MASTER THESIS IN MEDICAL BIOLOGY (MBI-3910)

Aminoglycoside resistance in clinical Gram-negative isolates from Norway



Bjørg C. Haldorsen

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Researchgroup for Host-Microbe Interactions	Reference Centre for Detection of Antimicrobial Resistance (K-res)
Department of Medical Biology Faculty of Health Sciences	Department of Microbiology and Infection Control
University of Tromsø	University Hospital of North-Norway

Intellectual growth should commence at birth and cease only at death

Albert Einstein

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The journey towards this master thesis started on Christmas day 2005. After a long talk with my sister-in-law Tone, the plan for further studies was launched. Maybe as a result of a mid-life crisis, the race towards a master degree was initiated.

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Summary

Aminoglycosides represent an important class of antimicrobial agents. The prevalence of aminoglycoside resistance among Gram-negative bacteria in Norway is low, but an increased prevalence among clinical isolates of *Escherichia coli* has been observed during the last years. The most prevalent resistance mechanism is aminoglycoside modifying enzymes. In addition, resistance may occur when bacteria produces 16S rRNA methylases, which causes high level and broad-spectrum aminoglycoside resistance.

In this study, we analysed the susceptibility pattern of different aminoglycosides in different Norwegian strain collections of *E. coli*, *Klebsiella* spp. and *Pseudomonas aeruginosa*. Among *E. coli* and *Klebsiella* spp. isolates from the Norwegian surveillance programme for antimicrobial resistance (NORM) 2009 the prevalence of reduced susceptibility to tobramycin (4.1%) was slightly higher compared to gentamicin (3.8), in blood culture isolates of *E. coli* and 2% and 1.4% in in blood culture isolates of *Klebsiella* spp., respectively. The prevalence of reduced susceptibility to amikacin was low in both species; 0.6% in *E. coli* and 0.4% in *Klebsiella* spp. In a collection of $ESBL_A$-positive Enterobacteriaceae isolates the prevalence of reduced susceptibility was much higher with 56% and 44% of the isolates showing reduced susceptibility to tobramycin and gentamicin, respectively. The prevalence of reduced susceptibility to amikacin (7%) was also lower than for tobramycin and gentamicin in the $ESBL_A$-positive isolates. The same pattern was also observed in the collection of carbapenem non-susceptible *P. aeruginosa* isolates.

In both Enterobacteriaceae collections the aminoglycoside modifying enzymes AAC(3)-II and AAC(6')-Ib were the dominating enzymes causing aminoglycoside resistance. The 16S rRNA methylases *rmt*B and *rmt*D were detected in one *E. coli* and one *P. aeruginosa* isolate resistant to all aminoglycosides tested, respectively.

Three different methods for detection of reduced susceptibility were used; Etest, EUCAST disk diffusion and VITEK2 AST. The results from the three methods were compared. Discrepancies were mainly observed when comparing Etest and EUCAST disk diffusion for detecting tobramycin resistance in Enterobacteriaceae, and comparing Etest and EUCAST disk diffusion for detecting gentamicin resistance in *P. aeruginosa.*

In conclusion, aminoglycoside resistance in Norway is low, but increasing. Worryingly, aminoglycoside resistance is coupled with other resistance mechanisms such as $ESBL_A$ resulting in multidrug resistance limiting treatment options. Method comparison indicates a need for evaluation and frequent maintenance of breakpoints.

1. Introduction

1.1 Antimicrobial agents

1.1.1 Brief historical background

Antimicrobial agents have since their discovery during the 20th century, saved lives and eased the suffering of millions of people. Many serious infectious diseases have been brought under control by these antimicrobial agents, and thus contributed to major gains in life expectancy experienced during the latter part of the last century.

In September 1928, Alexander Fleming by coincidence discovered the fact that the mold *Penicillum* inhibited the growth of *Staphylococci*, and decided to investigate this phenomenon further(17). This is considered the onset of the antibiotic era. But the findings of Fleming were actually a rediscovery of the observations of John Tyndall, an English physicist in 1875(17). During an experiment Tyndall wanted to find out whether bacteria were evenly dispersed in the atmosphere or aggregated in clouds, by incubating open test tubes containing broth. After incubation a number of the tubes remained clear, indicating that bacteria were not evenly dispersed in the atmosphere. But more importantly he observed a *Penicillum* on the surface of the broth in some of the tubes. A battle was in progress between the bacteria and the mold, and "in every case where the mold was thick and coherent, the bacteria died or became dormant and fell to the bottom as a sediment". Tyndall did not explore his observations any further, and the power of *Penicillum* remained unknown until Fleming observed the *Penicillin's* ability to kill bacteria(17). In the spring of 1940, Florey and Chain from Oxford University were able to make a small amount of yellowish powder from the mold discovered by Fleming. This initiated the first commercial production of penicillin. In 1945, the Nobel prize in medicine was awarded to Fleming, Florey and Chain(26).

During the years 1940-1960 many other antimicrobial agents were identified and developed, as Figure 1 shows. Aminoglycosides, other β-lactams than penicillin, tetracycline, macrolides, and glycopeptides are examples of new classes of antibiotics developed. But between the introduction of quinolones in 1962 and the next new structural class of antibiotic; the oxazolidinones, there was a gap of 40 years(48). Unfortunately, few large pharmaceutical companies are active in the antibacterial infectious disease arena, making the future unsure regarding development of new antibiotics.

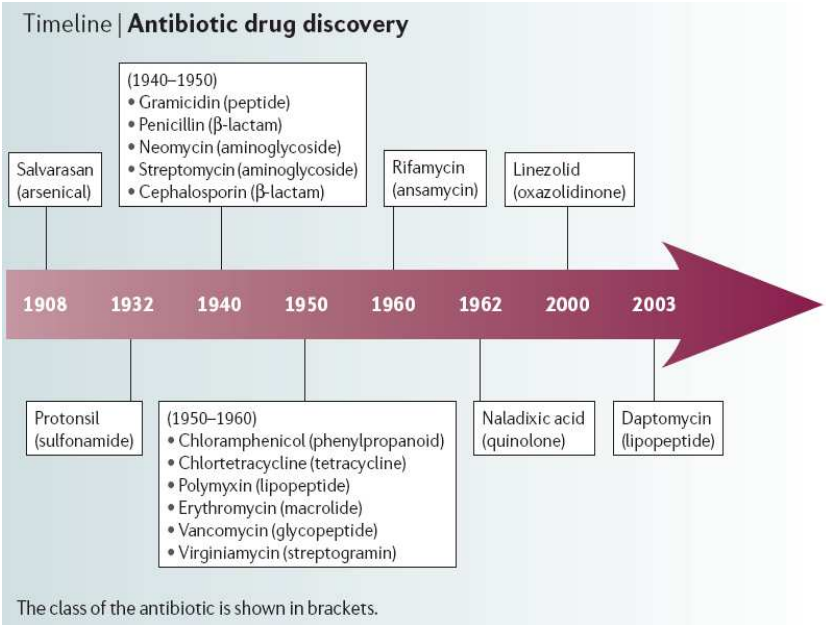


Figure 1. Timeline showing development of antimicrobial agents during the years 1908 – 2003(52).

1.1.2 Antimicrobial agents

Antimicrobial agents are molecules that stop microbes, both bacteria and fungi, from growing or kill them outright. They are often products of soil bacteria and fungi, with the major group of antibiotic-producing bacteria being the actinomycetes(50). The majority of antimicrobial agents in clinical use today are derivates from natural products of fermentation or chemically modified (i.e semi-synthetic) to improve their antibacterial and pharmacologic properties. In addition, some agents like the quinolones are totally synthetic(31).

Antimicrobial agents can be classified as bactericidal, exemplified by penicillin or bacteriostatic, exemplified by chloramphenicol. Bactericidal agents cause bacterial cell death, while bacteriostatic agents prevent the bacteria from growing(50). Classification of antimicrobials may also be done according to their mechanisms of action (Table 1); (I) interference with cell wall synthesis, (II) inhibition of protein synthesis, (III) interference with nucleic acid synthesis, and (IV) inhibition of a metabolic pathway. Disruption of the bacterial membrane structure may be a fifth mechanism of action, although it is less well characterised(45).

Table 1. Mechanisms of action of antimicrobial agents (adapted from Tenover 2006(45))

Mechanism of action	Antimicrobial agent(s)
1. Interference with cell wall synthesis	β-lactams: penicillins, cephalosporins, carbapenems, monobactams Glycopeptides: vancomycin, teicoplanin
2. Inhibition of protein synthesis:	Macrolides, chloramphenicol, clindamycin,
Binding to 50S ribosomal unit	quinopristin-dalfopristin, linezolid
Binding to 30S ribosomal unit	Aminoglycosides, tetracyclines
3. Interference with nucleic acid synthesis:	Fluoroquinolones, rifampicin
Inhibition of DNA synthesis	
Inhibition of RNA synthesis	
4. Inhibition of a metabolic pathway	Sulfonamides, folic acid analogous
5. Disruption of bacterial membrane structure	Polymyxins, daptomycin

1.2 Aminoglycosides

1.2.1 Discovery of aminoglycosides

Streptomycin was the first aminoglycoside to be identified and characterized by Selman Waksman in 1944. In contrast to penicillin which was isolated from fungi, streptomycin was the first antimicrobial to be isolated from a bacterial source. The discovery of streptomycin was a landmark in the history of antimicrobials, since it was the first effective treatment for tuberculosis, a disease that had caused tremendous human suffering for centuries(9).

A number of aminoglycosides have been isolated and tested during the years, but only the aminoglycosides investigated in this study will mainly be further discussed. The source and year of isolation of the aminoglycosides included in this study are listed in Table 2. In Norway only gentamicin and tobramycin are licensed for general use, while amikacin and netilimicin are not marketed.

Table 2. The source and year of isolation of the aminoglycosides included in this study. (adapted from Aminoglycoside antibiotics. From chemical biology to drug discovery(9))

Aminoglycoside	Isolated/synthesized from	Year of isolation
Streptomycin	*Streptomyces griseus*	1944
Kanamycin	*Streptomyces kanamyceticus*	1957
Gentamicin	*Micromonospora purpureochromogenes*	1963
Tobramycin	Synthesized from kanamycin	1967
Amikacin	Synthesized from kanamycin	1972
Arbekacin	Synthesized from dibekacin (syntethic derivative from kanamycin)	1973
Isepamicin	Synthesized from gentamicin	1975
Netilmicin	Synthesized from sisomicin (syntethic derivative from gentamicin)	1976

1.2.2 Classification of aminoglycosides

Aminoglycosides are a complex family of compounds and the classification can be based on the chemical structure. There are different structural classes of aminoglycosides, characterised by having an aminocyclitol nucleus (streptamine, 2-deoxystreptamine (DOS) or streptidine) linked to amino sugars through glycosidic bonds(35). There are two main classes of aminoglycosides; the streptomycin class (I) and the 2-deoxystreptamine class (II).

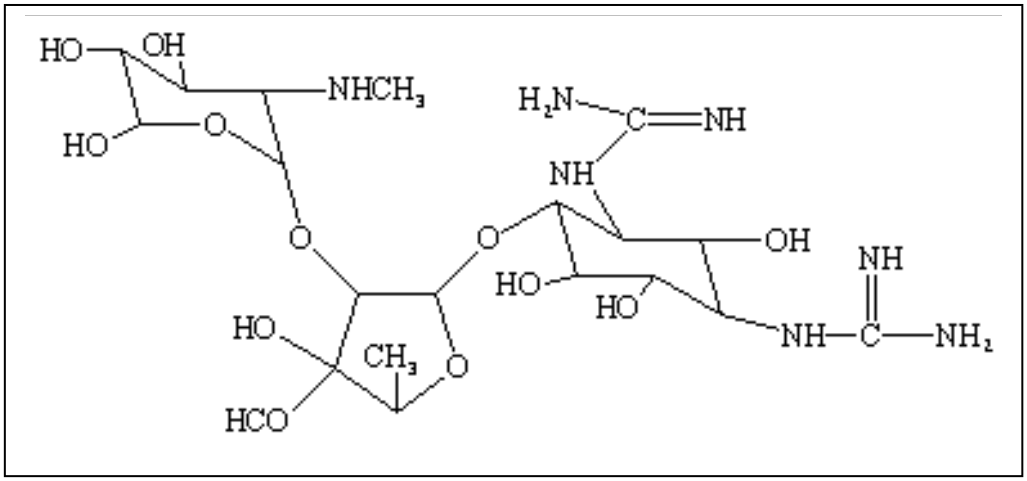


Figure 2. The chemical structure of streptomycin, including (from left to right) aminocyclitol nucleus (streptidine), pentose (streptose), and glucosamine
(www.textbookofbacteriology.net/themicrobialworld/streptomycin.gif)

(I) Streptomycin consists of three sugar constituents: an aminocyclitol (streptidine) connected to a pentose (streptose) which again is connected to glucosamine(49) (Figure 2). Derivatives of streptomycin have different semi-synthetic modifications as R-groups on the pentose. Streptomycin has HCO as the R-group, while derivates of streptomycin has other R-groups at this position.

(II) The deoxystreptamine class has the aminocyclitol nucleus streptamine placed in centre of the molecule. This class is divided into two groups according to whether the sugar constituents are placed at 4,5 or 4,6 position of the aminocyclitol nucleus(9). In kanamycin (Figure 3), an aminoglycoside belonging to the 4,6 substituted deoxystreptamine class, the aminocylitol deoxystreptamine is the central ring, with glucosamine connected at position 4 and a hexose is connected at position 6 of the aminocyclitol ring. Different R-groups placed

on different positions of the sugars, define derivates of kanamycin. Kanamycin was the first useful deoxystreptamine aminoglycoside, isolated in Japan in 1957(9).

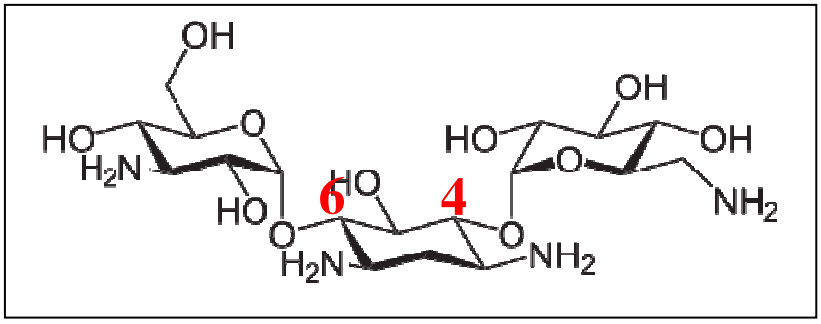


Figure 3. The chemical structure of kanamycin, with deoxystreptamine as the aminocyclitol nucleus, glucosamine connected at position 4 and hexose connected at position 6.
(http://upload.wikimedia.org/wikipedia/commons/thumb/d/d6/Kanamycin_A.svg/491px-Kanamycin_A.svg.png)

Streptomyces, Micromonospora, Bacillus, and other bacterial genera have been shown to produce aminoglycoside-aminocyclitol antibiotics(9). The compounds derived from *Streptomyces* are named with the suffix "-mycin" (e.g tobramycin), while compounds derived from *Micromonospora* are named with the suffix "-micin" (e.g. gentamicin). Gentamicin is special among the aminoglycosides as it is not a single molecule, but consists of three major and several minor components(24). The major components of the drug complex are gentamicins C_1, C_{1a} and C_2. The C_2 component consists of two stereoisomers; C_2 and C_{2a} (Figure 4).

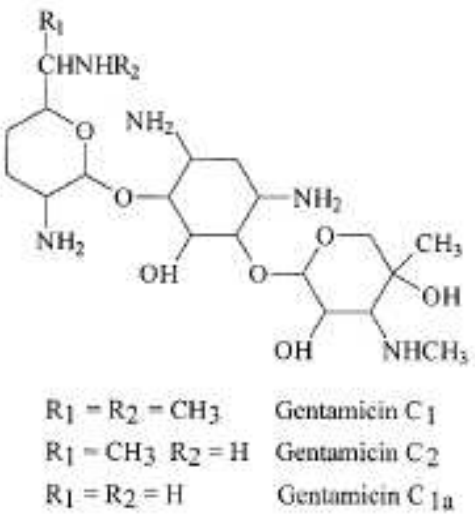


Figure 4. Chemical structure of gentamicins C_1, C_2 and C_{1a}(24).

1.2.3 Antibacterial characteristics and toxicity of aminoglycosides

Aminoglycosides exhibit *in vitro* activity against a broad-spectrum of clinically important Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* spp., *Pseudomonas* spp., *Shigella* spp., *Salmonella* spp., *Enterobacter* spp., *Citrobacter* spp., *Acinetobacter* spp., *Proteus* spp., *Serratia* spp., and *Morganella* spp as well as the Gram-positive bacteria *Staphylococcus aureus* and some streptococci(47). No *in vitro* activity has however been predicted against *Streptocccus pneumoniae*, *Neisseria gonorrhoeae, Burkholderia cepacia*, *Stenotrophomonas maltophilia,* and anaerobic microorganisms. Aminoglycosides only give adequate activity against enterococci as long as they are used synergistically with a cell wall-active antibiotic, such as a β-lactam or vancomycin.

Aminoglycosides exhibit several characteristics that make them useful as antimicrobial agents(47). The bactericidal activity of aminoglycosides depends more on their concentration than on the duration of bacterial exposure to inhibitory concentrations of the antimicrobial agent. The potential of aminoglycosides to kill bacteria depends on the concentration of the antibiotic, and increases with increasing concentrations. In addition, aminoglycosides continue to kill bacteria even after the aminoglycoside is detectable, exhibiting an important post-antibiotic effect. This is probably due to a strong, irreversible binding to the ribosome. The synergistic bactericidal activity in combination with antimicrobial agents inhibiting cell wall biosynthesis is another important characteristic of aminoglycosides. Synergism is probably due to the enhanced intracellular uptake of aminoglycosides caused by the increased permeability of bacteria after incubation with cell wall synthesis inhibitors.

Unfortunately, aminoglycosides can give toxic responses such as ototoxicity and renal toxicity. Streptomycin, among other aminoglycosides targets sensory hair cells of the inner ear and can lead to hair-cell degeneration and permanent hearing loss in up to five % of patients(9). Concentration-dependent bactericidal activity and post-antibiotic effect in combination with the risk of ototoxicity and renal toxicity are the major reasons for once-daily dosing with aminoglycosides in patients with normal renal function(47).

1.2.4 Mechanism of action of aminoglycosides

Aminoglycosides attack the bacteria in a two-step process. Firstly, uptake of aminoglycosides into the bacteria is an important process for their biological activity. Secondly, inside the bacterial cell the aminoglycoside binds to the ribosome and inhibits protein synthesis(47).

1.2.4.1 Bacterial uptake of aminoglycosides

The bacterial cell wall is penetrated by the aminoglycoside in a three-step process; an energy-independent step followed by two energy-dependent steps(35).

In the energy-independent step the aminoglycosides binds to the surface-anionic compounds of the bacterial cell wall such as lipopolysaccharide, phospholipids and outer membrane proteins in Gram-negatives and teichoic acids and phospholipids in Gram-positives(35). The binding to the anionic sites on the outer membrane, results in displacement of Mg^{2+} and Ca^{2+} ions that link adjacent lipopolysaccharide molecules. The effect on the bacterial cell is increased permeability that leads to the so-called “self-promoted uptake” penetration of aminoglycoside molecules into the periplasmic space.

The initial electrostatic surface binding is followed by the energy-dependent phase I. A small number of aminoglycoside molecules cross the cytoplasmic membrane in a process that requires a threshold transmembrane potential generated by a membrane-bound respiratory chain(35). This explains why anaerobes, which have a deficient electron transport system, are intrinsically resistant to aminoglycosides. The aminoglycoside molecules that reach the cytoplasm binds to the ribosome and results in misreading of the mRNA and production of misfolded membrane proteins. These membrane proteins cause damage to the integrity of the cytoplasmic membrane.

Finally, the loss of membrane integrity triggers the energy-dependent phase II. The damaged cytoplasmic membrane results in an accelerated rate of uptake of aminoglycoside molecules. The aminoglycoside accumulates rapidly in the cytoplasm and irreversibly saturates all ribosomes, resulting in cell-death. The higher concentration of the aminoglycoside, the more rapid is the onset of energy-dependent phase II and the death of the bacterial cell(35,47)

1.2.4.2 Molecular mechanism of action

The ribosome has an important role in translating mRNA into proteins in the bacterial cell, and consists of two subunits; designated 50S and 30S(47). The large subunit (50S) is made up by two RNA molecules; 5S and 23S RNAs and about 30 proteins. The small subunit (30S) is made up by 16S RNA and 20 to 21 proteins. During proteins synthesis, the ribosome decodes the mRNA and incorporates amino acids into the growing polypeptide chain.

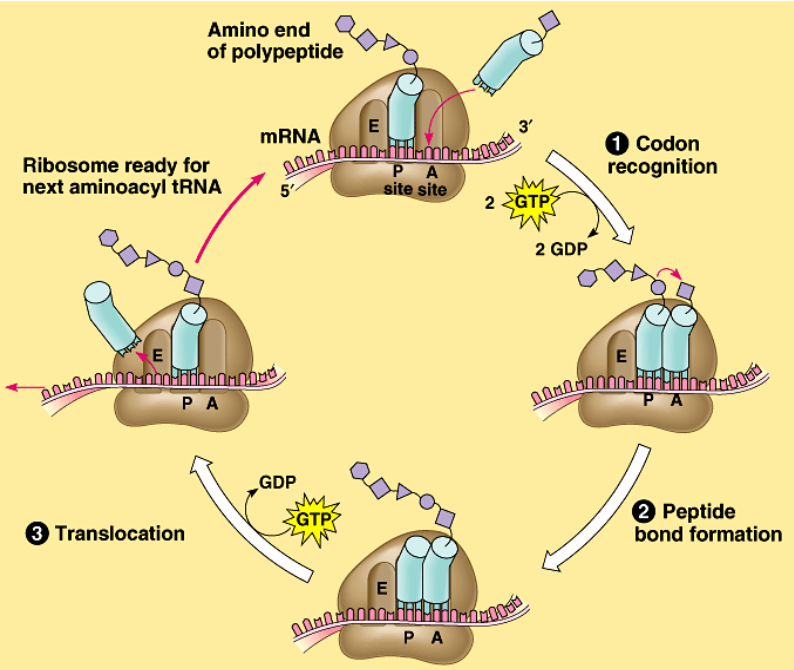


Figure 5. Protein synthesis where tRNA attaches to the A-site at the small ribosome unit and incorporates amino acids into the growing polypeptide chain. (kentsimmons.uwinnipeg.ca/cm1504/Image283.gif)

Transfer RNAs (tRNAs) are small stable RNAs to which specific amino acids are attached. The tRNA with the amino acid attached enters the ribosome and basepairs through its anticodon sequence with a 3-nucleotide codon sequence in the mRNA to insert the correct amino acid into the growing polypeptide chain(43) (Figure 5). The 30 S ribosome has three functionally important tRNA binding sites; A-site (for acceptor), P-site (for peptidyl) and E-site (for exit). The A-site has a great ability to discriminate correct and incorrect binding of tRNA, leading to a high fidelity of translation(47). The aminoglycosides bind to specific sites of the 30S ribosomal subunit and interfere with protein synthesis. Most aminoglycosides of the 2-deoxystreptamine aminocyclitol class (e.g. gentamicin) bind specifically to the A-site

(tRNA binding site) on the 16S rRNA. Streptomycin, belonging to the streptamine aminocyclitol class, also binds to the A-site, but in addition binds to rRNA and other proteins(9).

1.3 Antibiotic resistance

The major advances in antimicrobial drug development beginning through the middle of the 20th century, made a great difference in the battle between humans and the multitude of microorganisms that causes infection and disease. This made the humans believe they could win the battle. But almost as soon as the antimicrobials were taken into use, the bacteria responded by showing various forms of resistance. As antimicrobial usage has increased over the years, the bacteria have responded by developing different forms of resistance.

Microbes that are resistant to one or several of the antimicrobial agents available are emerging and disseminating worldwide. The World Health Organisation (WHO) launched in 2001, *WHO Global Strategy for Containment of Antimicrobial Resistance*, the first global strategy for combating the serious problems caused by the emergence and spread of antimicrobial resistance. The strategy recognizes that antimicrobial resistance is a global problem that must be addressed in all countries.
(http://www.who.int/mediacentre/factsheets/fs194/en/ Access date: 230910).

Bacteria may be intrinsically resistant to one or more classes of antimicrobial agents. In these cases, all strains of a bacterial species are resistant to those antimicrobial agents(45). Resistance can also be acquired by *de novo* mutations or by the acquisition of resistance genes from other organisms. Acquired resistance is not present in the entire species population, but may proliferate and spread under selective pressure.

There are several different mechanisms responsible for development of antibiotic resistance(45); (I) The bacteria may acquire genes encoding enzymes that inactivate the agent before it can have an effect, (II) the bacteria may acquire efflux pumps that pump the agent out of the cell before it reaches its target site, (III) the bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down-regulation of porin genes, and finally (IV) the bacteria may acquire several genes for a metabolic pathway which

produces altered cellular targets in the bacteria which no longer contain the binding site of the antimicrobial agent.

Normally susceptible populations of bacteria may become resistant to antimicrobial agents through mutations and selection, or by acquiring resistance-encoding genes from other bacteria(45). Bacteria that carry resistance-conferring mutations are selected by antimicrobial use, which allows the resistant strains to survive and proliferate, while the susceptible strains are killed. This is termed vertical gene transfer. Bacteria may also develop resistance through acquisition of new genetic material from other resistant organisms, and this is termed horizontal gene transfer.

1.3.1 Horizontal gene transfer

Horizontal gene transfer is an event in which an organism incorporates genetic material from another organism, without reproduction(43). It may occur between strains of the same species or between different bacteria or genera. DNA can be transferred among bacteria in three ways: transformation, conjugation and transduction (Figure 6). DNA is derived from a donor bacterium and taken up by a recipient bacterium, and the off-springs are designated transformants, transconjugants/exconjugants or transductants according to the mechanisms involved.

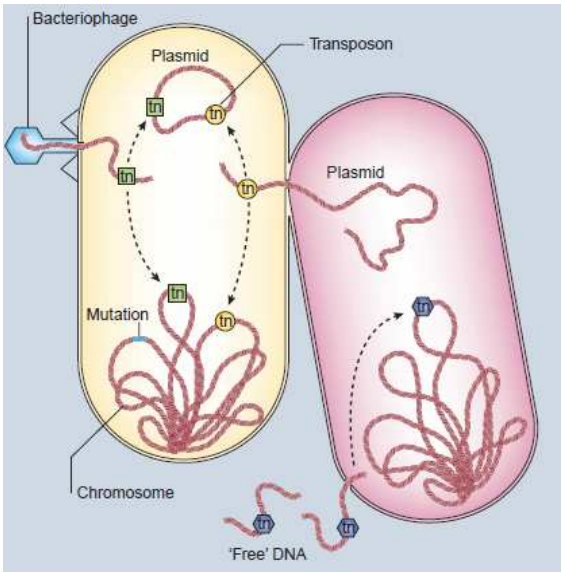


Figure 6. DNA may be transferred between bacteria through transformation, conjugation and transduction(27).

1.3.1.1 Transformation

Uptake of naked DNA from the environment is called transformation, and this mechanism of bacterial gene transfer was the first to be discovered(43).

The general steps of natural transformation differ depending on whether the bacterium is Gram-positive or Gram-negative, since the Gram-positive bacteria lack the outer membrane. In the Gram-negative bacteria: I) double stranded DNA (dsDNA) is bound to the outer surface of the bacterium, II) DNA is moved across the cell wall and outer membrane, III) one of the DNA strands is degraded by nucleases and IV) the single stranded DNA (ssDNA) is transferred into the cytoplasm across the inner membrane(43). The steps of transformation in Gram-positive bacteria are quite similar, except that transport through the outer membrane is not necessary. When inside the cytoplasm, the ssDNA might (I) synthesize the complementary strand and establish itself as a plasmid, (II) stably integrate into the recipients chromosome by homologous recombination or (III) be degraded. Bacteria made artificially competent can take up double stranded DNA (dsDNA). The role of natural transformation is thought to be DNA repair, nutrition and recombination to increase diversity.

1.3.1.2 Conjugation

The ability to transfer DNA through cell-to-cell contact is called conjugation, and was first observed by Joshua Lederberg and Edward Tatum in 1947(43). This process is associated with transfer of plasmids and chromosomal genetic elements.

The transfer systems of plasmids and chromosomal genetic elements are associated with *tra*-genes(43). These systems are similar between plasmids and chromosomal genetic elements. In plasmids *tra*-genes can also act on another plasmid in the same cell, they are trans-acting. In addition, the plasmid must have an *oriT* site for transfer. The *tra*-genes encode Mpf (Mating Pair Formation) components and Dtr (DNA Transfer and Replication) components. The function of the Mpf is to hold the donor and the recipient cell together during mating process, and to form a channel through which proteins and DNA are transferred during mating. It also includes proteins which communicate with the Dtr-system. The Dtr components prepare the plasmid for transfer. In brief, the conjugation process involves: I) The donor cell produces a pilus which makes contact with the recipient cell, II) the self-transmissible plasmid encodes a relaxase, which makes a single-stranded nick at the *oriT* site of the plasmid, III) a plasmid encoded helicase separates the strands of the plasmid DNA, IV)

relaxase attached to the 5' end of the ssDNA transports the strand into the recipient, V) inside the recipient, the relaxase recycles the ssDNA, and a complementary strand is made, and VI) a complementary strand of the remaining ssDNA in the donor cell is made.

1.3.1.3 Transduction

Transduction is the third known process of horizontal gene transfer, where DNA is transferred from one cell to another by a virus that infects bacteria, a so-called bacteriophage(43). Transduction is known to occur in a variety of bacteria, such as *Salmonella* spp., *Staphylococcus* spp., *Escherichia* spp., *Pseudomonas* spp., and *Desulfovibrio* spp.

In generalized transduction, almost any gene on the chromosome from the donor cell can be transferred to the recipient(43). When a bacterial cell is infected by a phage, the lytic cycle may be initiated. The lytic cycle is a series of steps where the virus replicates inside the host cell and cause lysis of the host. During the replication process, parts of host DNA might accidentally be packed into the virus genome. During cell lysis, these particles, called transducing particles, will be released. Transducing particles containing DNA from their previous host can then infect another cell. The accidentally packed DNA from the host bacteria can then undergo genetic recombination with the DNA of the new host.

Specialised transduction only occurs in some temperate viruses, such as phage lambda of *E. coli*(43). Here the phage DNA becomes integrated into the host DNA, and the phage enters the lysogenetic phase, where the viral DNA replication is under the control of the bacterial host chromosome. Upon induction, the viral DNA is excised from the host DNA and starts replicating. In some cases, the viral DNA is not excised correctly, and some of the adjacent host DNA is incorporated in the virus DNA.

1.3.1.4 Plasmids

Plasmids are DNA molecules that exist free of the chromosome in the bacterial cell and replicate independently of the chromosome(43). Most plasmids are circular, but some are linear. The number of plasmids in a cell may vary from one copy to hundreds of copies in a single cell and the cell can also harbour different plasmids. The size of plasmids can vary from a few hundred basepairs to almost the length of the chromosome. Plasmids do generally

not harbour genes essential to bacterial growth, but genes which are beneficial for the bacterium harbouring them, such as genes coding for antibiotic resistance.

Some plasmids are self-transmissible and are able to transfer themselves to other bacterial cells in the process called conjugation(43). Plasmids that encode functions needed for transfer of themselves are called self-transmissible or conjugative plasmids. Other plasmids do not encode all the genes required for transfer and consequently need the help of transferable plasmids to move between different bacteria. Self-transmissible plasmids probably exist in all types of bacteria, but the most studied bacteria are the Gram-negative *Escherichia coli* and the Gram-positive *Enterococcus* spp*., Streptococcus* spp*., Bacillus* spp*.*, *Staphylococcus* spp*.*, and *Streptomyces* spp(43).

Plasmid-associated resistance genes have been detected for almost all clinically available antimicrobials, and a single plasmid may mediate resistance to multiple antimicrobials and may be shared among different bacterial genera(43). In addition to carrying resistance genes, plasmids can serve as vehicles for other genetic elements important in antimicrobial resistance, such as transposons and integrons.

1.3.1.5 Transposons

Transposons are DNA sequences that are able to move from one place in DNA to a different place with the help of the enzyme transposase(43). This movement is called transposition. The smallest transposons in bacteria are IS elements, which contain only the genes required for their own transposition. Transposons are known to carry antibiotic resistance genes.

1.3.1.6 Integrons

Integrons are genetic systems responsible for the gathering of resistance determinants in mobile genetic elements such as plasmids and transposons(12). These mobile elements contain a gene for integrase and an *att* site for integration of gene cassettes, often coding for antibiotic resistance. A promoter is also present to allow transcription of cassette genes inserted into the *att* site(43).

1.4 Aminoglycoside resistance

Aminoglycoside resistance occurs by three different mechanisms; (I) modification of the rRNA and ribosomal protein targets, (II) reduced uptake and increased efflux, and (III) aminoglycoside-modifying enzymes(22). The latter mechanism has by far been the most prevalent resistance mechanism in clinical isolates(9). Figure 7 illustrates the different resistance mechanisms.

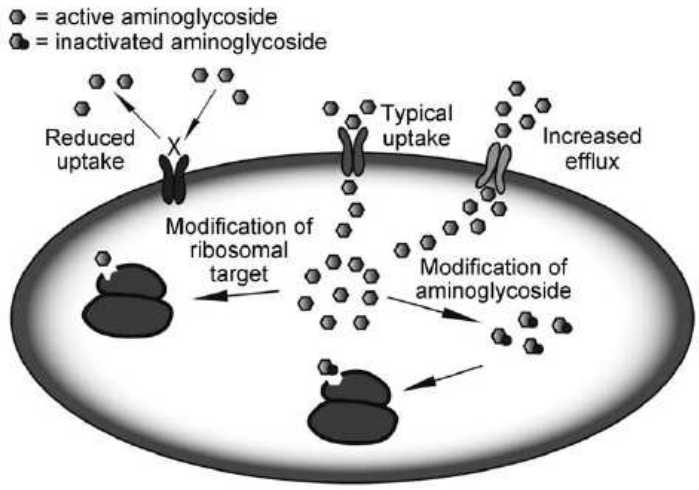


Figure 7. The various mechanisms of bacterial resistance to aminoglycosides(22).

1.4.1 Resistance by target modification

Resistance to aminoglycosides by target modification can occur in two ways: either by alteration of the target or by enzyme-catalysed target modification(9).

Aminoglycosides bind to the tRNA A-site of the ribosome where the codon-anticodon interactions occur. In most cases this will disrupt the ribosomal discrimination of cognate codon-anticodon pairs, leading to impairment of the genetic code and production of missense proteins. Most of the aminoglycosides of the 2-deoxystreptamine class (e.g. gentamicin) bind to the 16S rRNA of the 30S ribosomal subunit in the codon-decoding A-site. As a result, point mutations in the 16S rRNA can lead to impairment of codon-anticodon pairing and consequently lead to aminoglycoside resistance(9).

Streptomycin which belong to the streptamine class of aminoglycosides, also binds in the A-site at the 16S rRNA, but in addition streptomycin binds to other rRNA and ribosomal proteins. As a consequence, mutations in 16S rRNA and ribosomal proteins may result in high level resistance to streptomycin(9).

Many aminoglycoside-producing organisms, including *Streptomyces* spp. and *Micromonospora* spp. are capable of expressing 16S rRNA methylases, which are able to methylate nucleotides within the A-site of the 16S rRNA, conferring high level and broad-spectrum aminoglycoside resistance(16) (Figure 8). This is an efficient way of avoiding inhibition of their own protein synthesis, and several intrinsic 16S rRNA methylases have been described among actinomycetes. In addition, 16S rRNA methylases associated with mobile genetic elements such as plasmids and transposons have been identified in clinically relevant strains of Gram–negative bacteria, such as *Klebsiella pneumoniae*, *E. coli*, and *Pseudomonas aeruginosa*.

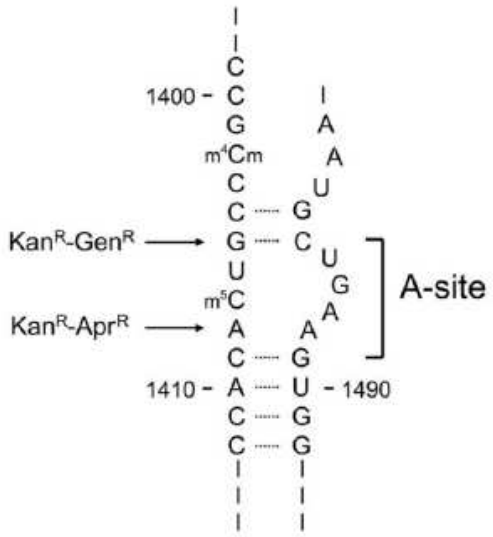


Figure 8. 16S rRNA methylases methylate nucleotides within the A-site of the 16S rRNA conferring aminoglycoside resistance by target modification(16).

1.4.2 Resistance by reduced uptake and increased efflux

Aminoglycoside resistance can also be due to mechanisms that limit the uptake of aminoglycosides to the cytoplasm. To gain access to the target ribosome the aminoglycosides have to traverse the plasma membrane and in the case of Gram-negative bacteria also the outer membrane(9). This uptake of aminoglycosides requires respiration, generated by an electrical potential across the cytoplasmic membrane. Thus, mutations in electron chain components reducing the electric potential can lead to reduced uptake of aminoglycosides and resistance.

In species such as *Pseudomonas, Burkholderia*, and *Stenotrophomonas* transmembrane efflux systems have been identified as a significant mechanism for aminoglycoside resistance(9). Different efflux systems have been described with the resistance-nodulation-division family (RND) as the dominant class responsible for pumping out aminoglycoside before they reach the ribosomes.

1.4.3 Aminoglycoside-modifying enzymes (AMEs)

The major mechanism of aminoglycoside resistance in clinical isolates of both Gram-negative and Gram-positive bacteria is enzymatic modification of amino- or hydroxyl-groups of the aminoglycosides(47). Enzymatic modification of aminoglycosides results in reduced or abolished binding of the aminoglycoside molecule to the ribosome and failure in trigging energy-dependent phase II.

Aminoglycoside-modifying enzymes (AMEs) can be divided into three families; aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-phosphotransferases (APHs), and aminoglycoside O-nucleotidyltransferases (ANTs). Many of the AMEs results in clinical relevant resistance, but in general only the APHs produce high level of resistance(47).

The different aminoglycoside modifying enzymes investigated in this study together with their antibiotic resistance profile are listed in Table 3.

Table 3. Aminoglycoside modifying enzymes investigated in this study, together with their antibiotic resistance profile.

AME	Subclass	Antibiotic resistance profile
AAC	AAC(6')-I	Amikacin, gentamicin C_{1a} and C_2
	AAC(3)-Ia	Gentamicin
	AAC(3)-II	Gentamicin, netilmicin, tobramycin
ANT	ANT(2'')-Ia	Gentamicin, tobramycin, kanamycin
	ANT(4')-IIb	Amikacin, tobramycin, isepamycin

An impressive number of AMEs has been identified to date. Unfortunately, there are inconsistencies in the nomenclature of genes and protein names of these enzymes(35). This study will follow the nomenclature proposed by Shaw and colleagues(41), where each enzyme family is given a three-letter identifier. Each of the families is further divided into classes, designated by the site of modification, indicated in parenthesis. The classes are further subdivided into enzyme types using Roman numerals, which specify unique resistance phenotypes. Enzymes of the same class and type that produce the same phenotype but are encoded by different genes, are designated by a lowercase letter. As example, AAC(6')-Ia represents an N-acetyltransferase that catalyses acetylation at the 6' position, and produces resistance to amikacin and gentamicins C_{1a} and C_2 in the same way as AAC(6')-Ib and AAC(6')-Ic.

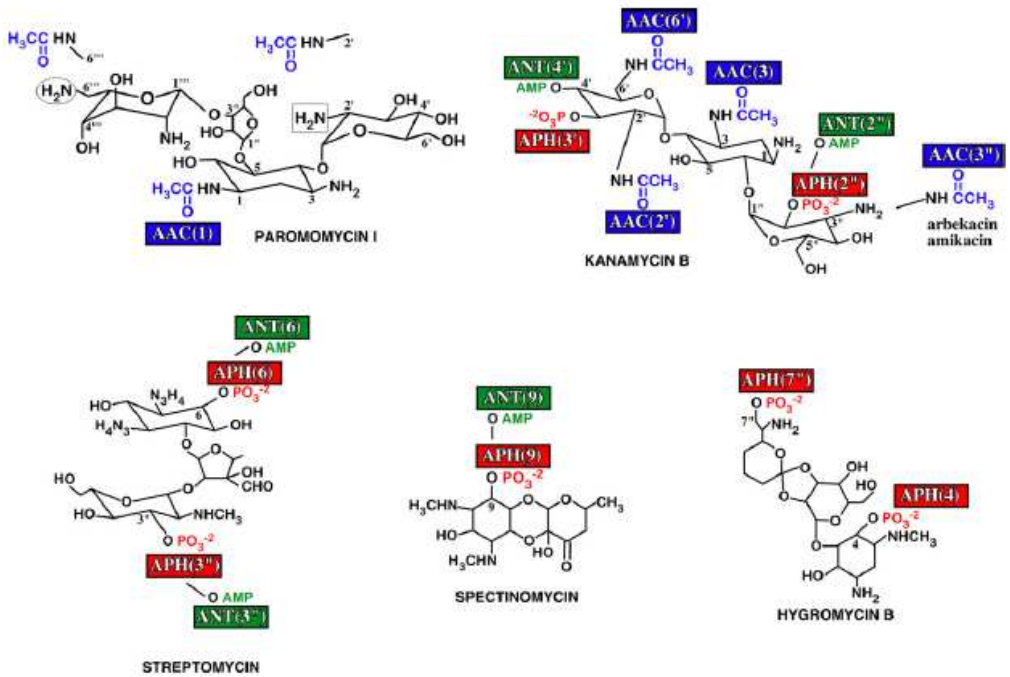


Figure 9. Representative aminoglycosides and modification sites by AAC, ANT, and APH(35).

1.4.3.1 Aminoglycoside N-acetyltransferases (AACs)

Aminoglycoside N-acetyltransferases (AACs) belong to a superfamily of proteins, which includes about 10,000 proteins. AACs catalyse the acetylation of $–NH_2$ groups in the aminoglycoside molecule, using acetyl coenzyme A as a donor substrate. There are four classes of AACs, and the acetylation occur in the 1 [AAC(1)], 3 [AAC(3)], 2' [AAC(2')], or 6' [AAC(6')] position of the aminoglycoside(35) (Figure 9).

AAC(6') enzymes are the most common enzymes among the AACs, and are present in both Gram-negative and Gram-positive bacteria. The genes coding for AAC(6') have been found located on plasmids and chromosomes and are often part of mobile genetic elements. There are two main subclasses; AAC(6')-I and AAC(6')-II. The first subclass, AAC(6')-I shows activity against gentamicin C_{1a} and C_2 and amikacin, and is probably the most clinically relevant acetyltransferase. AAC(6')-II shows high activity against all three forms of gentamicin (C_1, C_{1a} and C_2), but not amikacin(35). One enzyme variant of AAC(6')-I designated AAC(6')-Ib-cr also shows activity against fluoroquinolones, and can be considered a third subclass(35). AAC(6')-Ib-cr is probably the first enzyme discovered able to confer resistance to two different classes of antimicrobial agents of which one is purely synthetic(36).

The AAC(3) class of AACs consists of nine subclasses (I-X), and all of them are identified in Gram-negative bacteria. Subclass V was eliminated after confirmation that this enzyme was identical to AAC(3)-II. AAC(3)-I includes five enzymes (AAC(3)-Ia to AAC(3)-Ie) and confers resistance to gentamicin and other aminoglycosides such as sisomycin and fortimicin. These enzymes are present in a large number of Enterobacteriaceae and other Gram-negative clinical isolates and are all found encoded as part of gene cassettes in integrons. AAC(3)-II includes three enzymes AAC(3)-IIa, -IIb, and -IIc which confer resistance to gentamicin, netilmicin, tobramycin, sisomycin, and dibekacin, and their genes are located on plasmids in integrons as gene cassettes often associated with transposons. AAC(3)-II enzymes has been identified in different Gram-negative genera(35).

Other important AACs include AAC(1) enzymes which have been found in *E. coli*, *Campylobacter* spp. and an actinomycete, and AAC(2') enzymes that have been found in Gram-negative bacteria and *Mycobacterium*(35). The genetic location of AAC(2')

determinants is the chromosome, while the genetic location of AAC(1) encoding genes has not been determined. It has been suggested by Sunada *et al*(44) that the gene is located in the chromosome, but these results have not been confirmed.

1.4.3.2 Aminoglycoside O-nucleotidyltransferases (ANTs)

Aminoglycosides are inactivated by aminoglycoside O-nucleotidyltransferases (ANTs) through catalysation of the transfer of an adenosine monophospate (AMP) group from the donor substrate adenosine triphosphate (ATP) to a hydroxyl group on the aminoglycoside molecule. There are five classes of ANTs that catalyze adenlylation at the 6 [ANT(6)], 9 [ANT(9)], 4' [ANT(4')], 2'' [ANT(2'')], and 3'' [ANT(3'')] positions(35) (Figure 9).

ANT(3'') enzymes are the most commonly found enzyme of the ANT family, and at least 22 genes belonging to this class have been identified among both Gram-positive and Gram-negative bacteria. These genes confer resistance to spectinomycin and streptomycin, exist as gene cassettes and are part of a large number of integrons, plasmids and transposons(35).

ANT(2'') is present in enterobacteria and non-fermentative Gram-negative bacteria and mediates resistance to gentamicin, tobramycin, kanamycin, dibekacin, and sisomicin. The only enzyme in this class is ANT(2'')-Ia of which the gene is widely distributed as a gene cassette product in class 1 and 2 integrons commonly located on plasmids and transposon structures(35).

ANT(4') includes two subclasses, I and II. The ANT(4')-I gene has been found in plasmids in Gram-positive bacteria, while ANT(4')-II enzymes have been identified in Gram-negative bacteria. These enzymes confer resistance to tobramycin, amikacin and isepamicin, and subclass I also confers resistance to dibekacin. Two ANT(4')-II enzymes have been described; ANT(4')-IIa was identified encoded on plasmids from *Pseudomonas* and Enterobacteriaceae and ANT(4')-IIb was encoded in a *Pseudomonas aeruginosa* transposon(35).

ANT(6) enzymes are widely spread among Gram-positive bacteria and confer resistance to streptomycin. Genes coding for ANT(6) enzymes are found in plasmids,

transposons and integrons. In the ANT(9) class, two enzymes have been described in Gram-positive bacteria, conferring resistance to spectinomycin. The genes coding for these enzymes were located on a transposon(35).

1.4.3.3 Aminoglycoside O-phosphotransferases (APHs)

Aminoglycoside O-phosphotransferases (APHs) catalyse the transfer of a phosphate group to the aminoglycoside molecule. Seven classes of enzymes have been identified in clinical isolates and aminoglycoside-producing organisms. The phosphate group are introduced at the 4 [APH(4)], 6 [APH(6)], 9 [APH(9)], 3' [APH(3'), 3'' [APH(3'')], 2'' [APH(2'')], and 7'' [APH(7'')] position of the aminoglycoside molecule(35) (Figure 9).

The largest group of APH enzymes is the APH (3') class which can be divided in seven sub-classes I-VII. They have been identified in several different Gram-negative, Gram-positive and aminoglycoside-producing organisms, and their encoding genes are located on plasmids and chromosomes. The resistance profile differs in the different sub-groups(35).

Both APH(6) and APH(3'') confer resistance to streptomycin, and the genes coding for the enzymes are both located on chromosomes, transposons and plasmids. APH(4) and APH(7'') both confer resistance to hygromycin which is not clinically relevant. The enzymes in class APH(9) are encoded by genes located on the chromosome, and confer resistance to spectinomycin. In the APH(2'') class, the enzymes confer resistance to gentamicin in Gram-positive bacteria. This class can be divided into four subclasses I-IV, and the genes encoding these enzymes are located on plasmids and chromosomes(35).

1.5 ESBL (extended-spectrum β-lactamases)

As the genes encoding AMEs are often located on plasmids they can be associated with other types of resistance genes conferring resistance to other antimicrobial agents. This is particularly observed with genes encoding $ESBL_A$-enzymes resulting in isolates resistant to both aminoglycosides and β-lactams.

β-lactams represent almost 50% of the total antibiotic consumption in Norway (NORM). This class of antibiotics includes penicillins, cephalosporins, monobactams and carbapenems. There are three different mechanisms that confer resistance to β-lactams; (I) target modification, (II) efflux and impermeability and (III) β-lactamases. β-lactamases are enzymes which hydrolyse the β-lactam ring and inactivate the β-lactam and is by far the most common resistance mechanism. The genes encoding β-lactamases can be located both on the chromosome and on plasmids. There are different schemes for classification of β-lactamases, and Giske *et al* introduced in 2008 a redefined classification system; where the β-lactamases were grouped into $ESBL_A$, $ESBL_{M-C}$, $ESBL_{M-D,}$ $ESBL_{CARBA-A}$ and $ESBL_{CARBA-B(19)}$.

$ESBL_A$ was previously designated as ESBL, and are generally plasmid-mediated. $ESBL_A$ enzymes confer reduced susceptibility to penicillins, 1^{st}-4^{th} generation cephalosporins and monobactams, but not to carbapenems. These enzymes are inhibited by β-lactamase inhibitors such as clavulanic acid. The β-lactamase genes encoding $ESBL_A$ include CTX-M, TEM, SHV, VEB and PER, with CTX-M being the most prevalent(34).

1.6 Bacterial species investigated

As shown in Figure 10 *Escherichia coli* (30.2%) and other Enterobacteriaceae (15.7%) account for the vast majority of aerobic Gram-negative species in Norwegian blood cultures. *Klebsiella* spp. (8.6%) is the dominating species among the other Enterobacteriaceae(6).

E. coli are facultative anaerobe Gram-negative rods, which are motile and may appear with or without capsule(32). The normal habitat for *E. coli* is the gut of man and animals, but it may also colonize the lower end of urethra and vagina. Infection and spread of *E. coli* is by contact and ingestion (fecal-oral route), and may also be food-associated. Diseases caused by *E. coli* include urinary tract infections, diarrhoeal diseases, neonatal meningitis and septicaemia.

Klebsiella spp. are also Gram-negative rods. They are often capsulated and are capable of aerobic and anaerobic respiration(32). The normal habitat for *Klebsiella* spp. is the gut of man and animals and moist inanimate environments, especially soil and water. Infection may be endogenous or acquired by contact spread. Unlike *E. coli*, *Klebsiella* spp. are rarely

associated with infection except as opportunistic pathogens in compromised patients. *Klebsiella pneumoniae* is the dominating species among *Klebsiella* spp.

Pseudomonas aeruginosa are aerobic Gram-negative rods, which are motile and do not ferment carbohydrates. This pathogen is widespread in moist areas in the environment, and patients usually become infected by contact spread, directly or indirectly from these environmental sites(32). It is an important opportunistic pathogen in immunocompromised patients and can infect almost any body site given the right predisposing conditions. It may cause infections of skins and burns, is a major pathogen in cystic fibrosis, and can cause pneumonia in intubated patients. *P. aeruginosa* may also cause urinary tract infections, septicaemia, osteomyelitis and endocarditis.

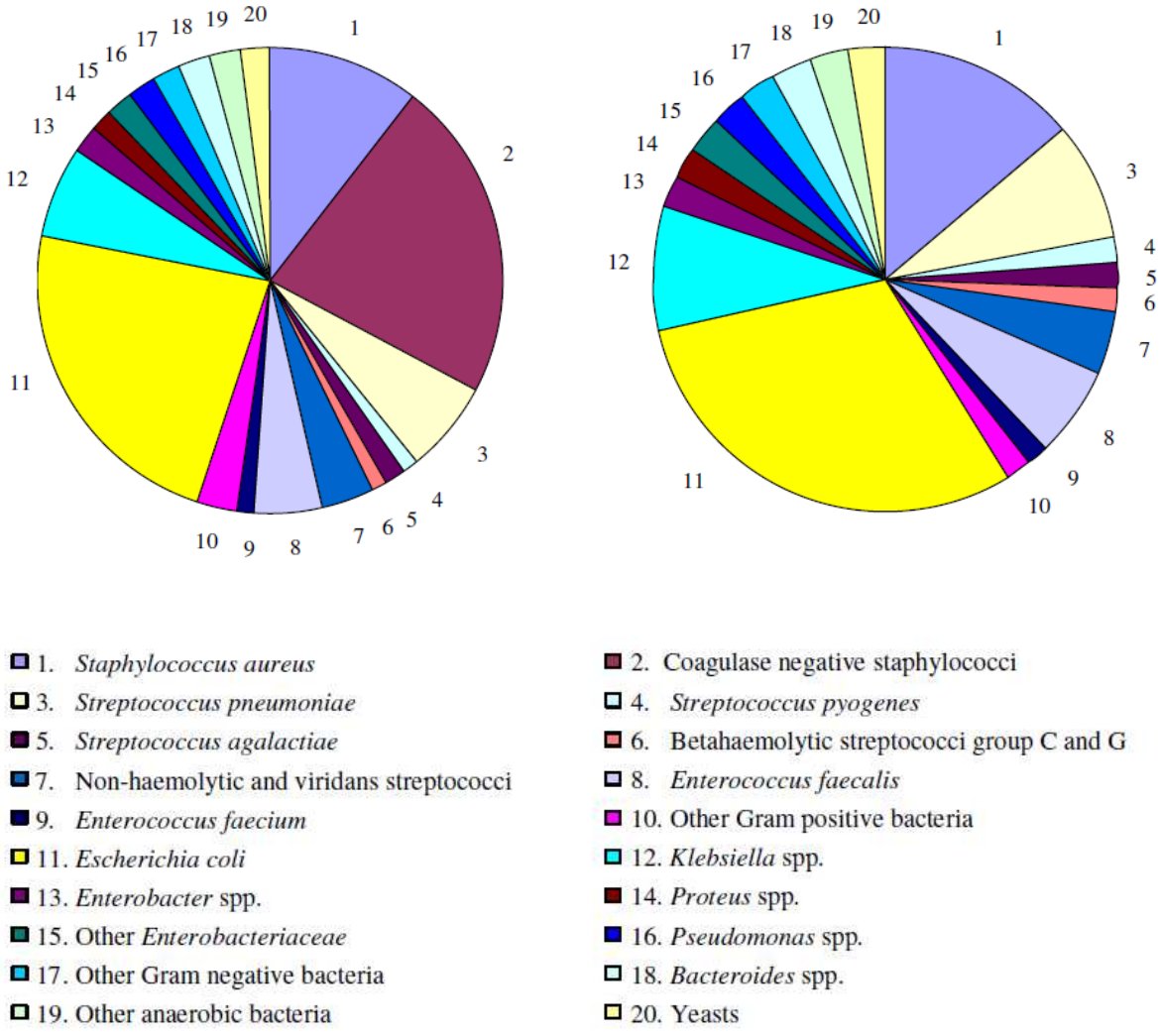


Figure 10. Distribution of all blood culture isolates (left) and blood culture isolates excluding common skin contaminants (right) from all Norwegian medical microbiology laboratories except one in 2009(6).

2. Aims of the study

According to the Norwegian surveillance programme for antimicrobial resistance in human pathogens (NORM 2009)(6), aminoglycosides represent only 0.4% of the total antimicrobial consumption in Norway. Nevertheless, aminoglycosides are important antimicrobials and are often the first choice for empirical treatment of septicaemia with unknown cause.

The prevalence of intermediate resistance and resistance to gentamicin in *E. coli* blood culture isolates in Norway is low, where 3.6% of blood culture isolates were reported as resistant in 2009. The numbers have nevertheless shown an increase during the years from 2000-2009(6) (Figure 11). In *Klebsiella* spp., 1.2% of blood culture isolates were resistant in 2009. The percentage of resistance was lower in urinary tract isolates than in blood culture isolates. In *E. coli* 2.1% of urinary tract isolates were resistant, while 0.6% of *Klebsiella* spp. isolates were resistant to gentamicin. In 2009, tobramycin was added to the surveillance scheme to further investigate aminoglycoside resistance. The level of resistance to tobramycin in *E. coli* blood culture isolates and urinary tract isolates were 2.4% and 1.5%, while the level of tobramycin resistant *Klebsiella* spp. were 1.4% and 0.3%, respectively (Table 4).

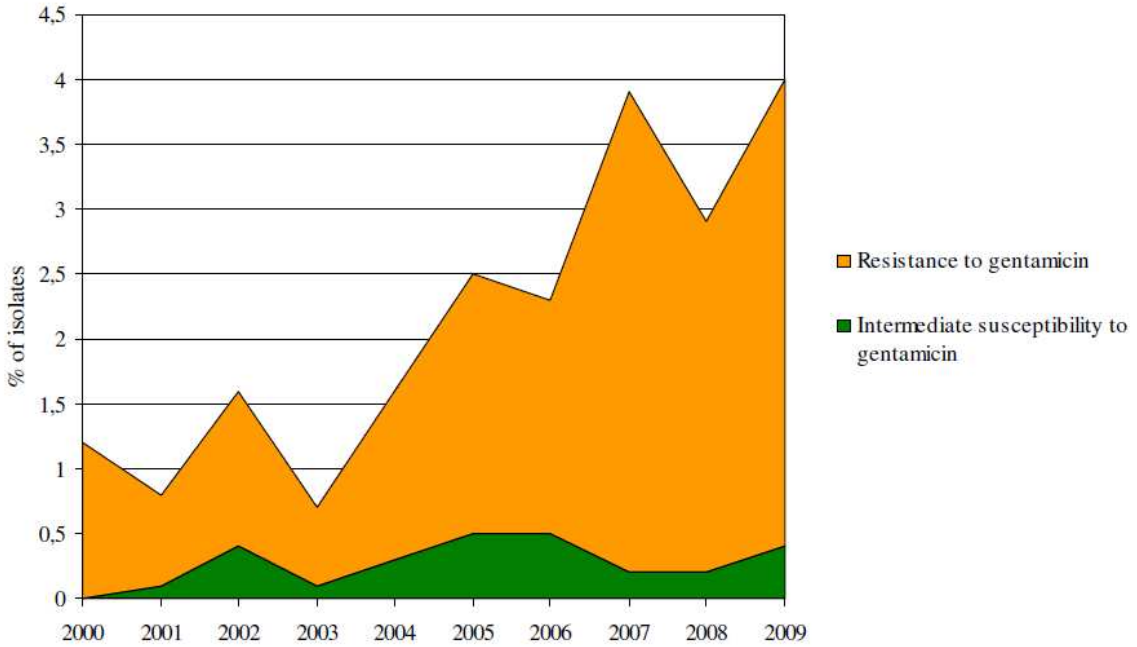


Figure 11. Prevalence of intermediate susceptibility and resistance to gentamicin in *E. coli* blood culture isolates 2000-2009(6).

Table 4. Proportion of *E. coli* and *Klebsiella* spp. isolates from blood culture and urine resistant to gentamicin and tobramycin in NORM 2009(6).

Species	Material	Aminoglycoside	% Resistant
E. coli	Blood (n=1379)	Gentamicin	3.6
		Tobramycin	2.4
	Urine (n=1126)	Gentamicin	2.1
		Tobramycin	1.5
Klebsiella spp.[1]	Blood (n=568)	Gentamicin	1.2
		Tobramycin	1.4
	Urine (n=1004)	Gentamicin	0.6
		Tobramycin	0.3

[1] *Klebsiella* spp. includes *Klebsiella pneumoniae* and *Klebsiella oxytoca*

The European Antimicrobial Resistance Surveillance System (EARS-Net) annual report for 2009(4) shows that the highest percentages of aminoglycoside resistance in invasive *E. coli* isolates are reported from the Southern and Eastern part of Europe, where most countries reported ~10% aminoglycoside resistance (Figure 12). During the last four years aminoglycoside resistance in *E. coli* has shown a significant increase in ten countries. This is consistent with the increase of all other resistance proportions for *E. coli.*

Countries in the Eastern part of Europe have reported between 25-50% aminoglycoside resistance in invasive *Klebsiella pneumoniae*, and three countries in this area reported aminoglycoside resistance above 50% (Figure 12). In the period of 2006-2009 a significant increase in aminoglycoside resistance in several countries has been observed(4).

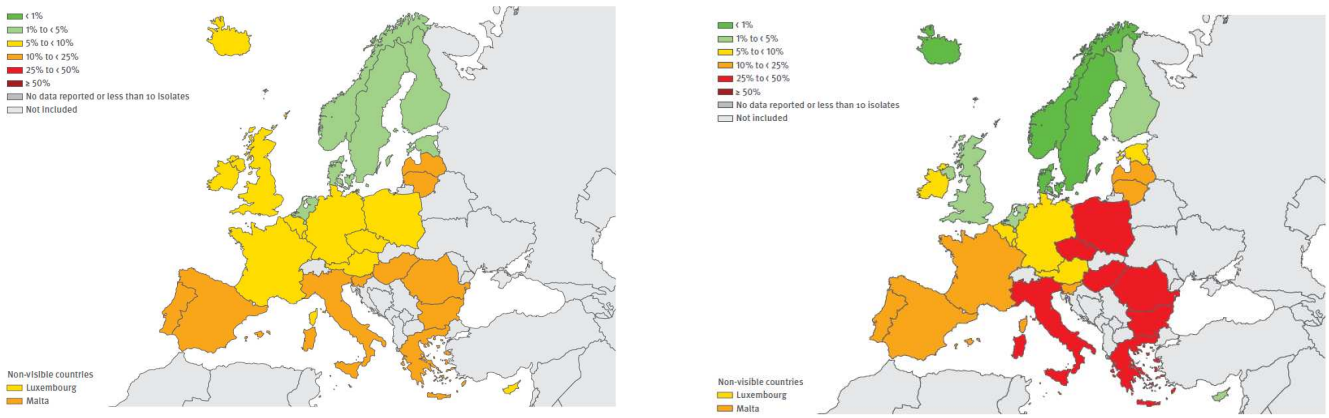


Figure 12. Proportion of invasive isolates of *E. coli* (left) and *Klebsiella pneumoniae* (right) in Europe with resistance to aminoglycosides in 2009 (EARS-NET)(4).

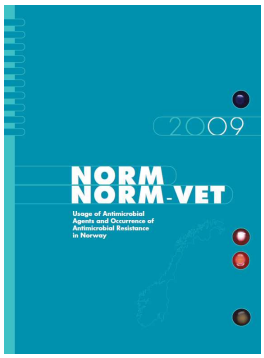
The aims of this study include:

Analysis of the susceptibility pattern of different aminoglycosides in clinically important Gram-negative isolates from Norway.

Evaluation of the prevalence of genes encoding clinically important aminoglycoside modifying enzymes (AMEs).

Evaluation of the prevalence of 16S rRNA methylase genes conferring high level broad-spectrum aminoglycoside resistance.

Compare different methods for phenotypic detection of aminoglycoside resistance.



3. Materials and methods

3.1 Strain collections

Two collections of *E. coli* and *Klebsiella* spp. were extracted from the Norwegian surveillance programme for antimicrobial resistance (NORM) year 2007-2009(2,5,6) as described below. Additionally a collection of *P. aeruginosa* was selected from isolates received at the Reference centre for detection of antimicrobial resistance (K-res).

3.1.1 Collection 1: *E. coli* and *Klebsiella* spp. from NORM 2009

A total of 2510 isolates of *E. coli* (blood *n* = 1381 and urine *n* = 1129) and 1578 isolates of *Klebsiella* spp. (blood *n* = 571 and urine *n* = 1007) were included in NORM 2009 (Table 5).

Table 5. The total number of *E. coli* and *Klebsiella* spp. included in NORM 2009 (collection 1), and distribution of isolates with reduced susceptibility to gentamicin and/or tobramycin.

Species and material	Total number of isolates	Number of isolates with reduced susceptibility to GEN[1] and/or TOB[1]
E. coli blood	1381	61
E. coli urine	1129	44
E. coli total	2510	105
Klebsiella spp. blood	571	17
Klebsiella spp. urine	1007	15
Klebsiella spp. total	1578	32

[1] GEN=gentamicin, TOB=tobramycin

Initial antimicrobial susceptibility testing was performed in each Norwegian laboratory contributing to NORM, using agar disk diffusion systems from Oxoid (Oxoid Ltd, Basingstoke, UK) or BD (Becton Dickinson, Cockeysville, MD, USA) in agreement with breakpoints from the Norwegian Working Group on Antibiotics (NWGA). Inhibition-zones detected by the disk-diffusion method from a broad panel of antibiotics, including the aminoglycosides gentamicin and tobramycin, were available in the NORM database (Appendix A; Table 1-2).

Using the breakpoints from the BD and Oxoid distributers (Table 6), a total of 137 isolates with reduced susceptibility to gentamicin and/or tobramycin (Table 5) were selected for further studies.

Table 6. Breakpoints for gentamicin and tobramycin from the BD and Oxoid distributers.

Distributor	Susceptible (S)	Intermediate (I)	Resistant (R)
BD	≥ 19 mm	17-18 mm	< 16 mm
Oxoid	≥ 21 mm	19-20 mm	< 18 mm

3.1.2 Collection 2: $ESBL_A$-positive *E. coli* and *Klebsiella* spp. from NORM 2007-08

A total of 68 PCR-confirmed $ESBL_A$-positive isolates, representing 60 *E. coli* (blood *n* = 32 and urine *n* = 28) and 8 *Klebsiella* spp. (all blood) isolates were identified in NORM 2007(2) and 2008(5). The different $ESBL_A$-types detected are listed in Table 7.

Table 7. Distribution of the $ESBL_A$-types included in collection 2.

	$ESBL_A$- type		
Species	**CTX-M gr.1**	**CTX-M gr. 9**	**SHV**
E. coli (*n* = 60)	30	26	4
Klebsiella spp (*n* = 8)	3		5

3.1.3 Collection 3: Carbapenem non-susceptible *P. aeruginosa* from K-res 2007-09

Between 2007 and 2009 K-res received a total of 121 *P. aeruginosa* isolates with reduced susceptibility to carbapenems (imipenem and/or meropenem). Forty-four of these isolates were isolated from cystic fibrosis patients, and not included in this study. The remaining collection of 77 isolates included eight metallo-β-lactamase (MBL)-positive isolates.

3.2 Phenotypic methods

3.2.1 Preparation of stock cultures

Stock cultures were prepared of all the clinical isolates examined in this study using glycerol as the osmotic protector.

Procedure:

1. Upon arrival at K-res, the isolates in collection 1 were cultured on green agar plates, while the isolates in collection 2 and 3 were cultured on green agar plates containing 100 mg/ml ampicillin (Appendix A; Table 8).
2. After overnight incubation at 35ºC, 8 to 10 colonies from the culture were transferred to 1 ml freeze broth (Appendix A; Table 8)
3. The broth were homogenized on a vortex mixer and stored at -70ºC.
4. Green agar plates were inoculated and incubated overnight at 35ºC as a contamination control.

3.2.2 VITEK2 identification and susceptibility testing

VITEK2 (bioMérieux, Marcy l'Etoile, France) is a fully automated systems for bacterial identification and susceptibility testing using fluorescence-based technology. Species identification of all three strain collections in this study was performed using the IDGN card (bioMérieux) of VITEK2 (bioMérieux) according to the manufacturers instructions. The VITEK2 Gram-negative susceptibility card AST029 (bioMérieux) evaluate a broad panel of antibiotic classes including: Gentamicin, tobramicin, ampicillin, cefotaxime, cefoxitin, cefpirome, cefpodoxime, ceftazidime, cefuroxime, ciprofloxacin, mecillinam, meropenem, nitrofurantoin, trimethoprim, trimethoprim/sulphamethoxazole, amoxicillin/clavulanic acid, piperacillin/tazobactam, aztreonam, cefalotin, cefuroxime axetil, and nalidixic acid. VITEK2 AST was performed on all three strain collections to evaluate level of resistance to other classes of antibiotics.

Procedure:

1. Colonies from an overnight culture grown on green agar plates (collection 1) or green agar plates containing 100 mg/ml ampicillin (collection 2 and 3) were suspended in 3 ml 0.45% NaCl (Appendix A; Table 8), and the turbidity was measured by a densitometer and adjusted to 0.52-0.63 McFarland.
2. The tube with the bacterial suspension was placed in a rack together with identification card IDGN and the anti susceptibility card AST029, respectively and scanned for registration.
3. The rack was placed in the VITEK2 where each test card was automatically filled with the bacterial suspension and automatic identification and susceptibility testing was performed by kinetic fluorescence measurement every 15 min.
4. The software then analysed the data and reported the results.

3.2.3. Oxydase test

The oxydase test is used to determine if a bacterium produces cytochrom c oxydase which can utilize oxygen for energy production with an electron transfer chain. The reagent will act as electron donor and when the reagent is oxidased, a dark blue colour will appear. Pseudomonadaceae produce cytochrom c oxydases and are oxydase positive bacteria. Enterobacteriaceae do not produce cytochrom c oxydases, and are oxydase negative (Ref. Mc Fadden). In this study the oxydase test was performed on the *P. aeruginosa* isolates in collection 3.

Procedure:

1. One drop of oxydase reagent (Appendix A; Table 8) was transferred to filter paper.
2. One colony from a fresh culture of the isolate was added to the oxydase reagent on the filter paper.
3. The test was read immediately.

3.2.4 EUCAST Disk Diffusion

During 2009-2010 The European Committee on Antimicrobial Susceptibility Testing (EUCAST) developed a disk diffusion test for routine antimicrobial susceptibility testing. The method is derived from the Kirby-Bauer method(11). Disk diffusion is a semi quantitative method used to examine microbes' susceptibility to specific antibiotics. It allows categorization of bacterial isolates as susceptible, intermediate or resistant to a variety of antimicrobial agents (www.eucast.org Access date: 120311). A micro-organism is defined as susceptible (S) by a level of antimicrobial activity associated with a high likelihood of therapeutic success. It is defined as intermediate (I) by a level of antimicrobial agent activity associated with uncertain therapeutic effect. And finally, a micro-organism is defined as resistant (R) by a level of antimicrobial activity associated with a high likelihood of therapeutic failure. Clinical breakpoints for relevant antimicrobials are established for everyday use in the clinical laboratory to advise on patient therapy.

Commercially prepared filter paper disks impregnated with a standard concentration of the antimicrobial agents are applied to the surface of an agar medium inoculated with the test organism. The antibiotic will diffuse into the agar, creating a high concentration close to the antibiotic disk and continuous lower concentrations with increasing distance to the disk. Following incubation, a bacterial lawn appears on the plate and zones of inhibition of bacterial growth appear around the antibiotic disk (Figure 13). The test is performed under standardized conditions and hence the size of the inhibition zone is dependent of the degree of sensitivity of the microorganism to the antibiotic(30).

In this study, susceptibility testing against the following aminoglycosides was performed on collection 1 and 2: amikacin 30 µg (Oxoid), gentamicin 10 µg (Oxoid), kanamycin 30 µg (Oxoid), netilmicin 10 µg (Oxoid), streptomycin 10 µg (Oxoid), tobramycin 10 µg (Oxoid), arbekacin 30 µg (BD), and isepamicin 30 µg (BD). Susceptibility testing against amikacin 30 µg (Oxoid), gentamicin 10 µg (Oxoid), netilmicin 10 µg (Oxoid), streptomycin 10 µg (Oxoid), and tobramycin 10 µg (Oxoid) was performed on collection 3.

Procedure:

1. A 0.5 McFarland suspension of the organism were made from an overnight culture grown on green agar plates (collection 1) or green agar plates containing 100 mg/ml ampicillin (collection 2 and 3) in 0.85% NaCl, and swabbed on a Mueller-Hinton (MH) II-plate (BD), (Appendix A; Table 8).
2. A panel of aminoglycoside disks were applied to the plate within 15-60 minutes.
3. The plates were incubated at 35ºC in ambient air for 18 ± 2 hours.
4. After incubation the inhibition-zones were measured using a slide calliper and recorded.
5. *E. coli* ATCC 25922 was used as reference strain in investigation of collection 1 and 2, while *P. aeruginosa* ATCC 25783 was used as reference strain in investigation of collection 3.

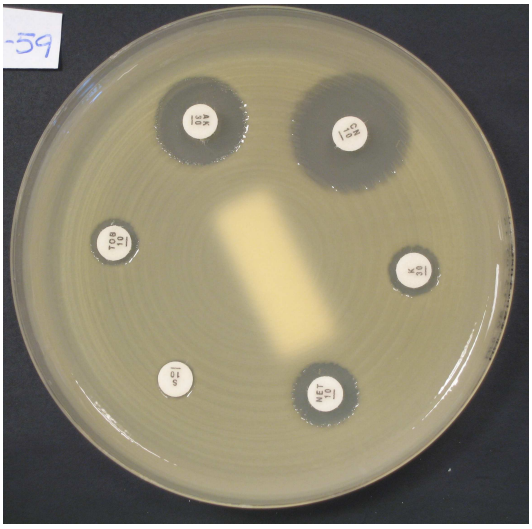


Figure 13. EUCAST disk diffusion showing a MH II-plate with six different aminoglycoside disks. Different zones of inhibition of bacterial growth are present around the antibiotic disks.

3.2.5 Etest susceptibility testing

Etest is a quantitative technique for determining the antimicrobial susceptibility of antimicrobial agents. An Etest is a thin plastic strip, containing a predefined, stable gradient of an antimicrobial agent which is calibrated to give stable gradient agar diffusion. After incubation, the position of the growth/inhibition edge for a particular bacterium/antimicrobial agent combination determine the minimal inhibitory concentration (MIC) of the agent

(http://www.abbiodisk.com/pdf/etm_html/03_etm.htm Access date: 18022011) (Figure 14). The MIC is the lowest concentration of the antimicrobial agent that results in inhibition of visible growth analogue with colonies on a plate or turbidity in broth culture(30).

MIC`s were determined for the isolates in collection 1 and 2 using the following panel of aminoglycoside Etests (bioMérieux): amikacin, gentamicin, kanamycin, netilmicin, streptomycin, and tobramycin. In collection 3 the MICs were determined on the same panel of aminoglycosides, except kanamycin.

Procedure:

1. A 0.5 McFarland suspension of the organism were made from an overnight culture grown on green agar plates (collection 1) or green agar plates containing 100 mg/ml ampicillin (collection 2 and 3) in 0.85% NaCl, and swabbed on a MH-II plate (BD).
2. The Etests were applied on the swabbed plates using the Etest applicator Simplex C76 (bioMérieux).
3. The plates were incubated at 35ºC in ambient air for 16-18 hours.
4. After incubation, the MIC values were determined and recorded.
5. *E. coli* ATCC 25922 was used as reference strain in investigation of collection 1 and 2, while *P. aeruginosa* ATCC 25783 was used as reference strain in investigation of collection 3.

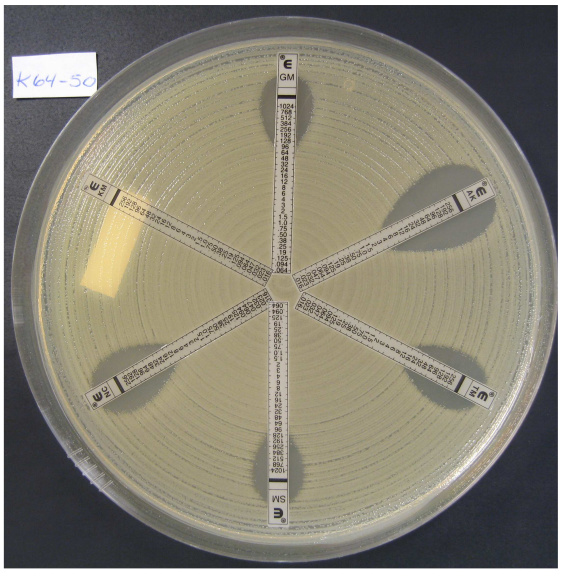


Figure 14. Etest susceptibility test showing a MH-II plate with six different aminoglycoside Etests. Minimum inhibitory concentrations (MIC) are measured where the inhibition edge intersect with the Etest.

3.3 Molecular methods

3.3.1 Isolation of DNA from bacteria

DNA from all the isolates of the three collections was isolated using the QIAgen GenoM-48 biorobot (QIAgen, Hilden, Germany) according to the manufacturer's instructions. In brief, DNA from lysed bacteria binds to the surface of magnetic particles. Several washing steps are performed, to improve the purity of the DNA. DNA is finally eluted in water(8).

Procedure:

1. 200 µl of a 0.5 McFarland suspension of the organism in 0.85% NaCl was prepared from an overnight culture and transferred to sample tubes.
2. The sample tubes were placed in the robot which performed all steps of the isolation, including sample lysis, binding to the GenoPrep beads, washing steps and elution with reagents from MagAttract DNA Mini M48 Kit (QIAgen).
3. Following the isolation, the DNA was stored at 2 - 8ºC.

3.3.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a rapid method for amplification of DNA *in vitro*. Target DNA is heated to separate the strands (denaturation), and DNA oligonucleotide complementary to the target DNA sequence, is added together with a heat-stable enzyme DNA polymerase. As the reaction cool down, the primers will bind to the target strands (annealing). DNA polymerase then extends the primers using the target strands as templates (elongation). After an incubation period, the mixture is again heated to separate the newly made strands, and the process is repeated. After 20-30 cycles, a 10^6-10^9–fold increase in the target sequence is obtained (Figure 15). Agarose gels are used for detection of the amplified PCR-products at the end-point of the PCR-reaction (see below).

In this study, hot-start PCRs were performed by using JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma Aldrich, St. Louis, USA) according to the manufacturer's instructions. The mix includes deoxynucleotides, polymerase, buffer, and loading dye.

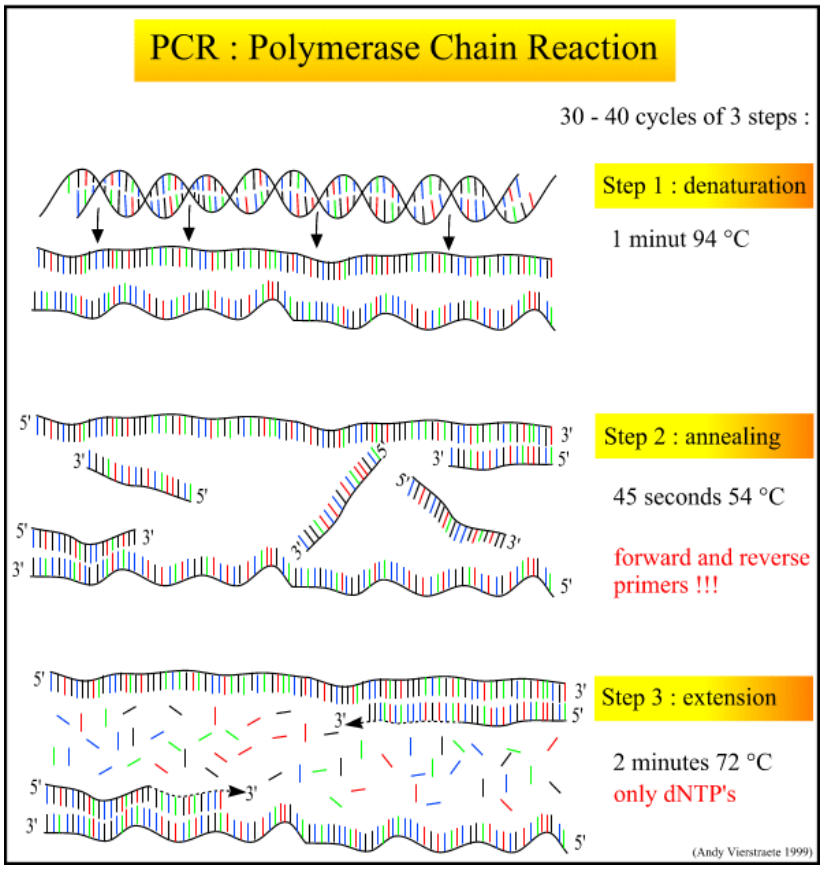


Figure 15. The different steps of PCR, including denaturation of the dsDNA, annealing of primers, and extension of new DNA strands. (http://users.ugent.be/~avierstr/principles/pcr.html. Access date: 230111)

General procedure for all PCRs:

1. For each reaction: 3 µl DNA was added to 22 µl PCR mastermix (Appendix A; Table 3,4-6).
2. Controls included, in addition to ddH_2O as a mastermix control, are listed in Table 8.
3. The PCR was run on a PCR thermocycler (Applied Biosystems, Foster City, Ca) according to program given in Appendix A; Table 5.

Table 8. Positive and negative PCR-controls used in this study.

PCR	Positive control	Negative control	Reference
16S rDNA	*Enterococcus faecalis* ATCC 29212 *E. coli* ATCC 25922	ddH_2O	ATCC[2]
aac(6')-Ib	*E. coli* A3-21	*E. coli* ATCC 25922	L. Poirel. Personal communication
aac(3)-II	*E. coli* K53-1	*E. coli* ATCC 25922	This study
aac(3)-Ia	P1658/97 pSEM		(56)
ant(2'')-Ia	*P. aeruginosa* K44-24	*E. coli* ATCC 25922	(39)
ant(4')-IIb	*P. aeruginosa* K44-36	*E. coli* ATCC 25922	This study
*rmt*A	Plasmid	ATCC 25922 *E. coli*	Arakawa Y. Personal communication
*rmt*B	A4-26	ATCC 25922 *E. coli*	(10)
*rmt*C	NA[1]	ATCC 25922 *E. coli*	
*rmt*D	NA[1]	ATCC 25922 *E. coli*	
*rmt*E	NA[1]	ATCC 25922 *E. coli*	
*arm*A	A4-25	ATCC 25922 *E. coli*	(10)
*npm*A	A4-47	ATCC 25922 *E. coli*	Arakawa Y. Personal communication

[1] NA; not available, [2] ATCC: American Type Culture Collection

3.3.3 Agarose gel electrophoresis

Agarose gel electrophoresis is a simple, rapid and highly sensitive method widely used to separate DNA and RNA molecules by size(28). The rates of which DNA/RNA- molecules migrate are inversely proportional to their molecular weight. Thus agarose gel electrophoresis may also be used to estimate the size of DNA or RNA molecules by comparing with the migration of molecules of known size (molecular ladders). Nucleic acid molecules are negatively charged, and will migrate in electric fields towards the positive electrode. The agarose molecules will delay the migration of larger molecules and smaller molecules will therefore move faster. As a result, molecules of different sizes will be separated in the gel.

Procedure:

1. 1% agarose (Seakem LE agarose, Lonza, Rochland, ME, USA) was dissolved in 0.75X Tris/Borate/EDTA-buffer (TBE) (Appendix A; Table 8) by boiling the solution in a microwave oven.
2. 0.5 µg/ml ethidium bromide (EtBr) (Sigma-Aldrich) was added for staining of the DNA molecules
3. The agarose-EtBr solution was poured into a geltray fitting the electrophoresis apparatus (BioRad laboratories, Hemel Hemstead, UK), containing the combs, and allowed to set for about 30 minutes
4. 3 µl of each PCR product was loaded into the gel wells.

5. 4 µl of 1 Kb+ DNA molecular size marker (Invitrogen, Carlsbad, CA, USA) (Appendix A; Figure 1) was loaded into the flanking wells.
6. The electrophoresis was run at 100 V for approximately 1 hour.
7. The DNA bands were visualized using the GelDoc system (BioRad).

3.3.4 PCR-based detection of 16S rDNA

The quality of the DNA extraction from the isolates in the three strain collections were all tested by 16S rDNA PCR. Strong 16S rDNA-PCR products indicate high quality of the DNA templates, while unsuccessful amplification of 16S rDNA reflects poor isolation of DNA, DNA degradation, or the presence of PCR inhibitors. Modified universal primers giving PCR-products of about 1500 bp, described by Weisburg *et al*., 1991, were used. (Appendix A, Table 6)

3.3.5 PCR-based detection of aminoglycoside modifying enzymes (AMEs)

Strain collection 1 and 2 were screened for the following AMEs: *aac*(6')-Ib, *aac*(3)-II, *aac*(3)-Ia, *ant*(2'')-Ia, and *ant*(4')-IIb using primers and conditions described in Appendix A, Table 3,5-6.

3.3.6 PCR-based detection of 16S rRNA methylases

Eight isolates from collection 1 and one isolate from collection 2, all resistant or intermediate resistant to amikacin, gentamicin, tobramycin, kanamycin, and netilmicin were screened for the following 16S rRNA methylases: *rmt*A, *rmt*B, *rmt*C, *rmt*D, *rmt*E, *arm*A, and *npm*A. Primers, conditions and mastermix are described in Appendix A, Table 3-6. Sixteen isolates from collection 3 with resistance to amikacin, gentamicin, tobramycin, and netilmicin were also screened for the same 16S rRNA methylases.

3.3.7 DNA sequencing reaction

The Sanger method is the most widely used method for determination of the precise nucleotide sequence of a DNA fragment(40). Briefly, the method is based on DNA synthesis in the presence of fluorescent labelled dideoxynucleotides (ddNTP), which differ from normal deoxynucleotides (dNTP) in that they lack a 3'-hydroxyl group (OH). A mix of dNTP and a small portion of the ddNTP are used. DNA synthesis proceeds normally until DNA polymerase inserts a ddNTP instead of a normal dNTP. When a ddNTP is added to the growing DNA strand, it terminates the chain elongation because there is no 3'-OH for the next nucleotide to be attached. In this manner various truncated chain lengths will be produced. Automated fluorescence detection machines will detect and record the fluorescence and the nucleotide order can be determined.

Before starting the sequencing reaction, it is important to remove access dNTP and DNA polymerase from the PCR-product. Two hydrolytic enzymes; Shrimp Alkaline Phosphatase (SAP) and Exonuclease I (Exo) will remove dNTPs and DNA polymerase, respectively and purify the PCR-product.

Sequencing in this study was performed using the BigDye v. 3.1 sequencing chemistry (Applied Biosystems). To confirm the PCR-results, 14 of the positive *aac*(6')-Ib isolates and eight of the positive *aac*(3)-II isolates were sequenced. In addition the positive PCR-products from the *ant*(2'')-Ia-, *rmt*B-, and *rmt*D-PCR were sequenced. The primers used in the sequencing reaction, was the same as for the PCRs.

Procedure:

A. Purification of the PCR-product.

1. 1 µl of Exo-SAP (USB, Cleveland, Ohio) was added to each PCR-product and the tubes were put into the PCR thermocycler (Applied Biosystems).
2. Cycling-programme

37°C	1 min
37°C	90 min
85°C	15 min
4°C	∞

3. The purified PCR-products were stored at -20°C until sequencing.

B. Sequencing PCR and analyses:

1. For each reaction, 1 µl template was added to 19 µl sequencing mastermix (Appendix A; Table 7).
2. The PCR thermocycler (Applied Biosystems) was programmed and according to program given in Appendix A; Table 5.
3. Nucleotide sequencing was performed at the Sequencing core facility at the University of Tromsø using ABI Prism 3130XL Genetic Analyser (Applied Biosystems)
4. Editing and alignment of the DNA sequences were performed using the SeqMan II software package (DNAStar Inc., Madison, WI, USA).
5. Searcing for nucleotide sequence homology was performed using BLAST available at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4 Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a method for resolving chromosomal DNA and is a well established molecular strain typing method. PFGE may be used for genotyping or genetic fingerprinting and has been considered a gold standard in epidemiological studies of pathogenic organisms. This method allows separation of whole genomes after enzymatic digestion by electrophoresis. DNA fragments from 100 bp to 10 Mbp can be separated.

Bacterial cells are moulded into agarose plugs, to protect the chromosome from fragmentation. The cells are then lysed by enzymes such as lysozyme. After washing-procedures, the plugs are treated with proteinase K, to inactivate nucleases that otherwise will degrade the DNA. The DNA in the agarose plugs are then digested with restriction enzymes that have relatively few recognition sites, resulting in a relatively small number of restriction fragments. The fragments are then separated in an agarose gel using electrical fields of changing orientation for defined periods.

Eighteen representative *aac*(3)-II-positive *E. coli* isolates with elevated gentamicin resistance from collection 1 from all over Norway were investigated by PFGE to investigate if they were part of a successful Norwegian clone. The isolates included in the PFGE are listed in Table 9.

Table 9. *aac*(3)-II positive *E. coli* isolates with elevated gentamicin resistance investigated by PFGE. Green = susceptible, yellow = intermediate resistant, and red = resistant.

				Etest MIC µg/ml				
Ref. no	**Species**	**Material**	**Location**	**AMK[1]**	**GEN[1]**	**TOB[1]**	**KAN[1]**	**NET[1]**
K64-13	*E. coli*	Blood	Aker	3	64	12	8	6
K64-17	*E. coli*	Blood	Bærum	1,5	128	16	12	16
K64-18	*E. coli*	Blood	Bærum	2	96	12	8	8
K64-22	*E. coli*	Blood	Drammen	3	96	8(8)	8	8
K64-43	*E. coli*	Blood	Haukeland	4	96	8	16	8
K64-44	*E. coli*	Blood	Haukeland	3	128	12	24	12
K64-46	*E. coli*	Blood	Haukeland	3	96	12	24	12
K64-49	*E. coli*	Urine	Haukeland	3	96	12	12	12
K64-55	*E. coli*	Blood	Kristiansand	3	96	8	8	12
K64-70	*E. coli*	Blood	Molde	3	128	12	12	12
K65-03	*E. coli*	Blood	Stavanger	3	96	12	12	8
K65-06	*E. coli*	Urine	Stavanger	3	128	16	12	8
K65-08	*E. coli*	Urine	Stavanger	2	96	12	12	12
K65-21	*E. coli*	Blood	Tromsø	3	96	16	8	12
K65-25	*E. coli*	Urine	Tromsø	3	96	12	12	8
K65-34	*E. coli*	Urine	Trondheim	3	96	16	12	16
K65-44	*E. coli*	Blood	Ullevål	3	128	12	12	12
K65-52	*E. coli*	Urine	Ullevål	3	96	16	12	16

[1] AMK=amikacin, GEN=gentamicin, TOB=tobramycin, KAN=kanamycin, and NET=netilmicin

In addition, PFGE was performed on 18 *E. coli* isolates from collection 1 were the Etest and the disk diffusion results for tobramycin did not correspond. Due to a change in EUCAST breakpoints after the analysis was performed, some of Etest/disk diffusion results were now corresponding. The isolates included in the PFGE are listed in Table 10.

Table 10. *E. coli* isolates with not corresponding tobramycin resistance investigated by PFGE, with their tobramycin MIC profiles, mm-zones, *aac*(6')-Ib, and *aac*(3)-II PCR results. Green = susceptible, yellow = intermediate resistant and red = resistant

				TOB[1]		AME	
Ref. no	**Species**	**Material**	**Location**	**Etest**	**Disk**	***aac*(6')-Ib**	***aac*(3)-II**
K64-01	*E. coli*	Blood	AHUS[2]	6	16	neg	pos
K64-03	*E. coli*	Urine	AHUS[2]	12	12	neg	pos
K64-23	*E. coli*	Blood	Drammen	8	13	neg	pos
K64-32	*E. coli*	Blood	Fredrikstad	12	14	neg	pos
K64-33	*E. coli*	Blood	Fredrikstad	8	12	neg	pos
K64-39	*E. coli*	Blood	Haukeland	6	12	neg	pos
K64-43	*E. coli*	Blood	Haukeland	8	12	neg	pos
K64-53	*E. coli*	Blood	Kristiansand	6	14	neg	pos
K64-54	*E. coli*	Blood	Kristiansand	6	15	neg	pos
K64-66	*E. coli*	Urine	Lillehammer	8	14	neg	pos
K64-67	*E. coli*	Urine	Lillehammer	4	17	neg	pos
K65-01	*E. coli*	Blood	Stavanger	6	15	neg	neg
K65-03	*E. coli*	Blood	Stavanger	12	12	neg	pos
K65-23	*E. coli*	Blood	Tromsø	8	14	neg	pos
K65-28	*E. coli*	Blood	Trondheim	8	14	pos	pos
K65-45	*E. coli*	Blood	Ullevål	8	12	neg	pos
K65-46	*E. coli*	Blood	Ullevål	8	14	neg	pos
K65-47	*E. coli*	Blood	Ullevål	6	14	neg	pos

[1] TOB=tobramycin, [2] AHUS= Akershus university hospital

Procedure:

A. Preparation of PFGE plugs

1. One bacterial colony from a fresh agar culture was transferred into 5 ml of BHI-medium (Appendix A; Table 8) and incubated overnight at 37°C, using shaking to increase the access of O_2.
2. 50 µl of the culture were transferred into to a new tube of 5 ml of BHI and incubated for 4 hours at 37°C, using shaking. When harvested after 4 hours, the bacterial cells should be in exponential growth.
3. The cells were centrifuged at 3500 rpm for 10 min.
4. The supernatant was removed and the bacterial cells were resuspended in 1 ml of cold PIV-buffer (Appendix A; Table 8).
5. 495 µl of the suspension were transferred to an eppendorf tube, and 5 µl lysozym (100mg/ml) (Sigma-Aldrich) were added. The cells were mixed well by vortexing, and equilibrated at 50°C in a water bath.
6. 500 µl 2% Megabase agarose (BioRad) dissolved in PIV-buffer and equilibrated at 50°C, were added to the bacterial suspension and mixed thoroughly. The mixture was transferred to the plug mould, and the plugs were left to solidify at 4°C for 15 min.

7. The solidified plugs were transferred to new tubes, and 2 ml of lysis buffer (Appendix A; Table 8) were added and incubated at 37°C for 2 hours, using slow shaking.
8. The lysis buffer was removed, and the plugs were washed in 1 ml ddH_2O for 15 min.
9. After removing ddH_2O, 1 ml of ESP buffer (Appendix A; Table 8) containing 50 µl/ml proteinase K-solution (20 mg/ml, VWR, West Chester, PA, USA) were added and incubated at 50°C in a water bath over night.
10. The ESP-solution was removed and the plugs were washed 3 x 30 min in 1 ml TE-buffer (Appendix A; Table 8).
11. The plugs were stored in TE-buffer at 4°C.

B. Restriction enzyme digestion of DNA in agarose plugs

1. A thin slice of the plug was cut and transferred to an eppendorf tube.
2. The plug was washed 3 x 30 min in 500 µl restriction enzyme buffer (Buffer 4, New England BioLabs, Ipswitch, Ma, USA) containing bovine serum albumin (BSA) (Appendix A; Table 8).
3. The restriction enzyme mix, 20 U *Xba*I (New England BioLabs) per 125 µl total enzyme mix, was prepared (Appendix A; Table 8).
4. The plugs was transferred to 125 µl restriction enzyme mix and incubated at 37°C over night.
5. The plug was washed 3 x 30 min in TE buffer.

C. Gel electrophoresis

1. 1% agarose (Seakem LE agarose) was dissolved in 0.5X TBE by boiling the solution in a microwave oven and equilibrated to 50°C in a waterbath.
2. The gel equipment was prepared, and the plugs were placed onto the gel comb and mounted directly into the gel by inserting the comb before the agarose was poured. A low range PFG marker (New England Biolabs) was included (Appendix A, Figure 2). The gel was left to solidify for 30 min at room temperature.
3. The electrophoresis chamber was filled with 1.8-2 l of 0.5X TBE, and the circulation was started to cool the system to 12°C.
4. The comb was released from the gel, and access gel was removed.
5. The gel was placed in the electrophoresis chamber, the program parameters were set, and the electrophoresis started.

Program parameters:

Pulstime:	1-20 sec
Total runtime:	21 hours
Voltage: 6.0V/cm=	200V
Angle:	120°
Temperature:	12°C
Gel running buffer:	0.5 x TBE

6. After the electrophoresis, the gel was stained in an EtBr-solution (Appendix A; Table 8) for 20 min and de-stained in dH_2O for 40 minutes.
7. The DNA bands were visualized using the GelDoc system (BioRad, USA).
8. Interpretation of DNA relatedness was computationally analysed in BioNumerics v.6.01 (Applied Maths, Sint-Martens-Latens, Belgium) using the band-based DICE similarity coefficient and the unweighted pairs geometric matched analysis (UPGMA) with a position tolerance of 1% and 1% for band comparison.

3.5 Plasmid transfer studies

Many plasmids containing antibiotic resistance determinants have the ability to move between bacterial cells by conjugation. In Gram-negative bacteria, conjugation involves cell to cell contact via sex-pili. In this study, 12 isolates showed an aminoglycoside resistance profile but were negative for the aminoglycoside modifying enzymes tested for, nine isolates in collection 1 (*E. coli* n = 6 and *Klebsiella* spp. n = 3) and three *E. coli* isolates from collection 2. Four of these isolates were chosen for further plasmid transfer studies to explore if the resistance marker were located on a transferable plasmid. The resistance profile of the donor isolates are shown in Table 11. A rifampicin resistant, aminoglycoside susceptible *E. coli* (J53-2) was used as the recipient.

Table 11. The donor isolates included in the plasmid transfer studies, including their MIC profile.

				Etest MIC µg/ml				
Ref. no	**Species**	**Material**	**Location**	**AMK[1]**	**GEN[1]**	**TOB[1]**	**KAN[1]**	**NET[1]**
K64-71	*E. coli*	Blood	Molde	3	32	64	6	32
K65-38	*E. coli*	Blood	Tønsberg	2	16	32	4	16
K64-31	*E. coli*	Blood	Fredrikstad	2	24	4	4	2
K65-35	*K. pneumoniae*	Urine	Trondheim	2	16	1,5	3	2

[1] AMK=amikacin, GEN=gentamicin, TOB=tobramycin, KAN=kanamycin, and NET=netilmicin

Procedure:

1. The donor and recipient strains were inoculated in LB-medium (Appendix A; Table 8) and incubated over night at 37°C with shaking.
2. The overnight cultures were diluted 1:100 in LB-medium and incubated at 37°C with shaking until the cultures were in exponential growth (OD_{600} = 0.3-0.5). OD was measured on a SprectraMax Plus (Molecular Devices, Sunnyvale, CA, USA).
3. The donor and recipient cultures were mixed in two different ratios:

   1:1 (2.5 ml donor and 2.5 ml recipient)

   1:9 (0.5 ml donor and 4.5 ml recipient)

   The mixed cultures were incubated for 2 hours at 37°C, with very slow shaking.
4. The transconjugants were diluted 10^{0}, 10^{-2} and 10^{-4} and 100 µl of the dilutions were spread on LB-plates containing rifampicin (100 µg/ml) and gentamicin (6 µg/ml) (Appendix A; Table 8). The donor and recipient cells were diluted 10^{-5} and 100 µl of the dilutions were spread on LB-plates containing gentamicin and LB-plates containing rifampicin, respectively (Appendix A; Table 8). All plates were incubated at 37°C overnight.
5. The next day, possible transconjugants, recipients and donors were counted and the conjugation frequencies were calculated.
6. From each donor, five possible transconjugants were spread on green agar plates, and incubated at 37°C overnight.
7. The next day Etest (bioMérieux) of gentamicin and/or tobramycin and netilmicin were performed.

3.6 Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) is a widely used typing method to determine the clonality of strains. This technique makes use of multiple housekeeping gene loci to characterise bacterial species. The loci sequences are given allelic numbers and the combination of seven housekeeping gene loci defines a sequence type (ST).

MLST schemes have been developed for different organisms, and in this study MLST schemes for *E. coli* and *P. aeruginosa* have been used. For *E. coli,* the housekeeping genes *adk, fum*C, *gyr*B, *icd, mdh, pur*A, and *rec*A have been sequenced. The housekeeping genes *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A, and *trp*E belong to the MLST scheme to determine the sequence types of *P. aeruginosa.* The collection of MLST data on *E. coli* is available online on http://mlst.ucc.ie/mlst/dbs/Ecoli/ and the *P. aeruginosa* collection are available on http://pubmlst.org/paeruginosa/.

PCR and sequencing of the housekeeping genes belonging to the different MLST schemes were performed according to the procedure previously described in this chapter. The different mastermix and PCR-conditions are described in Appendix A; Table 5-7. Sequence analyses were performed using SeqMan DNAStar Lasergene 8.1.3 (DNAStar Inc., Madison, WI). Sequences for each housekeeping gene were submitted to the online MLST databases for determination of allele number and sequence type.

In this study MLST was performed on the isolates harbouring 16S rRNA methylases, and partial MLST was performed on selected transconjugants to verify if the plasmid transfer had been successful.

3.7 Comparison of phenotypic methods

The comparison between the different methods was performed on Etest versus disk diffusion, Etest versus VITEK2 anti susceptibility testing (AST) and VITEK2 AST versus disk diffusion. The aminoglycosides looked into were tobramycin, gentamicin and amikacin in all three strain collections. In this comparison, the Etest results were considered as the key in comparison with disk diffusion and VITEK2 AST. When comparing VITEK2 AST and disk diffusion, VITEK2 AST was considered as the key. Disk diffusion or VITEK2 AST is the standard method for detecting reduced susceptibility in Norwegian microbiology laboratories. When the disk diffusion or the VITEK2 AST considers a isolate susceptible and the Etest shows resistance, this is considered a very major error since the result from the disk diffusion or the VITEK2 AST will be reported.

The comparisons of methods are classified as follows:

Very major error:	Disk diffusion/VITEK2 AST: S	Etest: R
Major error:	Disk diffusion/VITEK2 AST: R	Etest: S
Minor error:	Disk diffusion/VITEK2 AST: S	Etest: I
Minor error:	Disk diffusion/VITEK2 AST: I	Etest: S
Minor error:	Disk diffusion/VITEK2 AST: R	Etest: I
Minor error:	Disk diffusion/VITEK2 AST: I	Etest: R

In the cases of very major error, the isolates were retested.

4. Results

4.1 Prevalence of aminoglycoside resistance

Antimicrobial susceptibility testing on several different aminoglycosides was performed on the three different strain collections using Etest and disk diffusion test. In addition, gentamicin and tobramycin MIC values from the automated VITEK2 AST system were included. The clinical breakpoints defined by EUCAST or CLSI (Appendix B; Table 1) were used. The results of the resistance pattern used for investigation of the prevalence are based on the Etest results. The results from both the Etest and disk diffusion test are listed in Appendix B; Table 2-15). Because of no available breakpoints for streptomycin, arbekacin and isepamicin the resistance profiles for these aminoglycosides have not been analysed.

4.1.1 Collection 1: *E. coli* and *Klebsiella* spp. from NORM 2009

When testing the 137 selected isolates from collection 1, 28 (20 %) isolates did not show reduced susceptibility by Etest as reported in NORM 2009. 19 isolates (18%) were *E. coli* and nine isolates (28%) were *Klebsiella* spp. The Etest resistance profiles of blood culture and urinary tract isolates of *E. coli* and *Klebsiella* spp tested from NORM 2009, respectively are shown in Figure 16.

The results showed that the overall prevalence of reduced susceptibility to amikacin was low. In *E. coli* the level of reduced susceptibility to amikacin was 0.6% in blood culture isolates and 0.1% in urinary tract isolates. In *Klebsiella* spp. reduced susceptibility to amikacin was 0.4% in blood culture and 0.2% urinary tract isolates. The prevalence of reduced susceptibility to gentamicin were 3.8% and 2.5% in *E. coli* from blood culture and urinary tract isolates, respectively and 1.4% and 0.7% in *Klebsiella* spp. Regarding reduced susceptibility to tobramycin in *E. coli*, the level was 4.1% in blood culture isolates and 2.6% in urinary tract isolates. In *Klebsiella* spp. the figures were 2.0% and 1.0%, respectively. The level of reduced susceptibility to netilmicin also showed higher values in *E. coli* compared to *Klebsiella* spp. In *E. coli* from blood culture isolates the prevalence of reduced susceptibility netilmicin was 3.9%, while in *Klebsiella* spp. the level was 2.0%.

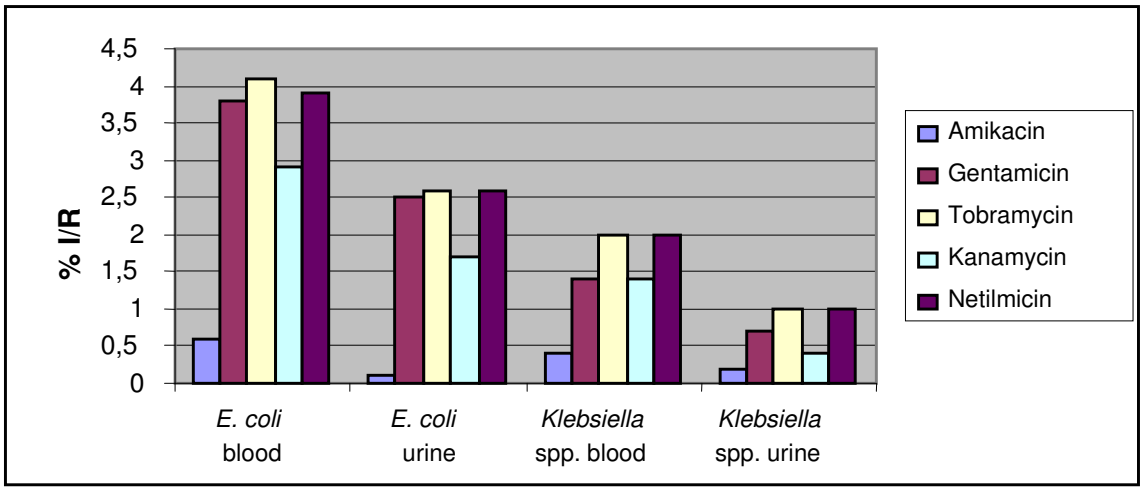


Figure 16. Level of reduced susceptibility to different aminoglycosides in collection 1, including the blood culture and urinary tract isolates of *E. coli* and *Klebsiella* spp.

4.1.1.1 Broad-spectrum aminoglycoside resistance in collection 1

With respect to broad-spectrum aminoglycoside resistance 1% of the isolates only showed reduced susceptibility to either gentamicin or kanamycin (Table 12). The prevalence of isolates with reduced susceptibility to both gentamicin and tobramycin was 1% in *E. coli.* No *Klebsiella* spp. isolates showed reduced susceptibility to both gentamicin and tobramycin (Table 12). A total of 46% of the isolates showed reduced susceptibility to gentamicin, tobramycin, netilmicin, and kanamycin. And finally, when also including amikacin altogether 5% of the isolates showed reduced susceptibility to all aminoglycosides investigated. The distribution of reduced susceptibility to several aminoglycoside in collection 1, including results for *E. coli* and *Klebsiella* spp. separately, are shown in Figure 17.

Table 12. Broad-spectrum aminoglycoside resistance in collection 1.

Reduced susceptibility to[1]	**Total (%) *n* = 109**	***E. coli* (%) *n* = 86**	***Klebsiella* spp. (%) *n* = 23**
KAN	1 (1)	0	1 (4)
GEN	1 (1)	0	1 (4)
GEN + TOB	1 (1)	1 (1)	0
GEN + TOB + KAN	2 (2)	2 (2)	0
TOB + NET + KAN	6 (5)	2 (2)	4 (18)
GEN + TOB + NET	35 (33)	25(29)	10 (44)
GEN + TOB + NET + KAN	50 (46)	47 (55)	3 (13)
AMK + TOB + NET + KAN	7 (6)	4 (5)	3 (13)
AMK + GEN + TOB + NET + KAN	6 (5)	5 (6)	1 (4)

[1]AMK=amikacin, GEN=gentamicin, TOB=tobramycin, KAN=kanamycin, and NET=netilmicin

The prevalence of reduced susceptibility to gentamicin, tobramycin and netilmicin combined were observed in 29% of the *E. coli* isolates and 44% of the *Klebsiella* spp. isolates. Finally, the prevalence of reduced susceptibility to all aminoglycosides tested, including amikacin was 6% in *E. coli* and 4% in *Klebsiella* spp.

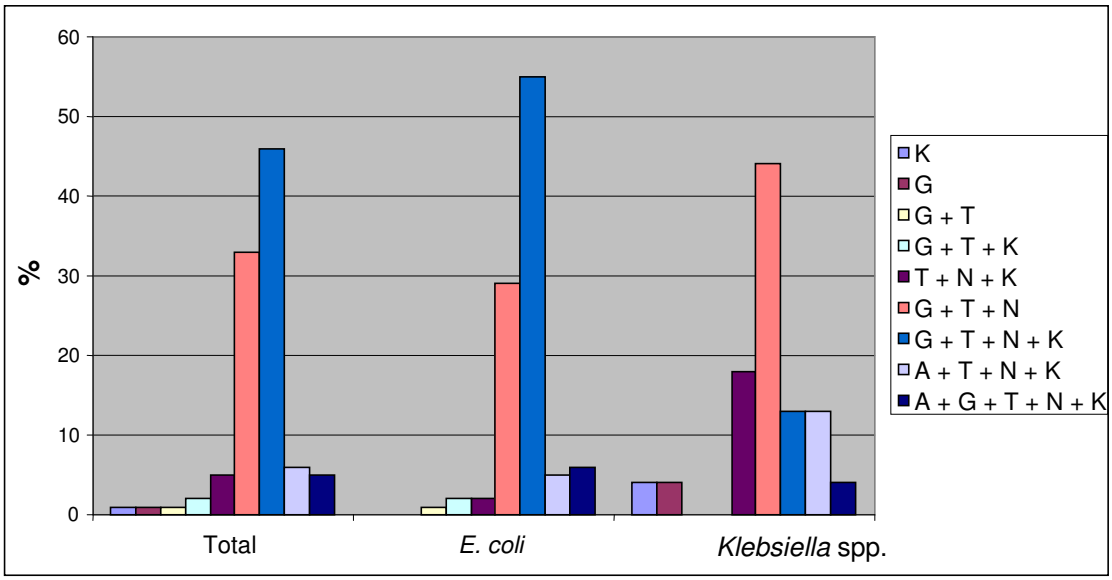


Figure 17. Distribution of broad-spectred aminoglycoside resistance in collection 1, including results for *E. coli* and *Klebsiella* spp. T = tobramycin, N = netilmicin, G = gentamicin, K = kanamycin, and A = amikacin.

4.1.1.2 Co-resistance to other classes of antibiotics

The results from the VITEK2 Gram-negative card AST029 were used to evaluate the level of resistance to other classes of antibiotics. A total of 36 (34%) of the isolates in collection 1 with reduced susceptibility to different aminoglycosides showed a phenotypic $ESBL_A$ or $ESBL_{M-C}$ profile while 58 (54%) were resistant to ciprofloxacin and 86 (80%) were resistant to trimethoprim/sulfamethoxazole. Two isolates were resistant to meropenem, and these isolates had previously been analysed at K-res. They turned out to be *Klebsiella pneumoniae* carbapenemase (KPC)-positive isolates, isolated from the same patient in two different hospitals(37). Twenty-one (20%) isolates had a phenotypic $ESBL_A$ or $ESBL_{M-C}$ profile, together with reduced susceptibility to aminoglycosides and resistance to ciprofloxacin and trimethoprim/sulfamethoxazole.

4.1.2 Collection 2: $ESBL_A$-positive *E. coli* and *Klebsiella* spp. NORM 2007-08

The Etest resistance profiles of the total number of isolates tested from collection 2 are listed in Table 13. The prevalence of reduced susceptibility to amikacin was 7% in collection 2. No resistance towards amikacin were observed in the eight *Klebsiella* spp. included in the collection, while 8% of the *E. coli* isolates showed reduced susceptibility to amikacin (Figure 18) and (Appendix B; table 10-13). The prevalence of reduced susceptibility towards tobramycin and gentamicin was 56% and 44%. The prevalence of species specific reduced susceptibility was 57% and 45% towards tobramycin and gentamicin in *E. coli* and 50% and 38% in *Klebsiella* spp., respectively. For kanamycin the prevalence of reduced susceptibility was 57% and for netilmicin 56%. The species specific results for *E. coli* showed 60% reduced susceptibility towards kanamycin and 57% reduced susceptibility towards netilmicin. The corresponding results for the *Klebsiella* spp. were 38% towards kanamycin and 50% towards netilmicin.

Table 13. Prevalence of reduced susceptibility to aminoglycosides in $ESBL_A$-positive *E. coli* and *Klebsiella* spp. in collection 2 (*n* = 68).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) *n* = (%)**	**Intermediate (I) *n* = (%)**	**Resistant (R) *n* = (%)**	**I + R *n* = (%)**
Amikacin	S ≤ 8 R > 16	63 (93)	4 (6)	1 (1)	5 (7)
Gentamicin	S ≤ 2 R > 4	38 (56)	1 (1)	29 (43)	30 (44)
Tobramycin	S ≤ 2 R > 4	30 (44)	0	38 (56)	38 (56)
Kanamycin[1]	S ≤ 6 R > 24	29 (43)	15 (22)	24 (35)	39 (57)
Netilmicin	S ≤ 2 R > 4	30 (44)	1 (1)	37 (55)	38 (56)

[1] Breakpoints from CLSI

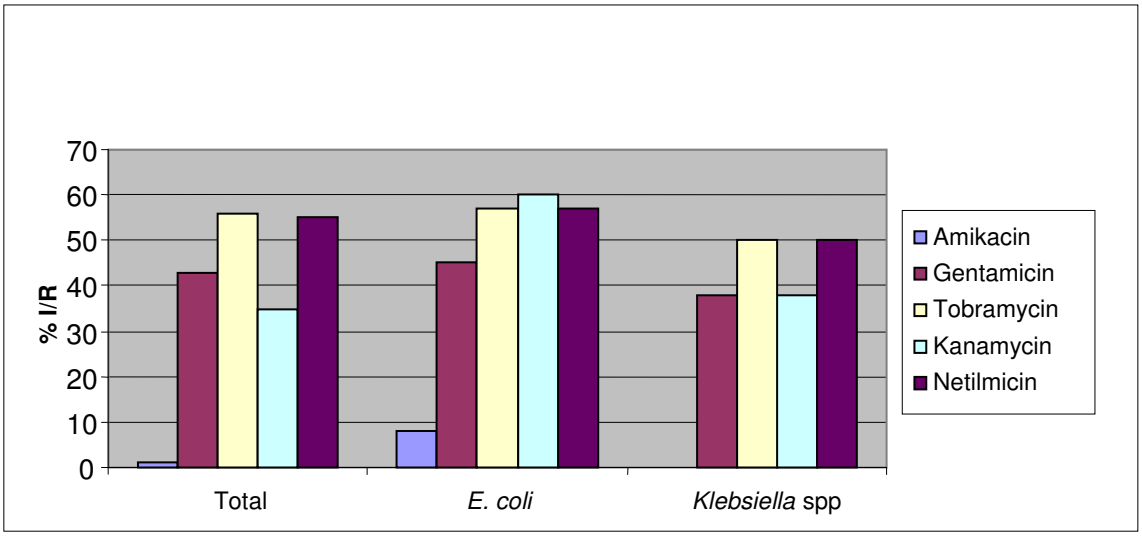


Figure 18. Total and species specific reduced susceptibility to aminoglycosides in the ESBL$_A$-positive *E. coli* and *Klebsiella* spp in collection 2.

4.1.2.1 Broad-spectrum aminoglycoside resistance in collection 2

In total the prevalence of isolates susceptible to all aminoglycosides in collection 2 were 40%, including 39% of the *E. coli* and 50% of the *Klebsiella* spp. isolates, as shown in Fig 19. Five percent of the *E. coli* isolates, but none of the *Klebsiella* spp. showed reduced susceptibility to kanamycin only. The prevalence of reduced susceptibility to tobramycin, netilmicin, kanamycin, and gentamicin were 40%. Only one *E. coli* isolates was observed with reduced susceptibility to all aminoglycosides tested.

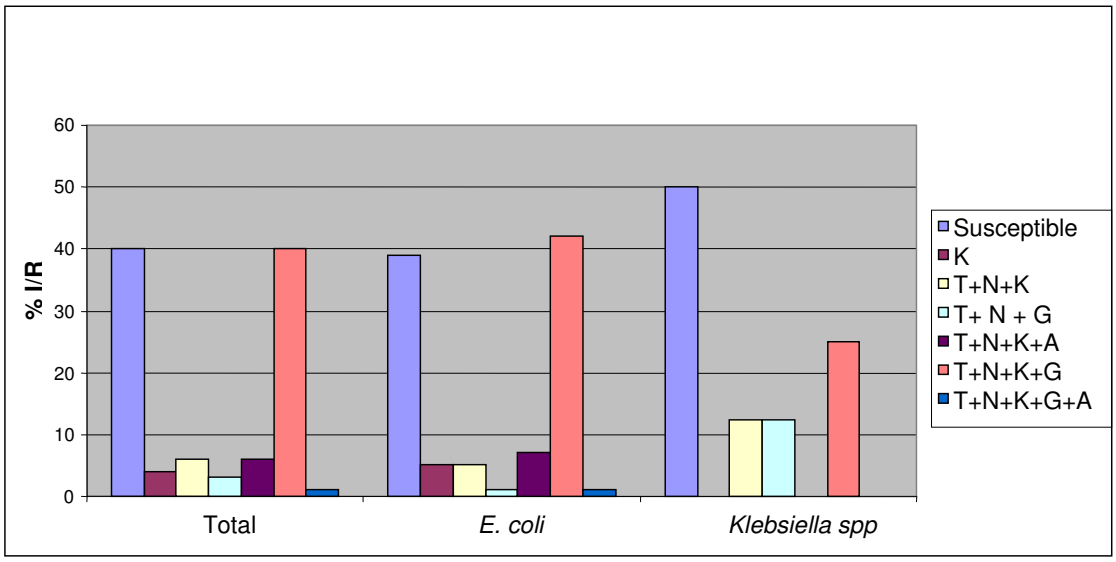


Figure 19. Distribution of broad-spectrum aminoglycoside resistance in the ESBL$_A$-positive isolates in collection 2, including results for *E. coli* and *Klebsiella* spp. T = tobramycin, N = netilmicin, G = gentamicin, K = kanamycin, and A = amikacin

4.1.3 Collection 3: Carbapenem non-susceptible *P. aeruginosa* from K-res 2007-09

The Etest resistant profiles for the *P. aeruginosa* isolates included in collection 3 are listed in Table 14. The highest prevalence of reduced susceptibility observed in collection 3 was 47% to netilmicin. The prevalence of reduced susceptibility to gentamicin and tobramycin was 38% and 18%, respectively. A total of 30% of the *P. aeruginosa* isolates showed reduced susceptibility to amikacin (Figure 20).

Table 14. Etest resistant profile for the *P. aeruginosa* isolates in collection 3 (*n* = 77).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) *n* = (%)**	**Intermediate (I) *n* = (%)**	**Resistant (R) *n* = (%)**	**I + R *n* = (%)**
Amikacin	S ≤ 8 R > 16	54 (70)	8 (10)	15 (20)	23 (30)
Gentamicin	S ≤ 4 R > 4	48 (62)	0	29 (38)	29 (38)
Tobramycin	S ≤ 4 R > 4	63 (82)	0	14 (18)	14 (18)
Netilmicin	S ≤ 4 R > 4	41 (53)	0	36 (47)	36 (47)

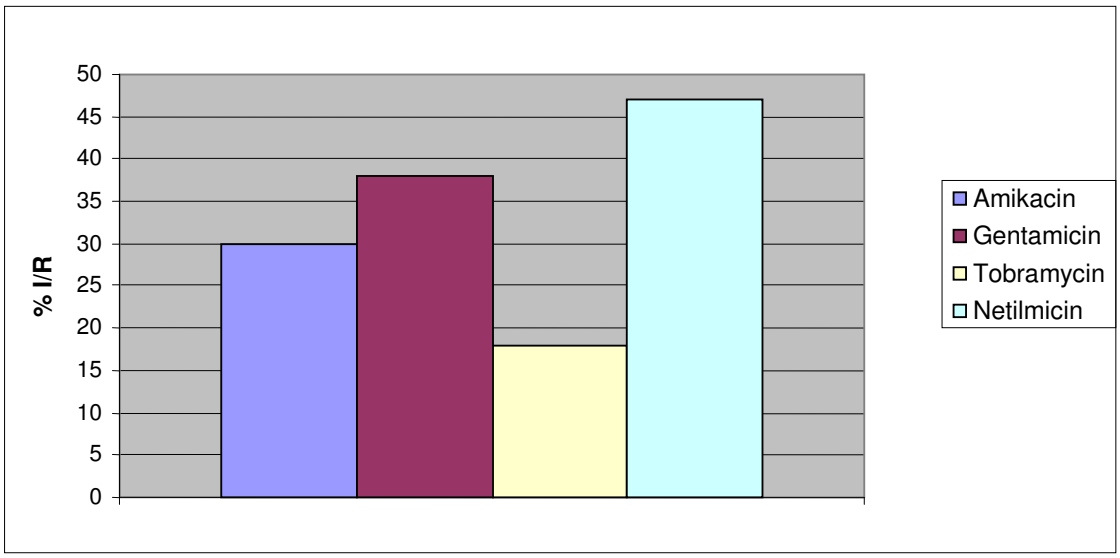


Figure 20. Reduced susceptibility to different aminoglycosides for the *P. aeruginosa* isolates in collection 3.

4.1.3.1 Broad-spectrum aminoglycoside resistance in collection 3

As shown in Figure 21, 53% of the *P. aeruginosa* isolates were susceptible to all aminglycosides tested. Resistance only to netilmicin was observed in 9% of the isolates, while 8% were resistant to both netilmicin and gentamicin. Twelve percent of the isolates were in addition resistant to amikacin, while 14% were resistant to all aminoglycosides tested.

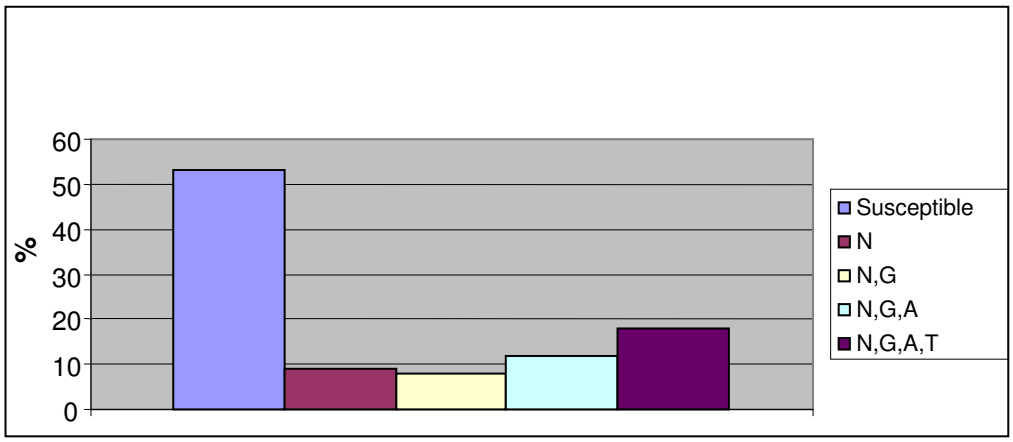


Figure 21. Distribution of broad-spectrum aminoglycoside resistance in *P. aeruginosa* in collection 3. T = tobramycin, N = netilmicin, G = gentamicin, and A = amikacin.

4.2 Prevalence of clinical important aminoglycoside modifying enzymes (AMEs).

4.2.1 Collection 1: *E. coli* and *Klebsiella* spp. from NORM 2009

The total prevalence of the AMEs in *E. coli* and *Klebsiella* spp. with reduced susceptibility to different aminoglycosides in collection 1 is shown in Fig 22. The figures are shown in Appendix B; Table 16. The prevalence of the AAC(6')-Ib enzyme, which confers resistance to amikacin and gentamicin C_{1a} and C_2 was 28% in collection 1; 24% in *E. coli* and 43% in *Klebsiella* spp. respectively. The prevalence of AAC(3)-II which confers resistance to gentamicin, tobramycin, and netilmicin was 79%. Both enzymes, AAC(6')-Ib and AAC(3)-II, were in total detected in 16% of the isolates; in 16% of the *E. coli* isolates and in 14% of the *Klebsiella* spp isolates.

Three percent of the *E. coli* isolates and none of the *Klebsiella* spp. isolates harboured the ANT(2'')-Ia enzyme, which confer resistance to gentamicin, tobramycin and kanamycin. AAC(3)-Ia which confer resistance to gentamicin and ANT(4')-IIb which confers resistance to amikacin and tobramycin, were not detected in any of the isolates. In nine isolates (*E. coli n* = 6 and *Klebsiella* spp. *n* = 3) with an aminoglycoside resistant profile, none of the enzymes tested were detected.

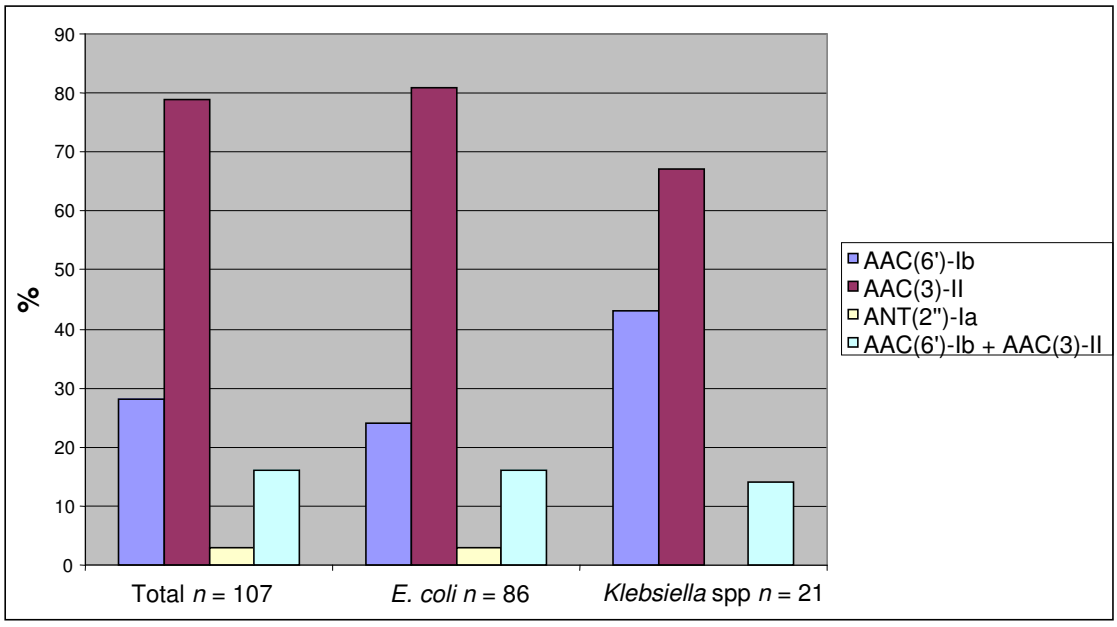


Figure 22. Prevalence of AMEs detected in *E. coli* and *Klebsiella* spp. with reduced susceptibility to aminoglycosides collected through NORM 2009 in collection 1.

4.2.2. Collection 2: ESBL$_A$-positive *E. coli* and *Klebsiella* spp. NORM 2007-08

Among the ESBL$_A$-positive isolates from NORM 2007-08 with reduced susceptibility to different aminoglycosides, 34% of the isolates harboured the *aac*(6')-Ib gene while the *aac*(3)-II gene was detected in 43% of the isolates. Both genes were detected in 21% of the isolates. The total prevalence of AMEs is shown in Figure 23 and the figures are shown in Appendix B; Table 17. Among *E. coli*, 37% of the isolates harboured *aac*(6')-Ib while *aac*(3)-II gene was detected in 43%. Both enzymes were present in 23% of the *E. coli* isolates. Among the *Klebsiella* spp. isolates the *aac*(6')-Ib gene was detected in 13% of the isolates, while the *aac*(3)-II gene was detected in 38% of the isolates. None of the *Klebsiella* spp. isolats were carrying both genes. *aac*(3)-Ia, *ant*(2'')-Ia and *ant*(4)-IIb were not detected in collection 2. Three *E. coli* isolates with an aminoglycoside resistant profile did not come out positive of any of the enzymes tested.

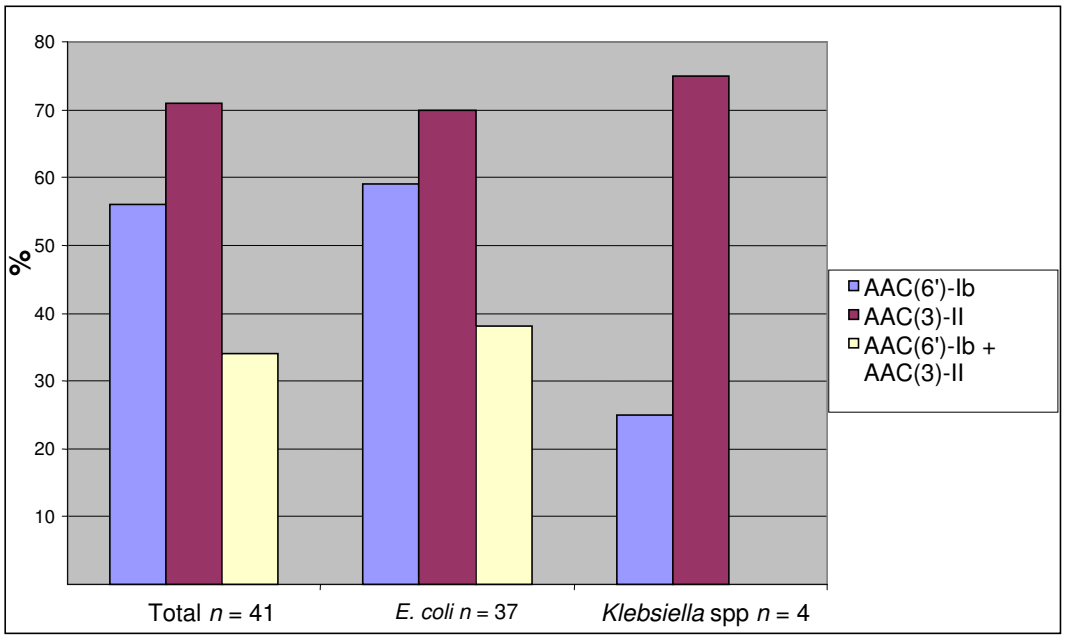


Figure 23. Prevalence of AMEs detected in ESBL$_A$-positive *E. coli* and *Klebsiella* spp. with reduced susceptibility to aminoglycosides in collection 2.

4.3 Detection of 16S rRNA methylases

Eight isolates from collection 1 and 2, with high-level and broad-spectrum aminoglycoside resistance were screened for seven different 16S rRNA methylases by PCR. The isolates and results of PCRs are listed in Table 15. One blood culture isolate of *E. coli* (K65-50) included in the NORM 2009 collection (collection 1) from Ullevål hospital was positive for the 16S rRNA methylase gene *rmtB*. This positive result was confirmed by sequencing, and MLST revealed sequence type 617 (Table 16).

Table 15. Results of screening for 16S rRNA methylases in isolates from collection 1 and 2 with high-level and broad-spectrum aminoglycoside resistance. Green = susceptible, Yellow = intermediate resistant and red = resistant.

				Etest MIC µg/ml					16S rRNA
Ref. no	**Species**	**Material**	**Location**	**AMK[1]**	**GEN[1]**	**TOB[1]**	**KAN[1]**	**NET[1]**	**methylases**
K64-02	*E. coli*	Blood	AHUS[2]	8	96	48	64	24	-
K64-06	*E. coli*	Urine	AHUS[2]	12	6	4	8	3	-
K64-07	*K. pneumoniae*	Blood	AHUS[2]	32	256	64	> 256	> 256	-
K64-80	*E. coli*	Blood	Stavanger	12	16	48	> 256	32	-
K65-01	*E. coli*	Blood	Stavanger	16	8	6	12	4	-
K65-48	*E. coli*	Blood	Ullevål	16	128	96	128	32	-
K65-50	*E. coli*	Blood	Ullevål	> 256	256	384	> 256	> 256	***rmt*B**
K53-37	*E. coli*	Blood	Molde	32	4	64	256	24	-

[1]AMK=amikacin, GEN=gentamicin, TOB=tobramycin, KAN=kanamycin, and NET=netilmicin
[2]AHUS= Akershus university hospital

Table 16. Allele numbers and sequence type for the *rmt*B-positive *E. coli.*

		Allelic profile							
Ref. no	**Species**	***adk***	***fum*C**	***gyr*B**	***icd***	***mdh***	***pur*A**	***rec*A**	**ST**
K65-50	*E. coli*	10	11	4	8	8	13	73	617

From strain collection 3, 16 *P. aeruginosa* isolates with high-level and broad-spectrum aminoglycoside resistance were screened for 16S rRNA methylases. The 16S rRNA methylase gene *rmtD* was detected in one isolate from Stavanger University Hospital (K52-27), a urine sample, and sequencing confirmed the result. The rest of the isolates were negative in the screening. The isolates and results of PCRs are listed in Table 17. MLST revealed K52-27 as sequence type 277 (Table 18).

Table 17. Results of screening for 16S rRNA methylases in *P. aeruginosa* isolates from collection 3 with high-level and broad-spectrum aminoglycoside resistance. Green = susceptible and red = resistant.

				Etest MIC µg/ml				16S rRNA
Ref. no	**Material**	**Location**	**Conclusion**	**AMK[1]**	**GEN[1]**	**TOB[1]**	**NET[1]**	**methylase**
K49-20	Sputum	Østfold	VIM[2]-2	> 256	> 1024	32	> 256	-
K36-45[4]	CVK[8]	AHUS[3]	VIM-4	> 256	192	128	> 256	-
K36-46[4]	Trachea	AHUS[3]	VIM-4	> 256	128	128	> 256	-
K45-32	Puss	RH[3]	VIM-2	96	6	48	4	-
K57-16	Decubitus	Vestfold	VIM-2	64	24	96	> 256	-
K61-69	Abscess	Stavanger	VIM-1	6	6	0,75	6	-
K44-24[5]	Urine	Vestfold	IMP[6]-14	> 256	> 1024	> 1024	> 256	-
K44-36[5]	Sputum	Vestfold	MBL[7]-pos	> 256	> 1024	> 1024	> 256	-
K52-27	Urine	Stavanger	Porinloss, efflux and AmpC-prod	> 256	> 1024	> 1024	> 256	*rmt*D
K61-73	Puss	UNN[3]	Porinloss, efflux and AmpC-prod	> 256	24	64	> 256	-
K71-59	Sputum	RH[3]	Porinloss, efflux and AmpC-prod	> 256	24	6	32	-
K44-57	Sputum	Innlandet	Porinloss, efflux and AmpC-prod	128	16	8	64	-
K44-79	Trachea	Haugesund	Porinloss, efflux and AmpC-prod	64	32	48	> 256	-
K44-80	Puss	Haugesund	Porinloss, efflux and AmpC-prod	64	32	48	> 256	-
K52-42	Urine	Innlandet	Porinloss, efflux and AmpC-prod	24	96	32	> 256	-
K52-63	Urine	AHUS[3]	Porinloss, efflux and AmpC-prod	24	6	1	8	-

[1]AMK=amikacin, GEN=gentamicin, TOB=tobramycin, and NET=netilmicin. [2]VIM= Verona imipenemase. [3]AHUS= Akershus university hospital, RH=Oslo university hospital, Rikshospitalet, UNN=University hospital of North Norway. [4] Isolates from the same patient. [5] Isolates from the same patient. [6] IMP= Imipenemase. [7] MBL= metallo-β-lactamase. [8] CVK= central veneous catheter

Table 18. Allele numbers and sequence type for the *rmt*D-positive *P. aeruginosa*.

		Allelic profile							
Ref. no	**Species**	***acs*A**	***aro*E**	***gua*A**	***mut*L**	***nuo*D**	***pps*A**	***trp*E**	**ST**
K52-57	*P. aeruginosa*	39	5	9	11	27	5	2	277

4.4 Pulsed-field gel electrophoresis (PFGE)

Eighteen *E. coli* isolates with elevated gentamicin resistance were selected for PFGE, to look for clusters that could suggest a dissemination of a successful Norwegian clone. PFGE banding patterns were obtained for 14 isolates (Figure 24). DNA from four of the isolates consistently autodigested and no banding patterns were obtained.

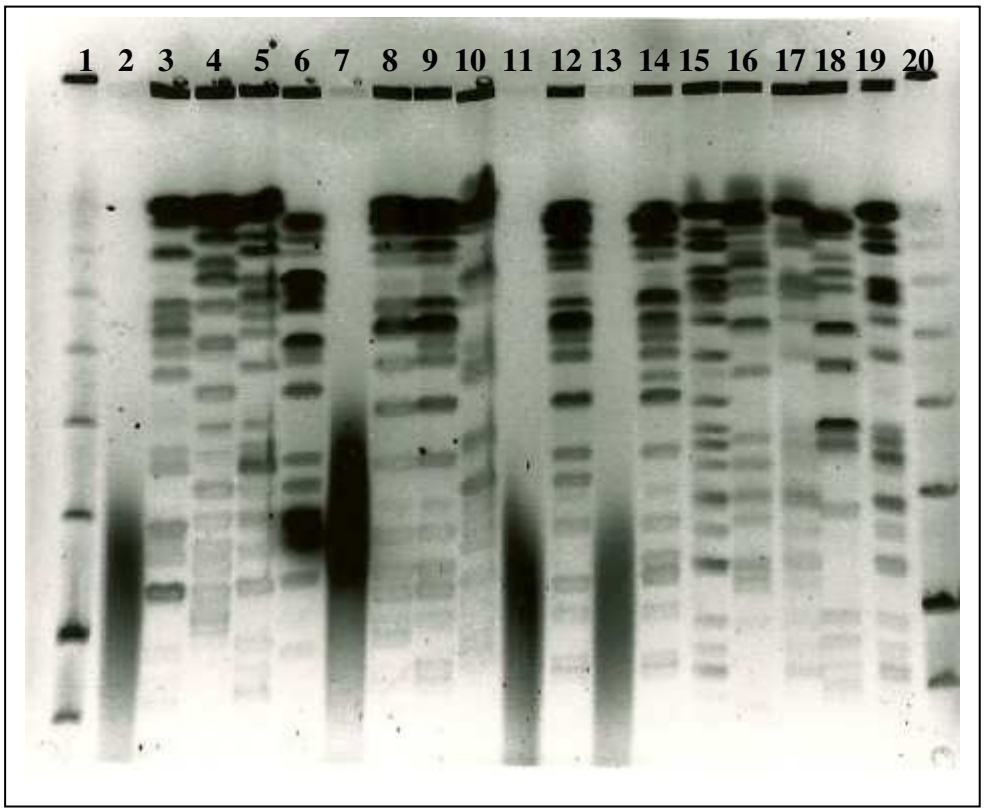


Figure 24. PFGE result of 18 *E. coli* isolates with elevated gentamicin resistance from different Norwegian hospitals. Lanes: 1 and 20, PFG marker; 2, K64-13; 3, K64-17; 4, K64-18; 5, K64-22; 6, K64-43; 7, K64-44; 8, K64-46; 9, K64-49; 10, K64-55; 11, K64-70; 12, K65-3; 13, K65-6; 14, K65-8; 15, K65-21; 16, K65-25; 17, K65-34; 18, K65-44; 19, K65-52.

A dendogram (Figure 25) was made to illustrate a potential relatedness between the different isolates. Similar band patterns represent in general relatedness between isolates, but this analysis did not reveal any relatedness or clonality between the chosen isolates.

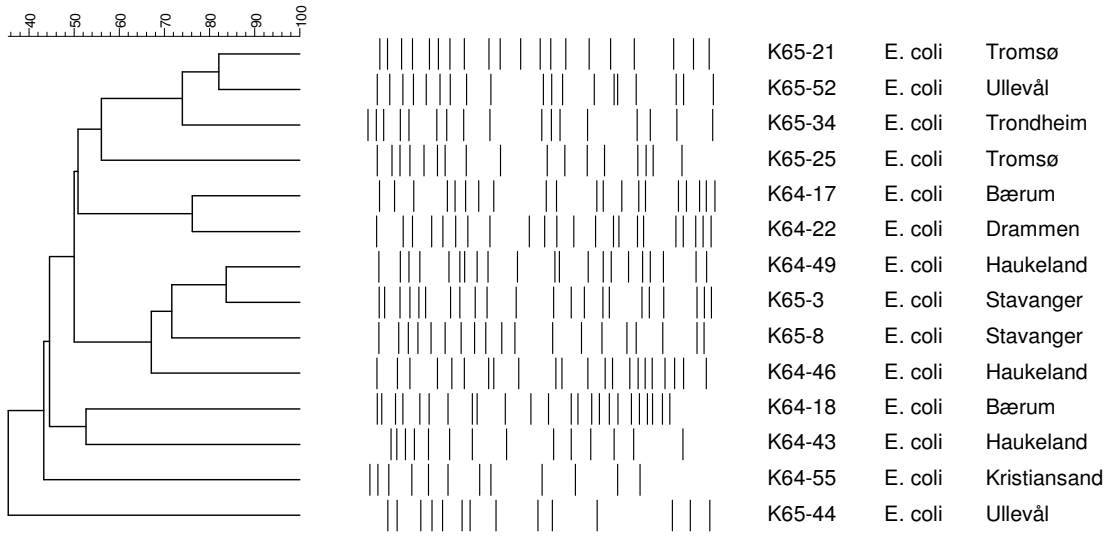


Figure 25. Dendogram to illustrate that there is no clonality between 14 *E. coli* isolates with elevated resistance to gentamicin. Percent similarities are shown on top of the dendrogram.

PFGE was also performed on 18 *E. coli* isolates from collection 1 were the Etest and the disk diffusion results for tobramycin did not correspond. Visual evaluation of the PFGE result showed diversity among the isolates (Figure 26). DNA from one isolate autodigested and no banding patterns were obtained. Since EUCAST breakpoints were changed after the analysis was performed, no further analysis was performed.

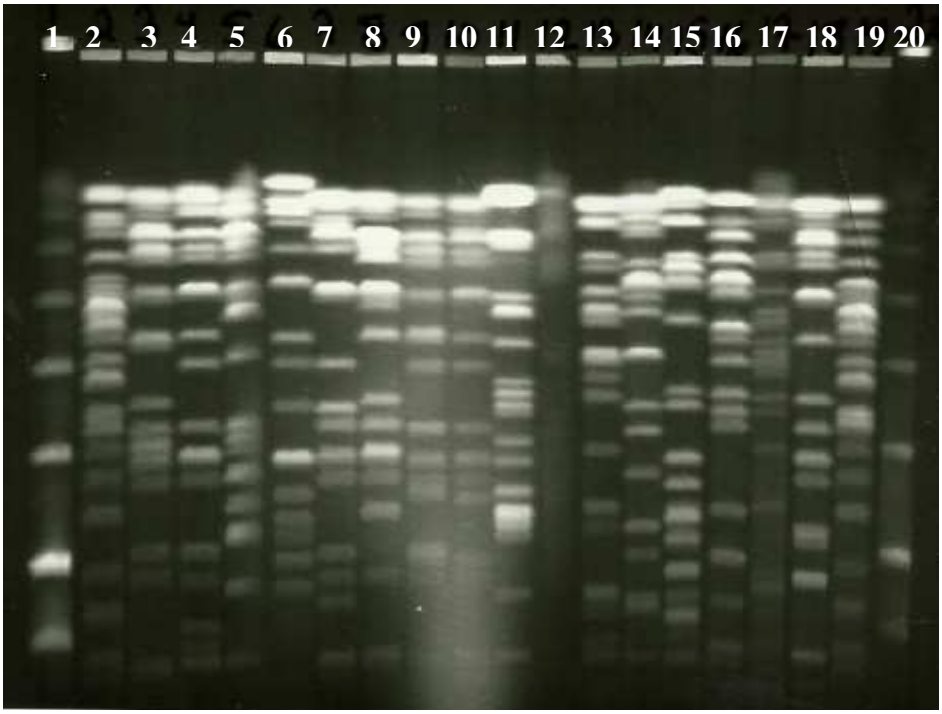


Figure 26. PFGE result of 18 *E. coli* isolates with non-corresponding Etest and disk diffusion results for tobramycin. Lanes: 1 and 20, PFG marker; 2, K64-1; 3, K64-3; 4, K64-23; 5, K64-32; 6, K64-33; 7, K64-39; 8, K64-43; 9, K64-53; 10, K64-54; 11, K64-66; 12, K64-67; 13, K65-1; 14, K65-3; 15, K65-23; 16, K65-28; 17, K65-45; 18, K65-46; 19, K65-47.

4.5 Plasmid transfer studies

PCR-based screening for different AMEs to give evidence for aminoglycoside resistance profile was negative in 12 isolates. Four isolates from collection 1 and 2 were chosen for plasmid transfer studies, to investigate if the resistance marker were located on a transferable element. Two of the isolates did not give transconjugants (K64-31 and K65-35). Transconjugants were obtained from K64-71 and K65-38, indicating a successful transfer of the resistance marker. All transconjugants from K64-71 and K65-38 were resistant to all aminoglycosides tested (Table 19). The transconjugation frequencies are shown in Appendix B; Table 18.

Table 19. MIC for donors, recipient and transconjugants in the plasmid transfer studies.

			Etest MIC µg/ml			
	Ref. no	**Species**	**GEN[1]**	**TOB[1]**	**NET[1]**	**RIF[1]**
Recipient	J53-2	*E. coli*	0,38	0,5	1	> 32
Donor 1	K64-71	*E. coli*	32	64	32	NA[2]
Donor 2	K65-38	*E. coli*	16	32	16	NA[2]
Transconjugant	K64-71 I	*E. coli*	12	24	16	> 32
Transconjugant	K64-71 II	*E. coli*	12	24	12	> 32
Transconjugant	K65-38 I	*E. coli*	12	24	16	> 32
Transconjugant	K65-38 II	*E. coli*	12	24	16	> 32

[1] GEN=gentamicin, TOB=tobramycin, NET=netilmicin, and RIF=rifampicin. [2] NA= not analysed

Table 20. Allelic profile and sequence type of recipient strain J53-2 and donor strain K65-38, and partial allelic profile of donor strain K64-71 and transconjugants.

			Allelic profile							
	Ref. no	**Species**	***adk***	***fum*C**	***gyr*B**	***icd***	***mdh***	***pur*A**	***rec*A**	**ST**
Recipient	J53-2	*E. coli*	10	11	4	8	8	8	2	10
Donor 1	K64-71	*E. coli*	6	4		16				
Donor 2	K65-38	*E. coli*	10	11	4	8	8	8	2	10
Transconjugant	K64-71 I	*E. coli*	10	11		8				
Transconjugant	K64-71 II	*E. coli*	10	11		8				
Transconjugant	K65-38 I	*E. coli*	10	11		8				
Transconjugant	K65-38 II	*E. coli*	10	11		8				

MLST was performed on the recipient and donor K65-38 while partial MLST (*fum*C, *adk*, and *icd*) was performed on donor K64-71 and the transconjugants (Table 20). One of the donor strains (K65-38) turned out to have an identical sequence type as the recipient (J53-2). In this case, it was not possible to distinguish between transconjugants and donor. However, the donor K64-71 had a completely different partial allelic profile compared to J53-2. Transconjugants from K64-71 had the same partial allelic profile as J53-2 indicating a successful transfer of the resistance marker.

4.6 Comparison of three phenotypic methods for detecting reduced susceptibility to aminoglycosides

A summary of the comparisons and retesting are listed in Appendix B. The resistant isolates are labelled R, the isolates with intermediate resistance are labelled I and the susceptible isolates are labelled S.

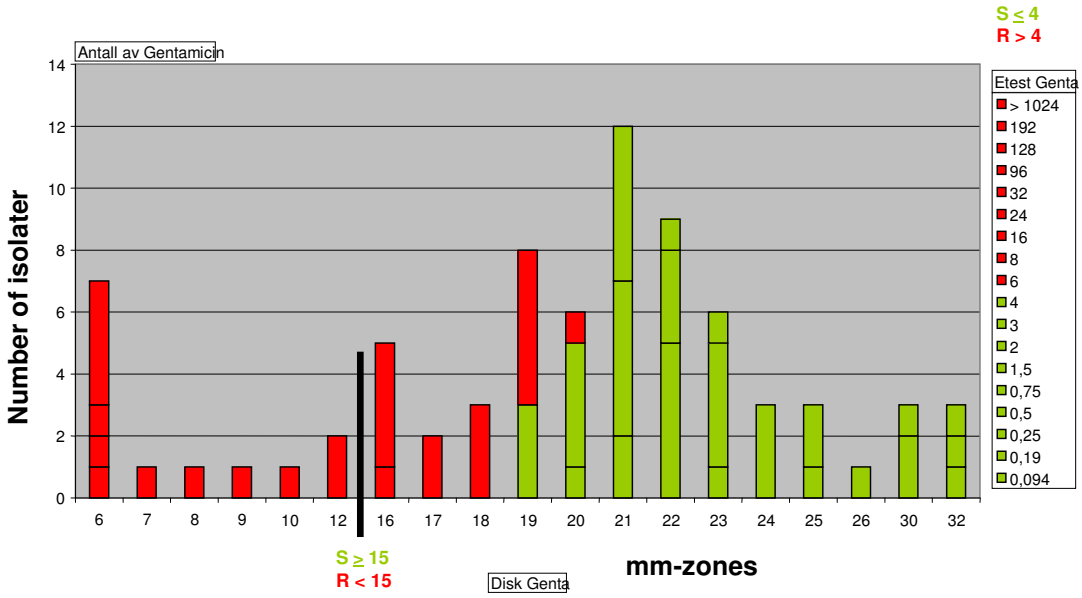


Figure 27. Comparison of Etest and disk diffusion results in detecting reduced susceptibility to gentamicin in collection 3 (*P. aeruginosa*).

4.6.1 Comparison of tests detecting reduced susceptibility to gentamicin

One very major error was detected in collection 1 when comparing Etest (R) versus VITEK2 AST (S), but this result was not reproduced. The retesting classified the difference as a minor error, with Etest R and VITEK2 AST I. No very major errors were detected in collection 2. Major errors were not detected in collections 1 and 2, while all together 6 minor errors were detected.

In collection 3, on the other hand, 16 isolates were classified as very major errors when comparing Etest (R) and disk diffusion (S), Figure 27. This result was reproduced in nine isolates, while six isolates gave identical results in both tests when retesting. Etest versus VITEK2 AST also gave 12 very major errors in collection 3 and seven of these errors were

reproduced, while five isolates became identical by retesting. Four isolates came out as very major errors, and reproduced when comparing VITEK2 AST and disk diffusion, Figure 28. No major or minor errors were observed in collection 3.

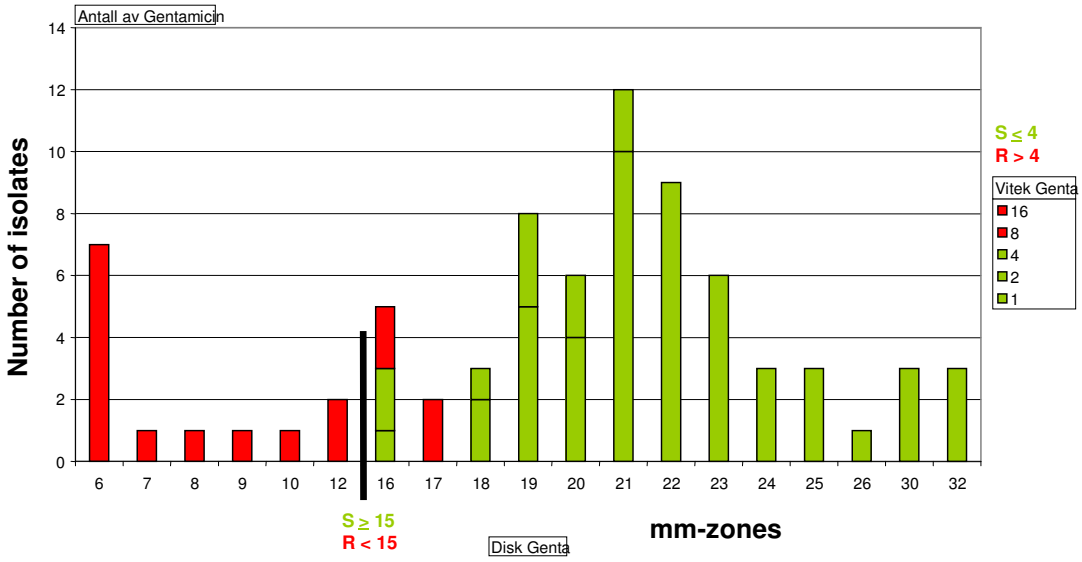


Figure 28. Comparison of VITEK2 AST versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 3 (*P. aeruginosa*).

4.6.2 Comparison of tests detecting reduced susceptibility to tobramycin

In collection 1, Etest detected one isolate as R while the disk diffusion test came out as S (Figure 29). This result was reproduced upon retesting, and considered a very major error. When comparing Etest and VITEK2 AST results (Figure 30), fourteen isolates were identified as R by Etest and S by VITEK2 AST also classified as very major errors. This result was reproduced in seven of these isolates upon retesting, five isolates were classified as minor errors after retesting while two isolates came out with no errors.

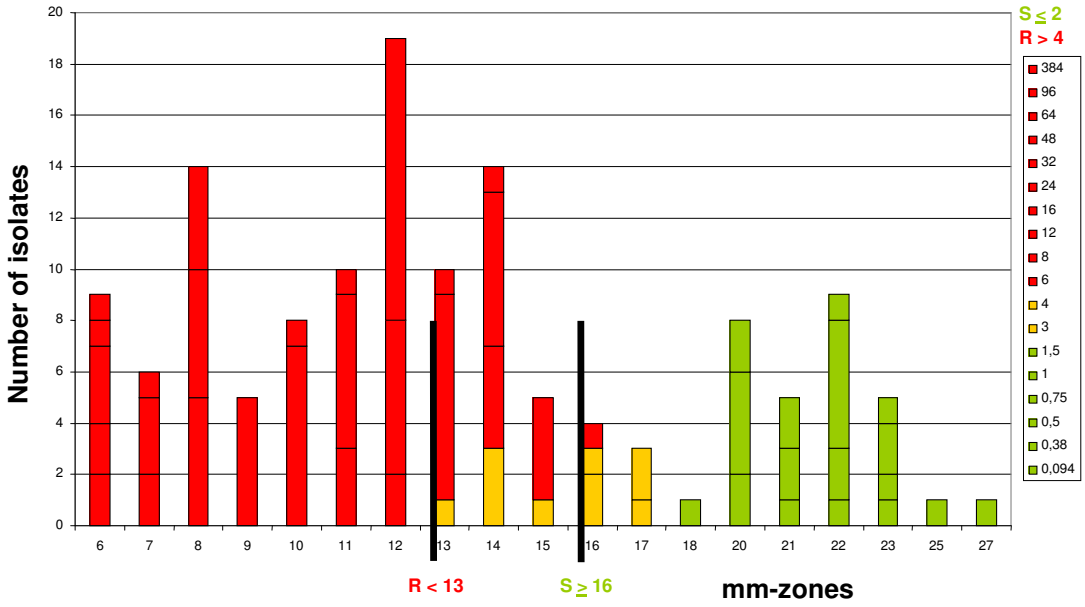


Figure 29. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 1 (NORM 2009).

No isolates in collection 2 gave very major errors. One isolate in collection 1 and one isolate in collection 2 were classified as major error, with VITEK2 AST S and disk diffusion R. Quite a number of isolates came out as minor errors. In collection 1, 24 isolates were reported as R by Etest and I by disk diffusion, and 17 isolates were R by Etest and I by VITEK2 AST. In collection 2, altogether 21 minor errors were demonstrated in the three comparisons.

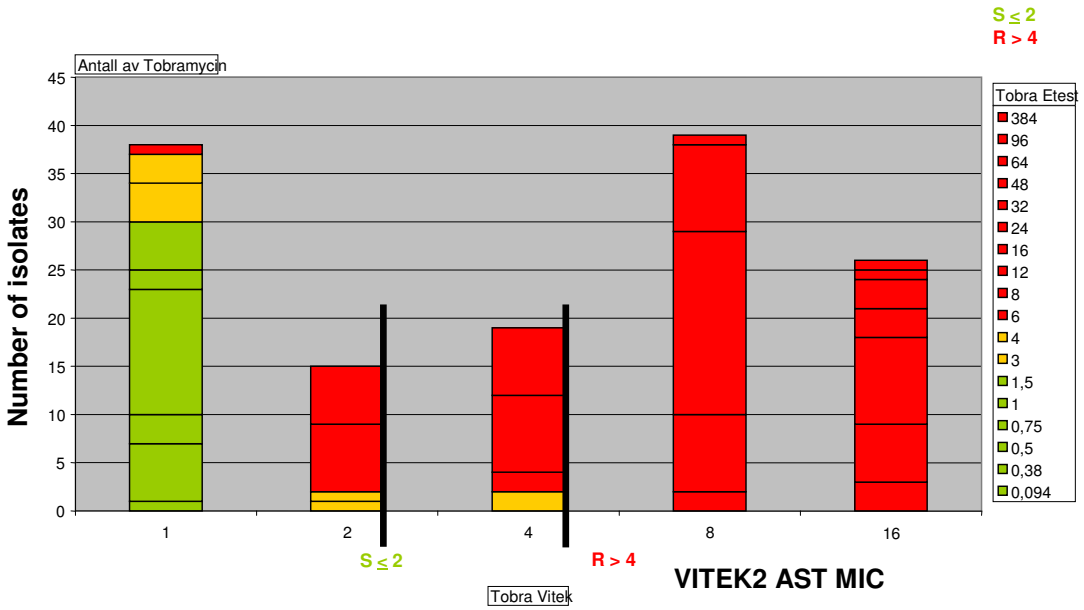


Figure 30. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to tobramycin in collection 1 (NORM 2009).

In collection 3, one *P. aeruginosa* isolate was classified as very major error, both with Etest (R) versus disk diffusion (S) and VITEK2 AST (R) versus disk diffusion (S). Retesting of this isolate gave Etest S, disk diffusion S and VITEK2 AST R. No minor errors were detected in collection 3.

4.6.3 Comparison of tests detecting reduced susceptibility to amikacin

MIC for amikacin was not available in the VITEK2 AST thus only Etest versus disk diffusion was compared. No very major errors or major errors were detected in collections 1 and 2 when comparing the results of Etest and disk diffusion, but all together 13 minor errors were observed. In collection 3, one very major error was observed but changed to a minor error after retesting. One major error and six minor errors were detected (Figure 31).

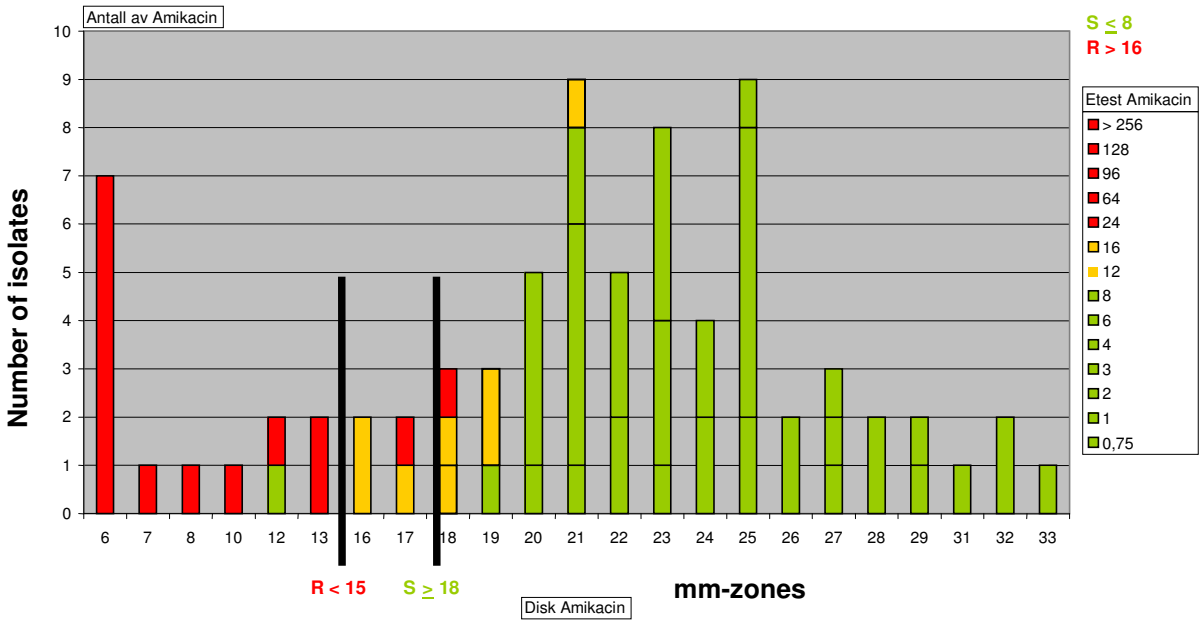


Figure 31. Comparison of Etest and disk diffusion results in detecting reduced susceptibility to amikacin in collection 3 (*P. aeruginosa*).

5. Discussion

Although aminoglycosides represent a small fraction of antibiotic consumption in Norway, it is still an important group of antibiotics in treating serious bacterial infections. Monitoring the level of aminoglycoside resistance is therefore an important task. The main mechanism causing aminoglycoside resistance is the presence of aminoglycoside modifying enzymes (AMEs). In addition, high level and broad-spectrum resistance to aminoglycosides is often associated with 16S rRNA methylases. Further, efflux mechanisms and impermeability of the cell wall may cause reduced susceptibility to aminoglycosides.

This study has focused on resistance to different aminoglycosides in clinically important isolates of Gram-negative bacteria from Norway, with emphasis on gentamicin, tobramycin, and amikacin. The prevalence of genes encoding clinically important AMEs and 16S rRNA methylases has also been evaluated. In addition, phenotypic methods for detecting reduced susceptibility to aminoglycosides have been compared.

5.1 Prevalence of aminoglycoside resistance

5.1.1 Aminoglycoside resistance in *E. coli* and *Klebsiella* spp. in Norway

An increase in the prevalence of resistance towards gentamicin has been observed in Norway over the past years. The Norwegian surveillance programme for antimicrobial resistance in human pathogens (NORM)(6) has reported an increased prevalence of reduced susceptibility to gentamicin from 1.2% in *E. coli* isolated from blood in 2000 to 4.0% in 2009 and from 0.8% to 1.8% in *Klebsiella* spp. In this study isolates with reduced susceptibility to gentamicin and/or tobramycin from NORM 2009 (collection 1) were collected and the phenotypic testing was repeated. Surprisingly, 20% of the strains in collection 1 could not be reproduced using the Etest method. Thus, in this study the prevalence of reduced susceptibility to gentamicin in *E. coli* was 3.8% in blood culture isolates and 2.5% in urine isolates. Our figures differed slightly from the figures reported in NORM, where 4.0% of blood culture isolates and 3.1% in urinary tract isolates showed reduced susceptibility to gentamicin. This was also observed for *Klebsiella* spp. urine isolates where the prevalence of reduced susceptibility to gentamicin in NORM 2009 was 0.9%, while our data show a

prevalence of 0.7%. For blood culture isolates the prevalence of reduced susceptibility to gentamicin was the same (1.4%).

The lack of reproducible results did affect the prevalence of tobramycin resistance to a greater extent than for gentamicin. In NORM 2009 the prevalence of reduced susceptibility to tobramycin in *E. coli* was reported to be 3.1% in blood culture isolates and 2.5% in urinary tract isolates. In our study the results were 4.1% in blood culture isolates and 2.6% in urinary tract isolates. For *Klebsiella* spp. the NORM 2009 figures were 1.8% in blood and 1.1% in urinary tract isolates, while our data showed 2.0% and 1.0%, respectively. When comparing the figures from NORM with our data, the major differences were in the intermediate resistant (I) category. This suggests that there is a difference between Etest and EUCAST disk diffusion in separating the different susceptibility categories (S-I-R). This was observed when comparing our Etest and EUCAST disk diffusion results (see below). In addition, the new EUCAST breakpoints for tobramycin were used in this study, while NORM 2009 is based on both the old method for disk diffusion and other breakpoints.

Although 20% of the results could not be reproduced the overall prevalences of gentamicin resistance compared to the prevalence of tobramycin resistance were similar. Tobramycin was included in the panel of antibiotics investigated in NORM for the first time in 2009. It will be interesting to follow the surveillance of tobramycin in the coming years.

There are no available national surveillance data regarding the prevalence of reduced susceptibility to neither amikacin nor netilmicin in Norway. Our study reveals low prevalence of reduced susceptibility to amikacin among the clinical isolates of *E. coli* and *Klebsiella* spp. ($\leq$0.6%). The rate of reduced susceptibility to netilmicin follows the same trends as for gentamicin and tobramycin with higher prevalence of reduced susceptibility in blood culture isolates (3.9% and 2.0%) compared to urinary tract isolates (2.6% and 1.0%) in *E. coli* and *Klebsiella* spp., respectively. However, the actual prevalence of reduced susceptibility to amikacin and netilmicin could be different as the isolates studied were selected based on reduced susceptibility to gentamicin and/or tobramycin.

The prevalence of aminoglycoside resistance in Norway is comparable to what is observed in other Scandinavian countries. The Swedish surveillance program SWEDRES(7) also reports an increasing trend in the prevalence of aminoglycoside resistance (gentamicin or

tobramycin). The prevalence of resistance in blood culture isolates in 2009 was 3.7% in *E. coli* and 1% in *Klebsiella pneumoniae* in Sweden, almost the same level as the Norwegian findings. In Denmark however, DANMAP(3), the Danish surveillance program, reports a prevalence of gentamicin resistance of 5% in *E. coli* blood culture isolates and 9% in *K. pneumoniae*.

In comparison with other European countries the prevalence of aminoglycoside resistance is low in Scandinavia. The annual report for 2009 from the European Antimicrobial Resistance Surveillance Network(4) (EARS-Net) reveals a significant increase in the prevalence of aminoglycoside resistance in clinical isolates of *E. coli* in the period 2006-2009 in 10 countries. Five of these countries (Hungary, Italy, Slovenia, Greece, and Spain) reported resistance rates higher than 10%. A significant increase in the prevalence of aminoglycoside resistance in clinical isolates of *K. pneumoniae* has been observed in 24 European countries. Even though the prevalence of resistance to aminoglycoside resistance in Norwegian isolates of *Klebsiella* spp. must be considered low, a significant increase has been observed in the period 2006-2009. Equivalent increase has been observed in Denmark, Estonia, France, and Portugal among other countries. Greece, Bulgaria and Lithuania report aminoglycoside resistance in *K. pneumoniae* above 50%. This suggests the likelihood that the prevalence of aminoglycoside resistance will continue to increase in Norway in the coming years.

PFGE analyses of a subset of gentamicin, tobramycin, and netilmicin resistant *E. coli* isolates selected from hospitals in different regions in Norway, revealed a diverse population. This indicates that the spread of aminoglycoside resistance is not associated with a specific clone.

5.1.2 Aminoglycoside resistance in $ESBL_A$-positive *E. coli* and *Klebsiella* spp. in Norway

The prevalence of reduced susceptibility among the confirmed $ESBL_A$-positive isolates in collection 2 were as expected high compared to the isolates in collection 1. Fifty-six percent were resistant to tobramycin, 44% were resistant to gentamicin, 55% were resistant to netilmicin while only 40% of the isolates tested were susceptible to all aminoglycosides. Seven percent of the isolates showed reduced susceptibility to amikacin.

This may suggest that amikacin could be a useful alternative drug in the treatment of infections with $ESBL_A$-positive Enterobacteriaceae.

The observed higher prevalence of aminoglycoside resistance in $ESBL_A$-positive isolates suggests a link between the resistance genes. Genes that encode resistance to aminoglycoside resistance are located on a wide range of genetic elements such as integrons or transposable elements which have been associated with different multidrug-resistant ESBL-plasmids. Further, clonal spread is one of the major causes of the increase in prevalence of $ESBL_A$-positive strains, and the $bla_{CTX\text{-}M}$ variant of ESBLs has been particularly successful(13). In Norway, CTX-M is also shown to be the dominant $ESBL_A$ enzyme (NORM 2009(6)) suggesting a link between $bla_{CTX\text{-}M}$ and aminoglycoside resistance genes. This is also shown in several studies which report a link between $ESBL_A$ and aminoglycoside resistance(20,29).

Of note, the prevalence of $ESBL_A$-positive *K. pneumoniae* increased from 5% in 2007 to 14.6% in 2009 in Denmark (DANMAP(3)). This rapid increase may explain the high level of aminoglycoside resistance in *K. pneumoniae*, compared to the other Scandinavian countries. Worryingly, an increase in the prevalence of $ESBL_A$ in Norway is also observed (NORM reports) suggesting that the prevalence of aminoglycoside resistance is likely to increase in the coming years.

Co-resistance to other antibiotic classes like fluoroquinolones, tetracyclines, and trimethoprim/sulfamethoxazole is frequently observed in $ESBL_A$-positive strains. Thus, increase in $ESBL_A$ will likely be associated with an increase in resistance to other antibiotics due to linked resistance.

5.1.3 Carbapenem non-susceptible *Pseudomonas aeruginosa*

P. aeruginosa is not included annually in the Norwegian surveillance system, but blood-culture isolates of *P. aeruginosa* from 2002-2003 were analysed in 2005 (NORM 2005(1)). The susceptibility to gentamicin, tobramycin and amikacin was tested. All isolates tested were susceptible to tobramycin, while 1.6% of the isolates were resistant to amikacin and 1.7% of the isolates were resistant to gentamicin.

In our study, including *P. aeruginosa* with reduced susceptibility to carbapenems, more than 50% of the isolates were susceptible to all aminoglycosides tested, while 18% revealed reduced susceptibility to all aminoglycosides tested. Tobramycin is considered the most effective anti-pseudomonal aminoglycoside. Our data show a higher prevalence of reduced susceptibility to gentamicin (38%) compared to tobramycin (18%) and 30% reduced susceptibility to amikacin. This shows that in a collection of *P. aeruginosa* with reduced susceptibility to carbapenems, the prevalence of aminoglycoside resistance is much higher that in the general *P. aeruginosa* population.

Our collection of *P. aeruginosa* was selected based on reduced susceptibility to carbapenems of which seven isolates were known to produce carbapenemase. In *P. aeruginosa,* different mutations may confer antibiotic resistance(46). Several mechanisms are known to confer carbapenem-resistance, such as (I) inactivation or down regulation of carbapenem specific porins, (II) upregulation of AmpC β-lactamases and (III) upregulation of efflux by MexAB-OprM and other pumps. The MexAB-OprM pump may also act against aminoglycosides, and may be the explanation of the relatively high level of aminoglycoside resistance along with different AMEs in collection 3. Five of the eight carbapenemase-positive *P. aeruginosa* strains included, showed high level and broad-spectrum aminoglycoside resistance. The genes encoding this resistance may be located on the same mobile genetic element harbouring the carbapenemase-gene.

5.2 Prevalence of clinically important aminoglycoside modifying enzymes (AMEs)

The AME AAC(3)-II, which confers resistance to gentamicin, tobramycin and netilmicin was the dominating enzyme (79%) detected in collection 1. All isolates with the *aac*(3)-II gene showed reduced susceptibility to gentamicin, tobramycin, netilmicin and kanamycin. AAC(6')-Ib is considered the most clinically relevant acetyltransferase and confers resistance to amikacin and gentamicin C_{1a} and $C_{2(35)}$. In collection 1, 28% of the strains was AAC(6')-Ib positive. However, in our collection many of the isolates were susceptible to amikacin. As many as 42/53 of the isolates harbouring the *aac*(6')-Ib gene had amikacin MIC categorised as susceptible. MICs detected were as low as 2 μg/ml, far below

the amikacin breakpoint at 8 μg/ml, This suggests that the susceptibility breakpoint for amikacin is not able to detect all isolates with AAC(6')-Ib or that the level of amikacin resistance caused by AAC(6')-Ib is low.

A variant of the AAC(6')-Ib enzyme, AAC(6')-Ib-cr, is responsible for antibiotic resistance to two different classes of antimicrobial agents; fluoroquinolones and aminoglycosides(36). This enzyme is found as a gene cassette in different integrons and is associated with reduced susceptibility to fluoroquinolones. The VITEK2 Gram-negative susceptibility testing in our study detected a phenotypic $ESBL_A$ or $ESBL_M$ profile in 20% of the strains in collection 1, together with resistance to ciprofloxacin and trimethoprim/sulfa. This supports that genes encoding aminoglycoside-, fluoroquinolone-, and β-lactam resistance, are carried together on mobile genetic elements. In a study on isolates from NORM 2005 with reduced susceptibility to ciprofloxacin, all isolates positive for *aac*(6')-Ib were of the *aac*(6')-Ib-cr variant(25). In strain collection 1 the prevalence of ciprofloxacin-resistance was 54%, indicating that the *aac*(6')-Ib-cr variant also could be present in a large proportion of isolates in our collection.

Also among the $ESBL_A$-positive isolates in collection 2, AAC(3)-II was the dominating AME, detected in 71% of the isolates with reduced susceptibility to aminoglycosides. This corresponds well with the fact that all isolates positive for the *aac*(3)-II gene in collection 2 showed reduced susceptibility to gentamicin, tobramycin, and netilmicin. AAC(6')-Ib was detected in 56% of the strains, and both enzymes were detected in 34%.

5.3 Transfer of aminoglycoside resistance

Transfer of antibiotic resistance genes is well known to occur between bacteria. One of the isolates with amonoglycoside resistant profile where no AMEs were detected (K64-71), gave successful transfer of the resistant marker to the recipient strain J53-2. This indicates that the resistance marker is located on a transferable element. Genes coding for fluoroquinolone resistance, *qnr*S and *qnr*B have successfully been transferred between bacteria by transconjugation(21,23). K64-71 was resistant to ciprofloxacin and the transfer of the resistance marker may imply that the genes coding for aminoglycoside resistance in the plasmid transfer studies are located on the same transferable element as the gene coding for

the fluoroquinolone resistance. Further studies are required to identify the enzyme which inactivates the aminoglycosides.

5.4 Detection of 16S rRNA methylases

Plasmid-mediated 16S rRNA methylases have been found worldwide in the members of the family Enterobacteriaceae, *P. aeruginosa* and *Acinetobacter baumannii*(55). The presence of 16S rRNA methylases is associated with high level (MIC typically $\geq$ 256 μg/ml) and broad-spectrum aminoglycoside resistance(16).In Gram-negative bacteria, seven enzymes have been described (ArmA, RmtA, RmtB, RmtC, RmtD, RmtE and NpmA)(14,18). *arm*A isolated from Enterobacteriaceae and *Acinetobacter baumannii* and *rmt*B isolated from Enterobacteriaceae appear to be the most widespread genes, and have been detected primarily in Europe and Asia. *rmt*A and *rmt*C have only been reported from Japan in *P. aeruginosa* and *Proteus mirabilis*, respectively, while *rmt*D has been detected in *P. aeruginosa* isolates in Latin America(18). *npm*A has been detected in a clinical isolate of *E. coli* in Japan(18) and *rmt*E has been reported from a bovine-origin *E. coli* from USA(14). In China, a high prevalence of *rmt*B in *E. coli* has been reported(53) and coexistence of *rmt*B and *arm*A has been detected in an isolate of *Klebsiella pneumonia*(54). There seems to be an association between 16S rRNA methylases and ESBL-coding genes, and the genes coding for these enzymes are sometimes located on the same conjugative plasmid.

In this study, the methylase *rmt*B was detected in an *E. coli* strain (K65-50). This strain revealed high-level resistance to all aminoglycosides tested, and was in addition harbouring the *ant*(2'')-1a gene coding for an aminoglycoside nucleotidyltransferase. However, no phenotypic $ESBL_A$ profile was detected in this strain, but the isolate was resistant to ciprofloxacin (MIC > 4 μg/ml). The 16S rRNA methylase gene *arm*A has been identified in a carbapenemase-producing isolate of *E. coli* isolated in a Norwegian hospital in 2009(38), but the 16S rRNA methylase *rmt*B has to our knowledge not been described in Norwegian isolates of Enterobacteriaceae previously. MLST analysis identified the *rmt*B positive *E. coli* as sequence type (ST)617, belonging to the sequence type complex STC10. Studies have revealed that the spread of $bla_{CTX\text{-}M}$ is attributed to different ST-types belonging to (STC)10, such as ST10, ST48, ST168 and ST617(33). STC 10 represents some of the major STs responsible for $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}14}$, and $bla_{CTX\text{-}M\text{-}9}$ dissemination.

In this study, disks of arbekacin and isepamycin (kindly donated by Mr Keiko Kawaguchi, BD, Japan) were included in the disk diffusion screening. Arbekacin, an aminoglycoside synthesized from dibekacin (syntethic derivate of kanamycin), is widely used in Japan and has shown a wide spectrum of antibacterial activity. Most noteworthy is arbekacin's activity against methicillin-resistant *Staphylococcus aureus*(47). A unique characteristic of 16S rRNA methylases is the high level resistance to arbekacin. Of all isolates tested (collection 1 and 2) only the isolate harbouring the *rmt*B gene displayed no inhibitory zone around the arbekacin disk confirming that arbekacin could be a useful substrate for detection of 16S rRNA methylases.

Among the *P. aeruginosa* isolates in collection 3, one isolate was positive for 16S rRNA methylases. The 16S rRNA methylase *rmt*D was identified in a urine isolate (K52-27). MLST analysis revealed the allelic profile of sequence type (ST) 277. No carbapenemases were identified in this isolate, and the reduced susceptibility to carbapenems was likely to be due to porin loss, efflux or increased AmpC-production. *rmt*D was firstly reported in Brazil in a clinical strain of *P. aeruginosa* which also produced the metallo-β-lactamase SPM-1 (Sao Paulo metallo- β-lactamase)(15). SPM-1 is carried on a class 1 integron which also harbours aminoglycoside resistance genes(51). Interestingly, Silva *et al* reported in a study from Brasil that 49/50 SPM-1 producing *P. aeruginosa* belonged to ST277(42).

5.5 Comparison of three phenotypic methods for detecting reduced susceptibility to aminoglycosides

Different phenotypic methods are available for detecting reduced susceptibility to antibiotics. In Norway the most common method used is the EUCAST disk diffusion method. In addition, automated systems like VITEK2 are often used. It is vital that the methods reveal comparable and consistent results.

When comparing the methods for detecting reduced susceptibility to the aminoglycosides investigated in the Enterobacteriaceae strains in collections 1 and 2, the main inconsistencies were related to tobramycin. Preliminary analyses of the comparison between Etest and disk diffusion revealed several very major errors. During the project period the

EUCAST disk diffusion breakpoints (mm-zones) were changed from R<12 and S≥15 to R<13 and S≥16. When comparing Etest and disk diffusion using the new breakpoints, the number of very major errors was reduced from five to one. Still, a considerable number of 24 minor errors were observed. In contrast, when the methods for detecting reduced susceptibility to gentamicin and amikacin were compared, only minor errors were found. This indicates better correlation between the methods, and also supports the current breakpoints. The majority of minor errors were observed when comparing amikacin Etest and disk diffusion.

Comparison of methods to detect reduced susceptibility to aminoglycosides in *P. aeruginosa* gave a different picture. In this species the methods to detect reduced susceptibility to tobramycin and amikacin mainly gave minor errors. However, the methods to detect reduced susceptibility to gentamicin showed a number of reproducible very major errors in all three comparisons. Interestingly, few minor errors were detected for gentamicin.

The high prevalence of very major errors in comparison of methods detecting reduced susceptibility to gentamicin (*P. aeruginosa*) and minor errors in methods detecting reduced susceptibility to tobramycin (Enterobacteriaceae) may indicate a need for evaluation of the breakpoints. The gold standard in establishing minimum inhibitory concentrations is micro broth dilution, and the isolates giving errors in this comparison should be retested using this method. In this manner, the minor errors could be confirmed or invalidated, and give support to a potential change in breakpoints. When comparing Etest and disk diffusion with the automated VITEK2 AST, several reproducible very major errors and minor errors were detected. This emphasises the importance of quality control of automated systems, and for the manufacturers to calibrate the anti-susceptibility cards regularly.

From a clinical point of view, very major errors in phenotypic methods for detecting reduced susceptibility to antibiotics may result in treatment failure. Incorrect reporting of susceptibility testing results may also contribute to development of resistance. It is therefore of utmost importance that medical microbiology laboratories perform appropriate testing. This study has also shown that breakpoints are not static, and have to be monitored and evaluated regularly.

6. Concluding remarks

- The level of aminoglycoside resistance in Norwegian clinical isolates of *E. coli* and *Klebsiella* spp. is still low, but has increased over the last years.
- 60% of the $ESBL_A$-positive *E. coli* and *Klebsiella* spp. isolates and 47% of the carbapenem non-susceptible *P. aeruginosa* isolates tested were resistant to at least one aminoglycoside. This shows that resistance mechanisms are linked together resulting in multi-drug resistance limiting treatment options.
- AAC(3)-II and AAC(6')-Ib are the dominating aminoglycoside modifying enzymes in Norwegian Enterobacteriaceae.
- 16S rRNA methylases (*rmt*B and *rmt*D) are present in Norwegian isolates of *E. coli* and *P. aeruginosa*.
- Plasmid transfer studies indicate that aminoglycoside resistance genes are located on transferable elements.

In order to overcome the worrisome development of increased resistance to antibiotic resistance in general and aminoglycoside resistance in particular, continued national and international surveillance programs are crucial. Norwegian medical microbiology laboratories have to ensure that methods used for detecting antibiotic resistance are validated. Hospitals have to ensure that appropriate infection control practices are implemented to limit continued spread of resistant microbes. Resources have to be made available for continued research on antibiotic resistance and development of new antimicrobial agents. Finally, prudent use of antimicrobial agents must be called for, and use of broad-spectrum antibiotics must be limited to severe infections.

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Appendix A

Table 1. Distribution of gentamicin susceptibility pattern of *E. coli* and *Klebsiella* spp included in NORM 2009. S = susceptible, I = intermediate resistant and R = resistant.

Gentamicin:	BD S	BD I/R	Oxoid S	Oxoid I/R	S	I/R (Total)
E. coli blood	409	1 / 18	917	5 / 31		
E. coli urine	257	2 / 10	837	9 / 14		
E. coli total	666	3 / 28	1754	14 / 45	2420	17 / 73 (90)
Klebsiella spp. blood	194	0 / 6	369	1 / 1		
Klebsiella spp. urine	315	1 / 2	683	2 / 4		
Klebsiella spp. total	509	1 / 8	1052	3 / 5	1561	4 / 13 (17)

Table 2. Distribution of tobramycin susceptibility pattern of *E. coli* and *Klebsiella* spp included in NORM 2009. S = susceptible, I = intermediate resistant and R = resistant.

Tobramycin:	BD S	BD I/R	Oxoid S	Oxoid I/R	S	I/R (Total)
E. coli blood	407	4 / 17	931	5 / 17		
E. coli urine	257	2 / 10	844	9 / 7		
E. coli total	664	6 / 27	1775	14 / 24	2439	20 / 51 (71)
Klebsiella spp. blood	191	2 / 7	365	5 / 1		
Klebsiella spp. urine	312	3 / 3	684	5 / 0		
Klebsiella spp. total	503	5 / 10	1049	10 / 1	1552	15 / 11 (26)

Table 3. General prescription for mastermix.

General Mastermix	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich)	500 µl
Primer F 50 pmole/ µl (Eurogentec S.A., Belgium)	8 µl
Primer R 50 pmole/ µl (Eurogentec S.A., Belgium)	8 µl
ddH_2O	384 µl

Table 4. Prescription for mastermix for 16S meth multiplex #1-PCR

16S meth multiplex #1 (rmtB, rmtC, armA) Mastermix	Amount
JumpStart™ REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich)	250 µl
rmtB F primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
rmtB R primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
rmtC F primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
rmtC R primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
armA F primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
armA R primer 50 pmole/ µl (Eurogentec S.A.)	4 µl
ddH_2O	176 µl

Table 5. PCR programs applied in this study.

PCR	Initial denaturation	Cycles	Denaturation	Primer annealing	Elongation	Final elongation
16S rDNA	95ºC / 1 min	30	95ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 7 min → 4°C → ∞
aac(6')-Ib	95ºC / 5 min	30	95ºC / 15 sec	58ºC / 15 sec	72ºC / 30 sec	72ºC / 4 min → 4°C → ∞
aac(3)-Ia	96ºC / 5 min	30	96ºC / 30 sec	55ºC / 30 sec	72ºC / 30 sec	72ºC / 4 min → 4°C → ∞
aac(3)-II	95ºC / 5 min	30	95ºC / 15 sec	58ºC / 15 sec	72ºC / 30 sec	72ºC / 4 min → 4 °C → ∞
ant(2'')-Ia	96ºC / 5 min	30	96ºC / 30 sec	58ºC / 30 sec	72ºC / 45 sec	72ºC / 7 min → 4 °C → ∞
ant(4')-IIb	96ºC / 5 min	30	96ºC / 30 sec	60ºC / 30 sec	72ºC / 30 sec	72ºC / 5 min → 4 °C → ∞
rmtA	96ºC / 5 min	30	96ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 5 min → 4 °C → ∞
rmtB/rmtC/ armA	96ºC / 5 min	30	96ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 5 min → 4 °C → ∞
rmtD	96ºC / 5 min	30	96ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 5 min → 4 °C → ∞
rmtE	96ºC / 5 min	30	96ºC / 30 sec	50ºC / 30 sec	72ºC / 1 min	72ºC / 7 min → 4 °C → ∞
npmA	96ºC / 5 min	30	96ºC / 30 sec	55ºC / 30 sec	72ºC / 1 min	72ºC / 5 min → 4 °C → ∞
MLST *E. coli*	95ºC / 2 min	30	95ºC / 1 min	54ºC / 1 min	72ºC / 2 min	72ºC / 5 min → 4 °C → ∞
MLST *P. aeruginosa*	96ºC / 1 min	30	96ºC / 1 min	55ºC / 1 min	72ºC / 1 min	72ºC / 10 min → 4 °C → ∞
Sequencing	96°C / 5min	25	96°C / 10 sec	50°C / 10 sec	60°C / 4 min	4°C → ∞

Table 6. Primers used in this study

Name	DNA sequence 5'-3'	Target site	Amplicon size (bp)	Reference from
16SrDNA-F	AGA GTT TGA TCM TGG CTC AG	16S *rDNA*	Ca 1500	Modified fD1 and fD2 (Weisburg *et al.*,1991)
16SrDNA-R	ACG GHT ACC TTG TTA CGA CTT			Modified from rP1, rP2, rP3(Weisburg *et al.*,1991)
aac(6')-lb-F	TTG CGA TGC TCT ATG AGT GGC TA	*aac*(6')-Ib	482	Park CH. AAC 2006
aac(6')-lb-R	CTC GAA TGC CTG GCG TGT TT			
aac(3)-Ia_F	ATG GGC ATC ATT CGC ACA TGT AGG	*aac*(3)-Ia	465	Hujer KM, 2006 AAC
aac(3)-Ia_R	TTA GGT GGC GGT ACT TGG GTC			
aac(3)-II-F	TGA AAC GCT GAC GGA GCC TC	*aac*(3)-II	370	Jensen et al JAC 2006:58.101-107
aac(3)-II-B	GTC GAA CAG GTA GCA CTG AG			
ant(2")-Ia_F	ATG GAC ACA ACG CAG GTC GC	*ant*(2'')-Ia	535	Hujer KM, 2006 AAC
ant(2")-Ia_R	TTA GGC CGC ATA TCG CGA CC			
ant(4')-IIb_F	TAT CTC GGC GGC GGT CGA GT	*ant*(4')-IIb	364	This study
ant(4')-IIb_R	CAC GCG GGG AAA CGC GAG AA			
rmtB-F	GCT TTC TGC GGG CGA TGT AA	*rmt*B	173	Doi Y and Arakawa Y. CID. 2007
rmtB-R	ATG CAA TGC CGC GCT CGT AT			
rmtC-F	CGA AGA AGT AAC AGC CAA AG	*rmt*C	711	Doi Y and Arakawa Y. CID. 2007
rmtC-R	ATC CCA ACA TCT CTC CCA CT			
armA-F	ATT CTG CCT ATC CTA ATT GG	*arm*A	315	Doi Y and Arakawa Y. CID. 2007
armA-R	ACC TAT ACT TTA TCG TCG TC			
rmtA-F	CTA GCG TCC ATC CTT TCC TC	*rmt*A	635	Doi Y and Arakawa Y. CID. 2007
rmtA-R	TTG CTT CCA TGC CCT TGC C			
rmtD-F	CGG CAC GCG ATT GGG AAG C	*rmt*D	401	Doi Y and Arakawa Y. CID. 2007
rmtD-R	CGG AAA CGA TGC GAC GAT			
rmtE-F	ATG AAT ATT GAT GAA ATG GTT GC	*rmt*E	818	Davis *et al*. AAC 2010
rmtE-R	TGA TTG ATT TCC TCC GTT TTT G			
npmA-F	CTC AAA GGA ACA AAG ACG G	*npm*A	640	Doi Y and Arakawa Y. CID. 2007
npmA-R	GAA ACA TGG CCA GAA ACT C			
adk-F	ATT CTG CTT GGC GCT CCG GG	*adk*	536	http://mlst.ucc.ie/mlst/dbs/Ecoli
adk-R	CCG TCA ACT TTC GCG TAT TT			
funC-F	TCA CAG GTC GCC AGC GCT TC	*fun*C	469	
funC-R	GTA CGC AGC GAA AAA GAT TC			
gyrB-F	TCG GCG ACA CGG ATG ACG GC ATC AGG CCT TCA CGC GCA TC	*gyr*B	460	
gyrB-R	ATC AGG CCT TCA CGC GCA TC			
icd-F	ATG GAA AGT AAA GTA GTT GTT CCG	*icd*	518	
icd-R	GGA CGC AGC AGG ATC TGT T			
mdh-F	ATG AAA GTC GCA GTC CTC GGC GCT GCT GGC GG	*mdh*	452	
mdh-R	TTA ACG AAC TCC TGC CCC AGA GCG TAT CTT TCT T			
purA-F	CGC GCT GAT GAA AGA GAT GA	*pur*A	478	
purA-R	CAT ACG GTA AGC CAC GCA GA			
recA-F	CGC ATT CGC TTT ACC CTG ACC	*rec*A	510	
recA-R	TCG TCG AAA TCT ACG GAC CGGA			
acsA-F	ACC TGG TGT ACG CCT CGC TGA C	*acs*A	390	http://pubmlst.org/paeruginosa
acsA-R	GAC ATA GAT GCC CTG CCC CTT GAT			
aroE-F	TGG GGC TAT GAC TGG AAA CC	*aro*E	492	
aroE-R	TAA CCC GGT TTT GTG ATT CCT ACA			
guaA-F	CGG CCT CGA CGT GTG GAT GA	*gua*A	373	

guaA-R	GAA CGC CTG GCT GGT CTT GTG GTA			
mutL-F	CCA GAT CGC CGC CGG TGA GGT G	*mut*L	442	
mutL-R	CAG GGT GCC ATA GAG GAA GTC			
nuoD-F	ACC GCC ACC CGT ACT G	*nuo*D	366	
nuoD-R	TCT CGC CCA TCT TGA CCA			
ppsA-F	GGT CGC TCG GTC AAG GTA GTGG	*pps*A	370	
ppsA-R	GGG TTC TCT TCT TCC GGC TCG TAG			
trpE-F	GCG GCC CAG GGT CGT GAG	*trp*E	443	
trpE-R	CCC GGC GCT TGT TGA TGG TT			

Table 7. General prescription for sequencing mastermix.

Sequencing Mastermix (pr. reaction)	**Amount**
Big Dye v 3.1 (Applied Biosystems)	1 µl
5x sequencing buffer (Applied Biosystems)	3 µl
Primer 3.2 pmole/ µl (Eurogentec S.A.)	1 µl
ddH_2O	14 µl

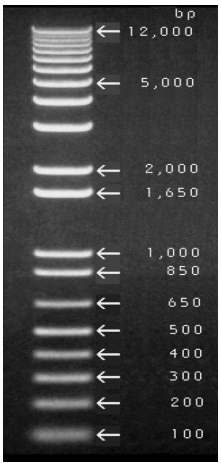

Figure 1. The sizes of the bands of the Kb+ ladder (Invitrogen)

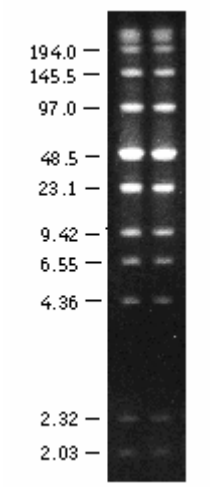

Figure 2. Low range PFG marker (New England Biolabs) labelled with the fragment sizes (kb)

Table 8. Bacterial growth media, reagents and solutions used in this study.

Green agar plates Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) ddH_2O	**Green agar plates w/ 100 mg/ml ampicillin** Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromthymol blue solution 0,2 % (Merck) Ampicillin (Bristol-Myers Squibb) ddH_2O
Freeze broth Brain Heart Infusion (Oxoid) Glycerol (Merck) ddH_2O	**Oxydase reagent** 50 mg of NNN'N'-tetrametyl-p-phenylene diamine dihydroklorid (Sigma-Aldrich) 5 ml dH_2O.
Mueller-Hinton (MH) II **agar** Mueller-Hinton II agar BBL (BD) ddH_2O	**0.85 % saline** 8.5 g Natrium chloride (Merck) ddH_2O to 1 litre
10X TBE buffer 108 g Tris Base (Sigma-Aldrich) 55 g Boric acid (Sigma-Aldrich) 40 ml 0,5 M EDTA pH 8.0 (Sigma-Aldrich) ddH_2O to 1 litre	**BHI-broth** 52 g Brain Heart Infusion (Oxoid) 15 g Bacto agar (Difco) ddH_2O to 1 litre
PIV-buffer 10 mM Tris-HCl pH 7.6 (Sigma-Aldrich) 1 M NaCl (Merck)	**0.45 % saline** 4.5 g Natrium chloride (Merck) ddH_2O to 1 litre
Lysis-buffer (for 5 plugs) 10 ml basic buffer 0.02 g sodium deoxycholate (Sigma-Aldrich) 0.5 g N-laurolsylsarcosine (Sigma-Aldrich) 0.01 g lysozyme (Sigma-Aldrich) 1 µl RNase One (10U/ µl) (Promega)	**Basic-buffer** 0,5 % Brij 58 (Merck) 1 M Tris-HCl pH 8 (Sigma-Aldrich) 0.5 M EDTA pH 8 (Sigma-Aldrich) NaCl (Merck) ddH_2O
ESP-buffer 0.5 M EDTA pH 9-9.5 (Sigma-Aldrich) 1 % N-laurolsylsarcosine (Sigma-Aldrich)	**TE-buffer** 10 mM Tris-HCl pH 8 (Sigma-Aldrich) 0.1 mM EDTA (Sigma-Aldrich)
***Xba* I mixture** Buffer 4 (10x) (New England BioLabs) BSA (100x) (New England BioLabs) *Xba* I (20U/µl) (New England BioLabs) ddH_2O	**Restriction enzyme buffer w/ bovine serum albumin (BSA)**. Buffer 4 (10x) (New England BioLabs) BSA (100x) (New England BioLabs) ddH_2O
LB-broth 25 g Difco LB Broth (BD) ddH_2O to 1 litre	**LB-agar w/ 100 µg/ml rifampicin** 40 g Difco LB Agar (BD) Rifampicin 100 µg/ml (Sigma-Aldrich) ddH_2O to 1 litre
LB-agar w/ 6 µg/ml gentamicin 40 g Difco LB Agar (BD) Gentamicin µg/ml (Sigma-Aldrich) ddH_2O to 1 litre	**LB-agar w/ 100 µg/ml rifampicin and 6 µg/ml gentamicin** 40 g Difco LB Agar (BD) Rifampicin 100 µg/ml (Sigma-Aldrich) Gentamicin µg/ml (Sigma-Aldrich) ddH_2O to 1 litre
EtBr-solution 50 µl EtBr (Sigma-Aldrich) 500 ml dH_2O	

Appendix B

Table 1. Clinical breakpoints used in this study.

	***E. coli* and *Klebsiella* spp.**		***P. aeruginosa***	
	MIC breakpoint (µg/ml)	**Zone diameter breakpoint (mm)**	**MIC breakpoint (µg/ml)**	**Zone diameter breakpoint (mm)**
Amikacin[1]	S ≤ 8 R > 16	S ≤ 8 R > 16	S ≤ 8 R > 16	S ≥ 18 R < 15
Gentamicin[1]	S ≤ 2 R > 4	S ≤ 2 R > 4	S ≤ 4 R > 4	S ≥ 15 R < 15
Tobramycin[1]	S ≤ 2 R > 4	S ≤ 2 R > 4	S ≤ 4 R > 4	S ≥ 16 R < 16
Kanamycin[2]	S ≤ 6 R > 24	S ≤ 6 R > 24		
Netilmicin[1]	S ≤ 2 R > 4	S ≤ 2 R > 4	S ≤ 4 R > 4	S ≥ 12 R < 12

[1] EUCAST breakpoints. [2] CLSI breakpoints

E. coli and *Klebsiella spp.* NORM 2009

Table 2. Prevalence of reduced susceptibility to aminoglycosides in **blood culture isolates** of ***E. coli*** collected through NORM 2009, performed by **Etest** (*n* = 1381).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) n =**	**Intermediate (I) n = (%)**	**Resistant (R) n = (%)**	**I + R n = (%)**
Amikacin[1]	S ≤ 8 R > 16	1373	7 (0.5)	1 (0.1)	8 (0.6)
Gentamicin[1]	S ≤ 2 R > 4	1329	0	52 (3.8)	52 (3.8)
Tobramycin[1]	S ≤ 2 R > 4	1324	3 (0.2)	54 (3.9)	57 (4.1)
Kanamycin[2]	S ≤ 6 R > 24	1341	21 (1.5)	19 (1.4)	40 (2.9)
Netilmicin[1]	S ≤ 2 R > 4	1327	11 (0.8)	43 (3.1)	54 (3.9)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 3. Prevalence of reduced susceptibility to aminoglycosides in **urinary tract isolates** of ***E.coli*** collected through NORM 2009, performed by **Etest** (*n* = 1129).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) *n* =**	**Intermediate (I) n = (%)**	**Resistant (R) n = (%)**	**I + R n = (%)**
Amikacin[1]	S ≤ 8 R > 16	1128	1 (0.1)	0	1 (0.1)
Gentamicin[1]	S ≤ 2 R > 4	1101	0	28 (2.5)	28 (2.5)
Tobramycin[1]	S ≤ 2 R > 4	1100	2 (0.2)	27 (2.4)	29 (2.6)
Kanamycin[2]	S ≤ 6 R > 24	1109	14 (1.2)	6 (0.5)	20 (1.7)
Netilmicin[1]	S ≤ 2 R > 4	1100	9 (0.8)	20 (1.8)	29 (2.6)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 4. Prevalence of reduced susceptibility to aminoglycosides in **blood culture isolates** of ***Klebsiella* spp.** collected through NORM 2009, performed by **Etest** (*n* = 571).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) *n* =**	**Intermediate (I) n = (%)**	**Resistant (R) n = (%)**	**I + R n = (%)**
Amikacin[1]	S ≤ 8 R > 16	569	1 (0.2)	1 (0.2)	2 (0.4)
Gentamicin[1]	S ≤ 2 R > 4	563	0	8 (1.4)	8 (1.4)
Tobramycin[1]	S ≤ 2 R > 4	560	2 (0.4)	9 (1.6)	11 (2)
Kanamycin[2]	S ≤ 6 R > 24	563	3 (0.5)	5 (0.9)	8 (1.4)
Netilmicin[1]	S ≤ 2 R > 4	560	6 (1.1)	5 (0.9)	11 (2)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 5. Prevalence of reduced susceptibility to aminoglycosides in **urinary tract isolates** of ***Klebsiella* spp**. collected through NORM 2009, performed by **Etest** (*n* = 1007).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S)** ***n* =**	**Intermediate (I)** ***n* =**	**Resistant (R)** ***n* =**	**I + R** **n = (%)**
Amikacin[1]	S ≤ 8 R > 16	1005	0	2 (0.2)	2 (0.2)
Gentamicin[1]	S ≤ 2 R > 4	1000	0	7 (0.7)	7 (0.7)
Tobramycin[1]	S ≤ 2 R > 4	997	4 (0.4)	6 (0.6)	10 (1)
Kanamycin[2]	S ≤ 6 R > 24	1003	2 (0.2)	2 (0.2)	4 (0.4)
Netilmicin[1]	S ≤ 2 R > 4	997	8 (0.8)	2 (0.2)	10 (1)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 6. Prevalence of reduced susceptibility to aminoglycosides in **blood culture isolates** of ***E. coli*** collected through NORM 2009, performed by **disk diffusion** (*n* = 1381).

		Number of isolates			
	EUCAST Breakpoints (mm)	**Susceptible (S)** ***n* =**	**Intermediate (I)** ***n* = (%)**	**Resistant (R)** ***n* = (%)**	**I + R** ***n* = (%)**
Amikacin[1]	S ≥ 16 R < 13	1378	2 (0.1)	1 (0.1)	3 (0.2)
Gentamicin[1]	S ≥ 17 R < 14	1329	1 (0.1)	51 (3.7)	52 (3.8)
Tobramycin[1]	S ≥ 16 R < 13	1326	13 (0.9)	42 (3)	55 (3.9)
Kanamycin[2]	S ≥ 18 R < 14	1344	22 (1.6)	15 (1.1)	37 (2.7)
Netilmicin[1]	S ≥ 15 R < 12	1335	22 (1.6)	24 (1.7)	46 (3.3)
Streptomycin[2]	S ≥ 15 R < 12	1326	13 (0.9)	42 (3)	55 (3.9)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 7. Prevalence of reduced susceptibility to aminoglycosides in **urinary tract isolates** of ***E. coli*** collected through NORM 2009, performed by **disk diffusion** (*n* = 1129).

		Number of isolates			
	EUCAST Breakpoints (mm)	**Susceptible (S)** ***n* =**	**Intermediate (I)** ***n* = (%)**	**Resistant (R)** ***n* = (%)**	**I + R** ***n* = (%)**
Amikacin[1]	S ≥ 16 R < 13	1128	1 (0.1)	0	1 (0.1)
Gentamicin[1]	S ≥ 17 R < 14	1101	1 (0.1)	27 (2.4)	28 (2.5)
Tobramycin[1]	S ≥ 16 R < 13	1102	8 (0.7)	19 (1.7)	27 (2.4)
Kanamycin[2]	S ≥ 18 R < 14	1103	17 (1.5)	9 (0.8)	26 (2.3)
Netilmicin[1]	S ≥ 15 R < 12	1103	16 (1.4)	10 (0.9)	26 (2.3)
Streptomycin[2]	S ≥ 15 R < 12	1102	8 (0.7)	19 (1.7)	27 (2.4)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 8. Prevalence of reduced susceptibility to aminoglycosides in **blood culture isolates** of ***Klebsiella* spp**. collected through NORM 2009, performed by **disk diffusion** (*n* = 571).

		Number of isolates			
	EUCAST Breakpoints (mm)	**Susceptible (S)** ***n* =**	**Intermediate (I)** ***n* = (%)**	**Resistant (R)** ***n* = (%)**	**I + R** ***n* = (%)**
Amikacin[1]	S ≥ 16 R < 13	570	1 (0.2)	0	1 (0.2)
Gentamicin[1]	S ≥ 17 R < 14	563	0	8 (1.4)	8 (1.4)
Tobramycin[1]	S ≥ 16 R < 13	562	3 (0.5)	6 (1.1)	9 (1.6)
Kanamycin[2]	S ≥ 18 R < 14	562	1 (0.2)	8 (1.4)	9 (1.6)
Netilmicin[1]	S ≥ 15 R < 12	564	4 (0.7)	3 (0.5)	7 (1.2)
Streptomycin[2]	S ≥ 15 R < 12	567	0	4 (0.7)	4 (0.7)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 9. Prevalence of reduced susceptibility to aminoglycosides in **urinary tract isolates** of ***Klebsiella* spp**. collected through NORM 2009, performed by **disk diffusion** (*n* = 1007).

		Number of isolates			
	EUCAST Breakpoints (mm)	**Susceptible (S) *n* =**	**Intermediate (I) *n* = (%)**	**Resistant (R) *n* = (%)**	**I + R *n* = (%)**
Amikacin[1]	S ≥ 16 R < 13	1005	0	2 (0.2)	2 (0.2)
Gentamicin[1]	S ≥ 17 R < 14	1000	0	7 (0.7)	7 (0.7)
Tobramycin[1]	S ≥ 16 R < 13	999	4 (0.4)	4 (0.4)	8 (0.8)
Kanamycin[2]	S ≥ 18 R < 14	1003	2 (0.2)	2 (0.2)	4 (0.4)
Netilmicin[1]	S ≥ 15 R < 12	1004	1 (0.1)	2 (0.2)	3 (0.3)
Streptomycin[2]	S ≥ 15 R < 12	999	3 (0.3)	5 (0.5)	8 (0,8)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Collection 2: ESBL-positive *E. coli* and *Klebsiella* spp. NORM 2007-2008 (*n* = 68)

Table 10. Prevalence of reduced susceptibility to aminoglycosides ESBL-positive *E. coli* and *Klebsiella* spp. in collection 2, performed by **Etest** (*n* = 68).

		Number of isolates		
	EUCAST Breakpoints (µg/ml)	**Susceptible (S) *n* = (%)**	**Intermediate (I) *n* = (%)**	**Resistant (R) *n* = (%)**
Amikacin[1]	S ≤ 8 R > 16	63 (93)	4 (6)	1 (1)
Gentamicin[1]	S ≤ 2 R > 4	38 (56)	1 (1)	29 (43)
Tobramycin[1]	S ≤ 2 R > 4	30 (44)	0	38 (56)
Kanamycin[2]	S ≤ 6 R > 24	29 (43)	15 (22)	24 (35)
Netilmicin[1]	S ≤ 2 R > 4	30 (44)	1 (1)	37 (55)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 11. Prevalence of reduced susceptibility to aminoglycosides ESBL-positive *E. coli* and *Klebsiella* spp. in collection 2, performed by **disk diffusion** (*n* = 68).

		Number of isolates		
	EUCAST Breakpoints (mm)	**Susceptible (S) *n* = (%)**	**Intermediate (I) *n* = (%)**	**Resistant (R) *n* = (%)**
Amikacin[1]	S ≥ 16 R < 13	67 (99)	1 (1)	0
Gentamicin[1]	S ≥ 17 R < 14	39 (57)	0	29 (43)
Tobramycin[1]	S ≥ 16 R < 13	30 (44)	10 (15)	28 (41)
Kanamycin[2]	S ≥ 18 R < 14	33 (49)	7 (10)	28 (41)
Netilmicin[1]	S ≥ 15 R < 12	35 (51)	23 (34)	10 (15)
Streptomycin[2]	S ≥ 15 R < 12	21 (31)	12 (18)	35 (51)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 12. The prevalence of reduced susceptibility to aminoglycosides in ESBL-positive *E. coli* in collection 2, performed by **Etest** (*n* = 60).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S)** ***n*** **= (%)**	**Intermediate (I)** ***n*** **= (%)**	**Resistant (R)** ***n*** **= (%)**	**I + R** ***n*** **= (%)**
Amikacin[1]	S ≤ 8 R > 16	55 (92)	4 (7)	1 (1)	5 (8)
Gentamicin[1]	S ≤ 2 R > 4	33 (55)	1 (1)	26 (44)	27 (45)
Tobramycin[1]	S ≤ 2 R > 4	26 (43)	0	34 (57)	34 (57)
Kanamycin[2]	S ≤ 6 R > 24	24 (40)	13 (22)	23 (38)	46 (60)
Netilmicin[1]	S ≤ 2 R > 4	26 (43)	1 (1)	33 (56)	34 (57)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Table 13. The prevalence of reduced susceptibility to aminoglycosides in ESBL-positive *Klebsiella* spp. in collection 2, performed by **Etest** (*n* = 8).

		Number of isolates			
	EUCAST Breakpoints (µg/ml)	**Susceptible (S)** ***n*** **= (%)**	**Intermediate (I)** ***n*** **= (%)**	**Resistant (R)** ***n*** **= (%)**	**I + R** ***n*** **= (%)**
Amikacin[1]	S ≤ 8 R > 16	8 (100)	0	0	0
Gentamicin[1]	S ≤ 2 R > 4	5	0	3	3 (38)
Tobramycin[1]	S ≤ 2 R > 4	4 (50)	0	4 (50)	4 (50)
Kanamycin[2]	S ≤ 6 R > 24	5 (62)	2 (25)	1 (13)	3 (38)
Netilmicin[1]	S ≤ 2 R > 4	4 (50)	0	4 (50)	4 (50)

[1] EUCAST breakpoints. [2] CLSI breakpoints

Collection 3: Carbapenem non-susceptible *Pseudomonas aeruginosa* from K-res 2007-2009 collection (*n* = 77)

Table 14. The prevalence of reduced susceptibility to aminoglycosides in carbapenem non-susceptible *P. aeruginosa* in collection 3, performed by **Etest** (*n* = 77).

		Number of isolates		
	EUCAST Breakpoints (µg/ml)	**Susceptible (S)** ***n*** **= (%)**	**Intermediate (I)** ***n*** **= (%)**	**Resistant (R)** ***n*** **= (%)**
Amikacin[1]	S ≤ 8 R > 16	54 (70)	8 (10)	15 (20)
Gentamicin[1]	S ≤ 4 R > 4	48 (62)	0	29 (38)
Tobramycin[1]	S ≤ 4 R > 4	63 (82)	0	14 (18)
Netilmicin[1]	S ≤ 4 R > 4	41 (53)	0	36 (47)

[1] EUCAST breakpoints

Table 15. The prevalence of reduced susceptibility to aminoglycosides in carbapenem non-susceptible *P. aeruginosa* in collection 3, performed by **disk diffusion** (*n* = 77).

		Number of isolates		
	EUCAST Breakpoints (mm)	**Susceptible (S)** ***n*** **= (%)**	**Intermediate (I)** ***n*** **= (%)**	**Resistant (R)** ***n*** **= (%)**
Amikacin[1]	S ≥ 18 R < 15	59 (77)	4 (5)	14 (18)
Gentamicin[1]	S ≥ 15 R < 15	64 (83)	0	13 (17)
Tobramycin[1]	S ≥ 16 R < 16	64 (83)	0	13 (17)
Netilmicin[1]	S ≥ 12 R < 12	61 (79)	0	16 (21)

[1] EUCAST breakpoints

Prevalence of clinical important aminoglycoside modifying enzymes (AMEs)

Collection 1: *E. coli* and *Klebsiella* spp. from NORM 2009

Table 16. The prevalence of AMEs detected in *E. coli* and *Klebsiella* spp. with reduced susceptibility to **aminoglycosides collected through NORM 2009 in collection 1.**

	***aac*(6')-Ib** ***n* = (%)**	***aac*(3)-II** ***n* = (%)**	***aac*(3)-Ia** ***n* = (%)**	***ant*(2'')-Ia** ***n* = (%)**	***ant*(4')-IIb** ***n* = (%)**	***aac*(6')-Ib + *aac*(3)-II** ***n* = (%)**
Total *n* = 107	30 (28)	84 (79)	0	3 (3)	0	17 (16)
E. coli n = 86	21 (24)	70 (81)	0	3 (3)	0	14 (16)
Klebsiella spp. *n* = 21	9 (43)	14 (67)	0	0	0	3 (14)

Collection 2: ESBL-positive *E. coli* and *Klebsiella* spp. NORM 2007-2008

Table 17. The prevalence of AMEs detected in ESBL positive *E. coli* and *Klebsiella* spp. with reduced susceptibility to aminoglycosides collected through NORM 2007-08 in collection 2.

	***aac*(6')-Ib** ***n* = (%)**	***aac*(3)-II** ***n* = (%)**	***aac*(3)-Ia** ***n* = (%)**	***ant*(2'')-Ia** ***n* = (%)**	***ant*(4')-IIb** ***n* = (%)**	***aac*(6')-Ib + *aac*(3)-II** ***n* = (%)**
Total *n* = 41	23 (56)	29 (71)	0	0	0	14 (34)
E. coli n = 37	22 (59)	26 (70)	0	0	0	14 (38)
Klebsiella spp. *n* = 4	1 (25)	3 (75)	0	0	0	0

Table 18. Transconjugation frequencies.

	Transconjugation frequency[1]	
Donor clinical isolate	Transconjugant per donor	Transconjugant per recipient
K64-71 (*E. coli*)	7×10^{-3}	6×10^{-3}
K65-38 (*E. coli*)	1.4×10^{-2}	7×10^{-3}

[1] Transconjugation frequencies were calculated by dividing the number of transconjugants by the number of donors or by the number of recipients

Summary of comparison of phenotypic methods

Collection 1: *E. coli* and *Klebsiella* spp. from NORM 2009 (*n* = 137)

Tobramycin:

	Very major error	Major error	Minor error	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	
	1 (1 reproduced)		24	
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	14 (7 reproduced) Retesting: 2 R/S – R/R 2 R/S – I/I 1 R/S – R/I 2 R/S – I/I		17	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK2 S – Disk R	VITEK2 R – Disk I	VITEK2 S – Disk I
		1	5	15

Gentamicin:

	Very major error	Major error	Minor error	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	
	0		2	
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	1 (0 reproduced) Retesting: R/S – R/I		0	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK2 S – Disk R	VITEK2 R – Disk I	VITEK2 S – Disk I
	0	0	0	2

Amikacin:

	Very major error	Major error	Minor error	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	Etest I – Disk S
	0		1	7

Collection 2: ESBL-positive *E. coli* and *Klebsiella* spp. NORM 2007-2008 (*n* = 68)

Tobramycin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	
	0		10	
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	0		4	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK2 S – Disk R	VITEK2 R – Disk I	VITEK2 S – Disk I
	0	1	7	0

Gentamicin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	Etest I – Disk S
	0		0	1
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	0		0	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK2 S – Disk R	VITEK2 R – Disk I	VITEK2 I – Disk S
	0	0	0	1

Amikacin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	Etest I – Disk S
	0		1	4

Collection 3: Carbapenem non-susceptible *Pseudomonas aeruginosa* from K-res 2007-2009 collection (*n* = 77)

Tobramycin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	
	1 (not reproduced)		0	
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	0		0	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK2 S – Disk R	VITEK2 R – Disk I	VITEK2 S – Disk I
	1 (1 reproduced)	0	0	0

Gentamicin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S		Etest R – Disk I	
	16 (9 reproduced) Retesting: 6 R/S – S/S 1 R/S – R/R		0	
Etest vs VITEK2	Etest R – VITEK2 S		Etest R – VITEK2 I	
	12 (7 reproduced) Retesting: 5 R/S – S/S		0	
VITEK2 vs disk	Disk S – VITEK2 R	VITEK S – Disk R	VITEK2 R – Disk I	VITEK2 S – Disk I
	4 (4 reproduced)	0	0	0

Amikacin:

	Very major error	**Major error**	**Minor error**	
Etest vs disk	Etest R – Disk S	Etest S – Disk R	Etest R – Disk I	Etest I – Disk S
	1 (0 reproduced) 1 R/S – I/S	1	1	5

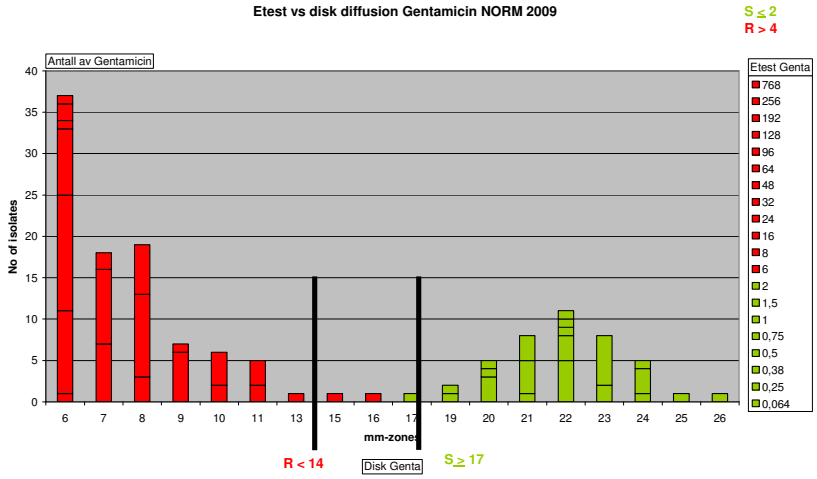

Figure 1. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 1

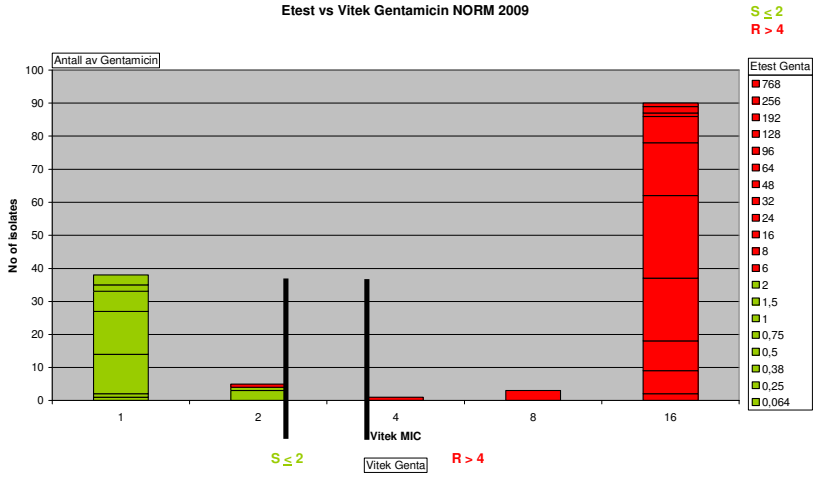

Figure 2. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to gentamicin in collection 1.

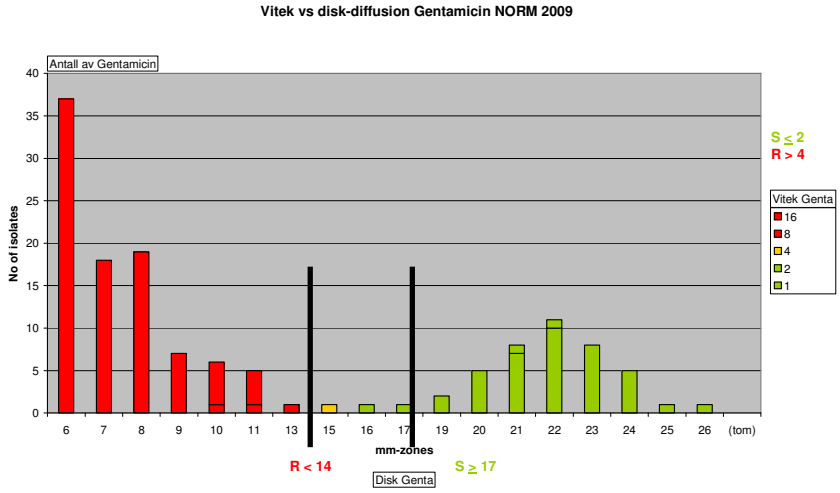

Figure 3. Comparison of VITEK2 AST versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 1

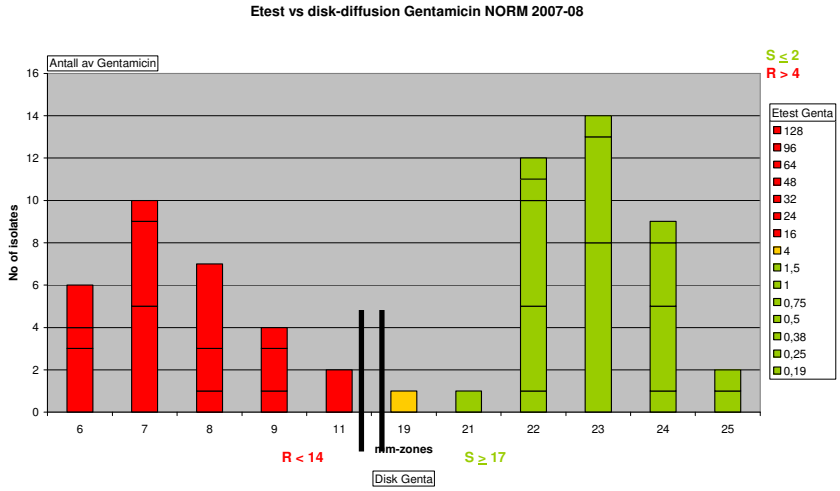

Figure 4. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 2.

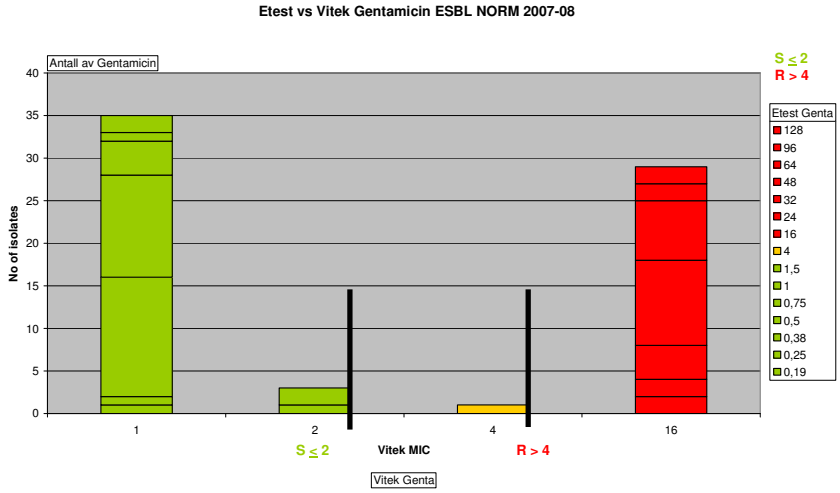

Figure 5. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to gentamicin in collection 2.

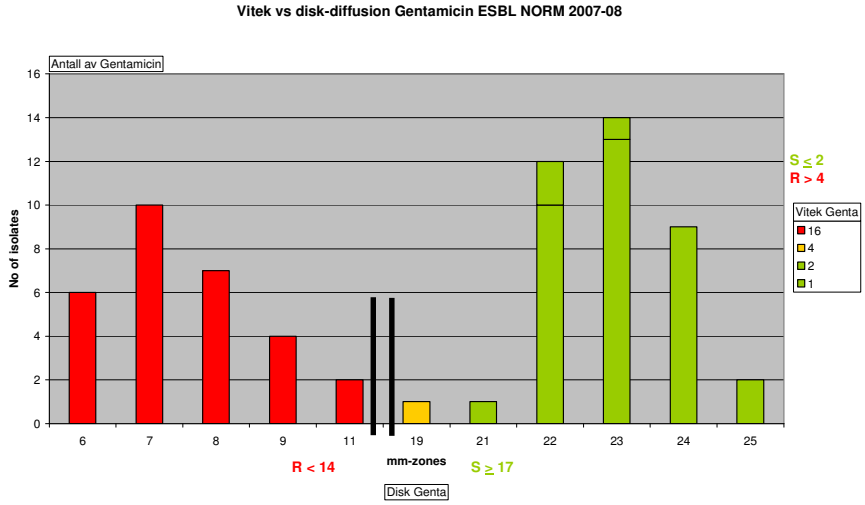

Figure 6. Comparison of VITEK2 AST versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 2.

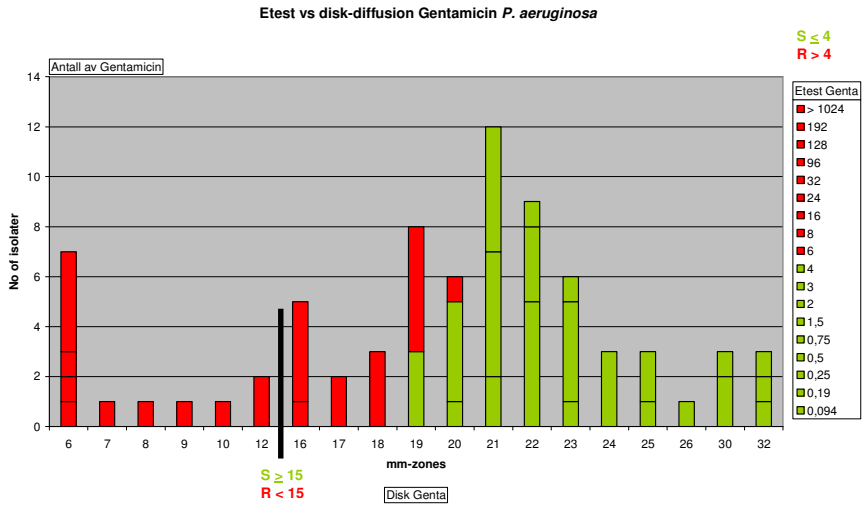

Figure 7. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to gentamicin in collection 3.

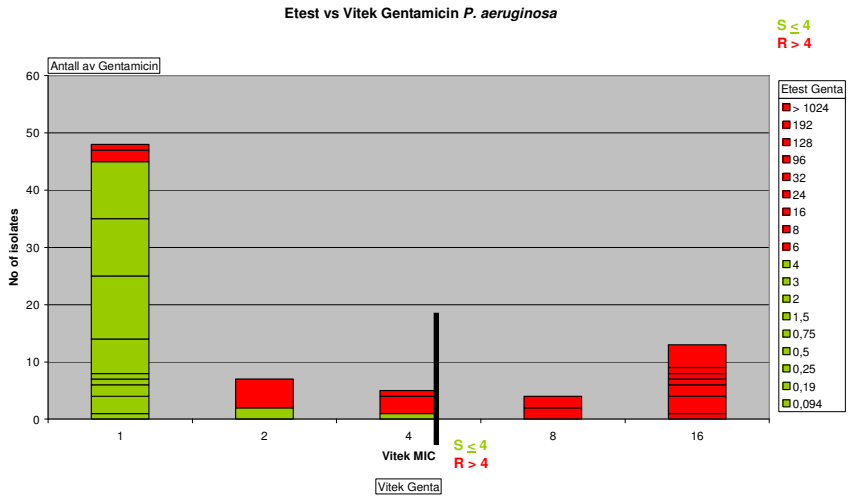

Figure 8. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to gentamicin in collection 3.

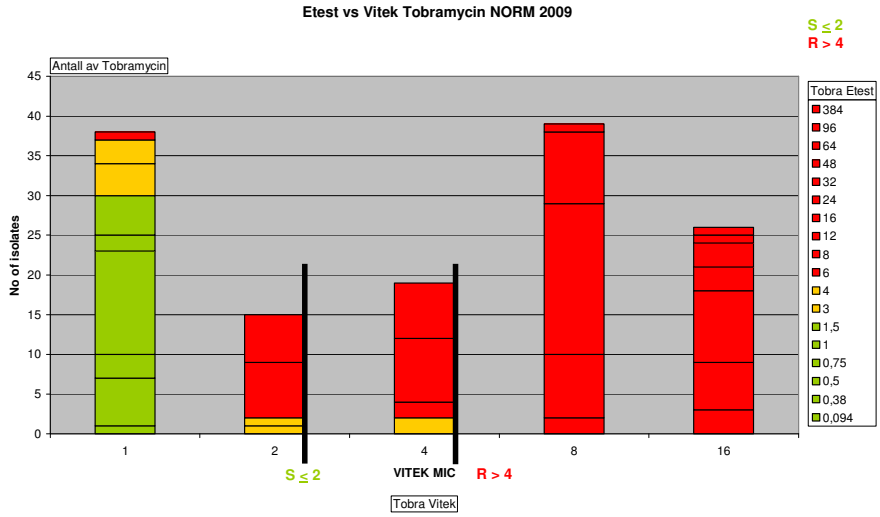

Figure 9. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to tobramycin in collection 1.

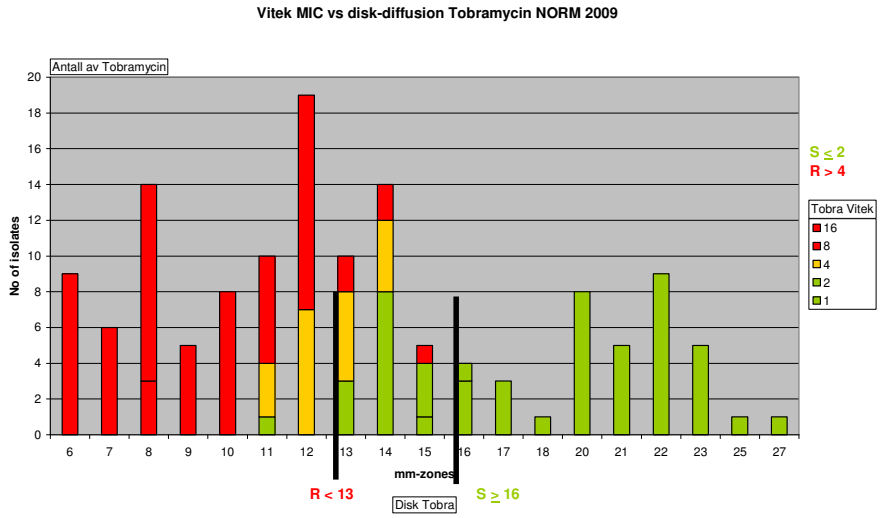

Figure 10. Comparison of VITEK2 AST versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 1.

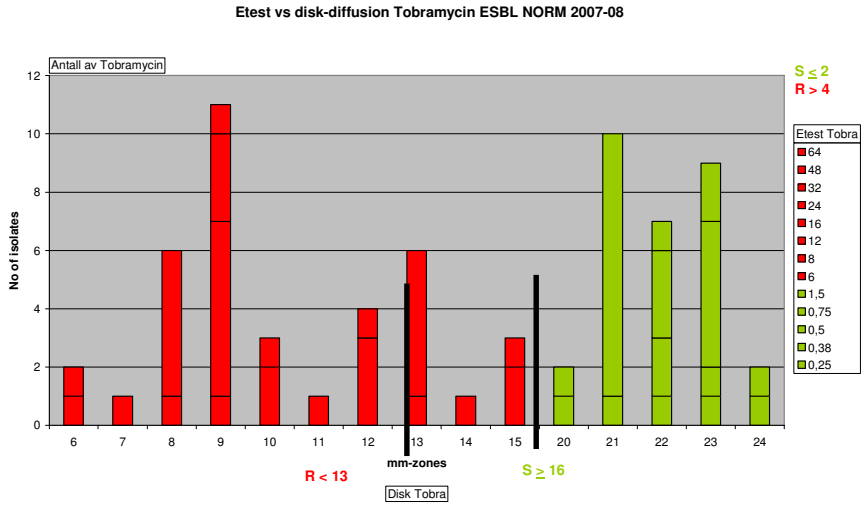

Figure 11. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 2.

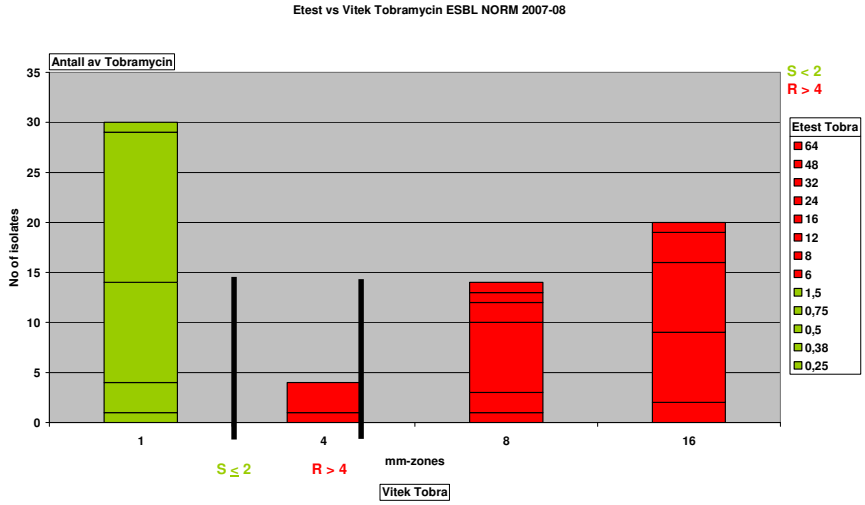

Figure 12. Comparison of Etest versus VITEK2 results in detecting reduced susceptibility to tobramycin in collection 2.

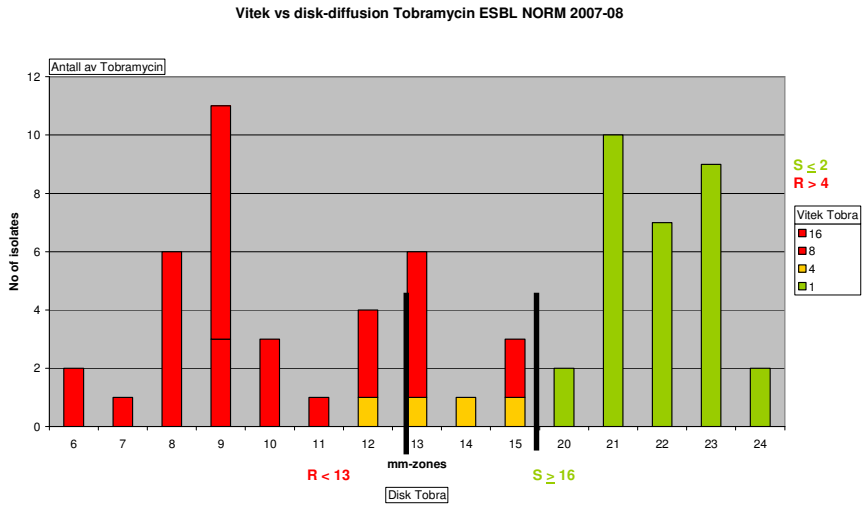

Figure 13. Comparison of VITEK2 versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 2.

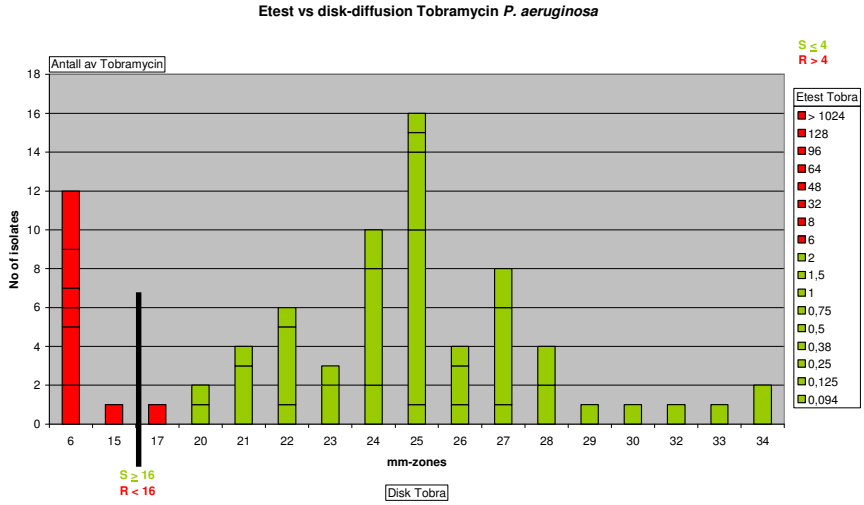

Figure 14. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 3.

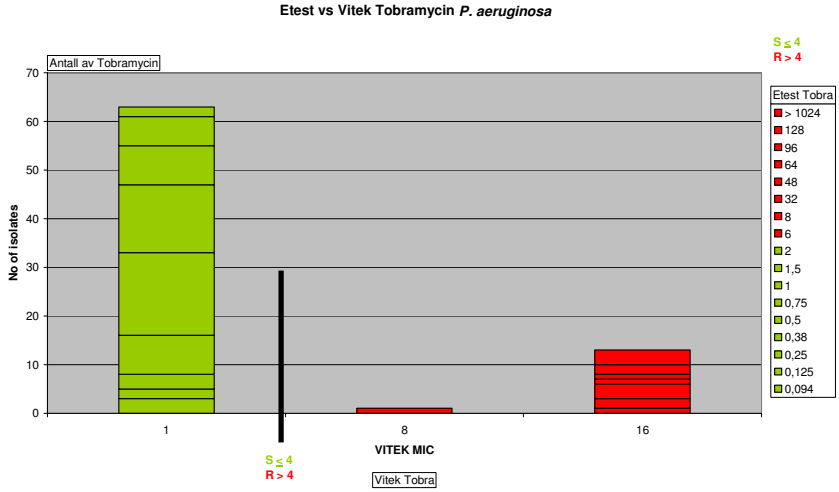

Figure 15. Comparison of Etest versus VITEK2 AST results in detecting reduced susceptibility to tobramycin in collection 3.

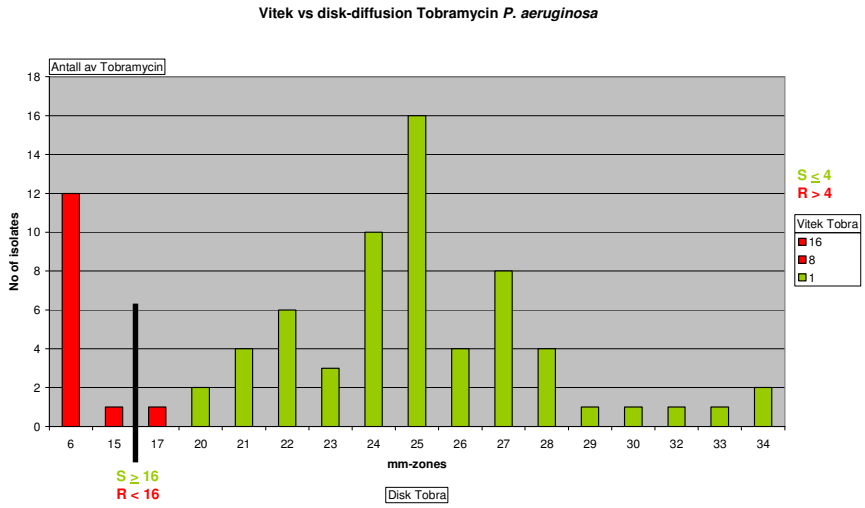

Figure 16. Comparison of VITEK2 AST versus disk diffusion results in detecting reduced susceptibility to tobramycin in collection 3.

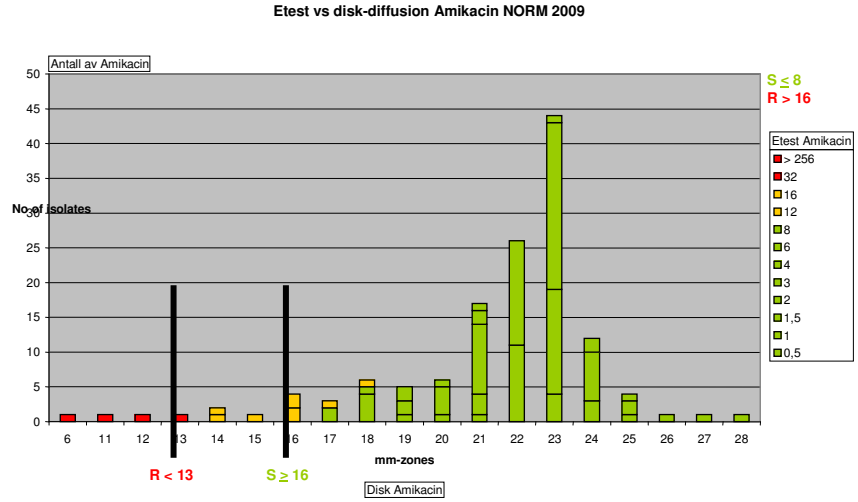

Figure 17. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to amikacin in collection 1.

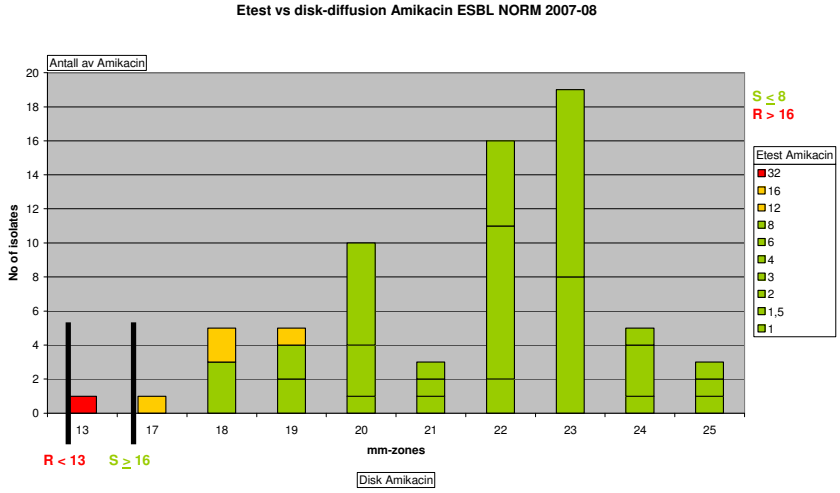

Figure 18. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to amikacin in collection 2.

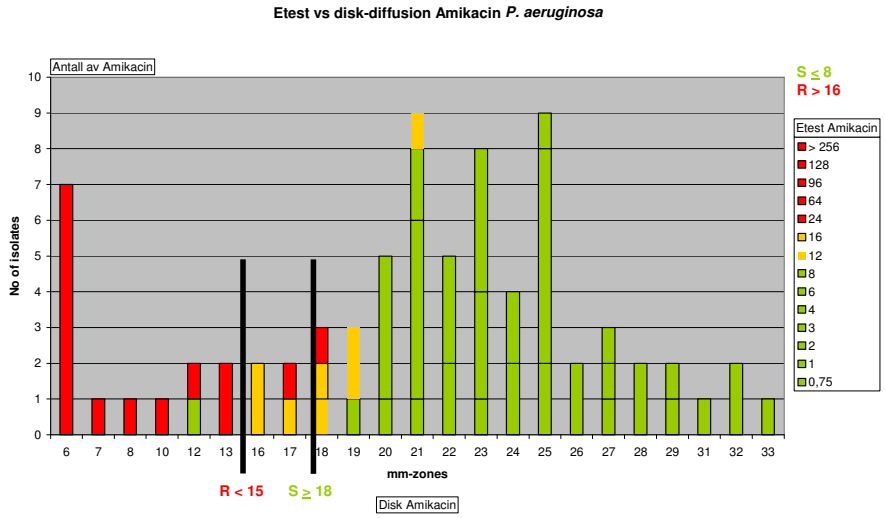

Figure 19. Comparison of Etest versus disk diffusion results in detecting reduced susceptibility to amikacin in collection 3.