in human and veterinary medicine, and in animal husbandry and agriculture. Within a collection of *Enterobacteriaceae* isolates collected between 1917 and 1954, only 2% were resistant to tetracycline [51]. Nowadays, 21-68% of *E. coli* isolated from healthy persons in Finland, Spain and Ghana are resistant to tetracycline [52-54]. In a study from Bolivia, among healthy children, 92% *E. coli* isolates were resistant to tetracycline [55]. Genes conferring resistance to tetracycline are often located on conjugative plasmids or transposons, which is a reason for spread of tetracycline resistance genes. Up to now at least 38 different *tet* (tetracycline) resistance genes are described [56].

Mechanism of tetracycline resistance

Efflux of drug

There are 23 *tet* efflux genes that code for membrane associated proteins, which export tetracycline out of the cell. Efflux genes are found in both gram-negative and gram-positive bacteria, but are more common in gram-negative genera. Of 23 efflux protein genes 16 have

gram-negative origin. *tetA*, *tetB*, *tetC*, *tetD* and *tetE* are the most widespread among gram-negative bacteria [3]. They are normally associated with large conjugative plasmids [57]. *tetK* and *tetL* are found in gram-positive bacteria and are associated with small mobilisable plasmids [4].

Ribosomal protection

Ribosomal protection proteins are cytoplasmic proteins that bind to the ribosomes. This binding causes an alteration in ribosomes, which prevents binding of tetracycline. Eleven genes code for ribosomal protection proteins and are found in both gram-positive and gram-negative genera [56]. *tetM*, *tetQ* and *tetW* are often associated with conjugative transposons and have a large host range. *tetO* and *tetS* are often located on plasmids and were primarily associated with gram-positive genera [58].

Inactivation of drug

Enzymatic inactivation of tetracycline is not a commonly found mechanism in tetracycline resistant bacteria. Three enzyme degrading genes are *tetX*, found exclusively in *Bacteroides*, *tet*(34) found in various genera and the recently found *tet*(37) in unknown genus [56].

THE INTESTINAL MICROBIOTA

The human normal microbiota consists of several ecosystems located on the skin, in the oral cavity, the upper respiratory tract, the gastrointestinal tract and the genital tract. The intestinal microbiota is a complex ecosystem, estimated to harbor > 10^{12} bacterial cells /g of faeces and more than 400 different bacterial species in an adult individual [59]. More than 99% of the bacteria are strictly anaerobic. *E. coli*, other enterobacteria and enterococci make up the majority of the aerobic microbiota, while the predominant anaerobes are *Bacteroides*, bifidobacteria, eubacteria, fusobacteria and anaerobic gram-positive cocci. The population level of each species appears to be directly regulated by the competition for nutrients and physical space [60]. The population levels and the species composition of the microbiota remain quite constant over time under natural conditions.

In the stable intestinal ecosystem, all natural niches are occupied by resident microorganisms, which persist over a prolonged period, and bacteria derived from food, water or skin that reach the intestine will usually not establish and colonize, but instead only pass through the intestinal tract [61]. In this way, the normal microbiota acts as a barrier against colonization by potentially pathogenic microorganisms, and also prevents overgrowth of microorganisms already present in the intestinal tract like yeasts or *Clostridium difficile*. The gut microbiota plays an important role in human health by exerting important metabolic functions (fermentation of non-digestible fibers, recovering energy as short-chain fatty acid, and production of vitamin K), and by stimulating the development of the immune system [62].

Establishment of the intestinal microbiota in infants

The gastrointestinal tract of a normal fetus is sterile. Microbes from the mother and surrounding environment colonize the infant during the birth process. The type of delivery has a significant effect on the establishments of the intestinal microbiota. Infants delivered vaginally are more likely to be colonized with microbes from the mother than infants born by caesarean section. The initial exposure of infants born by caesarean is mostly to environmental isolates from equipment, air, other infants and nursing staff [63, 64]. Later on, all neonates are continuously exposed to new microbes, e.g. via the breast milk and other feeds. The milk from healthy mothers contains up to 10^9 microbes/l [65]. Other colonizing bacteria are transferred from e.g. the hands of individuals who are in contact with the neonates.

E. coli and other enterobacteria, enterococci, and nowadays also staphylococci are the first to establish and form stable and numerous communities in the infant gut [66, 67]. As the neonatal intestine is rich in oxygen, these early aerobic colonizers initially reach high population counts in the intestine, up to 10^{11} / g faeces. They consume the oxygen and make way for the anaerobic bacteria, such as bifidobacteria, bacteroides and clostridia, which soon become dominant. After the introduction of solid foods, obligate anaerobes increase in numbers and diversity and a colonization pattern similar to adults is achieved by the age of two years [68]. In parallel with the establishment of successively more anaerobic bacteria, the population levels of the aerobic bacteria decline [60].

Role of microbiota in development of resistance

Antibiotic treatments do not only affect the pathogenic bacteria that are the target, but also the normal microbiota. Antibiotic treatment may cause ecological disturbances in the normal microbiota to a varying degree depending on the spectrum of the agents, the degree of absorption and the way of administration [69]. The ecological disturbance includes suppression of susceptible microorganisms, selection of resistant subpopulations and subsequently establishment of new resistant pathogenic and commensal bacteria [70]. The poorly absorbed drugs that reach the colon in an active form are likely to cause the worst disturbance of the ecological balance.

The use of antibiotics influences the intestinal colonization pattern of the neonate. Treatment with e. g. beta-lactam antibiotic and gentamicin leads to suppression of all anaerobic bacteria, with the exception of clostridia, and increased levels of *Klebsiella*, *Enterobacter*, *Citrobacter* and *Pseudomonas* and decreased levels of *E. coli* [71].

Escherichia coli

Escherichia coli was identified in 1885 by the German pediatrician, Theodor Escherich. *E. coli* is widely distributed in the intestines of humans and animals and is the predominant aerobic bacteria in the bowel.

In a given individual, different strains of *E. coli* can usually be isolated from the faeces at a given time point [72]. Some of these strains have the capacity to persist in the microbiota for extended periods, so called resident strains, while others, called transient, are not capable of long term colonization [73, 74].

E. coli colonizes the human gut shortly after birth and remains in our bowel throughout life, although individual strains come and go over time. Most of these strains are non-pathogenic, coexisting with their hosts. However, many *E. coli* strains can cause diseases if they reach extra-intestinal sites, such as the urinary tract, the meninges, and blood stream. *E. coli* strains causing extra-intestinal infections differ from other *E. coli* strains in that they are more likely to express several specific virulence factors, which increase their capacity to colonize and survive at extra-intestinal sites [75].

Urinary tract infection is the most common extraintestinal infection caused by *E. coli* [76]. Pyelonephritis is the most severe form of urinary tract infection where bacteria are

found in the kidneys, which may cause scaring, leading to hypertension and renal failure. The less severe form, cystitis, is caused by infection of the urinary bladder and is much more common. *E. coli* is also a major cause of septicemia and meningitis in the neonatal period [77]. In septicemia, *E. coli* reach the blood stream from the urinary tract, the intestines or from an infected wound [78].

Escherichia coli virulence factors

Fimbriae (pili)

Fimbriae are thin, hair-like, surface adhesive organelles composed of protein subunits. A number of different types have been described in *E. coli*, which are distinguished by their size (length and diameter) and their host target molecule. Fimbriae originate in the cytoplasm of the cell and project through the cell membrane and the cell wall. The most common fimbrial proteins are type 1, P, S fimbriae and Dr adhesin.

Type 1 fimbriae are produced by more than 80% of *E. coli* strains representing the single most commonly expressed virulence factor in this species [79]. Type 1 fimbriae are also found in many other species of the family *Enterobacteriaceae*. Type 1 fimbriae bind to mannose-containing carbohydrate moieties exposed e.g. on intestinal and urinary tract epithelial cells. Type 1 fimbriae may be of importance in cystitis caused by *E. coli* [76].

P fimbriae are the most important virulence factor for *E. coli* causing urinary tract infection [80]. They have been identified in 80% of pyelonephritic *E. coli* isolates [81]. The fimbriae bind to Galα1-4Galβ containing receptors in the colonic epithelium via its papG adhesin. The papG adhesion exist in three varieties, papG class I, II and III, with slightly different binding characteristics [76].

S fimbriae are expressed by some urinary *E. coli* strains. S fimbriae bind specifically to terminal sialyl residues on e.g. endothelial cells [82], urinary tract epithelial cells and brain endothelial cells [83]. S-fimbriated *E. coli* are associated with neonatal meningitis and septicemia [79, 84].

Dr adhesins are structurally distinct from other *E. coli* fimbrial adhesins [76]. They may appear as a fine mesh [85] or a filamentous capsular coating on the cell surface [86]. Dr adhesins bind to a specific membrane protein on the epithelial cells called decay-accelerating

factor (DAF) [87]. *E. coli* strains which express adhesins of the Dr family are associated with cystitis (30%–50%) in children and pregnancy associated pyelonephritis (30%)[80].

Aerobactin

Bacteria need iron for incorporation into enzymes and mediating oxygen transport. The amount of free iron in body fluids and secretions is low since almost all iron is bound in complex with host iron binding proteins such as transferrin and lactoferrin. Siderophores are bacterial iron chelators that have very high affinity for iron. In *E. coli*, the siderophore aerobactin is an effective system for iron acquisition [88]. Genes encoding aerobactin are found both on plasmids and on the bacterial chromosome. Chromosomal aerobactin is associated with other uropathogenic virulence factor genes, whereas the plasmid aerobactin system is often carried by plasmids encoding multiple antimicrobial agent resistance [89, 90].

Hemolysin

Hemolysin is a toxin pore forming protein which cause damage to e.g. blood cells and urinary tract epithelial cells. Water moves into the cell as a result of the increased intracellular osmotic pressure, causing the cell to swell and rupture. Lysis of e.g. erytrocytes release iron for bacteria [91]. The most common type of this toxin is α-hemolysin which is commonly produced by *E. coli* strains isolated from cases of human urinary tract infection and other extra intestinal infections [92]. Its production can be plasmid or chromosomally determined.

Capsules

More than 80 types of capsular polysaccharides have been described in *E. coli*. They coat the cell and protect the bacteria from phagocytosis [93]. K1 or K5 capsules are found in the majority of *E. coli* causing extraintestinal infections. 80% of *E. coli* isolates from neonates with meningitis carried K1 capsule [94]. *E. coli* expressing K5 is common in both sepsis and urinary tract infection.

Virulence factors as colonization factors

Certain colonization factors may enable persistence of bacteria in the commensal microbiota. *E. coli* adhesins P, S and type 1 fimbriae and Dr hemagglutinin all mediate binding to intestinal epithelial cells [95, 96]. In a rat model, strains with P fimbriae and K5 capsule have shown greater ability to colonize in the intestine, while S fimbriae did not contribute to the colonization [97-99]. Bacteria that inhabit a place close to the mucosal surface might have an advantage due to access to nutrients leaking from the tissues, while the colonic luminal contents is a very poor growth substrate [100].

In humans, the role of virulence factor for intestinal colonization and persistence has been studied in prospective studies. Expression of P fimbriae is more common in resident *E. coli* strains than in transient strains [96, 101, 102]. Genes for P fimbriae were significantly more common in resident than transient *E. coli* strains colonizing the microbiota of Swedish infants, Swedish school-girls and Pakistani infants [103-105]. Genes for type 1 fimbriae and hemolysin were significantly associated with persistence in *E. coli* strains from Swedish infants [104]. Genes for aerobactin were more common among resident as compared with transient strains from Swedish school-girls and Pakistani infants [103, 105]. Other virulence genes, like K5 and K1, were also found to be enriched among the resident strains isolated from Swedish school-girls [103-105]. The interpretation of these studies was that several virulence factors may, in fact, have evolved to enable persistence in the natural niche, the colon.

Phylogenetic classification of *E. coli* strains

E. coli segregates into four major phylogenetic groups, called A, B1, B2, and D [106]. These groups were defined using multilocus enzyme electrophoresis (MLEE) and multilocus sequences typing (MLST)[106-108]. More recently, a simple and rapid phylogenetic grouping technique based on triplex PCR was established [109]. The triplex method, which uses a combination of *chuA* gene (outer membrane hemin receptor gene), *yjaA* gene (unknown function) and an anonymous DNA fragment designated TspE4.C2, is more suitable for large-scale strain screening than MLEE or MLST.

Most extraintestinal pathogenic *E. coli* derive from phylogenetic group B2, and most extraintestinal virulence factors are concentrated in this group [77, 110]. Group B2 harbors

most of the virulent clones of *E. coli*, including O18:K1:H7, O4:K12:H5, O6:K2:H1 and several others [110, 111]. Interestingly, *E. coli* strains belonging to phylogenetic group B2 show enhanced capacity to persist in the colonic microbiota of Swedish infants and school-girls, partly due to their carriage of certain virulence factors [112, 113].

Group D is the second most common group in extraintestinal infections and typically have fewer virulence factor than group B2 [114, 115]. Group D contains several virulent clones, including O7:K1:H-, O15:K52:H1, and the recently described multi-drug-resistant clonal group A, CGA. CGA is a common cause of urinary tract infection [115, 116] and often exhibits a conserved antimicrobial resistance phenotype, i.e. resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and trimethoprim, which is conjugally transferable on a large plasmid [114, 117]. CGA is strongly associated with trimethoprim/sulfamethoxazolee resistance and exhibits an unusual O antigen (O11, O17, O73, and O77) [115].

Serotype O15:K52:H1 is another uropathogenic clone belonging to phylogenetic group D. The clone caused a large-scale epidemic of urinary tract infection, septicemia, and other serious extraintestinal infections in south London, in 1986 to 1987 [118]. In the 1990s, serotype O15:K52:H1 was recognized as the second most common serotype among *E. coli* bacteremia isolates at a Copenhagen hospital [119]. *E. coli* O15:K52:H1 strains accounted for 1.4% of *E. coli* isolates from Spanish patients with urinary tract infection and were typically positive for *papA* and *aer* and negative for *sfa* and *hly*. *E. coli* O15:K52:H1 often has a multiple antimicrobial resistance phenotype [120].

Strains belonging to group A or B1 rarely cause extraintestinal infections [110]. Those which do may represent exceptional members of these groups that have acquired virulence factors by horizontal gene transfer [111].

Despite the increasing prevalence of resistant *E. coli* some data suggest that they are less virulent than susceptible *E. coli* [121]. Picard *et al*. noted that *E. coli* of phylogenetic group B2 were more susceptible to antibiotics than strains of other phylogenetic groups [122]. Other studies have confirmed these results [123, 124]. It has also been reported that *E. coli* resistant to quinolones and fluoroquinolones were less virulent than susceptible strains and mostly belonged to phylogenetic group A [125-127]. Distribution of ESBL-producing *E. coli* among different phylogenetic groups has also been studied [128]. SHV and to a lesser extent TEM

were preferentially observed in strains of phylogenetic group B2, whereas the CTX-M type was associated with strains of phylogenetic group D [128].

ANTIBIOTIC RESISTANCE AND FITNESS IN MICROBIOTA

It is obvious that bacteria have advantages from the possession of antibiotic resistance genes when the antibiotic is present, but what happens when the antibiotic is absent? There is a dogma that the resistant microorganisms are less fit compared to susceptible ones and the resistant ones will be out-competed in the absence of selective pressure from antibiotics. The fact is that many resistant organisms survive well in the environment and keep their resistance genes in the absence of selective pressure [129].

To replicate and maintain antibiotic resistance genes is costly for the bacteria. Most studies have focused on fitness costs of antibiotic resistance genes resulting from chromosomal mutation, and have studied the burden of such chromosomal alteration on the bacteria during growth *in vitro* [130, 131] and some in *in vitro* as well as animal models [132-135]. The biological cost associated by resistance can be reduced by compensatory mutations. It means the burden of acquired mutations, leading to antibiotic resistance, can be compensated by new mutations in previously mutated genes or in other genes. For example, there are two essential genes in *Mycobacterium tuberculosis* that defend bacteria against oxidative stress. Mutations and subsequently elimination in one of these genes render bacteria resistance to the drug isoniazid. The loss of the first gene compensates by a second mutation in the other gene, resulting to hyperexpression of activity against oxidative stress. These secondary mutations are shown in clinical isolates of isoniazid resistant *M. tuberculosis* [136]. In another example, the fitness cost of fusidic acid resistance in clinical *S. aureus* isolates was partly or fully compensated by the acquisition of secondary intragenic mutations [134].

These studies concerned resistance resulting from mutations and not from acquisition of foreign DNA. However, the rapid spread of resistance is mostly mediated by mobile genetic elements such as plasmids or transposons. Drug resistance among *Enterobacteriaceae* and other gram-negative pathogens is often encoded on plasmids [137].

Among studies examining the impact of burden plasmid on fitness cost, some studies have shown that plasmids have a deleterious effect on bacterial fitness, and that the plasmid

is lost when the selective pressure is removed [138]. For example, in substrate-limited chemostat cultures, *E. coli* K12 lost a plasmid conferring resistance to tetracycline, ampicillin, sulfonamide and chloramphenicol, resulting in increased growth rates [139]. In expanded experiments by others it has been shown that resistance imposed by resistant plasmid initially reduce fitness, but the co-evolution of plasmid and the host can reduce or eliminate the fitness cost of plasmid carriage, which may explain why plasmid-born resistance is so difficult to eradicate [139-141]. The initial fitness cost on *E. coli* by plasmid was also reduced through genetic changes in both the plasmid and the bacterial chromosome, leading to that the plasmid was never lost [142].

Almost all studies on plasmid-born resistance cost have been done *in vitro* and only few studies have examined the cost of resistant plasmid in natural bacterial populations *in vivo*. An exception is a study by Johnsen et al, where two isogenic strains of *E. faecium* were examined during colonization in gnotobiotic mice one of which carried a plasmid coding for resistance to vancomycin. A low fitness cost was imposed by carriage of the *van*A-coding plasmid in the absence of antibiotic selection [143]. Recent studies by Enne *et al* in a pig model [144] showed that the fitness impact on wild-type *E. coli* imposed by various resistance elements was very low. The fitness cost in natural bacteria population colonizing in humans has not been examined.

DEMONSTRATING GENE TRANSFERS

Many studies have attempted to show the evidence for transmission of resistance genes between bacteria. These attempts have been made in different environments and by different methods:

DNA analysis

Transfer of genes has been assumed to occur when genes with similar DNA sequences in bacteria are found from different species.

1) Nucleotide-sequence similarity of kanamycin resistance genes, *aphA*, among the *Campylobacter coli*, *Streptococcus* and *Staphylococcus* suggested transfer of this gene between these bacteria [145].

2) Various tetracycline resistance genes, e.g. *tetK*, *tetL tetM*, *tetO*, were found to be common to many organisms isolated from urogenital tract by blot hybridization [146].

3) Tetracycline resistance gene, *tetQ*, was found to increase from 30% to 80% during the past three decades within the genus of *Bacteroides*, suggesting extensive gene transfer among bacteria in the human colon [147].

4) Plasmids conferring ceftriaxone resistance in clinical *Salmonella*, *E. coli* and *K. pneumoniae* isolated from different body sites of patients were found to have identical restriction patterns. This suggested the spread of plasmid carrying resistance to ceftriaxone between these species in hospitalized patients [148]

In vitro

1) The first evidence on gene transfer was reported in 1959 by Akiba T, *et a*l, and was performed by conjugation method [149, 150]. The researchers showed that multiple drug resistance to sulfonamide, chloramphenicol and tetracycline could be easily transferred between Shigella and *E. coli* by a mixed cultivation under laboratory condition.

2) Transfer of multiple drug resistance plasmids from various pathogens of human, animal, or fish origin to susceptible strains was demonstrated in different natural environments, e.g. hand towels, meat, fish, pig faeces and seawater [151].

Gnotobiotic animal experiments

Transfer of genes has been demonstrated in the intestines of animals kept under gnotobiotic conditions, i.e. in isolators.

1) An *Enterococcus faecalis* isolate could transfer a resistance plasmid to *Listeria monocytogenes* when both bacteria were colonizing the intestines of gnotobiotic mice [152].

2) Transfer of vancomycin and other resistance genes was shown to occur between two *Enterococcus faecium* strains colonizing the digestive tract of gnotobiotic mice [153].

3) Tetracycline resistance was transferred from a resistant *E. faecalis* to a sensitive *E. faecalis* strain in the gut of gnotobiotic rats [154].

3) Vancomycin resistance genes were transferred between two *E. faecium* strains in gnotobiotic mice [155].

Natural environments

A number of observations have indicated that transfer of antibiotic resistance genes may occur under natural conditions in farm animals and in the human.

1) Conjugative plasmids conferring resistance to apramycin were found in microbiota of six calves. One of these plasmids was present in three different *E. coli* strains, indicating the spread of resistance plasmid in the microbiota of calves [156].

2) In a study from farm inhabitants in Norway, spread of a multi-drug –resistant *E. coli* was examined. The same multi-resistance plasmid was isolated from cows, family members and veterinarians [157].

3) An O18 *E. coli* strain carrying a plasmid containing resistance genes to tetracycline, streptomycin and sulfonamide persisted in the microbiota in an individual for 9 months. Tetracycline was administered in the beginning of study for 10 days. On day 202 and 242 an O88 *E. coli* strain appeared transiently in the microbiota expressing the same resistance markers as strain O18. Plasmid analysis from O18 and O88 strains showed similarities in their restriction patterns. The authors suggested a plasmid transfer between two *E. coli* strains in the microbiota in the absence of antibiotic pressure. This study was performed as early as 1976. The authors could isolate the postulated transconjugant strain without proving the existence of recipient strain [158].

4) In 2002, a vancomycin resistant isolate of *S. aureus* was isolated from a catheter exit site of a kidney dialysis patient, who was previously treated with vancomycin due to foot ulceration and suspected catheter exit-site infection. *S. aureus* and *E. faecalis*, both resistant to vancomycin and both carrying the *vanA* gene, were isolated from the patient's foot ulcer. It was assumed that *E. faecalis* had transferred its resistance genes to *S. aureus* [159].

5) In a recently published study, a vancomycin resistant *E. faecium* of chicken origin and a vancomycin sensitive strain of *E. faecium* originating from a human were fed simultaneously to healthy volunteers. Both donor and recipient strains persisted transiently in the microbiota. Transconjugants were recovered from half of the individuals [160].

AIMS

The aim of the present study was to investigate the ecological consequences of antibiotic resistance in human commensal bacteria, more specifically focusing:

1- To study the prevalence of antibiotic resistance among *E. coli* colonizing the gut of healthy Swedish infants in comparison with urinary *E. coli* isolates.

2- To study the relation between antibiotic resistance, virulence gene factors and phylogenetic origin in *E. coli*.

3- To investigate the impact of carriage of resistance element on the *in vivo* fitness of *E. coli* and the stability of resistance gene carriage during colonization.

4- To examine the transmission of resistance genes between *E. coli* strains in the microbiota.

MATERIAL AND METHODS

INTESTINAL *E. COLI* STRAINS

A collection of intestinal *E. coli* strains from 128 Swedish infants was studied. The studied infants were born in 1998-2001 at the Sahlgrenska University Hospital in Göteborg and participated in the flora study, which investigated the relation between intestinal colonization pattern in infancy and later allergy development [67]. A diary was kept by the parents where feeding pattern, illnesses and the children's intake of antibiotics and other drugs were recorded. The records were checked by a study nurse who interviewed the parents by telephone at 6 and 12 months. Informed consent was obtained from the parents.

The strains were isolated as follows; a rectal swab was obtained and streaked onto Drigalski agar for isolation of *E. coli* 3 days after delivery within 24 h after collection. Faecal samples were obtained at 1, 2, 4 and 8 weeks and at 6 and 12 months of age (paper I and II) and also at 18 months and 3 years of age (paper III) to obtain a larger collection of strains. Freshly voided faeces was collected by the parents, brought to the lab under anaerobic conditions and processed within 24 h. Quantitative cultures of stool samples were performed as follows: a calibrated spoon-full of faeces was serially diluted in ten-fold steps in sterile peptone water and appropriate dilutions were plated on Drigalski agar plates that were incubated aerobically overnight at 37 °C. The level of detection was 330 ($10^{2.52}$) CFU (colony forming units) per g of faeces.

From the Drigalski agar plates, 1-10 colonies differing in size, shape, color or texture were regularly isolated. Each colony type was enumerated separately, gram-stained, subcultured for purity and speciated using API 20E identification strips (BioMerieux, Marcy-l'Etoile, France). Random Amplified Polymorphism DNA (RAPD) (see method section) was used to identify individual strain in a sample. Isolates with identical RAPD profiles were considered as belonging to the same strain and their counts were pooled.

URINARY *E. COLI* ISOLATES

A total of 205 urinary *E. coli* isolates were obtained from the Clinical Bacteriology Laboratory, Sahlgrenska University Hospital during the periods November 2002 - February 2003 and March 2004 - August 2005. These were consecutive isolates obtained from the first urinary cultures positive for *E. coli* of a child less than two years old seeking care at outpatients' clinics or the emergency ward at the Queen Silvia Children's Hospital in Göteborg. *E.coli* was identified using standard methods at the clinical laboratory and confirmed using API20E biotyping (BioMerieux) by us.

An overview of number of strains and their origin is presented in Table 3.

Table 3. Number of strains studied in each of the papers in the present thesis.

Paper	**Isolates**	
I	272 intestinal *E. coli* strains	From 128 infants participating in the flora study. Samples were obtained at 3 d, 1w, 2w, 4w and 2, 6 and 12 months of age
	205 *E. coli* isolates	Clinical urinary isolates from 205 infants 0-2 years obtained from the Clinical Bacteriology, Göteborg
II	309 intestinal *E. coli* strains	Same 128 infants as in Paper I, including also isolates obtained at 18 and 36 months of age
III	272 intestinal *E. coli* strains	Same as paper I
IV	Two *E. coli* strains	Infant no. 29 in the flora study
V	Two *E. coli* strains	Infant no. 117 in the flora study

CONTROL STRAINS

A number of strains were used as positive controls in the studies. Their characteristics and origin are shown in Table 4.

Table 4. Control *E. coli* strains used in the papers in the present thesis.

Control strains	Characteristics	Papers	References
EcoR 4	Phylogenetic group A	I, II, III, IV, V	[109]
EcoR 26	Phylogenetic group B1	I, II, III, IV, V	[109]
EcoR 40	Phylogenetic group D	I, II, III, IV, V	[109]
EcoR 54	Phylogenetic group B2	I, II, III, IV, V	[109]
233:4	*fimA*, *papC*, *sfaD/E*	I, II, III, IV, V	Kindly provided by Dr. Nowrouzian, University of Göteborg, [103]
C64	*fimA*, *draA*	I, II, III, IV, V	[103]
NF1	*hlyA*, *neuB*, *iutA*	I, II, III, IV, V	Kindly provided by Dr. Nowrouzian, University of Göteborg, [103]
RZ513	*hlyA*, *kfiC*, *iutA*	I, II, III, IV, V	[103]
CCUG 17620	Used in antibiotic susceptibility testing	I, II, III, IV, V	CCUG
PUTI26	CGA-positive strain	I, V	Kindly provided by Dr.Johnson, University of Minnesota
2P9	O15: K52: H1-positive strain	I, IV	[161]
K12 NC50078-02	*Tet*A-positive isolate	II	The Public Health Laboratory Service, London, UK
NC 50019	*Tet*B -positive isolate	II	The Public Health Laboratory Service, London, UK
K12 NC 50270-01	*Tet*C -positive isolate	II	The Public Health Laboratory Service, London, UK
ECO K12 J53-1 RA1 NC 50073-02	*Tet*D -positive isolate	II	The Public Health Laboratory Service, London, UK
ECO HB 101 pSL 1456 NC 50273-01	*Tet*E -positive isolate	II	The Public Health Laboratory Service, London, UK
CCUG 30600	*bla*$_{TEM}$-positive isolate	III	CCUG
CCUG 45421	*bla*$_{SHV}$-positive isolate	III	CCUG
CCUG 52541	*bla*$_{OXA}$-positive isolate	III	CCUG

STRAIN TYPING

Random amplified polymorphic DNA (RAPD)

RAPD was used for *E. coli* strain identification in samples obtained from the intestinal microbiota. Initially RAPD analysis was performed by picking a small amount of bacteria from colonies grown on TSA overnight [162]. Subsequently, in an attempt to obtain more clear and sharp RAPD profiles, DNA was extracted and an equal amount of DNA (200-300 ng) was used to RAPD analysis. The method used for DNA extraction has been published [163]. In brief, a loopful of bacteria was suspended in 20 µl of lysis buffer (0.25% sodium dodecyl sulphate, 0.05 N NaOH) and heated to 95°C for 5 min. The cell lysate was spun down by brief centrifugation (16,000 × g) and suspended in 180 µl of distilled water. Cell debris was removed by centrifugation at 16,000 × g for 5 min. The amount of DNA in the supernatant obtained was quantified by absorbance measurements at 260 nm (LKB Biochrom, Ultrospec). Supernatants were frozen at -20°C until further use.

For RAPD analysis, intact bacteria or isolated DNA were mixed with 6 µM of the primer "GTGATCGCAG", 2mM $MgCl_2$ and HotStarTaq Master Mix (Qiagen) in a final volym of 50 µL. The PCR was started with a 95°C 15 min heat activation step for the polymerase, and continued with the following temperature profile: 94°C for 45 s; 30°C for 120 s; 72°C for 60 s for four cycles followed by 94°C for 5s; 36°C for 30 s; 72°C for 30 s for 26 cycles (the extension step was increased by 1s for every new cycle). The reaction was terminated at 72°C for 10 min and cooled to 4°C. The PCR products were electrophoretically separated on 4-12% gradient polyacrylamide gels (TBE-gels, Invitrogen, Carlsbad, CA, USA) during 2 h at 90V and then silver-stained (Plus one DNA silver staining kit, GE Healthcare Bio-science, Uppsala, Sweden).

All *E. coli* isolates from one child were assayed together and their PCR products were separated on the same gel. Two isolates considered as belonging to the same strain if their RAPD profiles showed similarity regarding all major bands and variation in no more than 2-3 minor bands [164, 165].

Pulsed field gel electrophoresis (PFGE)

In selected cases, the strain identities determined by RAPD were confirmed by pulsed-field gel electrophoresis (PFGE) analysis. Briefly, bacteria were grown overnight in 5 ml tryptic

soy broth, and 250 µl of the suspension was prepared according to the method described by Gautom [166]. The DNA was digested with 20 units of *Xba* I enzyme in 200 µl of the appropriate restriction enzyme buffer and separated by electrophoresis performed with the Gene Path system (Bio-Rad Laboratories, Sweden). The program used comprised an initial switch time of 2.2 s, a final switch time of 54.2 s, a run time of 22 h, an angle of 120 °C, a gradient of 6.6 V/cm, a temperature of 14 °C and a linear ramping factor. The PFGE types were interpreted according to the method of Tenover *et al.* in which isolates whose PFGE patterns differ by ≤ 3 bands are defined as belonging to the same strain [167].

ASSESSMENT OF PHYLOGENETIC IDENTITY

Each strain was assigned to one of the phylogenetic groups A, B1, B2 or D using a triplex PCR as described previously by Clermont *et al* [109]. Briefly, a small amount of bacteria was added directly to the PCR mixture containing HotStarTaq Master Mix (Qiagen) and 20 pmol of each primer pair for the genes *ChuA* and *YjaA*, and for the DNA fragment TspE4C2, respectively. The PCR program was run as follows: 15 min at 95°C, 30 PCR cycles with DNA denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, primer extension at 72°C for 30 s and a final extension step of 7 min at 72°C. PCR products were separated by electrophoresis in 2% agarose gels and stained with ethidium bromide. The strains were assigned to a phylogenetic groups as follows: $chuA^+$ $yjaA^+$, group B2; $chuA^+$ $yjaA^-$, group D; $chuA^-$ $TspE4.c2^+$ group B1; $chuA^-$ $TsPE4.c2^-$ group A [109]. Control strains and primers used in each paper are presented in Table 4 and Table 5.

Detection of clonal group A (CGA) and O15:K52:H1 in *E. coli* strains

Two particular *E. coli* clones were identified: CGA and O15:K52:H1, both belonging to phylogenetic group D and have been associated with outbreaks of urinary tract infection [117, 120]. Two PCRs specifically identifying members of these clones due to single-nucleotide polymorphisms within the housekeeping gene *fumC*, i.e., C288T specific for CGA and G594A for O15:K52:H1, were run using previously described conditions [161, 168]. For both PCRs the procedures were as follows: boiled lysates were used as template DNA. Two µl of extracted DNA was added directly to 23 µl of PCR mixture containing HotStarTaq Master Mix (Qiagen) and 0.6 µM of each primer. The following PCR program was run: 10

min at 95°C, 25 PCR cycles each consisting of DNA denaturation at 94°C for 30 s, primer annealing at 63°C for 30 s, primer extension at 68°C for 3 min and a final extension step at 72 °C for 10 min. PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide.

SEROTYPING

Six strains were subjected to complete (O:K:H) serotyping at the Statens Serum Institute, Copenhagen, Denmark.

DETECTION OF VIRULENCE FACTOR GENES

The virulence gene carriage was determined using two sets of multiplex PCRs. The first set identified the genes for type1 fimbriae (*fimA*), P fimbriae (*papC*), S fimbriae (*sfaD/E*) and Dr hemagglutinin(*draA*), the second identified genes encoding the capsular antigens K1 (*neuB*) and K5 (*kfiC*), the iron-binding protein aerobactin (*iutA*) and cytolytic toxin hemolysin (*hlyA*). Briefly, bacteria were added directly to a mixture containing HotStarTaq Master Mix (Qiagen) and 0.45 μM of each primer pair in a final volume of 50 μL. The PCR programme was started for 15 min at 95°C in a thermocycler (Perkin-Elmer Cetus Model 480) to activate HotStarTaq DNA polymerase. After 4 min denaturing at 94°C, 25 PCR cycles followed consisting of denaturing at 94°C for 2 min, annealing at 65°C for 1 min and extension at 72°C for 2 min.

Table 5. Primers used in PCR detection of genes in the studies.

Gene	Primer sequences (5′ to 3′)	Product size (bp)	Reference
ChuA.1 ChuA.2	GAC GAA CCA ACG GTC AGG AT TGC CGC CAG TAC CAA AGA CA	279	[109]
YjaA.1 YjaA.2	TGA AGT GTC AGG AGA CGC TG ATG GAG AAT GCG TTC CTC AAC	211	[109]
TspE4C2.1 TspE4C2.2	GAG TAA TGT CGG GGC ATT CA CGC GCC AAC AAA GTA TTA CG	152	[109]
fimA(f) *fimA*(r)	CGA CGC ATC TTC CTC ATT CTT CT ATT GGT TCC GTT ATT CAG GGT TGT T	721	[103]
papC(f) *papC*(r)	GAC GGC TGT ACT GCA GGG TGT GGC G ATA TCC TTT CTG CAG GGA TGC AAT A	328	[169]
sfaD *sfaE*	CTC CGG AGA ACT GGG TGC ATC TTA C CGG AGG AGT AAT TAC AAA CCT GGC A	410	[169]
draA(f) *draA*(r)	GCC AAC TGA CGG ACG CAG CAC CCC CAG CTC CCG ACA TCG TTT TT	229	[103]
hlyA(f) *hlyA*(r)	AAC AAG GAT AAG CAC TGT TCT GGC T ACC ATA TAA GCG GTC ATT CCC GTC A	1177	[170]
iutA(f) *iutA*(r)	GGC TGG ACA TCA TGG GAA CTG G CGT CGG GAA CGG GTA GAA TCG	301	[171]
neuB(f) *neuB*(r)	CTA CCC CTT TTG ACG AAG AC ACA CAC CTG ACC CCA ATA C	493	[103]
kfiC(f) *kfiC*(r)	GCC ACC AAC TGT CGC AAA A TGT CGC CCA AAC AAA AAG ATT	809	[103]
*tet*Af *tet*Ar	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210	[57]
*tet*Bf *tet*Br	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659	[172]
*tet*Cf *tet*Cr	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418	[172]
*tet*Df *tet*Dr	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787	[172]
*tet*Ef *tet*Er	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278	[173]
bla$_{TEM}$f *bla*$_{TEM}$r	TTC TTG AAG ACG AAA GGG C ACG CTC AGT GGA ACG AAA AC	1150	[174]
bla$_{SHV}$f *bla*$_{SHV}$r	AGG ATT GAC TGC CTT TTT G ATT TGC TGA TTT CGC TCG	392	[175]
bla$_{OXA}$f *bla*$_{OXA}$r	ATA TCT CTA CTG TTG CAT CTC C AAA CCC TTC AAA CCA TCC	619	[175]
fumC C288Tf *fumC* C288Tr	GCT ATC TGG CAG ACT CGT GCA TCG CCG TTG GAA AG	175	[168]
fumC G594Af *fumC* G594Ar	GCT GCT GGC GCT GCG CAA GCA A CCG GAA ATC TCC TGT	175	[161]

ANTIBIOTIC RESISTANCE TESTING

Disc diffusion

The antibiotic susceptibility of *E. coli* strains was determined by agar disk diffusion as described by the Swedish Reference Group for Antibiotics [11]. Bacteria were suspended in phosphate-buffered saline (PBS) to a density corresponding to 0.5 McFarland. This suspension was diluted 1/100 in PBS and plated on Iso-Sensitest agar plates. The following antibiotic-containing discs (Biodisc, disc antibiotic named and concentration indicated within parentheses) were applied: ampicillin (AMP 10 µg), cefuroxime (CXM 30µg), cefoxitin (FOX 30 µg), mecillinam (MEL 10 µg), cefadroxil (CFR 30 µg), ceftazidime (CAZ 10 µg), chloramphenicol (C 30 µg), gentamicin (CN 30 µg), tobramycin (TOB 30 µg), nitrofurantoin (F 100 µg), nalidixic acid (NAL 30 µg), tetracycline (TE 30 µg), and trimethoprim (W 5 µg). The plates were incubated aerobically at 37°C for 16-24 h, whereafter the zone diameters were measured. The strains were defined as sensitive or resistant, according to the species specific zone diameter break points provided by the reference group [11].

E test for MIC determination

Strains with tetracycline clearance zones of <19 mm in diameter [11] and ampicillin clearance zones of <11 were further analyzed by E-test to determine the MIC value for tetracycline and ampicillin, as outlined by the Swedish Reference Group for Antibiotics [11]. A strip in concentrations ranging from 0.016 to 256 mg/l (AB Biodisk, Solna, Sweden) was placed on an Iso-Sensitest agar plate inoculated with bacteria at a concentration corresponding to 0.5 McFarland. The plates were read after 20 h at 37°C and the lowest concentration of tetracycline and ampicillin inhibiting bacterial growth was recorded.

DETECTION OF *TET* GENES (PAPER II)

The tetracycline resistance genes *tet*A, B, C, D and E were identified by multiplex PCR [176]. The following procedure was used: one bacterial colony was added to a sterile thin-walled reaction tube (Perkin Elmer, Foster City, CA) containing HotStarTaq Master Mix (Qiagen), primers, $MgCl_2$ and distilled water. The first multiplex PCR included primers for *tet*B and *tet*C (0.25 µM of each) and 1.5 mM $MgCl_2$. The second PCR included primers for *tet*A and *tet*E (1.0 µM of each) and *tet*D (3 µM), and 2 mM $MgCl_2$. The tubes were sealed with a drop of

mineral oil and heated to 95°C for 15 min in a thermocycler (Perkin-Elmer Cetus Model 480) to activate the HotStarTaq DNA polymerase. After 5 min DNA template denaturation at 94°C, 25 PCR cycles followed, with DNA denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1.5 min. Agarose gel electrophoresis was used for separating of PCR products by using 1.5-2 % agarose gel and visualized by staining with 0.5 μg/ml ethidium bromide and examined in UV-light.

DETECTION OF BETA-LACTAMASE GENES (PAPER III)

Ampicillin-resistant *E. coli* strains were assessed for carriage of the genes *bla*TEM, *bla*SHV and *bla*OXA using PCR. In brief, for amplification of *bla*TEM gene, one bacterial colony was picked from an agar culture and suspended directly in 25 μl HotStarTaq Master Mix (Qiagen) containing 0.2 μM of *bla*TEM primers [174] (Table 5). For amplification of *bla*SHV and *bla*OXA genes, a duplex PCR with 0.5 μM of SHV primers and 1.5 μM of OXA primers were used [175]. A negative (no template) control and positive controls (Table 4) were included in each PCR experiment. The PCR program was run as, 15 min at 95°C, 32 PCR cycles followed, with DNA denaturation at 94°C for 30 s, primer annealing at 54°C for 30 s, primer extension at 72°C for 1 min and a final extension step of 10 min at 72 °C. PCR products were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

SEQUENCING OF *BLA*TEM GENES

Strains positive for *bla*TEM genes were sequenced. *Bla*TEM-positive PCR products were purified with magnetic beads on the Biomek NX. Five sequence-PCRs were made using ABI PRISM Big Dye Terminator Cycle Sequencing v. 3.1 (Applied Biosystems) with five TEM-primers from published papers [177] to amplify both strands of the *bla*TEM gene. Sequence-PCR products were purified and then performed using an ABI PRISM 3730 DNA Sequencer (Applied Biosystems). The amino acid sequences were compared with those previously described for *bla*TEM genes in the GenBank database.

ISOELECTRIC FOCUSING (IEF)

Beta-lactamases of three *E. coli* isolates were characterized by isoelectric focusing on polyacrylamide gels (pH 3 –9) was used [178]. These analyses were performed at the Swedish Institute for Infectious Disease Control (SMI), Stockholm, Sweden.

CHARACTERIZATION OF PLASMIDS

For plasmid preparation (paper IV, V), bacteria were grown in Luria Broth (LB) (BD Difco, Sparks, USA) and total plasmid DNA was prepared using the Plasmid Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions for isolation of low-copy-number plasmids. Plasmid DNA was treated with *Hin*dIII, *Pst*I, *Eco*RI, *Sac*I and *Hha*I, separated on a 0.7% agarose gel, stained with 0.5 μg/ml ethidium bromide, and visualised under UV-light.

SOUTHERN HYBRIDIZATION

Plasmid DNA from different *E. coli* isolates was digested by *Hin*dIII. Cleavage products were separated by electrophoresis, denatured in neutral transfer buffer, transferred by capillary action onto a nylon membrane (Amersham, Uppsala, Sweden) and after pre-hybridization at 68°C overnight hybridized at 42°C for 30 min with digoxigenin (DIG)-labeled probe (Roche Diagnostics GmbH, Mannheim, Germany). The DIG-labelled probes were immunodetected with anti-digoxigenin-AP and visualized with a colorimetric substrate (Roche Diagnostics).

CONJUGATION EXPERIMENTS

Conjugation experiments were carried out by the broth method. Donor and recipient strains were pre-cultured separately in 35 ml LB broth at 37°C overnight. From these cultures, 4 ml was suspended in 35 ml of LB broth and incubated for 1 h at 37°C. One ml of donor culture was mixed with 5 ml of recipient culture and the mixture was incubated at 37°C overnight. Bacteria were spun down, resuspended in 100 μl PBS and plated in serial dilution onto LB agar containing two antibiotics (to determine transconjugant numbers) or one antibiotic (to measure donor numbers). The plates were incubated overnight at 37°C. Transfer frequency was defined as the proportion of transconjugants over total number of donors.

SELECTION OF ANTIBIOTIC RESISTANT *E. COLI* MUTANTS

To obtain the resistant recipient strains, sensitive strains were cultivated in 10 ml LB broth overnight, pelleted by centrifugation, suspended in 200 μl LB and spread onto BHI agar containing the selected antibiotic (nalidixic acid or streptomycin). After incubation at 37°C overnight, a single colony was chosen at random.

ASSESSMENT OF FITNESS COST

To assess the fitness cost of carriage of an antibiotic resistance plasmid by the host strain, pair-wise growth competition in Davis minimal medium with 25 mg/ml glucose (DM25) was performed and illustrated in the Fig. 17. Briefly, the strain with plasmid and the strain without plasmid were cultured separately in LB overnight and then inoculated at a ratio of 1:10,000 into fresh DM25. They were cultured separately for 48 h, during which time they were diluted at a 1:100 ratio into fresh DM25 every 24 h. The cultures were then mixed at a volumetric ratio of 1:1 and inoculated at a 1:100 dilution into DM25. After 24 h of growth, cultures were transferred at a ratio of 1:100 into fresh DM25. The experiment was continued for a total of six transfers. After the first 72 h period of growth and after each subsequent 24 h period, a sample of the culture was diluted appropriately and spread in triplicate onto Iso-Sensitest agar (Oxoid) and onto Iso-Sensitest agar containing antibiotic. Six replicates of each competition experiment were performed. For each competition experiment, the percentage per generation fitness impact of each resistance element was determined as described previously [131, 140].

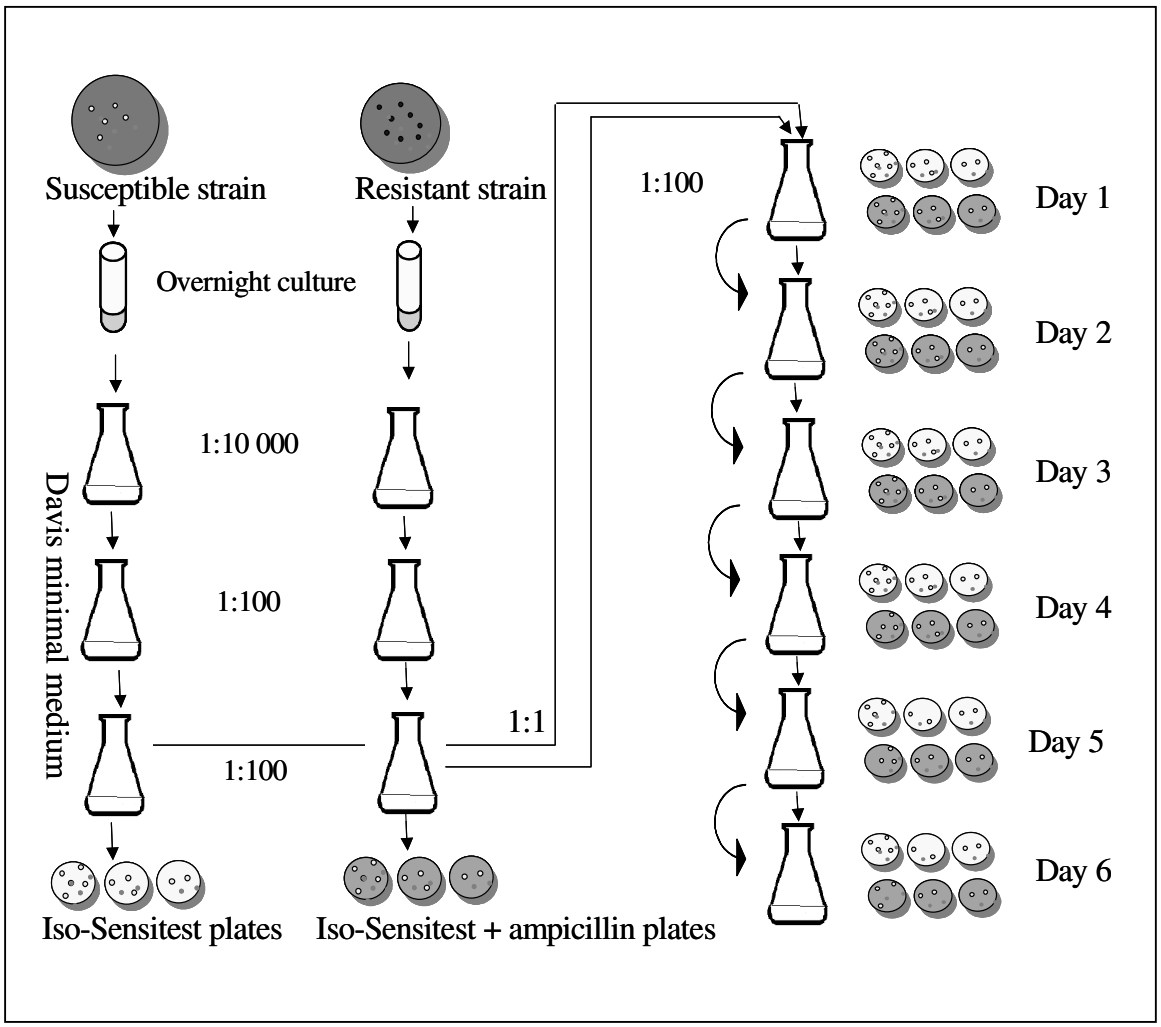


Figure 17. Schematic overview of growth competition experiment.

STATISTICAL METHODS

Comparison of proportions was performed using Fisher's exact test paper I, II and III.

MIC-values were compared using unpaired t-test (paper II). The time of persistence in the bowel flora of susceptible and resistant strains was compared using Mann-Whitney test (paper III). Population counts of susceptible and resistant strains colonizing the same individual were compared by the paired nonparametric test, Wilcoxon signed rank test (paper III).

RESULTS

FREQUENCY OF ANTIBIOTIC RESISTANCE IN INTESTINAL *E. COLI* STRAINS (PAPER I)

The overall prevalence of antibiotic resistance among 272 intestinal *E. coli* strains was 21% and resistance was most commonly observed towards ampicillin (12%), tetracycline (10%) and trimethoprim (8%). Resistance to nalidixic acid, chloramphenicol and nitrofurantoin was found in ≤ 1% of infantile bowel isolates. No strains were resistant to cefuroxime, cefoxitin, mecillinam, cefadroxil, ceftazidime, gentamicin and tobramycin. In paper II, a total number of 309 *E. coli* strains were tested, yielding a slightly higher tetracycline resistance (12%).

CHARACTERIZATION OF TETRACYCLINE AND AMPICILLIN RESISTANCE GENES (PAPER I AND II)

Tetracycline resistance genes; *tet*A, B, C, D and E were identified by using a multiplex PCR. *Tet*B and *tet*A were the most common tetracycline resistance gene, found in 51% (19/37) and 49% (18/37) of resistant strains, respectively. *Tet*C was found only in one strain, in combination with *tet*A. Neither *tet*D nor *tet*E were detected.

The MIC values of tetracycline-resistant strains ranged from 32 to 256 mg/l (median 128 mg/l), with significantly higher values for strains carrying *tet*B than for those carrying *tet*A (median: 256 *vs* 96 mg/l, $P < 0.0001$).

The carriage of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ genes among ampicillin resistant strains was determined by PCR. *Bla*$_{TEM}$ resistance genes were found in 84% (27/32) of ampicillin resistant strains, one of which also carried the *bla*$_{OXA}$ gene. The rest (n=5) had the *bla*$_{SHV}$ gene. All ampicillin resistant strains had a MIC-value of >256 µg/ml.

The structural genes and promoters in all *bla*$_{TEM}$ positive strains were sequenced and all were found to be of *bla*$_{TEM-1}$ varieties, namely *bla*$_{TEM-1b}$ (59%), *bla*$_{TEM-1c}$ (19%), *bla*$_{TEM-1a}$ (11%) and *bla*$_{TEM-1f}$ (7%). One strain had both *bla*$_{TEM-1c}$ and *bla*$_{TEM-1f}$. The weak *P3* promoter was found in 24 and the strong *Pa*/*Pb* promoter in three of these sequences. A decreased susceptibility to amoxicillin-clavulanic acid was observed in the three strains with the strong *Pa*/*Pb* promoter.

ANTIBIOTIC RESISTANCE COMBINATIONS

Phenotypic resistance

Eight percent of *E. coli* strains were resistant to more than one antibiotic and only 2% were resistant to at least three antibiotics. The most frequent combinations was resistance to ampicillin and trimethoprim.

Table 6: Profile combination of resistance among 272 *E. coli* strains resulting from disk diffusion susceptibility test.

	No of resistance (total resistant strains)	No. of strains
susceptible to all antibiotic tested	0	215
tetracycline Ampicillin trimethoprim nitrofurantoin nalidixic acid	1 (36)	16 15 3 1 1
tetracycline, trimethoprim ampicillin, trimethoprim ampicillin, tetracycline	2 (15)	4 8 3
ampicillin, tetracycline, trimethoprim ampicillin, chloramphenicol, trimethoprim	3 (4)	3 1
ampicillin, chloramphenicol, tetracycline, trimethoprim	4 (1)	1
ampicillin, chloramphenicol, nalidixic acid, tetracycline, trimethoprim	5 (1)	1
total		272

Association of various tetracycline and ampicillin resistance genes to other antibiotic resistance

Strains carrying *tet*A or *tet*B were more often resistant to other antibiotics than were tetracycline-susceptible strains (50% and 42% vs. 13%, P =0.0003 and P =0.002, respectively).

Strains carrying *bla*$_{TEM}$ were more often resistant to other antibiotics than were ampicillin susceptible strains (63% versus 12%, P=0.0017). *Bla*$_{SHV}$ were more often resistant to other antibiotics in addition to ampicillin than were *bla*$_{TEM}$ positive strains (100% versus 63%, P = 0.01).

ANTIBIOTIC CONSUMPTION

Thirty of the 128 infants (23%) received antibiotics at least once during their first year of life, six of whom were given ≥ 2 treatments. The prescribed antibiotics were as following, penicillin V or G (n=20), amoxicillin / ampicillin (n=12), trimethoprim (n=4), erythromycin (n=4), cefuroxime / cefibuten (n=2), nitrofurantoin (n=1), tobramycin (n=1) and ceftazidime (n=1). The diagnoses were *otitis media* (n=24), urinary tract infection (n=5), respiratory tract infection (n=4) and various other diagnoses (n=5). No child received tetracycline or related compounds.

IMPACT OF ANTIBIOTIC TREATMENT ON ESTABLISHMENT OF TETRACYCLINE AND AMPICILLIN RESISTANT *E. COLI* STRAINS

The colonization pattern of all 30 infants who received antibiotics at any instance during their first year of life is illustrated in Fig. 18

Although no infant was treated with tetracycline, tetracycline resistance was common among intestinal *E. coli*. Of 28 tetracycline resistant strains, only six (21%) derived from an infant treated with antibiotics. Three of these were found in children (No. 12, 17 and 26) before they received the antibiotic treatments. Two (No. 5 and 13) of three remaining tetracycline resistant strains were mono-resistant, while one was resistant to tetracycline and trimethoprim and harbored from an infant (No. 4) who received amoxicillin. Thus, in no case was a tetracycline-resistant strain established after treatment with antibiotics to which it was resistant.

We examined also the colonization of ampicillin resistant strains in relation to antibiotics, which could select for β-lactamase producing *E. coli*, i.e. amoxicillin or cephalosporins. Fourteen infants were treated with such antibiotics during their first year of life. Only one of these infants (No. 23) became colonized by an ampicillin resistant strain. This occurred at 12 months of age after treatment with amoxicillin. As 12% of all strains that were acquired at 12 months of age by children not treated with antibiotics were ampicillin resistant, we could not find any evidence of selection of ampicillin resistant strains by treatment with oral beta-lactams. In fact, 31 of 32 ampicillin resistant strains colonized infants who were not treated, or treated at a later time point during their first year of life.

Figure 18. Colonization pattern of the 30 infants, who treated with antibiotics at least once and colonized with *E. coli* strains during their first year of life. Each *E. coli* strain colonizing an infant is presented as a symbol. Unfilled symbols indicate fully susceptible strains, while filled symbols indicate strains resistant to tetracycline and trimethoprim (▲), tetracycline (■), ampicillin and trimethoprim (▼), ampicillin (●), nalidixic acid (✚), ampicillin, chloramphenicol and trimethoprim (◆) and trimethoprim (✳). A line connecting the strain symbols indicates persistence of a strain in the intestinal microbiota. A vertical dashed line marks antibiotic treatment and the antibiotics given are specified to the right. Abbreviation: AMX, amoxicillin; AMP, ampicillin; PEN, penicillins (PcV and PcG), TMP, trimethoprim; ERY, erythromycin; CXM, cefuroxime; TMP, trimethoprim; CTB, cefibuten; NIT, nitrofurantoin; CAZ, ceftazidime; TOB, tobramycin.

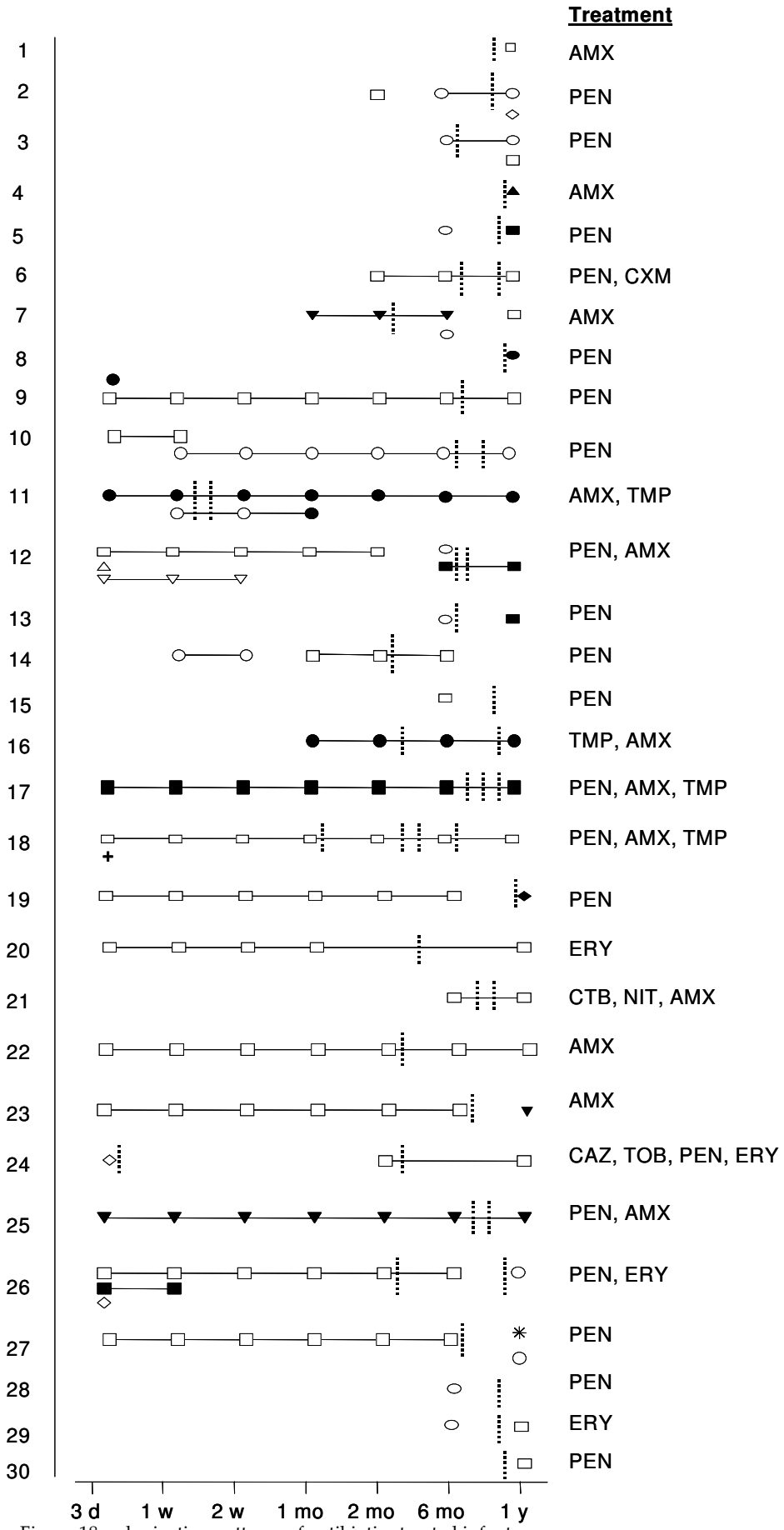


Figure 18. colonization patterns of antibiotics treated infants.

FREQUENCY OF ANTIBIOTIC RESISTANCE IN URINARY ISOLATES

E. coli were isolated from positive urine samples from children below two years of age, seeking out-patient clinics or a children's hospital emergency ward. Among the 205 urinary isolates tested, 40% were resistant to at least one antibiotic, resistance to ampicillin, tetracycline and trimethoprim being most common, followed by chloramphenicol. Resistance to nalidixic acid, gentamicin and mecillinam was observed in less than 3% of urinary isolates. No strains were found to be resistant to cefuroxime, cefoxitin, cefadroxil, ceftazidime, tobramycin and nitrofurantoin.

Resistance to ampicillin, tetracycline, trimethoprim and chloramphenicol were all significantly more prevalent among urinary isolates compared to faecal isolates (Table 7).

Table 7. The prevalence of antibiotic resistance among 205 urinary *E. coli* strains deriving from Swedish infants in comparison with 272 *E. coli* strains from the commensal intestinal microbiota of 128 infants.

	Urinary isolates (% resistant isolates)	*P*	Intestinal strains (% resistant strains)
Ampicillin	29	< 0.0001	12
Tetracycline	20	0.002	10
Trimethoprim	19	0.0006	8
Chloramphenicol	8	0.0001	1
Nalidixic acid	3	0.08	1
Gentamicin	1	-	-
Mecillinam	0.5	-	-
Total	40	< 0.0001	21

DISTRIBUTION OF PHYLOGENETIC GROUPS

Group B2 was most common among both faecal and urinary isolates (Fig. 19). However, a larger fraction among the urinary compared to intestinal strains belonged to the B2 group (72% vs 46%, P < 0.0001). Group A and B1 strains were more commonly found among intestinal isolates than among urinary isolates (*P* < 0.0001 and *P*= 0.0023, respectively), while group D was equally common among urinary and intestinal isolates.

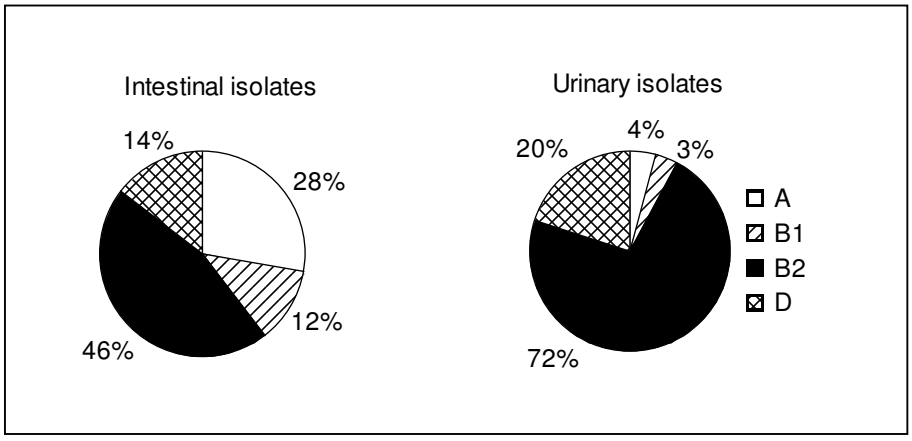


Figure 19. Distribution of four phylogenetic groups in 272 intestinal strains and 205 urinary isolates.

ANTIBIOTIC RESISTANCE AND PHYLOGENETIC GROUPS

We analyzed resistance to at least one antibiotic in relation to phylogenetic group origin in both faecal and urinary isolates. As shown in Fig. 20, urinary isolates were more resistant than faecal isolates within each phylogenetic group, the difference being highly statistically significant for the B2 and D isolates (*P*= 0.009 and *P* = 0.003, respectively).

Irrespective of origin, group D isolates were more often resistant than group B2 and group B1 isolates. This was true of faecal strains (*P*= 0.03 and *P*= 0.004, respectively), as well as urinary isolates (*P*< 0.0001 and P = 0.04, respectively).

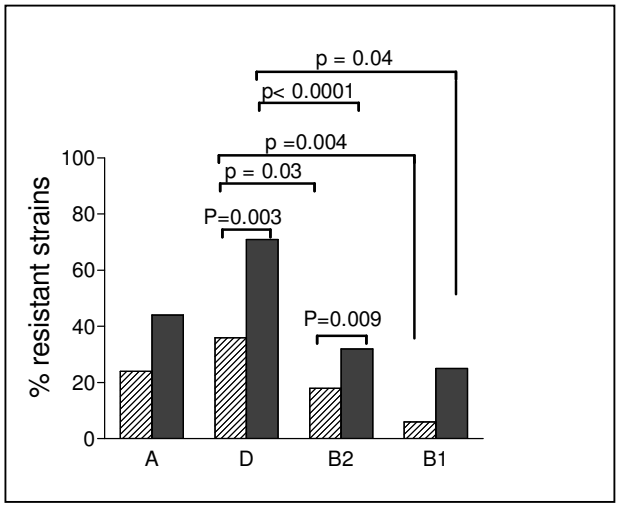


Figure 20. Distribution of resistance in each of the four phylogenetic groups A, D, B2 and B1 among commensal (striped bars) and uropathogenic *E. coli* (black bars).

We examined the occurrence of resistance to ampicillin, tetracycline and trimethoprim to the phylogenetic group identity. Among intestinal *E. coli* strains, group D strains were significantly more often resistant to ampicillin than strains belonging to all other groups combined ($P = 0.01$), while group A strains more often carried tetracycline resistance genes than strains belonging to the other groups combined ($P = 0.02$). Among urinary isolates, resistance to ampicillin, tetracycline, trimethoprim and chloramphenicol were all significantly more common among strains belonging to phylogenetic group D than among strains of the other groups combined ($P \leq 0.0002$, for ampicillin, tetracycline and trimethoprim, and $P = 0.05$ for chloramphenicol).

Rates of the CGA and O15: K52: H1 clones among intestinal and urinary isolates

The two globally spread clones CGA and O15: K52: H1 both belong to phylogenetic group D. We analyzed all group D strains for identity with these clones. Among intestinal *E. coli* strains, 2% (5/272) belonged to the CGA clone, representing 13% of intestinal group D strains.

Among urinary *E. coli* isolates, 14% (28/205) of isolates belonged to CGA, representing 68% of group D isolates. The distribution of virulence genes was for *fimA* (96%), *iutA* (89%) and *papC* (68%) in urinary isolates belonging to the CGA clone, which is in accordance with published data [115]. Resistance to various antibiotics among urinary strains

belonging to the CGA clone, other group D strains, and strains belonging to other phylogenetic groups is shown in Fig. 21. Resistance to several of the tested antibiotics was significantly more common among isolates belonging to the CGA clone than among all other isolates. CGA isolates also tended to be more resistant than other group D isolates, but this was only significant for tetracycline (P= 0.02). Furthermore, group D isolates tended to be more resistant than isolates belonging to other phylogenetic groups even after exclusion of the CGA clone isolates, but no significant differences were observed.

Only 1% (2/272) of intestinal strains belonged to O15: K52: H1 clone. Among urinary *E. coli* isolates, only one belonged to the O15: K52: H1 clone.

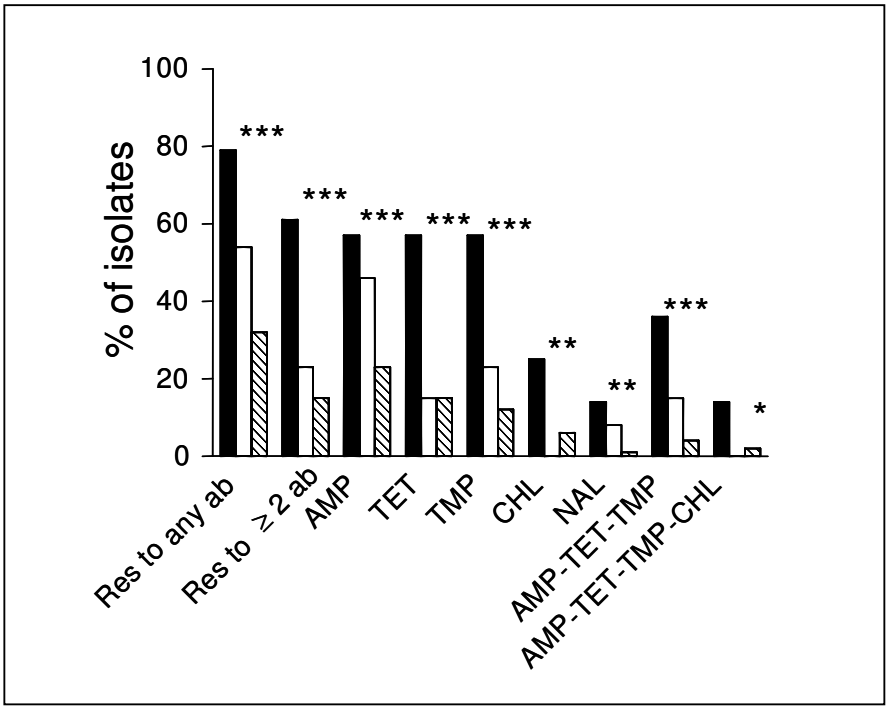


Fig. 21. Distribution of resistance profile among CGA isolates (black bars), group D isolates exclusive CGA (striped bars) and isolates from other groups A, B1, B2 (white bars). Significant levels were determined by comparing CGA isolates to other groups (A, B1, B2). Abbreviations: Res, resistance; ab, antibiotic; AMP, ampicillin; TET

ANTIBIOTIC RESISTANCE AND VIRULENCE FACTOR GENES

We investigated whether antibiotic resistance was related to carriage of virulence factor genes. Among both intestinal and urinary isolates, strains that carried *papC* or *iutA* were more often resistant than strain lacking these traits (Figure 22a and 22b).

Regarding resistance to individual antibiotics, carriage of *papC* was associated with resistance to ampicillin (P= 0.02) and *iutA* with resistance to tetracycline (P= 0.02) among intestinal isolates. Among urinary isolates, carriage of *iutA* was significantly associated with resistance to ampicillin, tetracycline and trimethoprim ($P \leq 0.002$ for all comparisons), and carriage of *papC* with resistance to ampicillin and chloramphenicol (P= 0.04 and P= 0.004, respectively).

As expected, urinary isolates showed significantly higher carriage rate of all virulence factors, with the exception of *fimA*, compared with intestinal strains, i.e., *papC* (72% vs. 29%, $P < 0.0001$), *sfaD/E* (45% vs. 27%, $P < 0.0001$), *hlyA* (45% vs. 22%, $P < 0.0001$), *iutA* (56% vs. 28%, $P < 0.0001$), *neuB* (31% vs. 22%, $P = 0.03$) and *kfiC* (15% vs. 7%, $P = 0.009$).

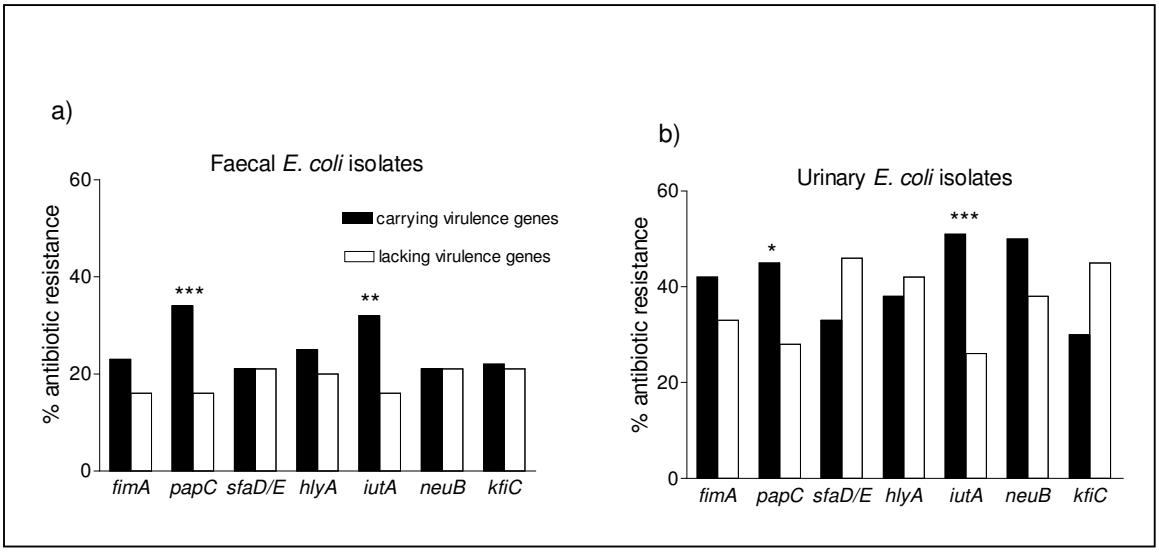


Figure 22. Relation between antibiotic resistance and virulence gene carriage in intestinal and urinary isolates.

STABILITY OF TETRACYCLINE AND AMPICILLIN RESISTANCE GENES

Tetracycline resistance

We examined the maintenance of resistance genes in resident strains obtained from the infants and determined the changes of population counts in these strains. Thirteen infants

each had a tetracycline resistant *E. coli* strains that persisted in the microbiota for at least three weeks. Ten of the strains kept their resistance genes, while three strains lost their resistance genes during the colonization period. The details of the three strains that changed resistance patterns during colonization are described in paper II. Briefly, the first strain colonized an infant at three days of age and persisted until six months of age. This strain had lost its aerobactin gene in the two month sample, which was associated with an increased population count from $10^{5.8}$ to $10^{7.6}$. In the six month's sample the strain had lost *tet*B gene. There was only a minimal change in population levels between the two and six month samples.

The second strain colonized an infant from one week to six months of age. At two months of age, two morphologically distinct isolates of the strain were recovered, one of which carried *tet*A, the other one lacking *tet* gene and being phenotypically susceptible to tetracycline. The tetracycline-susceptible variant had a population level more than 3 log units greater than the resistant variant ($10^{7.2}$ to $10^{9.9}$).

The third strain established in the microbiota of an infant when the child was one week old and persisted until six months of age. In the two month sample, two isolates of the strain with different susceptibility to tetracycline were recovered. The susceptible variant showed a lower population count compared to the resistant variant ($10^{9.4}$ to $10^{7.3}$).

In summary, loss of the *tet* gene resulted in retained population counts of the strain in one case, higher faecal population counts in one case, and lower population counts in one case.

Ampicillin resistance

We also studied the stability of ampicillin resistance genes in the cohort infants (paper III). Thirteen infants each had an ampicillin resistant *E. coli* strains that persisted in the microbiota for at least three weeks. Eleven of the strains kept their resistance genes during the entire colonization period. Two strains carrying bla_{TEM} lost their genes. The loss was associated with increased population counts in one strain and decreased counts in the other. The two strains simultaneously lost resistance to tetracycline and trimethoprim, suggesting loss of a multiple drug resistance plasmid.

FITNESS OF RESISTANT *E. COLI* STRAINS

Antibiotic resistance genes in resident and transient *E. coli* strains

The carriage of resistance genes was compared between resident and transient *E. coli* strains obtained from infants. Resident strains were defined as those persisting for at least three weeks in the intestinal microbiota. Transient strains were those persisting for shorter periods than three weeks. In paper II, among all strains, 109 could be defined as resident and 25 as transient. The proportion of resident strains was 64% (7/11) among *tet*A-positive strains, 86% (6/7) among *tet*B-positive strains and 83% (96/116) among tetracycline susceptible strains. Thus, at least *tet*B-positive strains seemed equally capable of long-term persistence in the infantile microbiota.

Among ampicillin resistant strains (paper III) 82% were resident, compared with 81% among ampicillin susceptible strains. Among strains carrying the ampicillin resistance gene, *bla*$_{\text{TEM}}$ 87% (13/15) were resident. Among the five *bla*$_{\text{SHV}}$ –positive strains, one strain was resident and one transient, while the three remaining strains could not be classified. Thus, *bla*$_{\text{TEM}}$ gene carrying ampicillin resistant strains were equally able to persist in the infantile microbiota as susceptible strains.

Fecal population levels of antibiotic resistant *E. coli* strains

The stool population counts of each strain were determined on each culture occasion, which permitted us to compare the population levels of resistant and susceptible strains. No significant differences in average population counts were observed at any time-point between *tet*A-positive, *tet*B-positive and *tet* susceptible strains, (e.g., $10^{8.42}$, $10^{8.34}$ and $10^{8.31}$ respectively, at 6 months of age). The faecal population levels of ampicillin resistant and ampicillin susceptible strains were also compared at different time points in infants who never received antibiotics during their first year of life. The faecal population counts of ampicillin resistant strains were no lower than those of ampicillin susceptible strains at any time point.

We examined the population counts of resistant and susceptible *E. coli* strains that colonized simultaneously the same child. Eleven infants carried one tetracycline-resistant strain and one tetracycline-susceptible strain at the same time point. Tetracycline-resistant *E.*

coli strains showed significantly lower population counts than tetracycline-susceptible strains in the same stool samples ($10^{7.60}$ versus $10^{8.46}$, P= 0.03, Fig. 23).

Eight infants had an ampicillin resistant strain and at least one fully susceptible strain in the same stool sample. The population counts were slightly lower for resistant than for susceptible strains present in the same stool sample ($10^{7.40}$ versus $10^{7.79}$), but the difference was not significant (P= 0.11, Fig. 23).

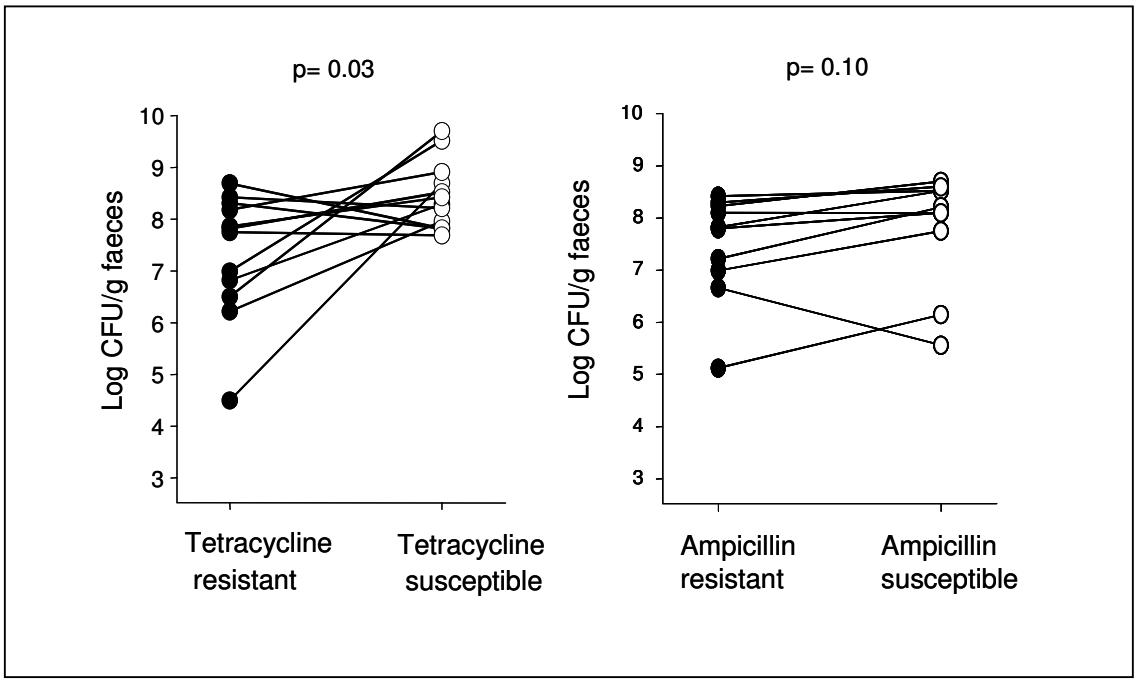


Figure 23. Faecal population counts (log 10-values) for tetracycline and ampicillin resistant strains and susceptible strains colonizing simultaneously in an infant. The *p* value was calculated by paired t-test.

TRANSFER OF RESISTANCE GENES

The acquisition of antibiotic resistance genes by an *E. coli* strain during intestinal colonization was discovered in two cases. These discoveries were made when we studied the stability of resistance genes in the infantile microbiota. During this screening, we analysed all consecutive isolates for antibiotic resistance and identified two *E. coli* strains that became resistant during the normal colonization of the human gut.

First case, infant no. 29

The first case of transmission was discovered in infant no. 29 in the flora cohort. This child received antibiotic treatment and as we analyzed all consecutive isolates of the *E. coli* strains colonizing antibiotic treated infants, we discovered acquisition of a resistance gene in a previously sensitive strain.

The infant was a boy delivered vaginally. At eight days of age, he was admitted to the hospital due to dehydration assumed to be the result of urinary tract infection. He received peroral treatment with trimethoprim for five days and a urinary culture showed growth of 100.000 *E. coli*/ml, the strain being resistant to ampicillin and piperacillin, but sensitive to other antibiotics. A second urinary culture was positive for *E. coli*, but also for enterococci, which was probably the reason why antibiotic treatment was switched to intravenous ampicillin for five days, followed by amoxicillin perorally for an additional eight days. Finally, trimethoprim was administered prophylactically for seven months according to clinical routines (Fig 24).

Faecal samples were obtained at 2, 9, 16 and 32 days and at 2, 6 and 12 months of age according to the flora protocol. Two faecal *E. coli* strains, 29A and 29B were isolated, strain 29A being present in all samples from two days until one year of age. All isolates of strain 29A were highly resistant to ampicillin and piperacillin, but sensitive to amoxicillin-clavulanic acid. The isolates obtained at 32 days and at 2, 6 and 12 months of age had a smaller zone diameter than those obtained earlier for piperacillin and amoxicillin-clavulanic acid. Serotyping showed that the strain had the O134:K1:H31 serotype. The strain found in the urine was found to be identical to 29A by RAPD typing, virulence factor gene carriage (*fimA*, *neuB*) and phylogenetic group identity (group D)

Strain 29B was present in the faecal samples obtained at nine, 16 and 32 days of age (Fig. 24). The isolates on the first two occasions were sensitive to all antibiotics tested (termed $29B^{S}$), while the isolate obtained at 32 days of age (termed $29B^{R}$) was resistant to ampicillin and piperacillin. Strain 29B had the O15:K52:H1 serotype, which is typical of a globally spread pyelonephritogenic clone. It belonged to phylogenetic group D and had the virulence genes *fimA* and *iutA*.

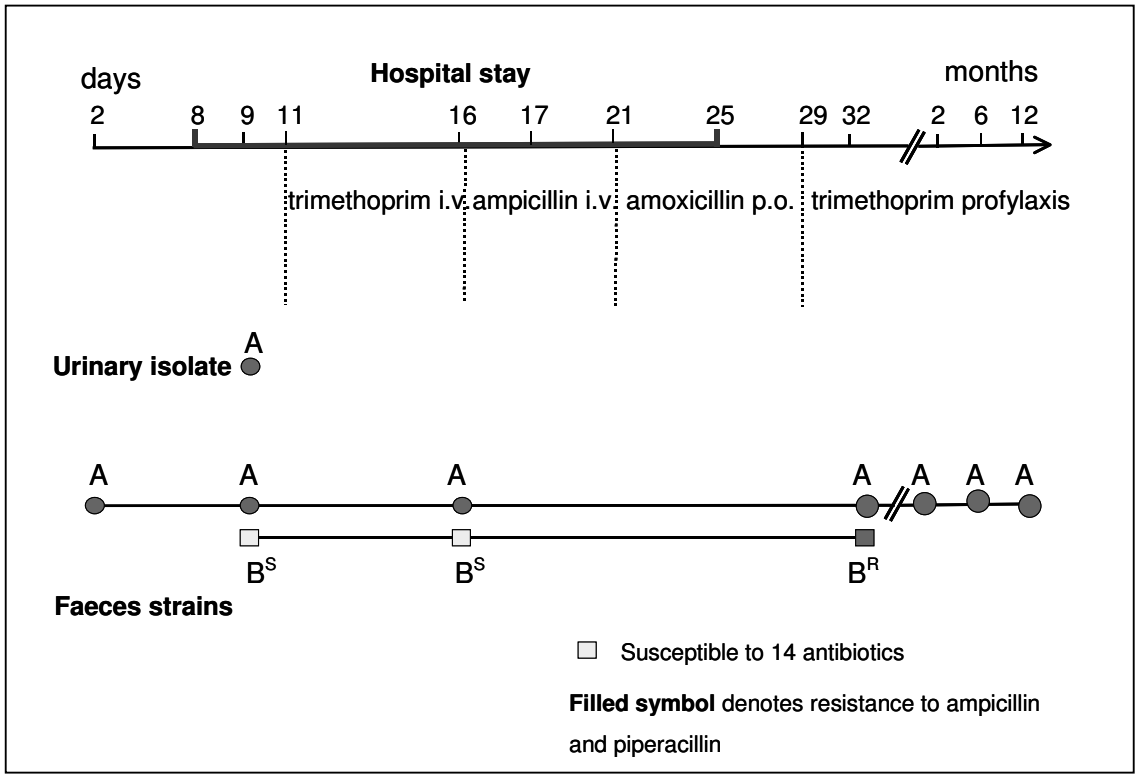


Figure 24. *E. coli* strains recovered from infant no. 29.

Characterization of beta-lactamases by isoelectric focusing revealed that strain 29A harbored two beta-lactamases, one of TEM-1 type (*pI*= 5.4) and one of the AmpC type (*pI*= 9). The isolate 29B^S contained a single beta-lactamase of AmpC type (*pI*= 8.6), while the isolate 29B^R had both AmpC (*pI*= 8.4/8.6) and TEM-1 (*pI*= 5.4) type beta-lactamases. AmpC is chromosomally encoded and found in all *Enterobacteriaceae*. It normally expressed at low levels but can be activated. In this case, we concluded that the AmpC genes found in both 29A and 29B were insufficient to mediate phenotypic resistance to ampicillin, but that transfer of aTEM-1 gene encoding beta-lactamase from strain 29A to strain 29B had rendered the latter resistant to ampicillin.

PCR and sequencing of the *bla*$_{TEM}$ gene carried by strains 29A and 29B^R revealed the same molecular variant, namely *bla*$_{TEM\text{-}1b}$. Strain 29B^S was PCR negative for TEM.

The promoters of the TEM genes were also sequenced. We found the weak variety of the promoter *P3* in strain 29A isolate recovered at two days of age, as well as in 29B^R. The strong *Pa*/*Pb* promoter was instead found in isolates of strain 29A recovered at 32 days and 6 months of age (Fig. 24). The promoters differ by a point mutation. The promoter shift

coincided with a decreased clearance zone for piperacillin and amoxicillin-clavulanic acid. The urinary variety of strain 29A had the weak P3 variety of the *bla*$_{TEM}$ promoter. We suspect that mutation of the promoter occurred in strain A between days 16 and 32 in response to the selective pressure afforded by antibiotic treatment.

Since TEM genes are often plasmid-encoded, we suspected that strain 29A had transferred a plasmid containing a *bla*$_{TEM1\text{-}b}$ gene to strain 29B. We isolated plasmid DNA from 29A, 29B^R and 29B^S. Three plasmid DNA fragments were detected in the 29A and 29B^R isolates that were absent in the 29B^S isolate. The plasmid fragments were subjected to Southern hybridization, using a specific probe. The probe hybridized at the same location to plasmid DNA from the strain 29A and the isolate 29B^R, but not to the isolate 29B^S. We assumed that strain 29A had transferred a plasmid of approximately 40 Kbp size to 29B and termed this plasmid pNKS29.

We tested whether pNKS29 could be transferred from 29A or 29B^R to the recipient strain *E. coli* DH5*α* using conjugation *in vitro*. The frequencies of transfer were 8×10^{-8} and 9×10^{-8} transconjugants per donor, respectively. In a second conjugation experiment, strain 29A was used as donor and a high-level streptomycin resistant mutant (induced by growth in the presence of streptomycin) of the isolate 29B^S used as a recipient. The frequencies of transfer was then 3×10^{-10} transconjugant per donor.

Strains 29A and 29B had similar fecal population counts at nine days of age. After trimethoprim treatment, the population levels of both strains were reduced. Once treatment switched to ampicillin and amoxicillin the fecal population counts of strain 29A rose dramatically to 10^{11} CFU/g. A strain 29B acquired ampicillin resistance, its population density also rose dramatically, i.e. to $10^{10.3}$ CFU/g of faeces (Figure 25a). The increase in *E. coli* faecal population counts coincided with a drop in anaerobic, especially bifidobacterial, population levels (Figure 25b). All bifidobacterial isolates retrieved from the infant were susceptible to ampicillin. Reduced competition from anaerobic bacteria most likely promoted the expansion of *E. coli* in the microbiota.

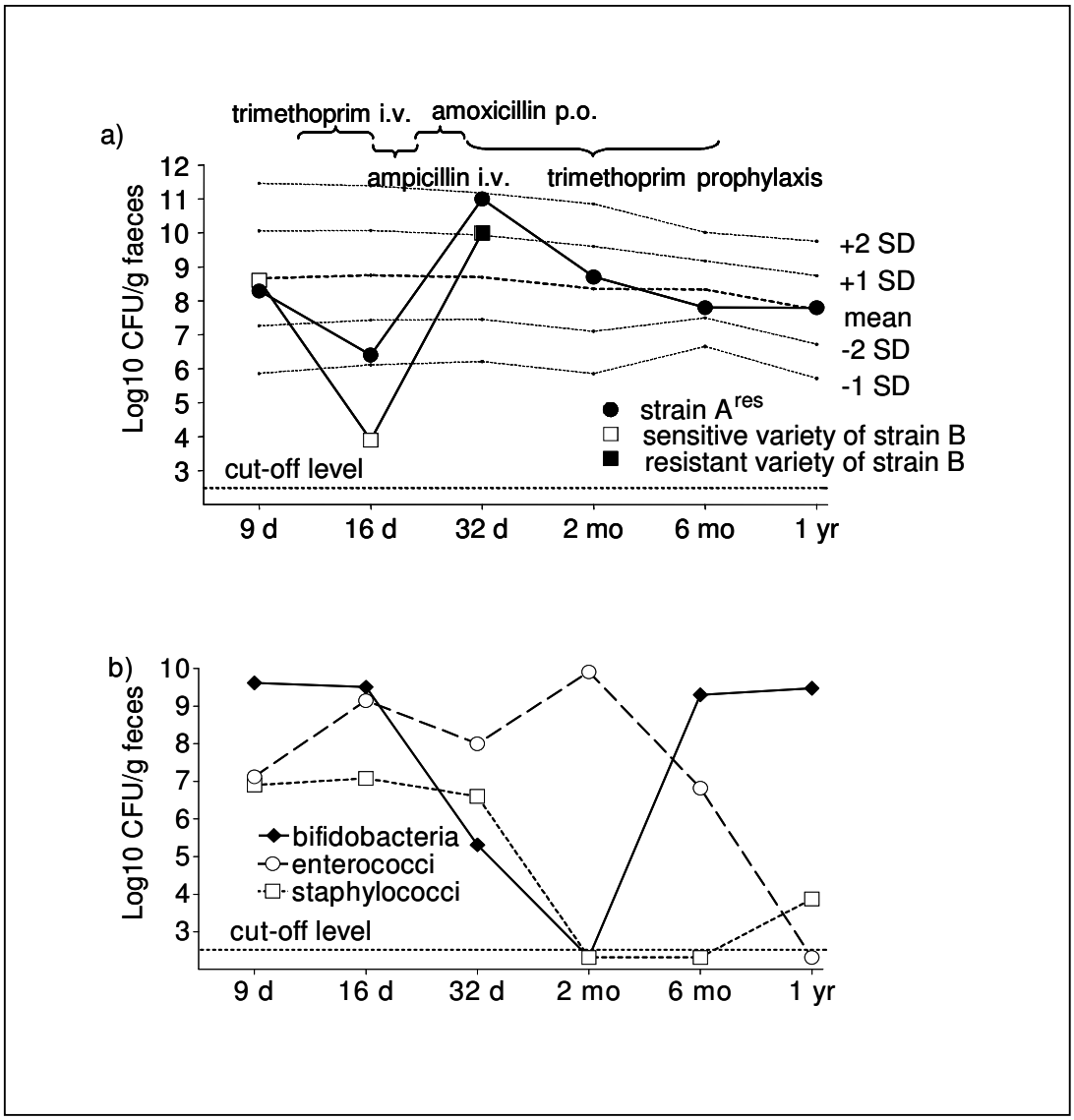


Figure 25. Fecal population counts of strain 29A and 29B at different time points.

The fitness cost of acquisition of the pNKS29 plasmid was calculated. When pNKS29 was introduced into *E. coli* DH5α a small fitness cost (-0.6% ± 2.2% /generation) was observed. When we calculated the fitness cost of the same plasmid into, 29B^{S}, a higher fitness cost (-4.9% ± 4.1% /generation) was observed. The difference in fitness costs associated with acquisition of the plasmid in the two strains was significant (P= 0.046).

Second case, infant no. 117

The second case of transmission was discovered in a girl, born in 2001. She harbored a strain, termed 117A, in a stool samples from two months of age until one year of age. Strain 117A belonged to the CGA clone and to phylogenetic group D and its virulence gene profile was *fimA* and *papC* (Fig. 26). All isolates of strain 117A were highly resistant to ampicillin, piperacillin, sulfonamide and streptomycin. Another *E. coli* strain, termed 117B, was first isolated at two weeks of age and was consistently present in the gut until 6 months of age. The strain belonged to the B2 sublineage and its virulence gene profile was initially *fimA*, *papC*, *sfaD/E*, *hlyA* and *iutA* but the *sfaD/E* gene was lost in the last isolate of the strain obtained at 6 months of age. The isolates of strain 117B obtained from the samples collected at 2 and 4 weeks of age were susceptible to all antibiotics tested, except trimethoprim to which it was intermediately sensitive. In contrast, the isolates of strain 117B obtained at 8 weeks and 6 months of age were resistant to ampicillin, piperacillin, sulfonamide, streptomycin and indeterminate to trimethoprim. The infant was never treated with antibiotics.

Strains 117A and $117B^R$ were both positive by PCR for carriage of the bla_{TEM} gene and sequencing revealed a same molecular variant of $bla_{TEM\text{-}1c}$ in these strain. Strain $117B^S$ was negative by PCR. We found the weak P3 promoter in both strain 117A and $117B^R$.

Five plasmids, four of small size and one of ≥ 40 kbp were isolated from strain 117A. The same large plasmid appeared to be present also in strain $117B^R$, but DNA from this plasmid was sheared during preparation, yielding a pattern which was not clear and easily interpreted. No plasmid bands were observed in $117B^S$. The plasmid DNA preparations of all strains were subjected to Southern hybridization, using a specific probe. The probe hybridized with the smear postulated to represent a large plasmid that had been sheared during preparation in strain 117A and $117B^R$. No hybridization signal was obtained using $117B^S$. We will try to optimize the condition for plasmid extraction to avoid shearing of DNA.

The resistance plasmid could be transferred from 117A as donor to a nalidixic acid resistant mutant of the $117B^S$ as recipient. The frequency of transfer was 50×10^{-6} transconjugants per donor.

We tested the *in vitro* fitness cost associated with carriage of the *bla*$_{TEM-1c}$ encoding the resistance plasmid. Acquisition of plasmid imposed an *in vitro* fitness cost on strain 117B of -6.3% ± 1.9% per generation.

The sensitive variety of strain 117B initially showed quite high population counts when recovered at 2 and 4 weeks of age, but the resistant variety found at 2 and 6 months had population levels more close to average for *E. coli* strains recovered from infants of this age. After acquisition of resistance, strain 117B persisted for at least four months in the microbiota. Strain 117A remained in the microbiota until 1 year of age, although its population counts decreased substantially over time. It kept its resistance profile during the entire colonization period.

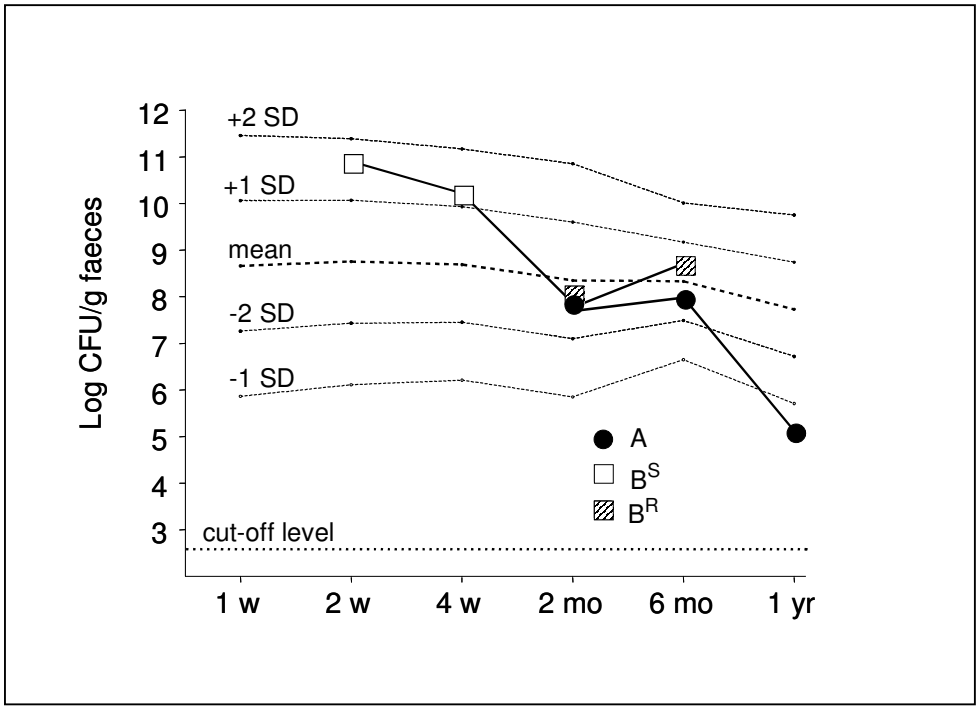


Figure 26. Fecal population counts of strain 117A and 117B different time points. The mean population levels (± 1 and 2 SD) of 272 *E. coli* strains in fecal samples from all 128 Swedish infants in the birth-cohort are shown for comparison.

DISCUSSION

In the present thesis, the prevalence of antibiotic resistance among *E. coli* colonizing the gut of healthy infants, the stability of resistance genes carriage, their impact on *in vivo* fitness and evidence of their transfer between strains co-residing in the gut microbiota were studied in Swedish children participating in the flora study. In this study, stool samples were obtained at specified intervals up to three years of age, which permitted longitudinal studies of the resistance pattern. Moreover, all medical treatment, including antibiotics was registered by the parents in a diary and reported to the study nurse at 6 and 12 months.

We also compared the resistance patterns, carriage of virulence genes and phylogenetic group identity of the faecal *E. coli* strains with *E. coli* positive urinary cultures obtained from children below two years of age. They obtained from the Sahlgrenska Hospital laboratory for Clinical Bacteriology

The commensal flora of the gut is considered as the most important reservoir for antibiotic resistance genes in the community [179]. Determination of the resistance profile in commensal *E. coli* strains may be of importance to judge the size of the pool of resistance elements in a society. Not only may resistant strains in the microbiota spread to other sites of the body, but it has also been speculated that resistance genes might be transferred between strains co-residing in the microbiota [180].

The prevalence of resistance to antibiotics in intestinal *E. coli* strains of the Swedish infants was moderate - the overall resistance was 21%. This may be compared to 89% among intestinal *E. coli* from healthy children in Mexico [181], 88% in Spanish children [53], and 44% in Greek children [182]. Generally, a higher rate of antibiotic resistance in commensal *E. coli* have been reported in the developing countries than developed countries [183, 184]. The reason might be due to free purchase of antibiotics [55], crowding [185], poor sanitation, or contaminated food [186]. We regard the moderate resistance prevalence in Sweden as a result of the lack of these factors, probably most importantly a restrictive policy for prescription of antibiotics.

Young populations may carry more antibiotic resistant *E. coli* than adults [53, 187], which might reflect the higher incidence of infections and more frequent use of

antimicrobials at young age. Data from the Swedish Reference Group for Antibiotics "STRAMA" shows that approximately thirteen times more amoxicillin clavulanic acid, three times more PcV and trimethoprim-sulfonamides is prescribed to 1000 persons per year in age group 0-6 years compared to the age group 60-79 in Sweden in the 2005 [11]. We have not found any Swedish study comparing the prevalence of resistance between children and older persons. Such studies might be of interest to perform in order to determine whether different consumption of antibiotics in different groups in a society result in different prevalence of resistance.

Our results did not support the notion that antibiotic use on the level of the individual was connected to increased risk of colonization with resistant strains in that particular individual. Only one of the infants who were colonized with an ampicillin resistant *E. coli* strain had received treatment with amoxicillin before acquisition and also non-treated infants acquired resistant strains. In fact, 31/32 ampicillin resistant strains colonized infants not treated with beta-lactam antibiotics and all tetracycline resistant strains colonized in the absence of selective pressure, since tetracycline is never given to children. In a study in Nepal, resistance rates were not correlated to the individual use of antibiotic, but instead to the community use of antibiotic [188]. Our results support the notice that resistant and susceptible *E.coli* strains originate from a common pool in society and might establish in the commensal microbiota of non-treated hosts, unhindered by their resistance.

Although the prevalence of resistance was low in intestinal microbiota of infants (21%), it was considerably higher than in a study of *E. coli* isolated from healthy Swedish school children performed between 1971 and 1974 (14%) [189]. This shows that antibiotic resistance increases slowly but relentlessly in the Swedish society.

Tetracycline resistance appeared to be unchanged between our study and that performed in the 1970s (10% vs 9%), despite the increasing consumption rate of tetracycline in out-patients by 50% from 1970 to 2005 in all age groups [11]. Tetracycline is still used frequently in human clinical practice and the consumption of tetracycline is second only to the usage of the beta-lactams. An explanation to why the tetracycline resistance did not increase, could relate to the new variety of this drug, namely doxyteracycline and minocycline that are absorbed to 95% from intestine and excreted in inactive form in the

faeces and urine, are opposed for the older varieties of tetracycline. The second explanation could be the reduced use of tetracycline in farming animals. Tetracycline has been used in human and veterinary medicine, but also in subtherapeutic levels as growth promoters in animal husbandry [190]. The use of tetracycline as a growth promoter for livestock was banned during the 1980s in Sweden and its use in veterinary medicine has also declined [191]. The third explanation could relate to the fact that *tet* genes were enriched among strains of phylogenetic group A. This phylogenetic group was dominant in the intestinal microbiota in studies performed in 1970s [113], while the B2 group appears to have become the predominant group among commensal intestinal *E. coli* in studies performed more recently [112]. Thus, the shift of sublineages of *E. coli* among commensal gut flora may play a roll in the stable level of tetracycline resistance.

The prevalence of ampicillin resistance has increased between the two studies (from 2% to 12% of faecal isolates being resistant), as has trimethoprim resistance (from 0% to 8%). During the 30 years which have passed between the studies, the Swedish sales of extended spectrum penicillins like ampicillin/amoxicillin for treatment of out-patients below 6 years of age increased three times, from 0.5 defined daily doses (DDD/1000) in 1970 to 1.5 in 2005 [10, 192]. Trimethoprim was introduced in Sweden in the 1970s and the consumption of this drug was 0.1 DDD in the age group 0-6 years in 2005. Thus, resistance to trimethoprim has increased quite dramatically, in relation to its quite moderate consumption, which shows that different antibiotics differ in their tendency to select for resistance.

Tetracycline resistant strains were assayed for their possession of the *tetA, B, C, D* and *E* genes, which are the most common tetracycline resistance genes in gram-negative bacteria. They all encode efflux system, which pump the antibiotic out from the bacterial cell. *TetA* and *tetB* were the genes most frequently encountered and all resistant isolates carried one of these two genes. *TetA* and *tetB* were equally common in our material, whereas *tetA* was the tetracycline resistant gene most frequently encountered in *E. coli* isolates from Spanish children, followed by *tetB* and *tetD* [53], and *tetB* dominated in Swedish urinary and Pakistani intestinal *E. coli* isolates (Unpublished data). *TetA* and *tetB* are also the genes most frequently encountered in tetracycline resistant *E. coli* of animal origin [193].The *tetB* gene conferred a higher level of resistance to tetracycline compared to the *tetA* gene, which has

also been noted elsewhere [194, 195]. The *tetB* efflux system might, thus, be more efficient than that of *tetA*.

Ampicillin resistant strains were assayed for their possession of the *bla*TEM, *bla*SHV and *bla*OXA genes. The TEM type of beta-lactamase was the most prevalent gene conferring ampicllin resistance (84%), while the SHV type was found in 16% of the ampicillin resistant isolates. *bla*OXA was found in a single isolate, in combination with TEM-1. There is limited information about the prevalence of beta-lactamases in commensal *E. coli*. However, in the Spanish study *bla*TEM was encountered in 96% of human *E. coli* isolates whereas the SHV type was not found [174]. *Bla*TEM1-b was the most frequent variant of *bla*TEM in both Spanish and our study respectively 50% and 59% of the *bla*TEM genes.

Beta-lactamases genes are preceded by a promoter which determines the rate of transcription. The promoter exists in different varieties which confer different levels of transcription and thereby resistance. The different promoter varieties differ in single nucleotides, and mutations are the reason for shift in promoters. The weak beta-lactamase promoter, called *P3*, was found in 24 strains carrying *bla*TEM-1 genes, while the strong promoter, termed *Pa*/*Pb*, was found in three strains. The strains with the *Pa*/*Pb* promoter all had decreased susceptibility to amoxicillin with clavulanic acid, a competitive inhibitor for beta-lactamase. Leflon-guilbout *et al*. [50] reported that reduced susceptibility to amoxicillin clavulanic acid often characterizes strains with the strong *Pa*/*Pb* promoter and that is caused by overproduction of TEM-1 β-lactamases, which overcomes the action of the competitive inhibition.

Antibiotic resistance was significantly more common among urinary than among intestinal isolates. This is in accordance with a study from Iran, showing that the frequency of resistance in commensal *E. coli* is usually lower, but related to, the frequency in clinical isolates from the same area [196]. In both intestinal and urinary isolates, resistance to ampicillin, tetracycline or trimethoprim was most common, and for urinary isolates also resistance to chloramphenicol. The finding of a very similar resistance pattern may reflect the fact that *E. coli* causing urinary tract infection originate in the patient's own bowel flora. The

question why uropathogenic *E. coli* are more resistant than commensal ones is not easy to answer.

Phylogenetic group D and to a lesser extent group A, were more often resistant to various antibiotics than group B2, which is in accordance with previous studies [122, 123]. Urinary isolates predominantly belong to the B2 and D phylogenetic groups. However, within each phylogenetic group, resistance was more common among urinary than commensal strains. Strains may differ between and within each phylogenetic group, in their capacity to take up genetic material from other strains and /or to keep their resistance genes. The genetic diversity within phylogenetic groups has been studied in a work by Zhang L, *et al*. [197]. They compared urinary *E. coli* isolates of the B2 and D groups obtained from college-age women with rectal isolates of the same phylogenetic groups using ERIC sequence typing. Urinary B2 and D isolates were found to be genetically more homogenous than the rectal B2 and D isolates. They suggested that certain groups of B2 and D are not equally pathogenic and maybe the most pathogenic and resistant fraction of these groups invade the other site of human body.

The CGA clone is a world-wide spread clone of uropathogenic *E. coli* that belongs to the phylogenetic group D [115-117]. We found that it represented about two thirds of the urinary isolates belonging to group D. We also found the clone among intestinal isolates, but in a considerably lower proportion (13% of intestinal group D strains). The vast majority of CGA isolates in the present study exhibited resistance to ampicillin, tetracycline, trimethoprim and chloramphenicol. It is evident that this clone can harbor multidrug resistance plasmids with little cost, because the clone has spread globally within the last seven years. Contribution from clone CGA isolates was one reason for the high rate of resistance among strains of phylogenetic group D. However, even when excluding this clone, group D strains tended to be more resistant than strains belonging to the other phylogenetic groups. One may speculate that group D strains have better capacity to act as recipients of resistance genes, or that they are more apt to keep acquired genes.

We also analyzed the association between resistance and carriage of virulence genes. Among both intestinal and urinary isolates, strains that carried *papC* or *iutA* genes, but not the other virulence factors genes, were more often resistant than strains lacking these traits. P

fimbriae and aerobactin are virulence factors for urinary tract infection [76, 80]. P fimbriae are the major virulence trait in human urinary tract infection. P fimbriae enable *E. coli* to bind to Galα1-4Galβ containing structures in urinary tract epithelium and thereby ascend into the urinary bladder and further up the urethers and into the kidney. 70% of *E. coli* isolates from pyelonephritis have the gene for P fimbriae [76]. Aerobactin is an iron-trapping compound that grants the bacterium access to iron necessary for its growth. Free iron is very limited in the urine, in the blood and tissues, since all iron is tightly bound to transport or storage proteins.

But both P fimbriae and aerobactin are also colonization factors in the human bowel. P fimbriae enable the bacteria to bind to Galα1-4Galβ containing receptors in the colonic epithelium, and in the populations we have examined (Swedish infants, Swedish school-girls, Pakistani infants [103-105]). *E. coli* strains with the gene for P fimbriae were more likely to persist in the microbiota than strains lacking this gene. It might appear logical that P-fimbriated *E. coli* strains are more often resistant to antibiotics as they more often cause disease and then become exposed to antibiotics. However, tetracycline is never given against urinary tract infection. It is therefore unlikely that the strains acquired such resistance due to treatment of urinary tract infection, rather, tetracycline resistance might have developed in commensal bacteria as a bystander effect when a patient is treated, e.g. for upper respiratory tract infection with tetracycline. In this case P fimbriated strains which are excellent colonizers of the human gut might often colonize humans and may therefore be more exposed to antibiotic given to humans.

The fitness cost associated with carriage of ampicillin and tetracycline resistance was examined in this thesis. On average, tetracycline and ampicillin resistant strains reached equally high population numbers in the stools as susceptible strains. Interestingly, when tetracycline-resistant and -susceptible strains were present in the stools simultaneously in an infant, the resistant variety showed slightly, but, significantly, lower population counts. There was no significant difference between ampicillin-resistant and -susceptible strains when competing in the gut of an infant. We are not aware of other study that measured the

population level in resistant strains and compared to population level of susceptible strains in the gut of human.

Another measure of colonizing success is the capacity to long-term persistence in the gut. Ampicillin resistant strains were equally capable of persisting in the microbiota as susceptible ones. This was also true for tetracycline resistant strains, despite their slightly lower population levels in direct competition with susceptible strains. Our findings suggest that antibiotic resistant strains once established might not be eliminated due to a low fitness cost. Long-term analyses of the intestinal microbiota and analysis of resistance have not been performed before, to the best of our knowledge. However, the long-term persistence of macrolide-resistant strains of *Staphylococcus epidermidis* for four years from the nostrils of five patients were demonstrated [198].

The human colon provides an environment that has been posed to be very suitable for horizontal gene transfer. The concentration of bacteria in the intestine is higher than of any other site in human body. The transfer of resistance genes are demonstrated in the gastrointestinal tract of gnotobiotic animal models [152-154] and volunteers who were fed bacteria [160], but a direct evidence of gene transfer in the human intestine, where donor, recipient and transconjugant being recovered have not demonstrated in the best of our knowledge. In this thesis, two cases of gene transmission from an ampicillin resistant *E. coli* strain to an initially sensitive *E. coli* strain are demonstrated. The donor and recipient strains colonized simultaneously in the bowel microbiota of two infants and transconjugants were recovered later. In the first case, the infant was treated with ampicillin, which enhanced the population counts of the donor strain and provided a selective pressure promoting the survival of the transconjugant strain. The transconjugants could not be recovered after removal of the selective pressure afforded by antibiotic treatment. The ampicillin treatment was also associated with mutation of the *bla*$_{\text{TEM}}$ promoter gene in the donor strain resulting in a more effective expression of the *bla*$_{\text{TEM}}$ gene. To our knowledge, demonstration of mutational changes of resistance genes during intestinal colonization is not shown before. In the first case, both donor and recipient strains belonged to phylogenetic group D.

In the second case, transfer of a plasmid conferring resistance to ampicillin, streptomycin and sulfonamide was demonstrated in an infant who was not treated with

antibiotics. The donor strain belonged to the CGA clone within phylogenetic group D, while the recipient belonged to phylogenetic group B2. Our results, thus, indicate that resistance genes are prone to spread among strains in the intestinal microbiota and that strains belonging to group D may be especially apt to participate in such gene transfer.

The recipient in the first case and the donor in the second case belonged to recognized uropathogenic clones, i.e the O15:K52:H1 clone and the CGA clone, respectively. We noted a very low frequency of these two clones in the intestinal microbiota, only 1% and 2% of strains, respectively. These clones are globally known as epidemic clones, but their strong potential involving in the horizontal gene transfer is not mentioned anywhere else.

During *in vitro* conjugation, we observed a lower frequency of transfer in the first case. This suggested transmission might only occur during strongly predisposing conditions i.e. treatment with antibiotic. Antibiotic depressed severely the population levels of anaerobic bacteria and enabled the population counts of donor to rise radically. In the second case, a higher transfer frequency was observed, indicating the transmission of plasmid might occur quite readily also *in vivo* in the absent of antibiotic pressure.

The fitness costs of carrying plasmids in both cases were measured upon the parent strains, i.e. plasmid free varieties. The fitness cost of plasmid in the first case was moderate and the fitness cost of plasmid in the second case was higher compared to the first case. In the first case, the strain that had acquired the resistance plasmid was eliminated from the microbiota when ampicillin treatment was terminated. In the second case, the strain which had acquired the resistance plasmid was not eliminated. Instead, it persisted for at least four months, despite the relatively high fitness cost found in *in vitro* measurements. The maintenance of plasmid in the bacterial host may be associated with other factors. Plasmid replication and gene expression may interfere with the bacterial growth and could not be maintained by the host.

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مقاومت میکروبی به آنتی بیوتیکها یک مشکل در حال رشد در تمام دنیا است . مقاومت نه تنها درمیکروبهای ایجاد کننده بیماری بلکه در میکروبهای فلور طبیعی بدن فرد در حال در مان نیز ایجاد می شود . یافته های اندکی در خصوص چگونگی ایجاد مقاومت میکروبی در باکتریهای فلور بدن در دست است . میکروب اشریشیا کلی جزو فلور طبیعی روده است اما میتواند ایجاد عفونت دستگاه ادراری یا سپتی سمی نیز بنماید . این مطالعه شیوع و پایداری مقاومت به آنتی بیوتیکها را در بین میکروبهای اشریشیا کلی کلونیزه شده در روده نوزادان سالم بررسی میکند . همچنین انتقال ژن ایجاد مقاومت در بین گونه های اشرشیا کلی موجود در فلور طبیعی روده مورد مطالعه قرار گرفت .

گونه های اشرشیا کلی مورد بررسی از مدفوع ۱۲۸ شیر خوار سوئدی جدا شده اند . برای مقایسه از ۲۰۵ شیر خوارزیر ۲ سال که مبتلا به عفونت دستگاه ادراری بودند نیز اشرشیا کلی جمع آوری گردید . هر یک از میکروبها بر اساس RAPD – فیلوژنتیک و حمل ژن ویرولانس طبقه بندی شدند . مقاومت کلینیکی به ١٤ آنتی بیوتیک بررسی شد و همچنین ژنهای کدگزارمقاومت به آمپی سیلین و تترا سیکلین شناسایی شدند .

حدود ۱۲٪ از سوش های اشرشیا کلی روده و ٤٠٪ اشر شیا کلی جدا شده از ادرار مقاومت داروئی نشان دادند.این مقاومت بیشترنسبت به امپی سیلین وتری متوپریم بود.

سوش های اشرشیا کلی مقاوم به تترا سیکلین و آمپی سیلین می توانند به مقدار برابر و یکسان با سوش های حساس فلور روده عمر کنند . ژن ایجاد کننده مقاومت به آنتی بیوتیک در تمام دوران کلونیزاسیون میکروب باقی مانده و از بین نمی رود . بیشتر سو ش های مقاوم در شیر خوارانی که قبلا هیچ آنتی بیوتیکی نگرفته بودند یافت شد . اکثر اشرشیا کلی های مقاوم به تترا سیکین متعلق به گروه فیلو ژنتیک A و سو ش های مقاوم به آمپی سیلین متعلق به گروه D بودند . ژن مقاومت به تترا سیکلین tet A با ژن ویرولانس (بیماری زائی) iut A و ژن آئروباکتین مرتبط می باشد درحالی که ژن tet B و ژن bla TEM که بتالاکتاماز را کد گزاری می کند با ژن pap C که مربوط به کد گزاری P فیمبریا می باشد همراه هستند .ما در ۲ مورد انتقال پلاسمید حامل ژنهای bla TEM را از سوش هلی اشرشیا کلی مقاوم به آمپی سیلین به سوش های حساس فلور روده شناسایی کردیم . در هر ۲ مورد سوش دهنده متعلق به گروه فیلو ژنتیک D می باشد .

مورد اول شیر خواری است که با آمپی سیلین درمان شده بود . مصرف آمپی سیلین باعث افزایش تعداد کلونی و جمعیت سوش دهنده و همچنین افزلیش مدت حیاط گونه در یافت کننده پلاسمید گشته بود . علاوه بر آن موتاسیون در ژن پروموتور blaTEM در سوش دهنده مشاهده شد که منجر به بروز بیشتر ژن blaTEM می شود .

در مورد دوم انتقال یک پلاسمید باعث ایجاد مقاومت به آمپی سیلین – استرپتومایسین و سولفونامید شده است .

این مورد شیر خواری است که قبلا با آنتی بیوتیک درمان نشده است . سوش دهنده متعلق به کلون پاتوژن سیستم ادراری CGA بود .

مطالعه ما بررسی پایداری و هزینه حمل ژن ایجاد مقاومت به آنتی بیوتیکها در باکتریهای فلور نرمال انسان می باشد . نتایج نشان داد که ژنهای ایجاد کننده مقاومت به راحتی در بین سوش های نرمال قابل انتقال و گسترش هستند . پایداری ژنهای ایجاد کننده مقاومت به آنتی بیوتیکها به دلیل هزینه اندک حمل ژن و نیز همراهی آنها با ژنهای کد کننده فاکتورهای کلونیزاسیون مانند P فیمبریا و آئروباکتین می باشد .