# **Aminoglycoside resistance mechanisms in *Enterobacteriaceae.***

An experimental study based on human clinical isolates from western Norway.

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# Summary

*Escherichia coli(E.coli)* belongs to the *Enterobacteriaceae* family of gram negative rods. It is the most common cause for bacterial sepsis. According to NORM (Norwegian system for surveillance of antibiotic resistance in microbes); *E.coli* is the main pathogen found in blood samples from humans submitted to Norwegian microbial laboratories (page 49 NORM report 2008)(1). *E.coli* is also the most common cause of human urinary tract infections (The Journal of the Norwegian Medical Association) (2).

Empiric treatment for sepsis and urosepsis in Norway is betalactam antibiotics in combination with aminoglycosides(3). In case of allergy, patients with urosepsis are treated with quinolones and aminoglycosides(4). There is concern related to recent years development of resistance against gentamicin (aminoglycoside) and ciprofloxacin (quinolone) among *E.coli* strains (page 52 NORM report 2008) (1).

This work was initiated as a first step to identify the most common mechanisms for aminoglycoside resistance in *Enterobacteriaceae* collected in western Norway. The focus for this work was to characterize antibiotic resistance and detect the presence of the genes encoding two aminoglycoside modifying enzymes. The genes chosen encodes two enzymes from the group aminoglycoside acetyltransferases (AAC`s); *aac(6`)-Ib* and *aac(3)-IIc* (*aacC2*).

The use of ciprofloxacin has increased markedly during the past few years. Recent studies have reported a variant of AAC(6`)-Ib characterized by two amino acid changes, Trp102Arg and Asp179Tyr. This variant called AAC(6`)-Ib-cr inflicts ciprofloxacin resistance in addition to aminoglycoside resistance. We suspected that increased use of ciprofloxacin could cause selection of isolates resistant to aminoglycosides by selecting the isolates that possess AAC(6`)-Ib-cr. We decided to examine the prevalence of *aac(6`)-Ib-cr* in isolates of *Enterobaceriaceae* resistant aminoglycosides, in order to provide either indirect support or rejection of this hypothesis.

# List of abbreviations

| | |
|---|---|
| A | Adenine |
| AAC | Aminoglycoside acetyltransferases |
| AAC(6`)-Ib | Aminoglycoside acetyltransferase(6`)-Ib |
| AAC(6`)-Ib-cr | Aminoglycoside acetyltransferase(6`)-Ib-ciprofloxacin variant |
| *aac(6`)-Ib* | The gene encoding AAC(6`)-Ib |
| *aac(6`)-Ib-cr* | The gene encoding AAC(6`)-Ib-cr |
| *AAC(3)-II* | Aminoglycoside acetyltransferase(3`)-II |
| *aac(3)-II* | The gene encoding AAC(3`)-II |
| AFA | Norwegian workgroup for questions related to antibiotics |
| AK | Amikacin |
| AmpC | Genes encoding extended spectrum beta lactamases |
| Arg | Arginine |
| Asp | Aspartic acid |
| BLAST | Basic Local Alignment tool |
| C | Cytosine |
| CTX-M | Genes encoding beta lactamases with extended spectra |
| DDD | Defined Daily Dose |
| DNA | Deoxyribonucleic acid |
| E.coli | *Eschericia coli* |
| ESBL | Extended spectrum beta-lactamase |
| EUCAST | The European comity on antibiotic susceptibility testing |
| G | Guanine |
| GM | Gentamicin |
| *gyrA* | Gene encoding the enzyme; DNA gyrase |
| *gyrB* | Gene encoding the enzyme; DNA gyrase |
| I | Intermediate resistant |
| K-res | Norwegian center for expertise for antibiotic susceptibility testing |
| KM | Kanamycin |
| MIC | Minimum inhibitory concentration |
| NC | Netilmicin |
| NCBI | Genome database of the National Center for Biotechnology Information |
| NORM | Norwegian system for surveillance of antibiotic resistance in microbes (Norsk overvåkningssystem for antibiotikaresistens hos mikrober) |
| NorPD | Norwegian prescription data base |
| *parC* | Gene encoding the enzyme; topoisomerase IV |
| *pare* | Gene encoding the enzyme; topoisomerase IV |
| PCR | Polymerase chain reaction |
| R | Resistant |
| RNA | Ribonucleic acid |
| S | Sensitive / Susceptible |
| SHV | Genes encoding beta lactamases ( some with extended spectra) |
| SM | Streptomycin |
| SNP | Single Nucleotide Polymorphism |
| *ssp* | Species |
| T | Thymine |
| TEM | Genes encoding beta lactamases ( some with extended spectra) |
| TM | Tobramycin |
| Trp | Tryphtophane |
| Tyr | Tyrosine |
| Qnr | Plasmid mediated quinolone resistance genes |

# Glossary

| | |
|---|---|
| Agar | A gelatinous material derived from certain marine algae. It is used as a base for bacterial culture media |
| Antimetabolites | A substance bearing a close structural resemblance to one required for normal physiological functioning, and exerting its effect by interfering with the utilization of the essential metabolite. |
| Chromosome | The genetic element in the cell that contains genes whose products is involved in essential cellular functions. |
| Efflux pumps | An active transport system for the removal of some antibiotics |
| Eukaryotic cell | Structurally large and complex cells containing membrane-enclosed organelles. Algae, fungi and protozoa, plants and animals are eukaryotes. |
| Genome | Total complement of genes in a cell; includes chromosomes and other genetic elements like plasmids, transposable elements etc. |
| Gram negative cell | A prokaryotic cell whose cell wall contains relatively little peptidoglycan but has an outer membrane composed of lipopolysaccharide, lipoprotein, and other complex macromolecules |
| Integrons | A set of genes and other DNA sequences that enable plasmids to capture genes from bacteriophages and other plasmids |
| Lethality | Causing death, Fetality |
| Metabolites | A substance produced by metabolism |
| Moiety | Portion, half |
| Morbidity | Incidence and prevalence of disease in a population, including both fatal and non fatal cases |
| Mutation | An inheritable change in the base sequence of the genome of an organism |
| Nephrotoxicity | Toxic to the kidneys |
| Nucleic acid | A polymer of nucleotides |
| Ototoxicity | Damaging to the hearing ability |
| Parenterally | Administered not through mouth. (intra venous, sub cutan etc.) |
| Pathogen | An organism, usually microorganism, that causes disease |
| Permeability | Being permeable, being porous, substances can sieve thorugh |
| Pharmacokinetics | The study of the process by which a drug is absorbed, distributed, metabolized, and eliminated by the body |
| Pharmacodynamics | The study of the action or effects of drugs on living organisms |
| Phenotype | The observable characteristics of an organism |
| Plasmid | Genetic elements that exist and replicate separately from the chromosome. They contain genes beneficial for the organism, but not genes for essential cellular functions. |
| Prevalence | Measurement of frequnecy |
| Prokaryotic | Structurally “simpler” cells lacking membrane-enclosed organelles. Bacteria and Arcaha are prokaryotes. |
| Proofreading | The 3`-5`-exonuclease activity possessed by some DNA polymerases which enables the enzyme to replace a misincorporated nucleotide |
| Ribosome | “The cell`s protein-synthesizing factory” |
| Rod | Describes the shape of the bacteria (curvy comma shaped) |
| Sepsis / Septicaemia | Infection of the blood stream by bacteria |
| Single Nucleotide Polymorphism | A point mutation that is carried by some individuals of a population |
| Template | The polynucleotide that is copied during a strand synthesis reaction catalyzed by DNA or RNA polymerase |
| Translation | The synthesis of protein using the genetic information in messenger RNA as a template |
| Transposon | A type of transposable element that, in addition to genes involved in transposition, carries other genes; often genes conferring selectable phenotypes such as antibiotic resistance. |
| Urosepsis | Sepsis secondary to urinary tract infection |

# 1. INTRODUCTION

## 1.1 Background

The discovery of antimicrobial agents has improved public health significantly. When the first sulphonamides came on the market in the middle of the 1930`s, they started a new era in the treatment of infectious diseases such as pneumonia, urinary tract infections and meningitis. These infectious diseases were previously associated with high lethality and long lasting morbidity. The most significant breakthrough in the development of antibiotics was probably the discovery and manufacture of Penicillin. Many different types of antimicrobials were marketed in the years to follow. Unfortunately not many years passed before antimicrobial resistance developed as a significant clinical problem. Resistance leads to loss off efficacy of antimicrobial agents. The continued development of new or improved agents has allowed us to maintain efficient antimicrobial therapy, but antibiotic resistance remains a challenge. Development of new agents is associated with increasing cost and effort(5). With few new agents coming on the marked, attenuating antibiotic resistance is of vital importance.

Use and misuse of antimicrobials is the main cause of antibiotic resistance development in microbes. New measures like prescription guidelines have been published to ensure correct use of antimicrobial agents. Surveillance of both antimicrobial resistance development and antimicrobial usage give valuable information for the prescription guidelines. Such surveillance data also makes it possible to evaluate any measures made towards the correct use of antimicrobial agents. In addition, this information can be used to increase our understanding on the relationship between the use of antimicrobial agents and resistance development.

Surveillance of antimicrobial resistant strains in Norway is performed by NORM (Norwegian system for surveillance of antibiotic resistance in microbes) since 1999. Data published in the NORM report of 2008 shows that *E.coli,* which belong to the family of *Enterobacteriaceae* is the predominant species isolated from blood cultures. (1) (figure 30, page 50) *E.coli* is also the predominant species causing urinary tract infections (The Journal of the Norwegian Medical Association) (2). *E.coli* has been reported to show a steadily increasing gentamicin resistance during 2004-2007 though a slight decrease was reported in 2008.There is also a contemporary increase in resistance against ciprofloxacin (fluoroquinolone) from 2004-2008 among *E.coli(1).*

In Norway, a combination of a penicillin and an aminoglycoside is the emperical treatment for septicaemia and if urosepsis (sepsis secondary to urinary tract infections) is suspected, the initial therapy is with either ciprofloxacin or cephalosporin, until the microorganism has been identified and possible until antimicrobial sensitivity is determined(6). Increasing resistance of *E.coli* for aminoglycosides or ciprofloxacin impose a serious threat to this standard treatment. The cause of the increasing resistance is disputed as few studies on this topic have been published. Recent reports suggest an association between the use of ciprofloxacin and development of aminoglycoside resistance, based on the identification of a variant of the aminoglycoside modifying enzyme AAC(6`)-Ib termed AAC(6`)-Ib-cr, which confers resistance to both aminoglycosides and fluoroquinolones.

These studies additionally report correlations between the presence of the gene for this enzyme and the presence of other plasmid mediated resistance genes, thus conferring multi-resistance.

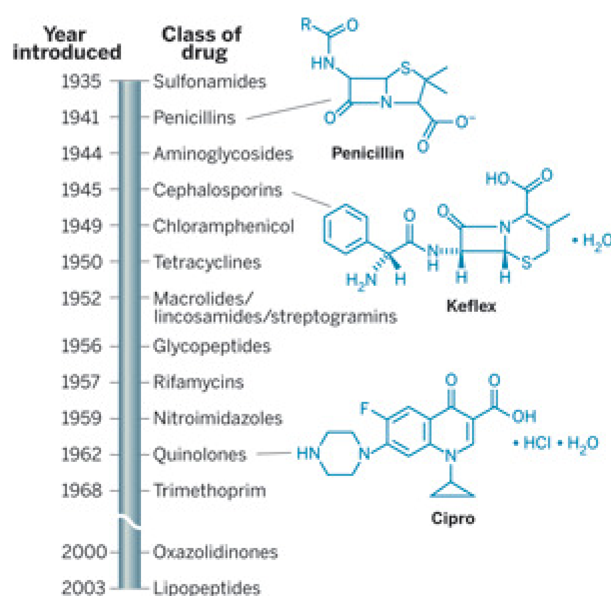
It is important that the national guidelines for use of antimicrobial agents are evaluated and modified as new insight into antibiotic resistance is gained. There is a need for research related aminoglycoside resistance in Norway to learn about mechanisms which has caused the increasing resistance during the past years.  The relevance of AAC(6`)-Ib-cr as the cause for increasing aminoglycoside resistance is not studied in Norwegian isolates. In this study, we wanted to look into this possibility by screening for the gene *aac(6`)-Ib-cr* in Norwegian clinical isolates and relating the results to their susceptibility patterns for aminoglycosides and ciprofloxacin. Our hypothesis is that there could be a strong association between the increased use of ciprofloxacin and selection of combined aminoglycoside and ciprofloxacin resistance due to the presence of the gene for this enzyme. In addition to AAC(6`)-Ib which is reported to be the most prevalent of all aminoglycoside acetyltransferases (AACs) (7), we looked for the presence of a second aminoglycoside acetyltransferase AAC(3`)-IIc (also known as aacC2). AAC(3)s  is reported to be the second largest group of aminoglycoside acetyltransferases conferring resistance to aminoglycosides(7).

## 1.2 Antibiotics

Antibiotics are microbial metabolites or synthetic analogs inspired by them that, in small doses, inhibit the growth and survival of microorganisms without causing serious toxicity to the host. Based the original definition all antibiotics are natural products or derivates based on them. Chemical modifications to the original structure have given rise to substances with broader antimicrobial spectrum, greater potency, lesser toxicity, more convenient administrations, etc. When fully synthetic agents for the use against infections were developed, they were classified as chemotherapeutic agents. A general term for both antibiotics and chemotherapeutic agents used against microbes is antimicrobial agents; this term also includes agents against viral, fungal and protozoan infections. When speaking of drugs used only against bacterial infection, the term antibiotic is now commonly used for antimicrobial agents of natural as well as synthetic origin.

The first truly effective antimicrobial agent was a chemotherapeutic agent which dates back as far as in the mid 1930`s. The trade name was Prontosil, and it belongs to the group of sufonamides. The first antibiotic came to use in the 1940`s; this was penicillin belonging to the group of beta-lactam antibiotics. In the years to follow many new agents were discovered or synthesized contributing to the treatment of infectious diseases.

**Figure 1.1:** *Timeline of discovered antimicrobial agents. (Figure adopted from:***(8)**)




Antibiotics are, because of availability, familiarity, generally low cost and relative safety, among the most misused of all medication(9). Misuse and consequent resistance development threatens their continued efficacy. Unfortunately we can no longer depend on discovery of new agents to keep the infectional diseases under control. Restriction and prudent use of these agents is critical for the sake of maintaining their efficacy(10).

### 1.1.1 Mechanisms

Bacteria have prokaryotic cell structure which is distinct from human cells. This facilitates design of drugs ensuring bacterial specificity without significant harm to the host cell. There are four major sites in the bacterial cell and aspects of its metabolic pathway that are sufficiently different from human cells to allow antimicrobial targeting.

- Cell wall (disrupts synthesis of cell wall).
- The bacterial ribosome (disrupts protein synthesis).
- The nucleic acid synthetic pathway
- The cell membrane (disrupts cell membrane).
- Antimetabolites (inhibit bacterial metabolic pathways).

### 1.2.3 Antimicrobial groups

**Table 1.1:** *Site of action for different antibiotics divided:*

| Cell wall | The bacterial ribosome | The nucleic acid synthetic pathway | The cell membrane | Antimetabolites |
|---|---|---|---|---|
| β-lactams | Aminoglycosides | Quinolones | Azoles | Trimetoprim |
| Glycopeptides | Tetracyclins | Rifampin | Polyens | Sulfonamides |
| Isoniazid | Erythromycin | | Polymyxins | |
| | Chloramphenicol | | Ergosterol inhibitors | |

### 1.2.3 Antibacterial efficacy

Antibiotics are classified clinically as bactericidal or bacteriostatic depending on their ability to kill microorganisms or prevent them from multiplying, when given in normal therapeutic dosages. Bactericidal drugs kill microorganisms. Bacteriostatic drugs will not kill the microorganisms; they are given in concentrations to interrupt the rapid growth of the organisms and are dependent on the host immune system be rid of the bacteria(10).

The efficacy of antibiotics depends on the microbe's sensitivity towards it and the concentration of the drug. Minimum inhibitory concentration (MIC) is a useful indicator for treatment. This is the lowest concentration of the drug that under certain conditions inhibit growth of the bacteria. Low MIC values are generally consistent with effective drugs, but pharmacokinetic and pharmacodynamic considerations play an important part in determining the dosage and dosage interval.

MIC-values are used to characterize the bacteria as sensitive (S), intermediate (I) or resistant (R) against the antibiotic according to the SIR system(11). AFA (Norwegian workgroup for questions related to antibiotics) annually updates guidelines for such classification, in collaboration with EUCAST (The European Committee on Antimicrobial Susceptibility Testing). These guidelines provide threshold MIC values used to identify a certain strain as **S**ensitive, **I**netermediate or **R**esistant.

**Figure 1.2:** *Antibiotic sensitivity as applied in the SIR-system*

| **S** (Sensitive) | **I** (Intermediate) | **R** (Resistant) |
|---|---|---|
| The bacterial isolate is sensitive for the drug. Clinical effect of the drug is expected with normal dosage, if the drug reaches infection focus. | The bacterial isolate is intermediate between sensitive and resistant. With increased dosage it might be possible to have clinical effect of the drug. | Clinical effect of the drug is not likely due to the bacterial isolate being resistant to the tested drug. |

There are various methods in use to determine bacterial sensitivity towards an antibiotic. The broth dilution method is the reference method but it is rarely used in routine laboratories; in this method a standardized amount of bacteria is grown in a fluid media or on agar media containing different concentrations of the specific agent. Usually the concentrations are 2-folds dilution which span over all concentrations clinically relevant for the agent. After overnight incubation at 35-37°C the minimum inhibitory concentration (MIC value) can be read as the lowest concentration of the drug that produces no visual growth. A similar approach, but easier in application is the E-test method. Assessing the susceptibility according to the size of the zones of bacterial inhibition around discs containing specific amounts of antibiotics (Disc diffusion methods) is inexpensive, easier to perform are is widely used in clinical laboratories.

**Disc diffusion method:**

The disc diffusion method for antibiotic susceptibility testing is called the Kirby-Bauer method. A filter paper disc impregnated with chemical is placed on agar and the chemical diffuse into the agar around the disc. For this method to give reliable results, the conditions and execution must be standardized including the medium, amount of microbes in dilution, concentration of chemical agent and incubation conditions. The antimicrobial drug diffuses into the agar, establishing a declining concentration gradient around the disc. Zones of no growth can be seen in the area where the concentration of the chemical is the same or higher than the microbes MIC. The result (distance from disc centre to the periphery of the zone of clearance) is measured in millimeters. A Norwegian national agreement is reached for stratification of these distances into a sensitive (S), intermediate (I) or resistant (R) organism.

**E tests**

E test is a method combining the principles of the disc diffusion method and the dilution method. An E-test is a plastic strip with specific concentrations of an antimicrobial agent on one side and a MIC scale printed on the other side. After spreading a standard amount of bacteria in dilution on the agar, the strip is placed with the MIC scale heading up. The antimicrobial agent will then diffuse into the agar, and establish a consistent and stable concentration gradient under the strip. After incubation, the antibiotic gradient gives rise to an elliptical-shaped inhibitory area around the strip. The MIC value is read where the ellipse intersects the strip.

## 1.3 Aminoglycosides

The first aminoglycosides were natural products of microorganisms. The very first aminoglycoside; streptomycin, was isolated from the bacterium *Streptomyces griseus* in the 1940`s and used clinically against tuberculosis. Several different types of aminoglycosides have *Streptomyces spp*. as their source; neomycin, tobramycin, paromycin, kanamycin, amikacin, arbekacin and spectinomycin. *Micromonospora spp*. is the source of gentamicin and netilmicin. Gentamicin is a mixture of several antibiotic components produced by fermentation of *Micromonospora purpurea* and other related soil microorganisms. Gentamicin and the other newer agents of the aminoglycoside family are so called semi synthetic agents. This implies that their natural basis has been synthetically modified to generate antimicrobial agents with expanded activities.

Aminoglycosides have a backbone structure consisting of an aminocyclitol ring (a pharmacophoric 1,3-diaminoinositol moiety) either streptamine, 2-Deoxystreptamin or streptidine. The diaminoinositol unit is connected through glycosidic linkages to various amino sugars. The chemistry, spectrum, potency, toxicity, and pharmacokinetics of these agents are functions of the identity of the diaminoinositol unit and the arrangement and identity of the attachments. (10)

**Figure 1.3:** (*Figure adopted from*(12))

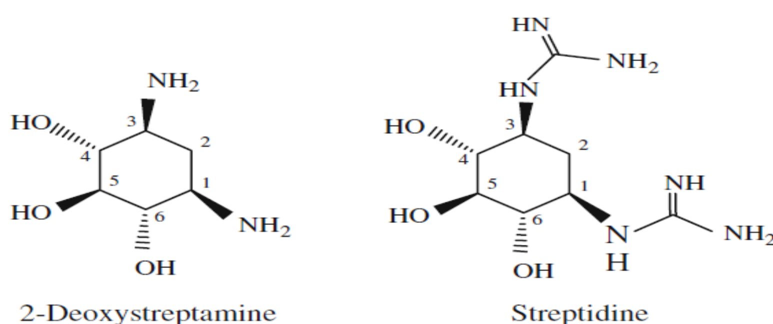


Fig. 1 Backbone structures of the aminoglycosides

Aminoglycosides are potent bactericidal antibiotics, mostly used against gram negative bacteria. They penetrate bacteria by binding to positively charged moieties of the cell membrane; this is believed to result in the displacement of $Mg^{2+}$ and $Ca^{2+}$ ions that link adjacent lipopolysaccharide molecules. The process damages the membrane and enhances its permeability(7). Inside the cells they inhibit bacterial protein synthesis by binding to the ribosome. The precise mechanism of their antimicrobial activity is still a subject of study but all aminoglycosides bind to the 16-S-ribosomal DNA portion of the 30S ribosomal sub particle impairing the proofreading function of the ribosome. This leads to mistranslation of RNA templates and the consequent selection of wrong amino acids and formation of so-called nonsense proteins. The most relevant of these unnatural protein syntheses is the effect on bacterial membrane and its function. The defective membrane allows for large additional quantities of aminoglycosides to enter the cell. With increased concentration within the cell, protein biosynthesis ceases all together. The combined effects cause bactericidal death(10).

The bactericidal effect of aminoglycosides is dependent on the drug concentration. The toxicity potential increases with increased concentration of the antibiotic. They exhibit post-antibiotic effect, which means that the bacterial death continues despite nil or very low drug levels. The concentration-dependent effect and post-antibiotic effect in addition to risk of nephrotoxicity and ototoxicity are the major reasons for once-daily dosing(7). Because of the toxicity issues of aminoglycosides, there has been a trend of using β-lactams and other less toxic agents against bacterial infections over the last years. Nevertheless aminoglycosides remain important drugs against microbes and especially against multiresistant strains that are still vulnerable to these agents.

Aminoglycosides are given parenterally because they are not absorbed in significant amounts from the gastrointestinal tract. There are several types of aminoglycosides with diverse antimicrobial spectra and therefore the choice of aminoglycoside for therapeutic purpose depends on the bacterial pathogen, for example gentamicin or netilmicin for gram negative intestinal bacteria (*Enterobacteriaceae*), tobramycin for *Pseudomonas spp* while streptomycin is usually reserved for *Mycobacterium tuberculosis*. Aminoglycosides are often combined with other antimicrobial agents when used. Aminoglycosides produce synergistic bactericidal activity when given together with other agents inhibiting cell wall biosynthesis(7). Gentamicin and β-lactam antibiotic is one combination used, but there is an interesting incompatibility problem between the two. Certain β-lactam antibiotics *N*-acetylate gentamicin on the C-1 position, inactivating both antibiotics(10). This makes it important not to mix the two components in the same solution and administer the two into different tissues compartments (usually one in each arm).(10)

### 1.3.1 Resistance against Aminoglycosides

Many bacteria have inherent resistance to antibiotics; such resistance existed long before antibiotics were introduced as pharmaceuticals. Since microorganisms produce antibiotics themselves, they must have mechanisms protecting against these. Some bacteria are therefore inherent resistant against certain antimicrobial agents. In addition to inherent resistant strains, bacteria have the ability to acquire resistance, either by mutation of their own genes or by the acquisition of extrinsic genes on mobile genetic elements.

Resistance can be defined as the ability of a microorganism to withstand the effects of a drug that are lethal to most members of its species. It can be caused by genetic changes in microbes that reduce or eliminate the effectiveness of drugs. Bacteria develop a variety of mechanisms to evade the lethal effect of antibiotics. With increasing exposure to antibiotics, new resistance mechanisms are developed or acquired by the organisms for their survival. Resistance against gentamicin in E.coli has increased in Norway over the last years according to surveillance data (see section 1.1).

Resistance against aminoglycosides is claimed to be caused by one or more of the following mechanisms.(13)

1) Aminoglycoside modifying enzymes (AME) that render the aminoglycosides incapable of binding to its ribosomal target.
2) Decreased cell membrane permeability limiting aminoglycoside uptake into the cell
3) Structural alteration in the ribosomal target hindering the attachment of the drug to its cite of action
4) Extrusion of the drug from the cell by efflux pumps.

AME`s are the most important mechanism of resistance to aminoglycoside antibiotics (7, 12). The enzymes inactivate aminoglycosides by transferring a functional group to the aminoglycoside structure. This makes the aminoglycoside unable to interact with the ribosome effectively. There are 3 types of enzymes that transfer different functional groups to the aminoglycoside structure:

1) Aminoglycoside nucleotidyltransferases (ANT`s) transfer a nucleotide triphosphate moiety to a hydroxyl group
2) Aminoglycoside acetyltransferases (AAC`s) transfer the acetylgroup from acetyl-CoA to an amino group
3) Aminoglycoside phosphotransferases (APH`s) transfer the phosphoryl group from ATP to a hydroxyl group

Each of the 3 families of enzymes is divided into classes according to the modification site on the aminoglycoside structure, indicated in parentheses. They are further divided according to their phenotypes (resistance profiles), indicated by roman numbers. Finally enzymes of the same type and phenotype but encoded by different genes are designated by a trailing lowercase letter (example AAC(6`)-Ib) (7). More than 85 aminoglycoside modifying enzymes have been identified(14). Illustrating an example of the nomenclature; each class (parentheses) within a group have different phenotypes (roman numbers). So AAC(6`)-I and AAC(6`)-II have different resistance phenotypes. While the lower case letter illustrates different genes, so AAC(6`)-Ib and AAC(6`)-Ia have identical phenotype but are encoded by different genes. When writing the name of the genes for the enzymes, the term is written with lowercase letters in italics (*aac(6`)-Ib).*

**Figure 1.4**: *Aminoglycoside acetyltransferase showing their modification sites and respective phenotypes (Figure adopted from (12))*

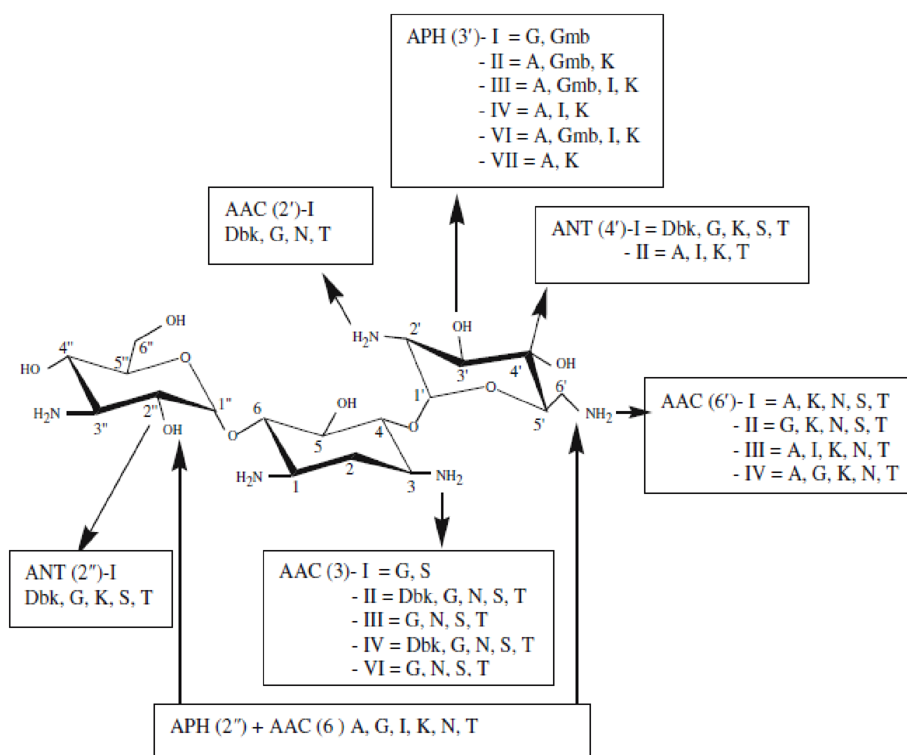



**Fig. 3** Aminoglycoside-modifying enzymes and their substrates. *A* amikacin, *Dbk* dibekacin, *G* gentamicin, *Gmb* gentamicin B, *I* isepamicin, *K* kanamycin A, *N* netilmicin, *S* sisomicin, *T* tobramycin

In this study we have included four classes of AAC`s; AAC(1), AAC(3), AAC(2`) and AAC(6`). We chose to investigate the presence of *aac(6`)-Ib* and *aac(3)-IIc*.

**Figure 1.5**: *Antibiotic resistance (phenotype) for each of the Aminoglycoside acetyltransferases. (Figure adopted from (7))*

Substrate profiles of aminoglycoside acetyltransferases

| Enzymes: | Substrate(s): |
|---|---|
| **AAC(6')** | |
| I (at least 24 enzymes) | Tobramycin, amikacin, netilmicin, dibekacin, sisomicin, kanamycin, isepamicin |
| II | Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, kanamycin |
| **AAC(3)** | |
| Ia, Ib | Gentamicin, sisomicin, fortimicin |
| IIa, IIb, Iic | Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin |
| IIIa, IIIb, IIIc | Tobramycin, gentamicin, dibekacin, sisomicin, kanamycin, neomycin, paromomycin, lividomycin |
| IV | Tobramycin, gentamicin, netilmicin, dibekacin, sisomicin, apramycin |
| VII | Gentamicin |
| **AAC(1)** | Paromomycin, lividomycin, ribostamycin, apramycin |
| **AAC(2')-Ia** | Tobramycin, gentamicin, netilmicin, dibekacin, neomycin |

Of all the known AAC(6`)s, AAC(6`)-Ib is the most prevalent among the gram-negative microorganisms. The gene *aac(6`)-Ib* has been detected on either transposoms or integrons. It is reasonable to assume that the location of *aac(6`)-Ib* on mobile genetic elements has facilitated its rapid dissemination in the presence of selective antibiotic pressure among a wide range of microorganisms(7).

AAC(3)s are widely distributed among different bacterial genera. They constitute the second largest group of aminoglycoside acetyltransferases, and are the second most common mechanism of aminoglycoside aacetyltransferase-mediated resistance in clinical isolates after AAC(6`)-I enzymes. *aac(3)-II* is widely distributed among gram negative bacteria(7).

Even though there are more than 85 aminoglycoside modifying enzymes, only a limited number of them have been selected to cause the majority of aminoglycoside resistance; ANT(2``)-I, AAC(6`)-I and AAC(3)-I + II + III + IV + VI.(14). Our chosen enzymes AAC(6`)-I and AAC(3)-II are among the limited number of enzymes selected to cause the majority of Aminoglycoside resistance.

Aminoglycoside resistance mechanisms have become complex over time. Combinations of different mechanisms occur frequently in the same isolate, broadening the spectrum of aminoglycoside resistance (15). Isolates may possess several mechanisms conferring a complex resistance phenotype against aminoglycosides.

### 1.3.2 Co-resistance with Fluoro-quinolones

Fluoro-quinolones and aminoglycosides are two different classes of antibiotics. The first generation of quinolones was introduced in the 1960`s, while the second generation, the fluoro-quinolones, were introduced to the marked in the 1980`s. Fluoro-quinolones are synthetic bactericidal agents. They inhibit DNA gyrase and topoisomerase IV. These key bacterial enzymes dictate the conformation of DNA. Inhibition of these enzymes makes the cell's DNA unstable and leads to cell death.

Bacteria are known to host enzymes providing resistance to antibiotics of natural origin. These enzymes have co-evolved with the antibiotics they inactivate and were thought to be specific for a single class of drugs or even selected members of a class (16). Fluoro-quinolones are as previously mentioned synthetic agents and thought to be spared for development of such a resistance mechanism (16). Independent mutations seemed to be the only threat. Since two or more resistance mutations are needed to reduce susceptibility to fluoro-quinolones and independent mutations take place once per $10^7$ cell division or less, the threat seemed negligible(17). Despite the presumption that fluoro-quinolones seemed to be unlikely to develop resistance easily, resistance against fluoro-quinolones has become common and widespread(17).

Quinolone resistance is mainly due to generation of efflux pumps, loss of porin channels or multiple chromosomal mutations in genes encoding quinolone target enzymes. The chromosomal genes encoding quinolone targets are; *gyrA* and *gyr B* encoding DNA gyrase, *parC* and *parE* encoding topoisomerase IV. Target alteration most frequently occur due to mutations in the *gyrA* gene, particularly in a region of the gene called QRDR ("Quinolone resistance-determining region")(18).

Lately plasmid mediated quinolone resistance (PMQR) determinants have been reported (17-19). The determinants include; Qnr-proteins which belongs to the pentapeptide repeat family that protects DNA gyrase and Topoisomerase IV from the action of quinolone agents including newer fluoro-quinolones(18, 19), AAC(6`)-Ib-cr which is described in the following section and QepA, an fluoro-quinolone specific efflux pump protein(19). PQMR confer low resistance on their own, but act additively with other resistance mechanisms (17, 19).

**Enzyme conferring resistance to both aminoglycosides and quinolones:**

Recent publications report the identification of a variant of the aminoglycoside modifying enzyme AAC(6`)-Ib, as a common occurrence in clinical gram-negative bacteria isolates(17, 20, 21). This variant called AAC(6`)-Ib-cr (cr for ciprofloxacin variant) has the ability to inactivate selected fluoro-quinolones in addition to its original activity on aminoglycosides. The gene *aac(6`)-Ib-cr* may have a role in the frequently observed but incompletely understood strong epidemiologic association between ciprofloxacin and aminoglycoside resistance (16).

The gene for this enzyme is characterized by two single nucleotide polymorphisms (SNP`s) giving rise to two amino acid substitutions, Trp102Arg and Asp179Tyr; both substitutions have been reported to be necessary for the acetylation and inactivation of ciprofloxacin (20). In another mechanistic and structural analysis of the enzyme; Asp179Tyr mutation was shown to be the main contribution for the fluoroquinolone acetylation (22).

### 1.3.3 Co-resistance with extended spectrum beta-lactamases (ESBL)

Beta-lactamases are enzymes cleaving the 4 atom ring (beta-lactam ring) and such deactivating the antimicrobial property of beta-lactam antibiotics. Their increasing prevalence in certain bacterial families like *Enterobacteriaceae,* and the spread to other bacteria led to the development of cephalosporins in the early 1980`s (23). Not long after cephalosporins were introduced to clinical practice, genes encoding **e**xtended **s**pectrum **b**eta **l**actamses (ESBL) were discovered. The genes showed nucleotide mutations in their parent genes encoding beta-lactamases, leading to extension of the substrate spectra of the enzymes to include even the 3$^{rd}$ and 4$^{th}$ generation cephalosporins (23).

ESBLs are capable of conferring bacterial resistance to penicillin, first-, second-, and third-generation cephalosporins and aztreonam (but not cephamycins and carbapenems)(23).

SHV-2 is a gene encoding an ESBL. It differs from the SHV-1 gene encoding a regular beta-lactamase by replacement of glycine by serine at the 238 position. This mutation accounts for the extended spectrum properties and bacteria with these genes were probably selected due to the pressure of the increased use of third-generation cephalosporins after their introduction to clinical practice. A large number of TEM (beta-lactamse gene) derivates has been described over the past years, the majority of them being ESBL. Also CTX-M (beta-lactamase gene) types beta-lactamases with extended spectra exist, and these are the most frequent ESBL worldwide(23). There are other genes, but these 3 classes are the most important genes encoding ESBL in Norway(24).

Many genes for ESBLs are found on plasmids, often large plasmids with multiple resistance genes, and studies imply that their rapid spread is caused either independent through effects of the antibiotic pressure or by plasmid transfer from organism to organism (23). These plasmids coding for multiresistance, frequently carry genes for ESBL in addition to genes encoding resistance against aminoglycosides and trimethioprim/sulfamethoxazole(23). Quinolone resistance mechanisms are also associated with ESBL.(23)

### 1.3.4 Different enzymes located on the same transposable genetic element (plasmids/transposomes/integrons)

A recent publication of complete nucleotide sequences of plasmids present in 2 variants of internationally prevalent *E.Coli* (O25:H4-ST131) shows that the plasmids encode several ESBL and AAC(6`)-Ib-cr(25). Other observational studies present reports where the majority of the isolates with *aac(6`)-Ib-cr* also show an ESBL phenotype(20).

Other publications of complete nucleotide sequences of plasmids shows that plasmids encode ESBL and aac(3)-II (aacC2)(26, 27).

A study from Norways report that prevalence of plasmid mediated quinolone resistance genes; *qnr* and *aac(6`)-Ib-cr* is higher among ESBL producing isolates (19).The association between the presence of *aac(6`)-Ib-cr* in isolates with *qnr* is observed in Korea (21). This association was not found in previous observational studies from the United States (20, 28).

Qnr was first found on Plasmid pMG25 (29), and its gene sequence was later reported(30). This plasmid also encodes AmpC, ESBL and FOX-5. Many plasmids have been sequenced over the years and plasmids with with *qnr* genes are reported to also contain multiple resistance genes like *aac* genes, *bla* genes (AmpC), FOX-5, DHA-1, OXA-30 and SHV-7 (beta lactamses), *cat* gene (chloramohenicol), QepA (efflux pumps), *sul*1 (sulphonamide) and AmpR(transcriptional regulator, beta-lactamases)(18)

The coexistence of multiple resistance determinants on a single transmissible plasmid indicate that any drug to which resistance occurs would preserve resistance to the other antibiotics on that plasmid.

Co transmission of plasmids with plasmid mediated quinolone resistance (*qnr*), aminoglycoside modifying enzymes (*aac(6`)-Ib*) and different extended spectrum beta lactamases can accelerate the pace at which we drive dangerous multidrug resistance development(17).

## 1.4 Patterns of antibiotic prescription in Norway

Sale of pharmaceutical agents in Norway is monitored and trends can be statistically analyzed. Antibacterial agents are sold thorough pharmacies and a prescription from a physician, dentist or veterinarian is required. The Norwegian prescription data base (NorPD) contains data for all prescription drugs handed out through pharmacies from 2004. Data from NorPD can be used to monitor and trace changes in use of pharmaceuticals. Specific numbers on antibacterial use in hospitals can be retrieved from “The hospital pharmacies drug statistics database”. Epidemiological surveillance of the consumption of antibacterial agents before 2004 is based on wholesaler’s statistics. The wholesalers database cover the total sale of medicaments from drug wholesalers to pharmacies and hospitals in Norway collected since the beginning of the seventies.

Usually drug consumption is measured in DDD (Defined Daily Dosage). The measurement unit DDD is defined as the assumed average maintenance dose per day for a drug used for its main indication in adults. It is important to be aware that in many cases the prescribed dose may deviate from the DDD.

## 1.5 Study of resistance genes

### 1.5.1 Introduction; Genes and gene transcription:

Genes are located on the bacterial genome in prokaryotic cells and are structurally composed of DNA (deoxyribonucleic acid), which is a polymer of 4 distinct nucleotides linked together. The sequence of these nucleotides is fairly specific for the genes and determines the structure of the encoded proteins. The nucleotides consist of a phosphate group, sugar (2`-Deoxyribose) and a nitrogenous base. The nitrogenous base differentiate the nucleotides and can be either Adenine (A), Cytosine (C), Guanine (G) or Thymine (T). The nucleotides are linked together with phosphodiester bonds forming polymers. Two polymers are assembled in the structure of a double helix linked together by base pairing rules with hydrogen bonds and π-π interactions.

Genes represent single units of information which is expressed to construct and maintain a living example of an organism. The resistance genes in focus are protein-coding genes whose biological information is required by the cell at a particular time. The genes are transcribed into RNA and further translated into proteins. Transcription is executed by DNA-dependent RNA Polymerases after strict base pairing rules. Translating RNA is more complicated, and uses the genetic code to specify which amino acids will be included in the specific sequence. The genetic code consists of triplets of nucleotides (Codons), defining which amino acid will be inserted. One amino acid may be coded by 1 or up to 6 codons. The biological properties of the protein are determined by the order of the amino acids in addition to its folded structure and spatial arrangement of its chemical groups on the surface.

The enzymes carrying out translation and transcription have characteristics making them able to carry out their jobs with great accuracy. Even so, errors do occur like single nucleotide polymorphisms (SNP). SNP is a position in the DNA where one nucleotide is different from what base-pairing rules demands. An SNP can be irrelevant, but sometimes the SNP in a gene alters the structure and the function of the protein that it encodes. In the case of AAC(6`)-Ib; two SNPs alter the amino acid composition, giving rise to a new variant of the enzyme AAC(6`)-Ib-cr, with additional characteristics.

### 1.5.2 Prokaryotic genome

The physical organization of the prokaryotic genomes is different from the eukaryotic genomes. Most of what is known of the organization of DNA in prokaryotes is from studies of *E.Coli*. The genome of *E.Coli* is a single circular DNA molecule, supercoiled to a compact structure localized in the cytoplasm of the cell. This is called the bacterial chromosome, even though the structure has few similarities with eukaryotic chromosomes.

Prokaryotes have plasmids which are small pieces of DNA, often but not always circular, that coexist with the chromosome in bacterial cells. Some plasmids are able to integrate within the chromosome, but others are thought to be permanently independent. Plasmids carry genes that are not usually present in the main chromosome and they are generally beneficial for the organism. Often these genes encode antibiotic resistance(31).Plasmids can also be conjugative, which means that they can move between bacterial species(32).

Prokaryotes also have genetic elements like transposable elements. These are molecules of DNA that can move from one site on the chromosome to another. Transposons are one example of a transposable element and they can confer drug resistance. The important feature of transposons is the presence of a gene encoding transposase, an enzyme necessary for transposition, and the presence of a short *inverted terminal repeat* (IS) at the end, which is involved in the transposition process. Transposons can also be conjugative (see below). Integrons are transposable elements that capture and express genes from other sources. These are unlike transposons highly selective in their insertion site. One important feature of integrons is the presence of a gene encoding the protein integrase, and the presence a specific DNA sequence allowing the intergrase to insert cassettes of genes along with a promotor. Integrons can become a part of chromosomes, transposones and plasmids(32).

The content of the prokaryotic genome with its transposable elements and plasmids that allows for transmission of genes complicate the understanding of resistance development.

### 1.5.3 Transferability to other bacteria

There are three mechanisms for genetic exchange; transduction, transformation and conjugation. Transduction is transfer of genes from cell to cell by a bacterial virus (bacteriophages). Transformation is a process where free DNA is incorporated into a recipient cell due to some prokaryotic cells being capable of taking up free DNA from the environment. This happens naturally only in a few bacterial species.

The main mechanism of transfer of plasmids is conjugation. This is a replicative process, where both cells end up with copies of the plasmid. A region of genes called *tra* region is present if plasmids are transmissible. These genes encode proteins that function in transfer and replication, and other that function in mating pair formation(32).

Bacterial conjugation is genetic transfer which involves cell-to-cell contact. A copy of a plasmid is transferred to a new host and sometimes other genetic elements can tag along. The genes in the *tra* region of the conjugative plasmid are involved in the synthesis of sex pili on the surface structure. They allow pairing to take place between a donor cell and a recipient cell. DNA synthesis of complementary strand begins, and at the end of the process, both the donor and the recipient would possess completely formed plasmids(32).

These 3 processes are mechanisms of horizontal gene transfer.

Resistance plasmids infectious nature permits their rapid spread through cell populations. Resistance plasmids are now a major problem in clinical medicine (32). The same plasmids are sometimes found in bacteria that belong to different species

### 1.5.4 Techniques to study DNA

Techniques to study DNA was assembled in the 70`s and 80`s when different enzymes were purified and the reactions they catalyzed studied. Examples of enzymes are; DNA polymerases who synthesize DNA, DNA nucleases which can remove nucleotides from the end (exonucleases) or cut the sequence into shorter fragments (endonucleases), DNA ligases that join DNA fragments together again. The purified enzymes were adapted as tools for manipulating DNA molecules forming the basis of recombinant DNA technology. In recombinant DNA technology enzymes are used as tools cutting DNA molecules in to shorter fragments and joining them together. There can be combinations made that do not exist in nature. New or "recombinant" DNA molecules are constructed from pieces of naturally occurring chromosomes and plasmids.

Recombinant DNA technology led to the development of gene cloning, in which short DNA fragments are inserted into a plasmid or a virus chromosome and then replicated in a bacterial or eukaryotic host. The next major breakthrough was Polymerase chain reaction (PCR), which gave the opportunity for repeated copying of a short segment of DNA. PCR techniques are now generally well established and easy accessible, and has become immensely important in many areas of research.

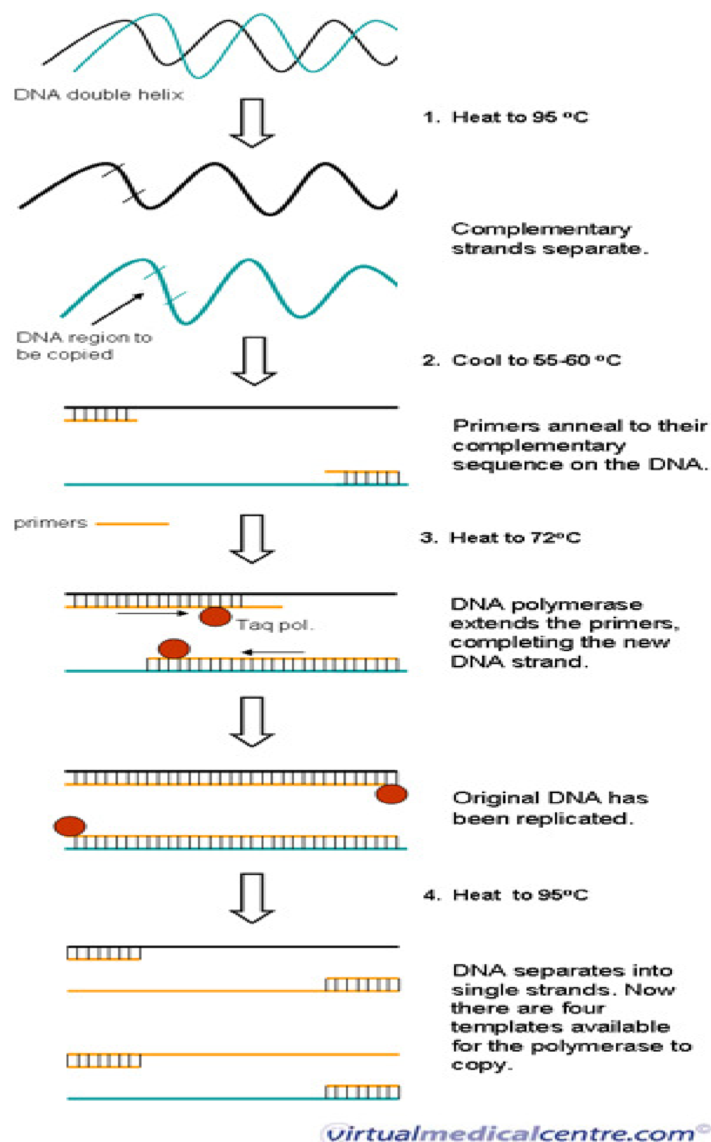
### 1.5.5 Polymerase chain reaction (PCR)

PCR is the repetitive copying of a targeted region of a DNA molecule. It has become increasingly important in biological research. Some of its areas of use are screening of human DNA for mutations associated with genetic diseases, obtaining sequences from trace amounts of DNA in forensic investigations or from archaeological sites, and it can be used to detect microbial pathogens in various infections. The technique has certain limitations, the main limitation being the need to know the genetic base sequence, or at least parts of the sequence, to be able to synthesize copies of it.

The copying is carried out by a thermostable DNA polymerase that synthesizes DNA. The polymerase is dictated by the sequence being copied, via base-pairing rules. The polymerases also have proofreading ability, making them able to correct errors by removing a nucleotide that has been inserted incorrectly. Polymerases always synthesize in 5`-3`-direction. In order for the polymerase to work there must be a short double stranded region to provide a 3`-end onto which the enzyme will add new nucleotides. In PCR, primers are used for this purpose. Primers are short synthetic oloigonucleotides, usually about 20 nucleotides in length. They attach to the target DNA at either side of the segment that is to be copied. To design correct primers the sequence of the target gene segment must be known.

Two types of platforms for PCR exist; Conventional PCR and Real-time PCR. The principles behind the amplification methods are the same, but the difference lies in the detection of the amplified product. In conventional PCR the results are assessed on agarose gel, while real-time PCR allows for detection while the reaction is ongoing. Conventional PCR is therefore a more time consuming method due to laborious post PCR methods. Real-time PCR is chosen as method for this thesis.

**Figure 1.6:** *The Polymerase chain method. (Figure adopted from (33):*

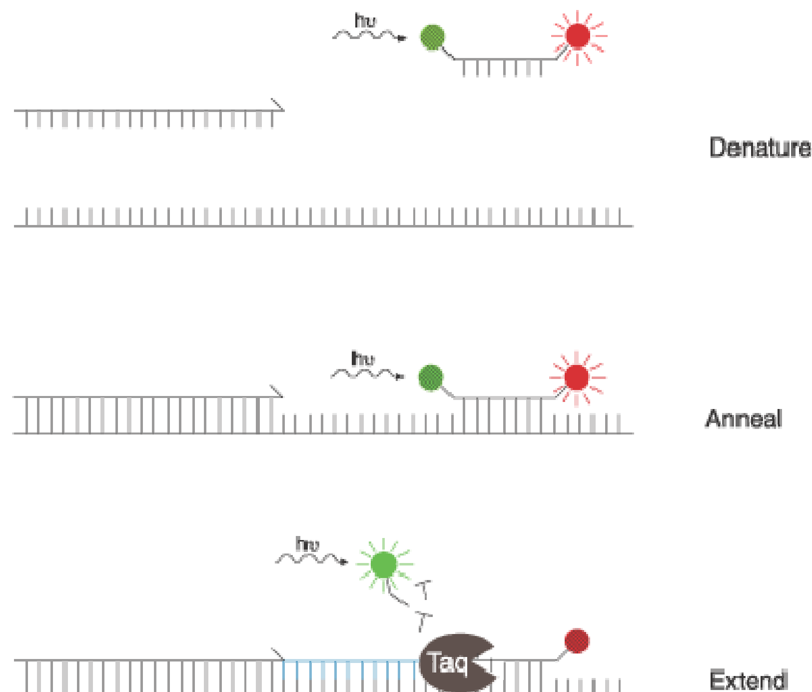


### 1.5.6 Detection of PCR product (specific probes/SYBRgreen)

Different chemistries allow detection of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

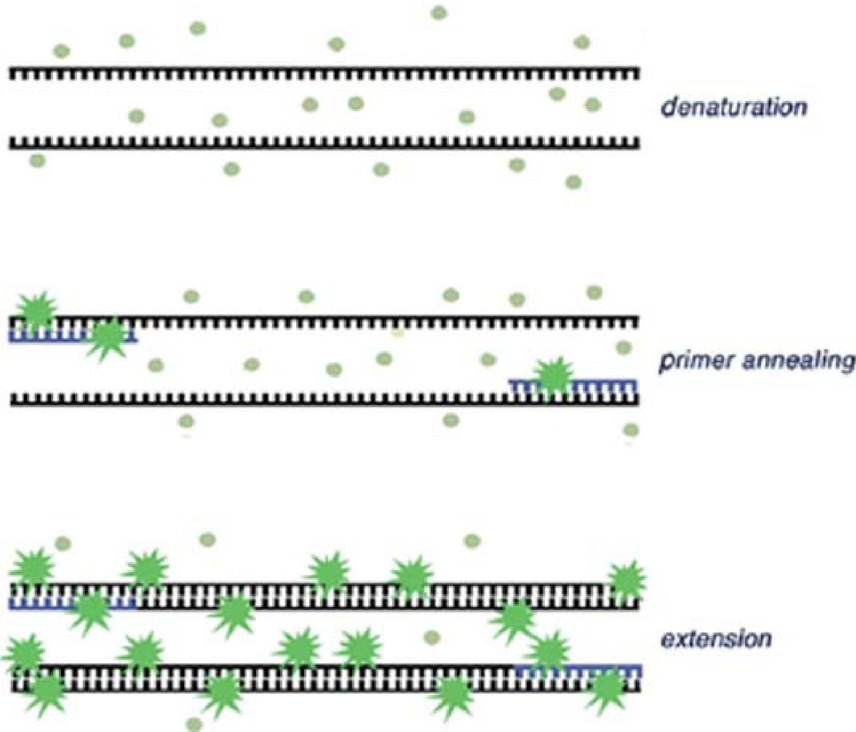
A Taqman probe has a reporter dye on one end and a quencher dye on the other end of the sequence. It is designed to have an annealing temperature 8-10°C higher than that of the primers; so it anneals to the sequence before the primers do. This ensures detection of a specific variant since Taq polymerase has a 5`-exonuclease activity (proofreading ability) as well as polymerase activity Due to this 5`-exonuclease activity Taq polymerase hydrolyzes the probe on encounter, releasing the reporter dye from the quencher. Once the probe is displaced, the polymerase continues amplification. The cleaved reporter gives rise to an increased fluorescent emission which is detected by the instrument. The signal increases proportionally to the amount of product amplified.

**Figure 1.7:** *Illustration of the principle behind specific Taqman probes. (Figure adopted from (34):*



SYBRgreen is used in an alternative detection. It binds to the minor grooves of any double stranded DNA and when bound it emits a fluorescent signal. The intensity of the fluorescent emission is proportional to the amount of double stranded DNA present. Thus, amplification could be monitored be monitored by increase of the fluorescent signal.

**Figure 1.8:** *Illustrating the principle behind SYBRgreen (Figure adopted from (35))*



### 1.5.7 Restriction enzymes

Restriction enzymes are as mentioned in section 1.5.2 used in recombinant DNA technology. They can also be used to study single nucleotide polymorfs (SNP`s) in DNA.

A restriction endonuclease binds to a specific sequence in the DNA molecule and makes a double stranded cut. Because of the specificity, the positions of the cut can be predicted and specific fragments will occur. After treatment with restriction endonucleases, the resulting DNA fragments can be examined by agarose gel electrophoresis to determine their size. Agarose gel electrophoresis is a method to separate DNA molecules of different length. Electrophoresis is the movement of charged molecules in an electric field. DNA molecules are negatively charged molecules and will migrate to the positively charged electrode. The pores of the gel impede larger molecules from travelling towards the electrode. Molecules of different length are therefore separated and shown as bands in the gel. The fragment produced by the use of endonucleases should always be the same due to its specificity of the cut, but this is not true if an SNP is present. If the restriction sites are polymorphic, then only the DNA sequence with the “correct” allele will be recognized and cut while the sequence with mutation will not be cut.

### 1.5.8 Sequencing the amplicons

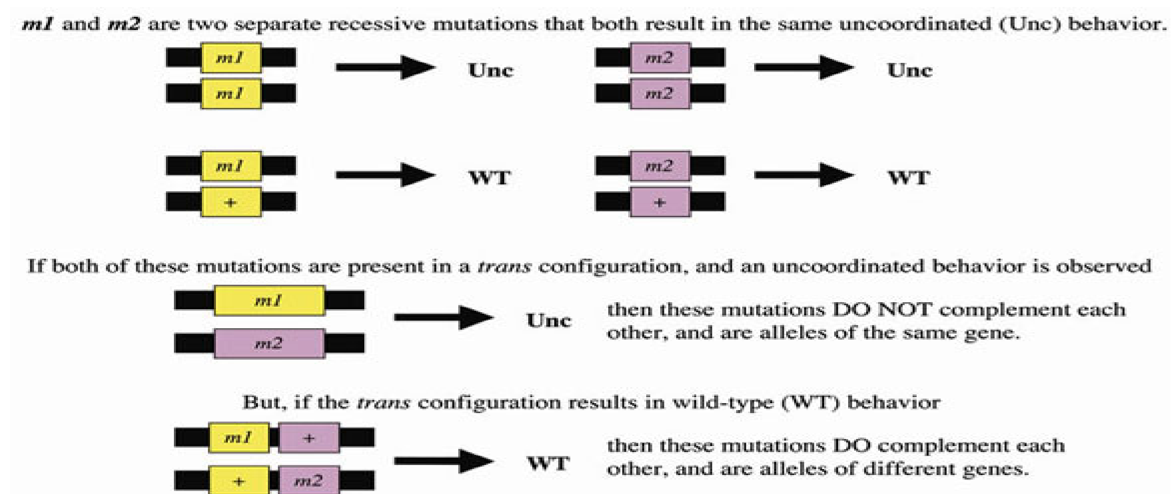
To gain information on the sequence of the nucleotides encoding a gene, several procedures are available. The traditional method is the chain termination method. After the discovery of thermostable DNA polymerases, which also led to PCR, a new methodology called thermal cycle sequencing was developed. The difference between the traditional method and the thermal cycle sequencing method is the material needed. The thermal cycle method requires very little template DNA and uses double stranded DNA as template, while the traditional method requires single-standed DNA and the necessity of cloning to get enough material.

Thermal cycle sequencing is carried out in a similar way to PCR, but just one primer to anneal to the same position in each molecule is needed. The reaction mixture contains DNA polymerase, deoxyribonuclease triphosphates (dNTP`s, dATP`s,dCTP`s,dGTP`s and dTTP,s) and a small amount of dideoxynucleotide triphosphates (ddNTP`s, ddATP`s,ddCTP`s,ddGTP`s and ddTTP`s). Each of these 4 last dideoxynucleotides is labeled with a different fluorescent marker. The polymerase enzyme does not discriminate between deoxynucleotides and dideoxynucleotides. Every time a dideoxynucleotides is incorporated into the newly synthesized sequence the elongation is blocked. Because of the different amounts added to the solution, many sets of molecules of different lengths are synthesized, each with an ending dideoxynucleotide at the terminal end whose identity indicates the nucleotides A, C, G or T. The DNA mixture is loaded into wells of polyacrylamide slab gel, or into a tube of capillary gel system, and electrophoresis carried out separates the molecules according to their length. After separation, the molecules are run past a fluorescence detector capable of discriminating the labels attached to the dideoxynucleotides. The detector determines which nucleotide each molecule ends with, and the results can be analyzed.

### 1.5.9 Knockout/contemplation studies

Complementation studies are experiments that can detect whether two mutations are in different regions of the same gene. This is a test for allellism and is also called *cis-trans* test. It is used to determine whether two mutant sites are in the same functional unit of the gene. The mutations must be recessive for this test to work. Basically if a wild-type phenotype is observed after mating two mutant strains, the two mutants are alleles of different genes. Each mutant has a functional version of the other gene. But if wild type function is not seen, the mutations are combined *trans* with one another and it is concluded that these are alleles of the same gene which means that neither allele produces a product that can restore wild-type function.

**Figure 1.9:** (*Figure adopted from* **(36)**):




*Two different mutations on opposite chromosomes will not complement each other, while two mutations on the same chromosome show a wild-type phenotype*

# 2 Aim of the study

In our material of aminoglycoside resistant clinical isolates of *Enterobacteriaceae* collected in western Norway, the following objectives were addressed:

1. Determine the susceptibility pattern to 6 different aminoglycosides; gentamicin, kanamycin, amikacin, streptomycin, netilmicin and tobramycin.

2. Screening for the genes *aac(6`)-Ib* , *aac(6`)-Ib-cr* and *aac(3)-II* encoding aminoglycoside modifying enzymes, reported to be common in *Enterobacteriaceae*

3. Relating the results of the susceptibility patterns for aminoglycosides and the presence of the selected genes

4. Relating the presence of resistance phenotype for AAC(6`)-Ib-cr to ciprofloxacin resistance and the presence of -cr variant.

**Hypotheses:**

1. Possible association between increased use of ciprofloxacin and selection of combined aminoglycoside and ciprofloxacin resistance due to the selection of isolates possessing *aac(6`)-Ib-cr.*

2. Plasmid mediated resistance genes like ESBL and *aac(6`)-Ib-cr* being co-transferred horizontally in our isolates.

# 3 Material and Methods

## 3.1 Material

### Bacterial isolates

**Clinical strains:**
A total of 132 *Enterobacteriaceae* isolates where included in this study, from two localisations; Blood (n=50) and isolates from various localisations, but mainly form urine (n=82) with ESBL/AmpC or other mechanisms conferring resistance agaist 3$^{rd}$ generation cephalosporins.

All isolates had shown reduced sensitivity or resistance to the tested aminoglycoside (gentamicin or netilmicin) on routine testing at the bacteriology department, Haukeland University Hospital. The blood culture isolates were from January 2000-June 2009 and the ESBL/AmpC/ other mechanisms conferring resistance agaist 3$^{rd}$ generation cephalosporins isolates were from 2006.
(The ESBL/AmpC/ other mechanisms conferring resistance agaist 3$^{rd}$ generation cephalosporins isolates are for future reference shortened to ESBL isolates)

Detailed description of routine examinations of the included isolates together with results from this study is listed in APPENDIX 1-4.

**Control strains:**
The control strains used were given to us by K-Res (The Norwegian center of expertise for antibiotic susceptibility testing) .
Quality control strains were given to us by the bacteriology department, Haukeland University Hospital.

Table 3.1: Reference strains

| | Specimen number | Strain | Origin |
|---|---|---|---|
| Quality control (E-test, MIC Test Strips) | ATCC®25922 | *E.Coli* | ATCC |
| Positive control (Aac(6`)-Ib-cr | K-37-80 | *E.Coli* | K-res |
| Positive control (Aac(6`)-Ib | K-38-23 | *E.Coli* | K-res |

## Media:

Table 3.2: Bacterial growth media used in this study:

| **Medium** | **Content** |
| --- | --- |
| Blood agar | Distilled water (RO-water), Colombia Blood Agar Base, Sheep's blood, Isovitalex without glucose. |
| Lactose agar | Distilled water (RO-water), Lab Lemco Powder, Bacteriological Peptone, Sodiumchloride (NaCl), Agar No.1, di-sodiumhydrophosphate dihydrate ($Na_2HPO_4x2H_2O$), Lactose ($C_{12}H_{22}O_{11}xH_2O$), Sodiumthiosulphate Penthydrate ($Na_2S_2O_3xH_2O$), 0,2% Bromothymol blue ($C_{27}H_{28}Br_2O_5S$), 0,1% Chrystal violet ($C_{25}H_{30}ClN_3$) |
| Mueller Hinton | Distilled water (RO-water), BBL Müeller Hinton Agar II, Sodiumchloride (NaCl) |
| Greaves solution | Distilled water (RO-water), L-Glutaminacid sodiumsalt, Bovine Serum Albumin, Glycerol (waterfree) |

## 3.2 Methods:

### 3.2.1 MIC

**E-tests and other types of strips**

**Table 3.3:** *E-tests and MIC test strips used:*

| Method | Chemical | Manufacturer |
|---|---|---|
| E-test | Gentamicin<br>Etest strips with range<br>0,016-256 µl/mL | AB Biodisc, Solna, Sweeden |
| | Kanamycin<br>Etest strips with range<br>0,016-256 µl/mL | AB Biodisc, Solna, Sweeden |
| | Streptomycin<br>Etest strips with range<br>0,064-1024 µl/mL | AB Biodisc, Solna, Sweeden |
| | Amikacin<br>Etest strips with range<br>0,016-256 µl/mL | AB Biodisc, Solna, Sweeden |
| | Netilmycin<br>Etest strips with range<br>0,016-256 µl/mL | AB Biodisc, Solna, Sweeden |
| | Tobramycin<br>Etest strips with range<br>0,016-256 µl/mL | AB Biodisc, Solna, Sweeden |
| MIC test strips | Gentamicin<br>MIC Test strips with range<br>0,016-256 µl/mL | LIOFILCHEM, Italy |
| | Kanamycin<br>MIC Test strips with range<br>0,016-256 µl/mL | LIOFILCHEM, Italy |
| | Streptomycin<br>MIC Test strips with range<br>0,064-1024 µl/mL | LIOFILCHEM, Italy |
| | Amikacin<br>MIC Test strips with range<br>0,016-256 µl/mL | LIOFILCHEM, Italy |
| | Netilmycin<br>MIC Test strips with range<br>0,016-256 µl/mL | LIOFILCHEM, Italy |
| | Tobramycin<br>MIC Test strips with range<br>0,064-1024 µl/mL | LIOFILCHEM, Italy |

## Preparing the bacteria:

The isolates were kept in the freezer at – 90 °C in Greaves solution. A scoop of bacteria were swabbed on blood agar and incubated overnight at 35°C. These cultured bacteria were used for further analyses. A Quality control strain was similarly incubated.

## E-test and MIC Test Strips:

All blood culture isolates were tested with E-test and defined as **s**ensitive, **i**ntermediate **r**esistant or resistant using epidemiological cutoffs obtained from AFA/EUCAST. The procedure adhered to the guidelines issued in "E-TEST Technical Manual (ETM)" at AB Biodisc homepage(37): 5) Enterobacteriaceae (EAS 004), gram negative aerobes updated 2007-06.

Procedure:

1) The bacteria were suspended in 0,85 % sodiumchloride to 0,5 McFarland (1 McFarland for mucoid strains).
2) Quality control strain identically suspended.
3) These suspensions were swabbed on Mueller Hinton agar within 30 minutes after making.
4) The Agar was left to dry for at least 15 minutes before E-tests were applied with a vacuum-pen.
5) Incubation over night at 35 °C for 16-20 hours.
6) The results of each test were agreed by 2 observers.

All Urine isolates where tested with MIC Test Strips and defined as **s**ensitive, **i**ntermediate **r**esistant or resistant using epidemiological cutoffs obtained from AFA/EUCAST. The procedure was based on the procedure recommended by Liofilchem in a leaflet sent with the product.

1) Morphologically similar colonies were suspended 0,85 % Sodiumchloride
2) A quality control strain was identically suspended
3) The turbidity was compared to the appropriate McFarland standard. (0,5 McFarland)
4) The suspensions were swabbed on Mueller Hinton agar within 30 minutes after making.
5) The Agar was left to dry for at least 15 minutes before E-tests were applied with tweezers.
6) Incubation over night at 35 °C for 16-20 hours.
7) The results of each test were agreed by 2 observers.

### 3.2.2 Selected genes

***aac(6`)-Ib* and *aac(6`)-Ib-cr***

The sequence for the genes *aac(6`)-Ib* and *aac(6`)-Ib-cr* was searched for using NCBI`s Nucleotide database search. The search was done within the strain *E.Coli* to reduce the number of hits. Basic Local Assignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used to align two nucleotide sequences at the time. First the only focus was finding the two SNP responsible for the difference between the two genes causing amino acid change; Trp102Arg and Asp179Tyr. A total of 8 sequences for the gene *aac(6`)-Ib* and 6 sequences for the gene *aac(6`)-Ib-cr* was found (see APPENDIX 5).

Due to several spotted differences between the base sequences, a map was created over all base variations to better find a suited area for primers and the probe (see APENDIX 6). This was done using the FASTA sequence from *Escherichia coli* strain 2098EC (GenBank number: FJ619242), encoding AAC(6`)-Ib and search in the nucleotide selection among *Enterobacteriaceae* species to find highly similar matches. The variations spotted were manually outlined in the FASTA sequence, and the areas of variations were avoided when choosing appropriate primers and probes.

***aac(3)-II***

The inclusion criteria used in this study was isolates reported resistant against gentamicin or netilmicin from routine investigations. In *E.coli*, gentamicin resistance is reported to be most commonly mediated by AAC(3)s and ANT(2``)-I enzymes(14). AAC(3)s are frequently found in Gram negative bacteria (7). This led us to screen for the presence of *aac(3)-II* as a possible cause for gentamicin resistance in the selected isolates.

The gene *aac(3)-II* has several subtypes. The primers chosen from publication would detect subtypes *aac(3)-IIa* and *aac(3)-IIc* according to the software DNA star Laser gene Seqman. A comparison of a total of 47 published gene sequences was performed prior to choosing a suitable pair of primers.

The theoretical specificities of all the chosen primers and probe sequences were tested with the Basic Local Assignment Search Tool.

### 3.2.3 Extraction of DNA

The procedure used was based on standard laboratory procedures for gram negative aerobes (Enterobacteriaceae) in use at Haukeland University Hospital, department of Microbiology. The process listed below lyses the cells and releases the DNA for PCR reactions.

Procedure:

1) 8-15 morphologically similar colonies where mixed in 0,5 ml sterile distilled water in an Eppendorf tube. Each sample was mixed on a Vortex mixer.
2) The tubes were heated on hot stones. To release the DNA from the bacteria the heat was kept at 100 °C for 5-10 minutes.
3) After cooling the suspension down to room temperature, the samples where centrifuged for 5 min at 13 000 rpm. The supernatant containing the DNA were kept frozen until usage.

These DNA samples are termed “template” in the procedural descriptions below.

### 3.2.3 PCR amplification

***aac(6`)-Ib***

**Initial screening for all *aac(6`)-Ib:***

The primers and probe were designed using Applied Biosystem`s Primer Express 3.0

**Table 3.4:** *Oligonucleotide primers and probe used for PCR*:

| Gene target: | Primer / Probe: | Sequence (5`-3`): | Producer: | Reference: |
|---|---|---|---|---|
| *aac(6`)-Ib* (Screening) | Forward Primer | CCGACACTTGCTGACGTACAG | Sigma-Aldrich, Leirdal, OSLO | This study |
| | Reverse Primer | TGACGGACTCTTGCGCTAAA | Sigma-Aldrich, Leirdal, OSLO | This study |
| | MGB Probe (6-FAM) | AAC AGT ACT TGC CAA GCG | Applied Biosystems, Warrington, UK | This study |

**Table 3.5:** *Kits used in this study*:

| Method | Reagent | Manufacturer: |
|---|---|---|
| RT-PCR<br>(Screening for *aac(6`)-Ib*) | TaqMan®Universal PCR Master Mix.<br>Contains:<br>-AmpliTaq Gold DNA Polymerase<br>-AmpErase UNG,<br>-dNTPs with dUTP,<br>-Passive Reference<br>-Optimized buffer components | Applied Biosystems, Bleiswijk, Netherlands |

Screening for *aac(6`)-Ib* was carried out by real-time PCR using specific primers and a probe designed for this study. PCR was done with a final volume of 20 µl; using 5 µl template, 10 µl TaqMan®Universal PCR Master Mix, 250nM probe and 900 nM of each primer. The instrument used was Applied biosystem 7500/7500 Fast real-time PCR system and standard cycling parameters was used; holding phase a; 50 °C for 2 min, holding phase b; 95 °C for 10 min to activate the enzymes, then cycling parameters; 95 °C for 15 sec and 60 °C for 1 min for 40 cycles.

### Amplification of longer segment to identify *aac(6`)-Ib:*

The primers used were from the published study; Prevalence in the United States of *aac(6`)-Ib-cr* Encoding a Ciprofloxacin-Modifying Enzyme; Chi Hye Park, Ari Robicsek et.al.(28) with a modification in the forward primer.

The probe was designed using Applied Biosystem`s Primer Express 3.0.

**Table 3.6:** *Oligonucleotide primers and probe used for PCR:*

| Gene target: | Primer / Probe: | Sequence (5`-3`): | Producer: | Reference: |
|---|---|---|---|---|
| *aac(6`)-Ib* | Forward Primer | TTGCGATGCTCTATGRGTGGCTA | Sigma-Aldrich, Leirdal, OSLO | Based on publication, with modification (28) |
| | Rewerse Primer | CTCGAATGCCTGGCGTGTTT | Sigma-Aldrich, Leirdal, OSLO | From publication (28) |
| | MGB Probe (6-FAM) | ACGGATGGTGGGAAGA | Applied Biosystems, Warrington, UK | This study |

**Table 3.7:** *Kits used in this study:*

| Method | Reagent | Manufacturer: |
|---|---|---|
| RT-PCR (*aac(6`)-Ib*) | TaqMan®Universal PCR Master Mix. Contains: -AmpliTaq Gold DNA Polymerase -AmpErase UNG, -dNTPs with dUTP, -Passive Reference -Optimized buffer components | Applied Biosystems, Bleiswijk, Netherlands |
| | *Premix Ex Taq*™ (Perfect Real Time) Contains: -*TaKaRa Ex Taq* ™ HS, -dNTP Mixture, -$Mg^{2+}$. Comes with separate tubes with: ROX™ Reference Dye ROX™ Reference Dye II | Takara BIO INC, AH Diagnostics, OSLO |

Detecting *aac(6`)-Ib* was carried out by real-time PCR using specific primers and a probe designed to detect only *aac(6`)-Ib* and not *aac(6`)-Ib-cr.* . The first instrument used was Applied biosystem 7500/7500 Fast real-time PCR system and was done with a final volume of 20 µl; using 5 µl template, 10 µl TaqMan®Universal PCR Master Mix, 250nM probe and 900 nM of each primer. Special cycling parameters was used; first a holding phase ; 95 °C for 10 min to activate the enzyme AmpliTaq Gold DNA Polymerase, then cycling parameters; 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 45 sec for 34 cycles as used in the article from where the primers sequences were found. Later standard cycling parameters was tried; holding phase a; 50 °C for 2 min, holding phase b; 95 °C for 10 min to activate the enzymes, then cycling parameters; 95 °C for 15 sec and 60 °C for 1 min for 40 cycles.

The second instrument used was a Smartcycler® with a final volume of 25 µl; using 2 µl template, 12,5 µl TaqMan®Universal PCR Master Mix or *Premix Ex Taq*™ (Perfect Real Time), 220 nM probe and 450 nM of each primer. The cycling parameters used was; holding phase ; 94 °C for 15 sec, then cycling parameters; 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 45 sec for 34 cycles as used in the article from where the primers sequences were found.

***aac(3)-II***

**Screening for all *aac(3)-II:***

The primers used were from the published study; Occurrence of antibiotic-resistant enterobacteria in agriculture foodstuff, Sybille Boehme, Guido Werner et.al(38)
The primers suitability to detect subtypes of *aac(3)-II* was assessed using BLAST and the software DNA Star Lasergene Seqman. They will detect the following subtypes: *aac(3)-IIa*, *aac(3)-IIc*

**Table 3.8:** *Oligonucleotide primers and probe used for PCR:*

| Gene target: | Primer / Probe: | Sequence (5`-3`): | Producer: | Reference: |
|---|---|---|---|---|
| *aac(3)-II* | Forward Primer | TGAAACGCTGACGGAGCCT | Sigma-Aldrich, Leirdal, OSLO | From publication(38) |
| | Rewerse Primer | GTCGAACAGGTAGCACTGAG | Sigma-Aldrich, Leirdal, OSLO | From publication(38) |

**Table 3.9:** *Kits used in this study:*

| Method | Reagent | Manufacturer: |
|---|---|---|
| RT-PCR (*aac(3)-II*) | SYBR® *Premix Ex Taq*™ (Perfect Real Time) | Takara BIO INC, AH Diagnostics, OSLO |

Detection of *aac(3)-II* was carried out using Smartcycler® with SYBRgreen reagents with a final volume of 25 µl using; 2 µl template, 12,5 µl SYBR® *Premix Ex Taq*™ (Perfect Real Time) and 200nM of each primer. The cycling parameters used was; holding phase ; 95 °C for 120 sec, then cycling parameters; 95 °C for 40 sec, 58 °C for 20 sec and 72 °C for 30 sec for 35 cycles.

### 3.2.4 Sequencing of all *aac(6`)-Ib*

Duplicate amplification of all positive isolates from "*screening for aac(6`)-Ib*" was performed. The PCR product from "*amplification of longer segment to identify aac(6`)-Ib-cr* "could not be directly sequenced due to the presence of a probe in the product. Probes interfere with sequencing and the cleaning kit used; ExoSAP-IT is not recommended for cleaning probes from a PCR product. ExoSAP-IT will however clean primers and unconsumed dNTP`s from a PCR product.

The primers used for the duplicate amplification were the same as used in "*amplification of longer segment to identify aac(6`)-Ib".*

**Table 3.10:** *Kits used in this study:*

| Method | Reagent | Manufacturer: |
|---|---|---|
| RT-PCR *(aac(3`)-II)* | SYBR® *Premix Ex Taq*™ (Perfect Real Time) | Takara BIO INC, AH Diagnostics, OSLO |
| Screening preparation | ExoSAP-IT for PCR Product Clean-Up Contains: -Exonuclease I -Shrimp Alkaline Phosphatase -Specially formulated buffer | USB, Affymetrix |
| | ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit 1.1 Contains: • Ready Reaction Mix • pGEM®-3Zf(+) double-stranded DNA Control Template • –21 M13 Control Primer (forward) • BigDye Terminator v1.1/3.1 Sequencing Buffer (5X) | Applied Biosystems, Bleiswijk, Netherlands |

Duplicate amplification of *aac(6`)-Ib* was carried out by real-time PCR using specific primers with detection by SYBRgreen. The instrument used was Smartcycler® and the reaction was done with a final volume of 20 µl; using 2 µl template, 12,5µl SYBR® *Premix Ex Taq*™ (Perfect Real Time) and 200nM of each primers.

Product clean up was perfomed in a DNA Engine Dyad® Peltier Thermal cycler by incubating a mix of template and ExoSAP-IT for 15 min. at 37°, thereafter deactivate the enzymes by incubating the mix at 80°C to for 15 min. Cycle sequencing was performed by using the forward primer.

The sequences were read on Chromas Pro version 1.5

## 3.3 Statistics

Prevalence of Ciprofloxacin resistance in different isolates were compared with Fisher Exact test (Five or less observations in each group) or Chi-square analysis. P-values <0.05 were considered statistically significant.

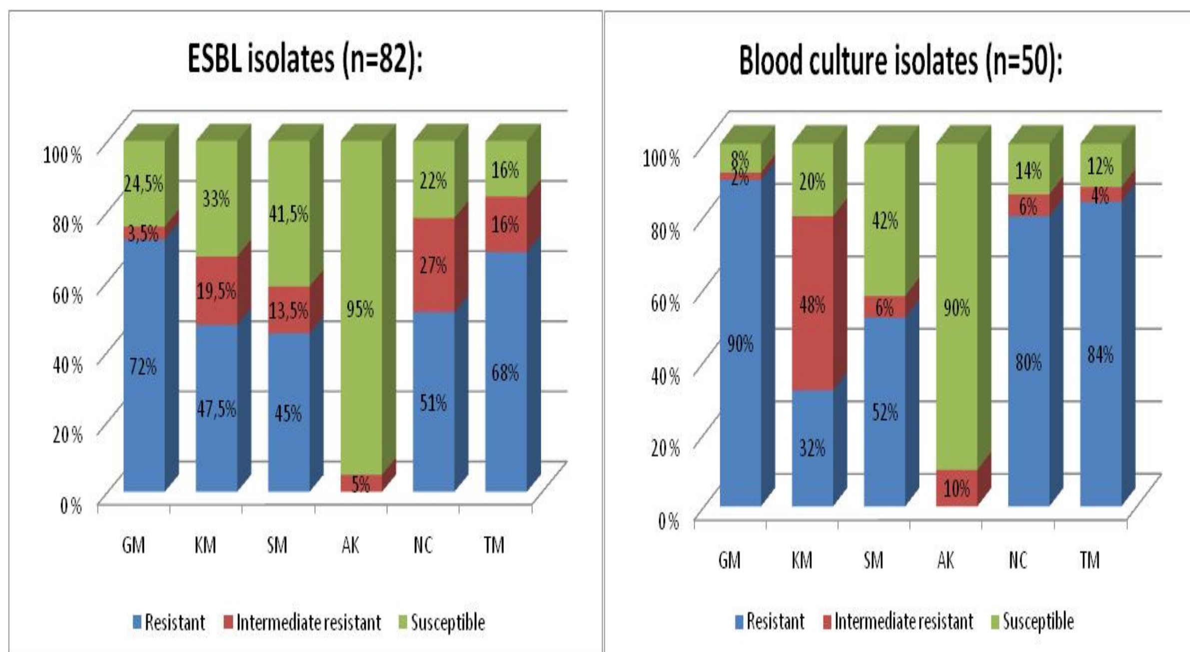
# 4. Results

See APPENDIX 1-4 for complete results listed in tables. The tables contain:

- Previously obtained data from routine practice.
- Resistance profiles based on MIC values.
- Data collected from preformed Polymerase chain reactions.

## 4.1 Susceptibility tests:

***Figure 4.1***: *Proportion of isolates **R**esistant, **I**ntermediate resistant or **S**usceptible to each Aminoglycoside tested. See Methods for details regarding categorization. (GM = Gentamicin, KM = Kanamycin, SM = Streptomyin, AK = Amikacin , NC = Netilmycin, TM = Tobramycin)*



**Presence of combination of antibiotic resistance previously reported to be associated with AAC(6`)-Ib and AAC(6`)-Ib-cr phenotypes:**

A total of 9 isolates were intermediate resistant (I) to amikacin in addition to resistance or intermediate resistance against kanamycin, netilicin and tobramycin consistent with the AAC(6`)-Ib profile. It's noteworthy that none of the isolates were resistant (R) to amikacin.

5 of these 9 isolates had previously been tested (Routine laboratory testing, Department of Microbiology, Haukeland University Hospital) resistant for ciprofloxacin and nalidixic acid, while 4 were reported resistant to ciprofloxacin alone. All of these isolates (100%) were resistant to the tested quinolones.

**ESBL isolates:**

~61 % of the ESBL isolates (50 of 82) were resistant (R) or intermediate resistant (I) against kanamycin, netilmicin and tobramycin. Of these; 8% (4 isolates) were additionally intermidate resistant to amikacin.

Only 3 isolates were exclusively R/I to kanamycin, amikacin, netilmicin and tobramycin matching the phenotypic profile for AAC(6`)-Ib. The rest were R/I also to one or both of the two remaining aminoglycosides tested. 40 isolates (73%) were additionally gentamicin R/I. 30 isolates (55%) were additionally streptomycin R/I.

**Blood culture isolates:**

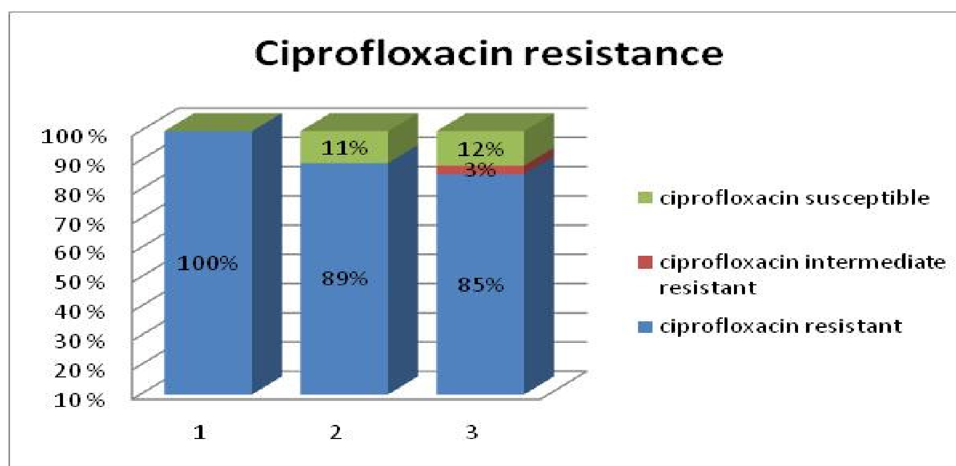
78 % of the blood culture isolates (39 of 50) were R/I against kanamycin, netilmicin and tobramycin. Of these 13% (5 isolates) were additionally amikacin intermediate resistant.

None of the isolates were exclusively R/I to kanamycin, amikacin, netilmicin and tobramycin matching the phenotypic profile for AAC(6`)-Ib. All showed resistance to one or both of the two remaining aminoglycosides tested. All 39 isolates (100%) were additionally gentamicin R/I. 25 (64%) isolates were additionally streptomycin R/I.

**Ciprofloxacin susceptibility results from routine work at Haukeland University hospital: relevant results**

Co-resistance of fluoroquinolone and the three aminoglycosides kanamyin, netilmycin and tobramycin, but without reduced susceptibility to amikacin was frequently observed in the isolates of the present study. Of the 46 ESBL isolates that showed this aminoglycoside resistance phenotype, 41 isolates were resistant to ciprofloxacin. Of the 34 blood culture isolates, 29 isolates were resistant and 1 isolate was intermediate resistant to ciprofloxacin (figure 4.3).

**Figure 4.2:** *Column **1** shows the proportion of ciprofloxacin resistance among 9 isolates with resistance phenotypes consistent with aac(6`)-Ib. The two rightmost columns shows ciprofloxacin resistance co-existing with resistance or intermediate resistance to kanamycin, netilmicin and tobramycin but not to amikacin among ESBL isolates (coloumn **2**) and blood culture isolates (column **3**).*



**Presence of antibiotic resistance associated with AAC(3)-II phenotype:**

A total of 95 isolates were intermediate resistant (I) against gentamicin, netilicin and tobramycin consistent with the AAC(3)-II profile.

**ESBL isolates:**

64% of the ESBL isolates (52 of 82) were R/I against gentamicin, netilmicin and tobramycin, only 8 isolates were exclusively resistant or intermediate resistant against these aminoglycosides matching the phenotypic profile for AAC(3)-II. The remaining isolates showed additional resistance (R) or intermediate resistance (I) to one or more of the 3 remaining aminoglycosides tested. 40 isolates (77%) were additionally kanamycin R/I. 30 isolates (58%) were additionally streptomycin R/I.

**Blood culture isolates:**

86 % of the blood culture isolates (43 of 50) were R/I against gentamicin, netilmicin and tobramycin, but only 4 isolates were exclusively resistant or intermediate resistant against these aminoglycosides matching the phenotypic profile for AAC(3)-II. The rest showed resistance (R) or intermediate resistance (I) to one or more of the 3 remaining aminoglycosides tested. 5 isolates are additionally amikacin I (12%), 39 isolates (91%) are additionally kanamycin R/I. 25 isolates (58%) were additionally streptomycin R/I.

## 4.2 Identification of the gene *aac(6`)-Ib*

### 4.2.1 Initial screening for *aac(6`)-Ib gene*.

A total of 47 of 132 isolates were positive for the presence of the gene *aac(6`)-Ib*. 12 out of 50 isolates form blood culture and 35 out of 82 ESBL isolates.

***Figure 4.3.:*** *Presence of aac(6`)-Ib in the initial screening. Blood culture (n = 50) Positive = Presence of gene, Negative = gene not detected.*

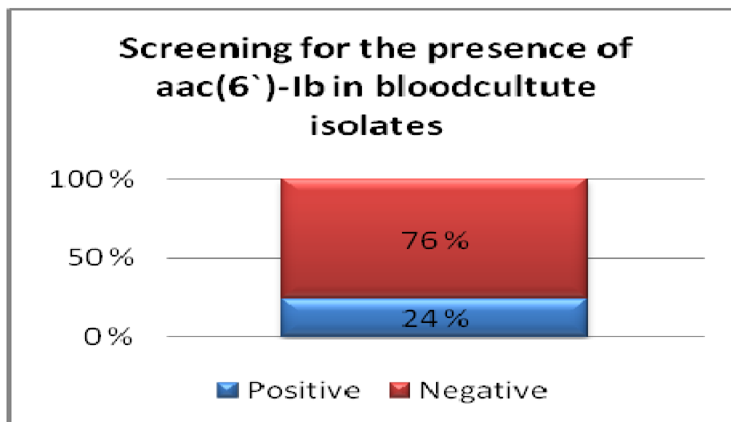
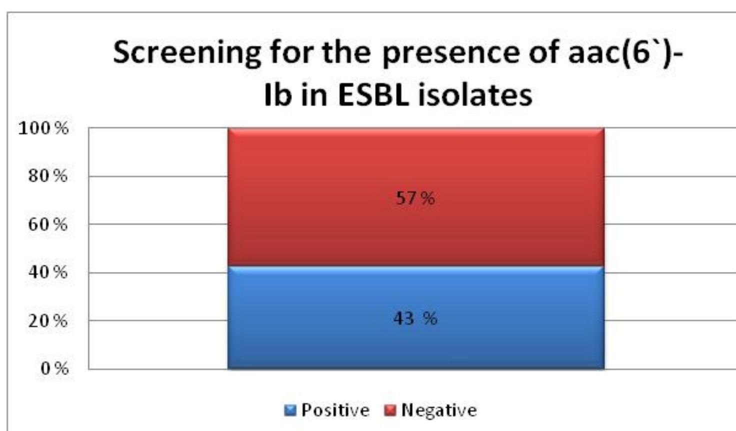



***Figure 4.4:*** *Presence of aac(6`)-Ib in the initial screening. ESBL (n = 82) Positive = Presence of gene, Negative = gene not detected*



**Association with antibiotic resistance (AAC(6`) phenotype):**

A total of 89 of 132 isolates were resistant (R) or intermediate resistant (I) against kanamycin, netilmicin and tobramycin. Of these; only 9 isolates were amikacin intermediate resistant matching the resistance profile for AAC(6`)-Ib.

Of these 9 isolates with matching resistance profile indicating the presence of AAC(6`)-Ib-cr; 4 were ESBL isolates and 5 were from blood culture isolates. All 4 (100%) of the ESBL isolates had the gene *aac(6`)-Ib*. Only 3 of the 5 isolates (60%) from blood culture had the gene *aac(6`)-Ib*

**Table 4.1:** *The 9 isolates that are R/I to kanamycin, amikacin, netilmycin and tobramycin matching the resistant profile for aac(6`)-Ib. Only 7 have the gene encoding* AAC(6`)-Ib *or its variants.*

*The isolates are divided after their phenotype (resistance profile). The phenotype is described with capital letters if resistant and small letters if intermediate resistant.*
*(G/g = Gentamicin, K/k= Kanamycin, S /s= Streptomyin, A/a = Amikacin, N/n = Netilmycin, T/t = Tobramycin.)*

| ESBL | | |
|---|---|---|
| Phenotype | ***aac(6`)-Ib or -cr variant*** | **no gene** |
| K a n T | 3 | 0 |
| K s a N T | 1 | 0 |

| Blood culture | | |
|---|---|---|
| Phenotype | ***aac(6`)-Ib or -cr variant*** | **no gene** |
| G K S a N T | 1 | 0 |
| G K a N T | 2 | 2 |

**Summary- initial screening test:**

70 % (30 out of 50) of the isolates from the ESBL isolates with resistance or intermediate resistance to kanamycin, netilmicin and tobramycin had the gene *aac(6`)-Ib*. (Including the 4 isolates intermediate resistant to amikacin and the 26 that were sensitive to amikacin.)

31% (12 out of 39) of the isolates from the blood culture isolates with resistance or intermediate resistance to kanamycin, netilmicin and tobramycin had the gene *aac(6`)-Ib*. (These include the 5 isolates intermediate to resistant to amikacin and the 7 that were sensitive to amikacin.)

The majority (~64%, 30 out of 47 isolates) of the isolates with *aac(6`)-Ib* were resistant to at least 3 of the relevant aminoglycosides; kanamycin, netilmicin and tobramycin. The rest of the isolates with the gene *aac(6`)-Ib* were only intermediate resistant to one or more of the aminoglycosides in focus.

92 % (32 of 35) ESBL isolates positive for *aac(6`)-Ib* were kanamycin resistant. All of the isolates from blood culture positive for *aac(6`)-Ib* were kanamycin resistant.

### 4.2.2 Subsequent amplification of longer segment to find aac(6`)-Ib.

As detailed in methods, the probe used in the subsequent procedure should detect only *aac(6`)-Ib* and not its variants with influence on the ciprofloxacin susceptibility. The presumption was that the procedure should exclude isolates with *aac(6`)-Ib-cr* variants from amplification.

Results from real-time PCR using Applied Biosystem ingredients resulted in weak signals even for the positive control, and we could not ascertain whether the tested isolates had *aac(6`)-Ib* or not. Different approaches were made to improve the signal on the same machine; changing temperature settings, time intervals, concentrations of the ingredients etc, but all our attempts failed.

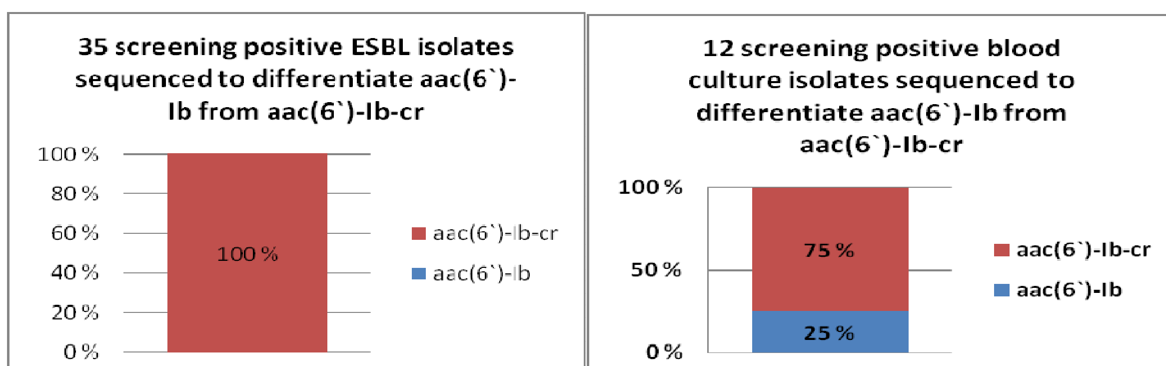
An alternative approach was tried, screening the material on a Smartcycler® machine with different reagents. However, no signal was obtained from the positive control for *aac(6`)-Ib* using the same reagents previously used on the Applied Biosystem machine. However, a positive signal was obtained when using Takara reagents. All isolates which were positive for *aac(6`)-Ib* with initial screening (n=47) gave positive signal with the probe for *aac(6`)-Ib*, even the positive control for the variant of *aac(6`)-Ib-cr*. This excluded the probe for further use, since it could not differentiate the gene from its variants. See discussion for possible explanations for these results.

Due to the inconclusive results, the amplicons were sequence analysed out in order to identify *aac(6`)-Ib* and its variants.

### 4.2.3 Results of genetic sequence analysis.

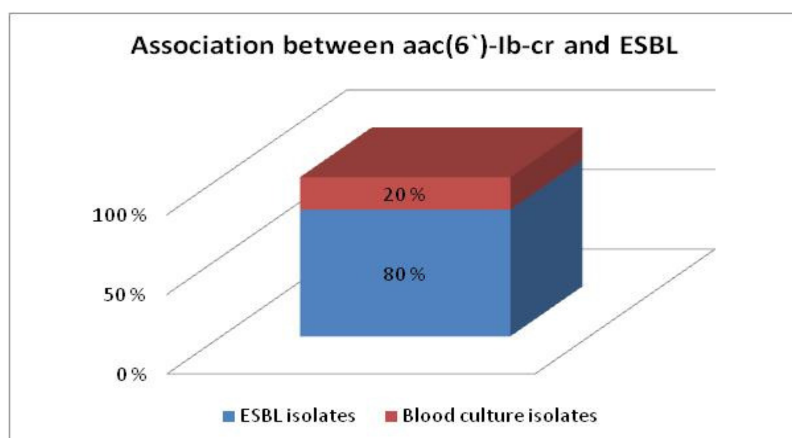
The majority of the isolates initially screened positive for *aac(6`)-Ib* had the variant *aac(6`)-Ib-cr* (44 of 47). The two single nucleotide polymorfs that differentiat *aac(6`)-Ib* and *aac(6`)-Ib-cr* was found in each isolate replacing two T bases with C and G respectively.

**Figure 4.5:** *A total of 44 isolates have the gene encoding aac(6`)-Ib-cr. All of the isolates with ESBL (100%) and 9 isolates (75%) collected from blood culture. Only 3 blood culture isolates have the gene encoding aac(6`)-Ib.*



Of the total of 132 isolates 44 have the gene *aac(6`)-Ib-cr*. 34 of these 43 isolates with *aac(6`)-Ib-cr* possess ESBL and 1 possess plasmid AmpC.

**Figure 4.6:** *80 % of the 43 isolates with the gene aac(6`)-Ib-cr also possess ESBL*




**Association between the presence of *aac(6`)-Ib* and its variant and the phenotypic resistance profile**

9 isolates had the phenotype for *aac(6`)-Ib-cr* being intermediate resistant (I) to amikacin in addition to resistant or intermediate resistant (R) to kanamycin, netilmicin, tobramycin and ciprofloxacin. Only 7 of these were screening positive for the presence of the gene. The sequencing results state that all of these 7 isolates have the variant of the gene *aac(6`)-Ib-cr*.

**Table 4.2:** *The 7 screening positive isolates with matching resistance profile to* AAC(6`)-Ib-cr *have the gene encoding this enzyme.*

| ESBL | | | Blood culture | | |
|---|---|---|---|---|---|
| Phenotype | ***aac(6`)-Ib-cr*** | ***aac(6`)-Ib*** | Phenotype | ***aac(6`)-Ib-cr*** | ***aac(6`)-Ib*** |
| K a n T | 3 | 0 | G K S a N T | 1 | 0 |
| K s a N T | 1 | 0 | G K a N T | 2 | 0 |

**Table 4.3:** *Phenotypic resistance profiles for the sequenced isolates which are susceptible to amikacin.*

| ESBL | | | Blood culture | | |
|---|---|---|---|---|---|
| Phenotype | *aac(6`)-Ib-cr* | *aac(6`)-Ib* | Phenotype | *aac(6`)-Ib-cr* | *aac(6`)-Ib* |
| G K S N T | 10 | | G K S N T | 3 | 3 |
| G k S N T | 1 | | G k S N T | | |
| G K S n T | 3 | | G K S n T | | |
| G K N T | 9 | | G K N T | 3 | |
| G K n t | 1 | | G K n t | | |
| G k n T | 1 | | G k n T | | |
| K S N T | 1 | | K S N T | | |
| K N T | 1 | | K N T | | |
| K S n T | 2 | | K S n T | | |
| K n T | 2 | | K n T | | |

**Association between sequence results and ciprofloxacin resistance**

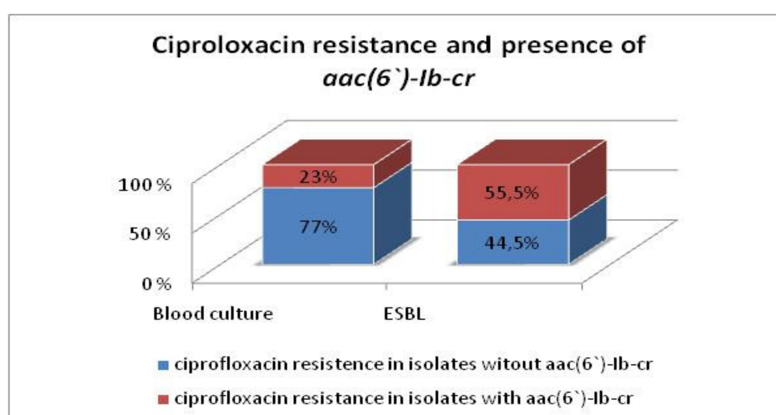
34 out of 35 screening positive ESBL isolates with the variant *aac(6`)-Ib-cr* were resistant to ciprofloxacin and nalidixic acid. All 9 screening positive from blood culture having the gene for *aac(6`)-Ib-cr* are resistant to ciprofloxacin according to the susceptibility performed as part of routine work at department of Microbiology, Haukeland Hospital. 2 out of 3 blood culture isolates having the gene *aac(6`)-Ib* are ciprofloxacin resistant.

The prevalence of ciprofloxacin resistance was significantly higher in isolates with *aac(6`)-Ib-cr* compared to isolates without this particular gene (98% vs 67%, P<0,001)

Ciprofloxacin resistance was not significantly different between the isolates with the *aac(6`)-Ib* and variant gene.

There was observed a high prevalence of ciprofloaxacin R/I among blood culture isolates without the gene encoding AAC(6`)-Ib-cr (n=30)( 77%). See figure below.

**Figure 4.7:** *Presence of aac(6`)-Ib-cr and ciprofloxacin resistance in the isolates*



## 4.3 AAC(3)-II

A total of 95 of 132 isolates were positive for the presence of the gene for *aac(3)-II*. 40 out of 50 blood culture isolates, and 55 out of 82 ESBL isolates.

***Figure 4.8:*** *Presence of aac(3)-II in blood culture isolates(n=50). Positive = Presence of gene, Negative = gene not detected.*

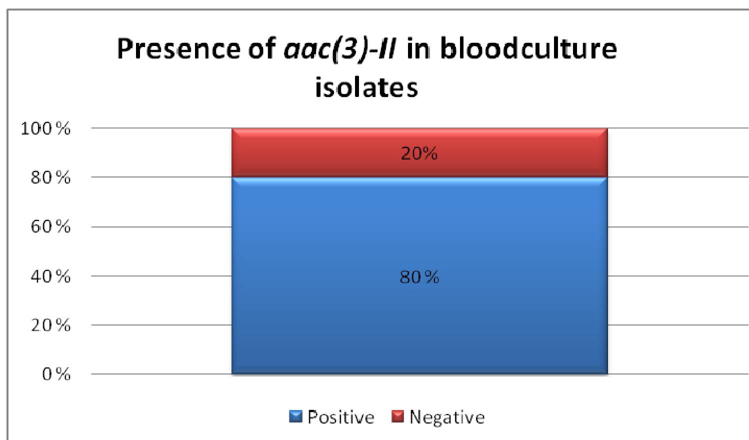



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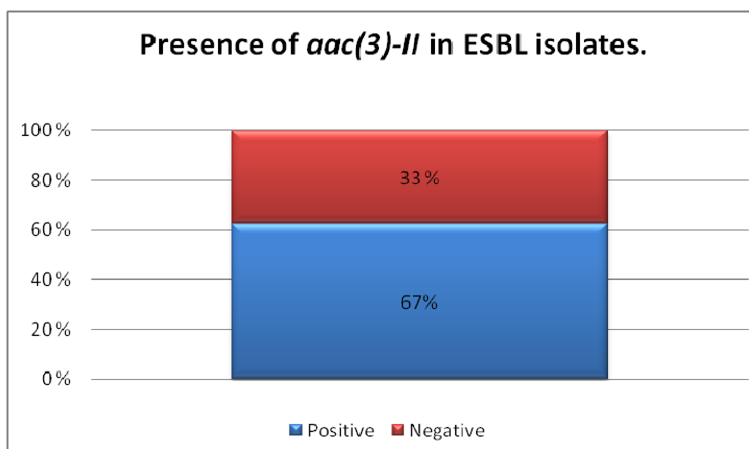
***Figure 4.9:*** *Presence of aac(3)-II in ESBL isolates(n=82). Positive = Presence of gene, Negative = gene not detected.*



**Resistance phenotype associated with the presence of AAC(3)-II**

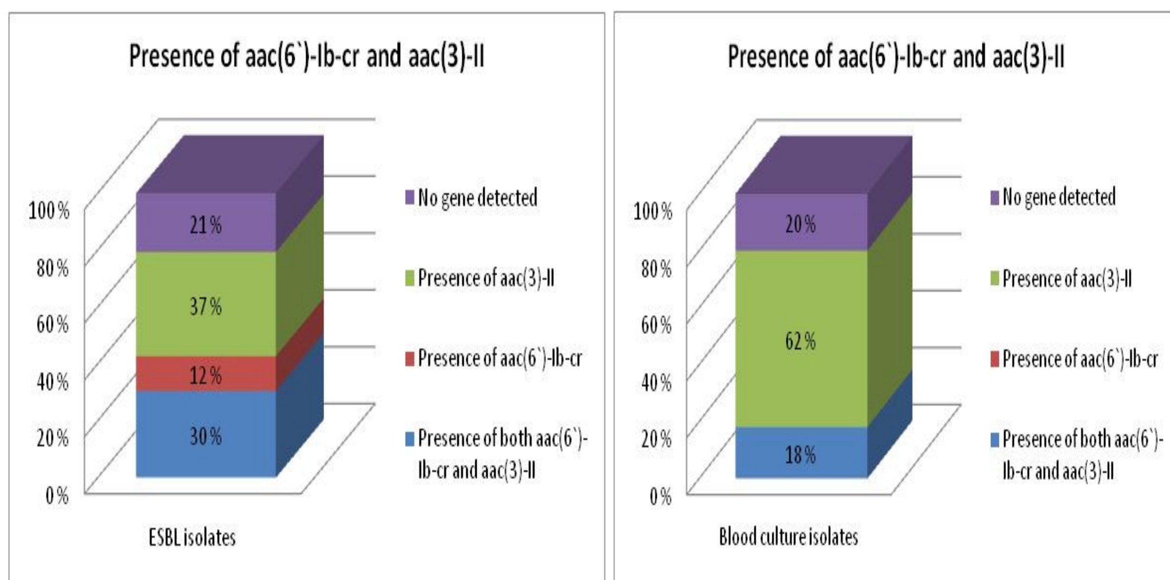
52 isolates with the gene *aac(3)-II* from the ESBL isolates were resistant or intermediate resistant to gentamicin, netilmicin and tobramycin consistent with the phenotype for AAC(3)-II. The majority were additionally resistant to one or more of the other tested aminoglycosides. The remaining 3 isolates does not fit the phenotypic profile for AAC(3)-II due to susceptibility to one or more of the aminoglycosides in focus.

All of the 40 of the isolates with the gene *aac(3)-II* from blood culture were resistant or intermediate resistant to gentamicin, netilmicin and tobramycin. The majority were additionally resistant to one or more of the other tested aminoglycodsides.

## 4.4 Association between presence of the genes *aac(6`)-Ib-cr* and *aac(3)-II*

There was a high association between *aac(6)-Ib-cr* and *aac(3)-II* as 34 of 44 isolates with *aac(6`)-Ib-cr* have both genes. All 34 with both genes present were gentamicin resistant, while none of the remaining 10 isolates with only *aac(6`)-Ib-cr* were resistant to gentamicin.

**Figure 4.10:** *Prevalence of the combination aac(6`)-Ib-cr and aac(3)-II among the isolates.ESBL*




The ESBL and blood culture isolates compared different with respect to prevalence of *aac(6`)-Ib-cr* and *aac(3)-II* genes (Fig 4.12, P<0.01). The combination of *aac(6`)-Ib-cr* and *aac(3)-II* genes were present in 30% of the ESBL isolates and 18% blood cultures, but this difference did not reach statistical significance

## 4.5 Data from the Norwegian prescription register and numbers from wholesalers statistics.

**Norwegian prescription data base (39):**

**Aminoglycosides:**

**Table 4.4**: *Data from NorPD (Gentamicin and Tobramycin) The number includes the use amongst people of all ages, both sexes in the entire country of Norway.*

| J01G Aminoglycosides | Turnover by dosage (DDD) |
|---|---:|
| 2004 | 25 432 |
| 2005 | 31 509 |
| 2006 | 33 472 |
| 2007 | 35 862 |
| 2008 | 33 724 |
| 2009 | 34 721 |

*The use of Aminoglycosides has increased slightly over the last 6 years.*

**Quinolones:**

**Table 4.5**: *Data from NorPD. The number includes the use amongst people of all ages, both sexes in the entire country of Norway.*

| J01MA02 Ciprofloxacin | Turnover by dosage (DDD) |
|---|---:|
| 2004 | 555 748 |
| 2005 | 606 853 |
| 2006 | 674 026 |
| 2007 | 748 975 |
| 2008 | 805 226 |
| 2009 | 822 339 |

*The use of Ciprofloxacin has increased considerably over the last 6 years.*

**Wholesalers statistics (40-42):**

**Table 4.6:** *Total sale of aminoglycosides and quinolones in Norway both to community pharmacies and hospitals/nursing homes.*

| | | DDD/1000 inhabitants/day | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ATC** | **ATC level name (DDD value)** | **1998** | **1999** | **2000** | **2001** | **2002** | **2003** | **2004** | **2005** | **2006** | **2007** | **2008** | **2009** |
| | | | | | | | | | | | | | |
| ***J01G*** | ***Aminoglycosides*** | ***0,05*** | ***0,05*** | ***0,04*** | **0,06** | **0,07** | **0,07** | **0,06** | **0,07** | **0,07** | **0,07** | **0,07** | **0,07** |
| J01GB01 | Tobramycin (0,24 g P/ 0,3 g inhal.sol.) | 0,03 | 0,03 | 0,02 | 0,03 | 0,04 | 0,04 | 0,03 | 0,03 | 0,03 | 0,03 | 0,03 | 0,03 |
| J01GB03 | Gentamicin (0,24 g) | 0,006 | 0,006 | 0,006 | 0,008 | 0,017 | 0,03 | 0,03 | 0,03 | 0,04 | 0,04 | 0,04 | 0,04 |
| J01GB07 | Netilmicin (0,35 g) | 0,02 | 0,02 | 0,02 | 0,02 | 0,01 | 0 | 0 | 0 | 0 | 0 | | |
| | | | | | | | | | | | | | |
| ***J01M*** | ***Quinolones*** | ***0,3*** | ***0,33*** | ***0,35*** | ***0,4*** | ***0,43*** | ***0,47*** | ***0,52*** | **0,57** | **0,62** | **0,67** | **0,7** | **0,7** |
| J01MA01 | Ofloxacin (0,4 g) | 0,06 | 0,06 | 0,05 | 0,05 | 0,05 | 0,05 | 0,05 | 0,05 | 0,04 | 0,04 | 0,04 | 0,03 |
| J01MA02 | **Ciprofloxacin** (1 g O/ 0,5 g P) | 0,23 | 0,26 | 0,29 | 0,34 | 0,38 | 0,42 | 0,47 | 0,52 | 0,57 | 0,62 | 0,66 | 0,67 |
| J01 M B02 | **Nalidixin syre** (4g) | 0,01 | 0,01 | 0,01 | 0,01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

The use of ciprofloxacin has increased considerably according to wholesalers statistics. The use of Tobramycin has not increased, but gentamicin consume has increased slightly.

It is important to keep in mind that these figures should be regarded as maximum figures based on the assumption that all products sold are actually consumed. The actual drug consumption is probably somewhat lower.

# 5 Discussion

### 5.1.1 Observations around phenotypic characterization

After phenotypic characterization by interpreting MIC values for the six aminoglycosides using epidemiological cutoffs, it became clear that we could not easily determine which aminoglycoside modifying enzyme (AME) was present from the MIC results alone. Most isolates showed resistance to a variety of aminoglycosides indicating the presence of several AME`s and/or possible other resistance mechanisms. The results from PCR verified this by the detection of both *aac(3)-II* and *aac(6`)-Ib/aac(6`)-Ib-cr* in several isolates.

One of the observations related to the MIC values was the low prevalence of amikacin resistance among our isolates. Due to structurally differences among aminoglycosides, inactivation of amikacin is reserved to only a few aminoglycoside modifying enzymes.

Another observation was that the gene *aac(6`)-Ib* or *aac(6`)-Ib-cr* was detected in several of the isolates and their presence was not associated with resistance against amikacin but only reduced susceptibility in 9 out of 47 isolates. The reason for this deviation could be either the use of different breakpoints in the published literature, reduced expression of the protein encoded by *aac(6`)-Ib /aac(6`)-Ib-cr* or modified enzyme protein with alterations in its substrate specificity, in our isolates. Our findings challenge the published information about the substrate specificity, as regards amikacin, of this enzyme.

As ciprofloxacin resistance was observed in isolates without the variant *aac(6`)-Ib-cr*, additional mechanisms must exist for fluoro-quinolone in these isolates. *gyrA* is the most prevalent in *E.coli* and might be the cause for ciprofloxacin resistance among isolates where *aac(6`)-Ib* was not found(43).The focus of this study was not to determine the cause of ciprofloxacin resistance, but to observe if there was a strong association between presence of *aac(6`)-Ib-cr* and ciprofloxacin resistance. Several studies state that PQMR`s like *aac(6`)-Ib-cr* show a low resistance expression on their own, but act additively with other resistance mechanisms like with *qnrA(28)* and that the presence of *aac(6`)-Ib-cr* is frequently associated with increase in the frequency of chromosomal mutations(21). A more comprehensive gene screening would be needed to investigate whether *aac(6`)-Ib-cr* increase resistance in isolates with other resistance mechanisms like *qnrA*. In addition ciprofloxacin susceptibility data in this study is based on VITEK results, VITEK state the isolates as resistant with MIC value >4 towards ciprofloxacin. MIC tests with E-rest strips for ciprofloxacin susceptibility should have been carried out to see if there was a higher degree of resistance among isolates with *aac(6`)-Ib-cr*.

The aminoglycoside resistance phenotypes most likely arise as a result of multiple resistance mechanisms. We recognize the limitations of the present study with respect to this. Resources has limited the number of aminoglycosides tested as well as the number of genes tested. In addition it is important to bear in mind that the presence of the gene sequence, does not necessarily mean that the organism has a transcribed functional protein AAC(6`)-Ib or AAC(6`)-Ib-cr.

### 5.1.2 Aac(6`)-Ib

### (Initial screening)

Several primers from previous publications were assessed for the *aac(6`)-Ib* screening PCR . Due to numerous polymorphisms discovered within the sequence, we chose to design primers with annealing sites without any single nucleotide polymorphisms, using Basic Local Assignment Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). When designing the primers and probe; amplicon length, primer length, % GC, 3`end for the primers, 5`end for the probe and annealing temperature for the primers and probe were all compatible with the design guidelines given by Applied Biosystem. Of all considered primers and probe sets, the selected set was the best choice.

The important factor is that the probe should bind to the sequence before the primers do. The probe should have an annealing temperature 10 °C or more, higher than the primers. In this case the temperature difference was 14 °C ensuring annealing of the probe to target well before the primers do. This amplification and detection proceeded as expected.

The results showing the majority of the isolates with *aac(6)-Ib* among the isolates with ESBL. This indicates a high prevalence of the variant *aac(6)-Ib-cr* since there are strong associations between ESBL and –cr variant in previous publications (19-21).

### 5.1.3 *aac(6`)-Ib*

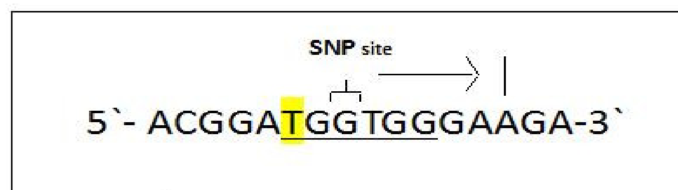
#### (Subsequent amplification of longer segment to find *aac(6`)-Ib*).

The probe to detect *aac(6`)-Ib* was designed to cover one of the areas where a single nucleotide polymorph differentiate *aac(6`)-Ib* from *aac(6`)-Ib-cr*. We chose the area with the single nucleotide polymorph causing the amino acid substitution Trp102Arg for the probe. The second area with the amino acid substitution Asp(179)Tyr was not suited as the probe site due to several nucleotide polymorphisms around this area.

The reason for choosing a probe to detect *aac(6`)-Ib* and not *aac(6`)-Ib-cr* was the discovery of a third SNP after using Basic Local Assignment Tool (BLAST). Both the base A and C were present as SNP`s in sequences for *aac(6`)-Ib-cr*. The codons AGG and CGG code for the same amino acid substitution in the protein sequence (Trp102Arg). This was checked with a translation tool (http://www.expasy.ch/tools/dna.html). To avoid having to use two probes in case of clinical isolates having A or C instead of T, we decided to design one probe detecting *aac(6`)-Ib* where T is the only option. This way we could subtract the number of isolates with *aac(6`)-Ib* from the total number of positives in the initial screening process to get the number of isolates with *aac(6`)-Ib-variants*.

Because of the limitations set by the location of the SNP the probe designed was not ideal. The probes length, % GC and its 5`end were considered in the designing, according to the guidelines from Applied Biosystems manual. Meeting the design guidelines on the 5`end gave us trouble. The probe can not have a G residue on the 5 `end due to its ability to quench of the reporter fluorescence, even after cleavage. There were several G residues present near the 5`end in the sequence. These G`s could not be included in the probe sequence leaving the SNP location too far to the left from what the design guidelines recommend. (see figure 5.1).

**Figure 5.1:** *Illustrates the recommended SNP site is according to Applied Biosystems manual. It should be situated in the middle third position or further to the right. This site is found by dividing the probe in 3 parts, followed by counting to the third base in the middle sequence. As our sequence consists of 16 bases each part consists of ~5 bases leaving base number 8 from the left side as the ideal position for the SNP as marked in the picture. Any one of the following bases to the right of this base can be the position for the SNP, but not the 2 last bases on the right end. Our SNP is situated too far left of the recommended area, as illustrated by the yellow marking.*



In addition to the failure of meeting the guidelines for the SNP position, the annealing temperatures were not ideal and the amplicon length exceeded the recommended number of bases by 334 bases. The recommendation is that a probe should have an annealing temperature between 68-70 °C, while primers should have annealing temperatures between 58-60 °C. Our probe has the annealing temperature; 68 °C, while the primers have the annealing temperatures 62,3 °C and 62 °C. This leaves a difference between the probe and the primers annealing temperatures of only 6 °C and the consequent risk of the probe not annealing before the primers do as it is recommended that the difference should be 10 °C or higher.

There were numerous point mutations discovered after the 3`end of the probe. Ideally the amplicon length should lie between 50-150 bases. This could not be done because of the presence of the variations in the sequence of the gene, thereby making it impossible to design primers targeting a shorter region.

Several published primers for the sequence were considered, and one primer set stood out as the most suited. These primers amplify a sequence of 484 bases and come from the published article; "Prevalence in the United States of aac(6`)-Iv-cr Encoding Ciprofloxacin-Modifying enzyme, C.Park, A.Robicsek et.al.(28). This targeted region includes both SNP`s necessary for ciprofloxacin resistance reported in variants, and therefore they could be identified by sequencing of the PCR product. Unfortunately their annealing temperature was not separated by 10 °C or more from the designed probe as mentioned above, and an SNP discovered in the forward primers annealing site, led to the change of that specific base to an ambiguous base, capable of annealing to more than one specific base. In this case the base is called R and is capable of annealing to both A and G(44).

Using the designed probe with the modified primers failed to give results on the Applied Biosystems machine. The real-time PCR showed indication of a signal, but the signal was weak and the positive control for *aac(6`)-Ib* could not be differentiated from the positive control for *aac(6`)-Ib-cr.* Several measures were made to try to increase the signal strength. The temperature settings were increased 2°C at the time, from the annealing temperature used in the article from where the primers where obtained (55°), to 60°C which is just above the annealing temperature according to the design program used. The time intervals were increased and different concentrations of both template and mix were tested without any improvement in signal strength. To rule out an instrument error being the cause, we tested the same ingredients on a smart cycler where we got no signal.

One possible explanation as to why the signal was low was the presence of AmpErase® uracil-N-glycosylase which is activated at temperatures as low as 50°C. Our amplification temperature was 55°C, and the possibility that the enzyme was active still could not be ruled out. This enzyme prevents re-amplification of carryover products by removing any Uracil incorporated into single or double stranded DNA. It is not active at 60°C which is standard amplification temperature. The possibility that the probe could work in a different mix led us to try a mix from Takara®, this gave strong signals but even the positive control for *aac(6`)-Ib-cr* came out as positive, making it clear that the probe under the Takara conditions, could not differentiate the two gene variants that is differed by a single base pair mismatch. This could be due to the specificity of the probe being dependent on the use of the commended ingredients. Using Sigma-Aldrich primers, Applied Biosystem probe and Takara mastermix might have binding of the probe despite the single base pair mismatch.

A well designed probe is capable of differentiating sequences where only one SNP differentiates between to variants. A probe must have certain properties which are described in the manual for the design program we used. The limitations set by the location of the SNP made it difficult to design a probe meeting all these demands of the design guidelines. This led to the design of a probe not ideal for the real-time PCR technique used. The designing of a probe specific for the SNP failed, as the probe could not differentiate the two variants. Restriction enzyme could have been used to differentiate the variants but we proceed with sequencing the amplicons. All screening positive isolates (n = 47) was screened to identify the variants of the enzyme.

## *aac(6`)-Ib*

## (Sequencing results).

The majority of the isolates had the gene *aac(6`)-Ib-cr* compared to *aac(6`)-Ib,* and they were mainly found among our ESBL strains (35 of 44). The gene for this variant is associated with plasmids which can explain the high prevalence due to spread between bacteria (18, 20, 25). The gene is reported on plasmids with genes encoding several ESBL(25), in addition to being frequently observed in isolates with ESBL(19, 21). The results verifies observations in these previous publications, that there is a strong association between *aac(6)-Ib-cr* and presence and ESBL, both plasmid mediated resistance mechanisms. The high prevalence of this gene among ESBL producing strains (43%) also supports it being an infectious plasmid mediated resistance mechanism.

There was noted a high prevalence of ciprofloxacin resistance especially among isolates from blood culture without the gene *aac(6`)-Ib-cr,* even so there was a statistically significant higher prevalence of ciprofloxacin resistance among the isolates with the *aac(6`)-Ib-cr*.

Assumptions can be made regarding chromosomal resistance mechanisms being present in these blood culture isolates due to lack of other plasmid mediated resistance mechanisms like ESBL. Further investigations regarding the presence chromosomal resistance genes like gyrA, parC, which are frequently found in Enterobacteriaceae, need to be done before opposing the reasons behind the resistance. (43).

### *5.1.2 Aac(3)-II*

There is a high prevalence of the gene *aac(3)-II* in the included isolates which could explain the high frequency of gentamicin resistance. The susceptibility tests conferred a resistance phenotype among the isolates consistent with presence of AAC(3)-II according to reports on this subject (see section 1.3). There are reports claiming this enzyme to be a common resistance mechanism in gram negative bacteria (7), but more comprehensive gene screening would be needed to investigate this in further detail. In addition the presence of the gene encoding this enzyme does not by itself prove a causative relationship to gentamicin resistance.

Sequencing of the isolates are needed to further investigate which variants of the genes encoding AAC(3)-II is present.

Among the 44 isolates having the gene encoding the enzyme AAC(6`)-Ib-cr, there was a high frequency of the *aac(3`)-II* gene. (34 out of 44 isolates with *aac(6`)-Ib-cr* also possessed *aac(3)-II*).All of these isolates were gentamicin resistant. The remaining 10 isolates with *aac(6`)-Ib-cr* lacked gentamicin resistance. This observation led to the assumption that *aac(6`)-Ib-cr* and *aac(3)-II* could be transferred coexistent by plasmids.

AAC(3)-II also termed aacC2 may be located on with plasmids(26, 45). It is associated with gentamicin resistance. Gentamicin resistance could be a secondary effect due to selection of multi resistant plasmids caused by the increasing ciprofloxacin use. The resistance genes for ESBLs, *aac(6`)-Ib-cr* and *aac(3)-II* are all associated with plasmids in several independent studies, and we wanted to address the possible association between these genes .

A high prevalence of both of *aac(6`)-Ib-cr* and *aac(3)-II* genes in the ESBL isolates, compared to the blood culture isolates, would be expected if these antimicrobial resistance genes was transferred together on plasmids encoding multiresistance. Indeed this combination was present in 30% of the ESBL isolates compared to 18% of the blood cultures, but this difference did not reach statistical significance. Care should be taken interpreting this statistics since test power was low ($\beta$=0.29) and a Type II error is possible.

# 6 Conclusion

The susceptibility pattern of the tested aminoglycosides indicated the presence of multiple aminoglycoside resistance mechanisms in the *Enterobacteriaceae* isolates from western Norway. Since genes for either other aminoglycoside modifying enzymes or other mechanisms may be present in each isolate, a more comprehensive gene screening is required to characterize this further.

The susceptibility pattern of the tested aminoglycosides in the *Enterobacteriaceae* isolates from western Norway also showed a low prevalence of amikacin resistance.

The observed low resistance against amikacin among isolates with genes encoding AAC(6`)-Ib`s challenge the published information about the substrate specificity of this enzyme. Further, the low prevalence of resistance against amikacin provides us with an aminoglycoside which can be used against multiresistant *Enterobacteriaceae*.

The screening for the genes *aac(6`)-Ib/ aac(6`)-Ib-cr* and *aac(3)-II* encoding aminoglycoside modifying enzymes in the isolates showed a high prevalence of the genes. *aac(6`)-Ib-cr* was more prevalent among ESBL isolates suggesting its location on plasmids along with *bla* genes conferring the ESBL phenotype.

The high prevalence of aac(6`)-Ib-cr in our isolates probably results from selection of isolates with this enzyme conferring reduced susceptibility to ciprofloxacin. A possible explanation for such a selection would be the increased prescription and use of ciprofloxacin over the past years. This study can not provide conclusive answers explaining the increasing resistance for gentamicin in Norwegian isolates. However, with a possibility of selection of gentamicin resistant isolates, it is reasonable to advocate restrictions in ciprofloxacin prescription.

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# 8 Appendices

**Abbreviations used in the following appendices:**

ECOFF/R = Epidemiological cut off values / Resistance

GM = Gentamicin (MIC values gained from E-tests and MIC test strips from this work)

KM = Kanamycin (MIC values gained from E-tests and MIC test strips from this work)

SM = Streptomycin (MIC values gained from E-tests and MIC test strips from this work)

AK = Amikacin (MIC values gained from E-tests and MIC test strips from this work)

NC = Netilmycin (MIC values gained from E-tests and MIC test strips from this work)

TM = Tobramycin (MIC values gained from E-tests and MIC test strips from this work)

NAL = nalidixic acid (Quinolone) (Resistant if >32, numbers gained from clinical routine data)

CIP = Ciprofloxacin (Quinolone) (Resistant if >4, numbers gained from clinical routine data)

GEN = Gentamicin (Aminoglycoside) (Resistant if >16, numbers gained from clinical routine data)

TOB= Tobramycin (Aminoglycoside) (Resistant if >16, numbers gained from clinical routine data)

Res.code: Phenotypic resistance described by using:

– when sensitive (S)

small letters when intermediate resistant (I) and

big letters when resistant(R).

Screening aac(6`)-Ib: positive = presence of *aac(6`)-Ib* , detected by using primers and a probe designed for this work.

Sequencing: Show witch variant of the gene was present: *aac(6`)-Ib* or *aac(6`)-Ib-cr*

*aac(3)-II:* positive = presence of *aac(3)-II*, detected by using primers from publication.

Extra comment: see list of abbreviations (page 7).

# APPENDIX 1: ESBL

| ECOFF/R | | ≤2/≥8 | ≤8/≥32 | ≤16/≥64 | ≤8/≥32 | ≤2/≥8 | ≤2/≥8 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GM | KM | SM | AK | NC | TM | NAL | CIP | GEN | TOB | Extra comment | Res.code | Screening aac(6`)-Ib | Sequence | aac(3`)-II |
| 1 | E.Coli 1126974-1 M1 | 0,5 | 48 | 96 | 4 | 6 | 32 | > 32 | > 4 | 2 | > 16 | ESBL | -KS-nT | Positive | aac(6`)-Ib-cr | |
| 2 | E.Coli 1137335-1 M2 | 0,5 | 64 | 4 | 12 | 6 | 16 | > 32 | > 4 | 2 | >16 | ESBL | K-anT | Positive | aac(6`)-Ib-cr | |
| 3 | E.Coli 1066997-1 M1 | 1 | 4 | 24 | 0,75 | 0,38 | 3 | > 32 | > 4 | 4 | 2 | ESBL | --s--t | | | |
| 4 | E.Coli 1069854-1 M1 | 3 | >256 | >1024 | 0,5 | 0,25 | 4 | > 32 | > 4 | 4 | 2 | Hyperproduction TEM1/TEM2 | gKS--t | | | |
| 5 | E.Coli 1069854-1 M2 | 2 | >256 | >1024 | 0,38 | 0,19 | 1,5 | > 32 | > 4 | 4 | 4 | ? | -KS--- | | | |
| 6 | E.Coli 1124307-1 M1 | 1 | 2 | 8 | 2 | 0,75 | 0,75 | > 32 | 1 | 4 | 2 | ESBL | ------ | | | |
| 7 | Mmorg 1129620-1 M1 | 1 | 3 | >1024 | 3 | 1 | 1 | > 32 | > 4 | 4 | 2 | AmpC | --S--- | | | |
| 8 | Pvulg 1148554-1 M2 | 0,5 | 2 | 8 | 2 | 0,75 | 0,5 | 4 | <0,25 | 4 | 2 | none | ------ | | | |
| 9 | Cfreu 1162535-1 M1 | 1 | 4 | 2 | 8 | 1 | 1 | 4 | <0,25 | 4 | 2 | Plasmid AmpC | ------ | | | |
| 10 | E.Coli 1167885-1 M1 | 4 | 8 | 24 | 1,5 | 0,38 | 3 | > 32 | > 4 | 4 | 4 | AmpC | g-s--t | | | |
| 11 | Koxyt 1193623-1 M1 | 0,38 | 1 | 4 | 0,75 | 0,5 | 0,38 | 4 | <0,25 | 4 | 8 | Hyperproduction | ------ | | | |
| 12 | Prot 1006848-1 M1 | 1,5 | 6 | 32 | 6 | 1,5 | 1,5 | <2 | <0,25 | <1 | 8 | Hyperproduction | --s--- | | | |
| 13 | Cfreu 1012517-1 M1 | 0,75 | 3 | 4 | 2 | 4 | 1,5 | > 32 | 1 | <1 | 4 | AmpC | ----n- | | | |
| 14 | E.Coli 1017826-1 M1 | 0,5 | 48 | 64 | 6 | 12 | 24 | > 32 | > 4 | <1 | >16 | ESBL | -KS-NT | Positive | aac(6`)-Ib-cr | |
| 15 | E.Coli 1116888-1 M1 | 0,75 | 48 | 12 | 4 | 8 | 24 | > 32 | > 4 | <1 | >16 | ESBL | -K--NT | Positive | aac(6`)-Ib-cr | |
| 16 | E.Coli 1154788-1 M1 | 0,5 | 48 | 16 | 12 | 6 | 16 | > 32 | > 4 | <1 | >16 | ESBL | -K-anT | Positive | aac(6`)-Ib-cr | |
| 17 | E.Coli 1156773-1 M1 | 0,38 | 32 | 4 | 4 | 4 | 16 | > 32 | > 4 | <1 | >16 | ESBL | -K--nT | Positive | aac(6`)-Ib-cr | |
| 18 | E.Coli 1164398-1 M1 | 0,5 | 24 | 6 | 4 | 4 | 12 | > 32 | > 4 | <1 | >16 | ESBL | -K--nT | Positive | aac(6`)-Ib-cr | |
| 19 | E.Coli 1194307-1 M1 | 0,5 | 48 | 8 | 12 | 6 | 16 | > 32 | > 4 | <1 | >16 | (from Blood) ESBL | -K-anT | Positive | aac(6`)-Ib-cr | |
| 20 | E.cloa 1012331-3 M1 | 256 | 32 | 128 | 3 | 8 | 24 | 8 | 0,5 | >16 | >16 | ESBL | GKS-NT | Positive | aac(6`)-Ib-cr | Positive |
| 21 | KPNPN 1012331-3 M2 | 48 | 32 | 128 | 4 | 12 | 24 | 16 | > 4 | >16 | >16 | ESBL | GKS-NT | | | Positive |
| 22 | E.Coli 1012331-3 M3 | 32 | 32 | 1024 | 4 | 8 | 32 | > 32 | **> 4** | **>16** | **>16** | (secretion from wound) ESBL | GKS-NT | Positive | aac(6`)-Ib-cr | Positive |
| 24 | Prett 1014464-1 | 24 | 24 | 768 | 2 | 0,5 | 32 | > 32 | > 4 | >16 | 8 | ESBL | GkS--T | | | |
| 25 | E.Coli 1014457-1 M1 | 64 | 8 | >1024 | 1 | 12 | 12 | > 32 | > 4 | >16 | 8 | ESBL | G-S-NT | | | Positive |
| 26 | E.Coli 1021613-1 M1 | 96 | 24 | 32 | 2 | 32 | 16 | > 32 | > 4 | >16 | 8 | ESBL | Gks-NT | | | Positive |
| 27 | E.Coli 1048046-1 M1 | 12 | 3 | 24 | 1 | 1 | 2 | > 32 | 0,5 | >16 | <1 | ESBL | G-s--- | | | Positive |
| 28 | E.Coli 1041884-1 M1 | 32 | 8 | 48 | 1,5 | 6 | 6 | > 32 | > 4 | >16 | 8 | (from Blood) K-res AmpC | G-s--- | | | Positive |
| 29 | E.Coli 1042728-1 M1 | 64 | 16 | 128 | 2 | 8 | 8 | > 32 | > 4 | >16 | 8 | K-res AmpC | GkS-NT | | | Positive |
| 30 | E.Coli 1052086-1 M1 | 48 | >256 | 384 | 1,5 | 4 | 6 | > 32 | > 4 | >16 | 4 | ESBL | GKS-nt | | | Positive |
| 31 | E.Coli 1054763-1 M1 | 64 | 48 | 64 | 3 | 12 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positive | aac(6`)-Ib-cr | Positive |
| 32 | Sstan 1055260-1 M1 | 24 | 2 | 256 | 0,75 | 4 | 2 | 4 | <0,25 | >16 | 4 | (Fæces) K-res Plasmid AmpC | G-S-n- | | | |
| 33 | E.Coli 1059115-1 M2 | 32 | 8 | 48 | 0,75 | 6 | 8 | > 32 | 1 | >16 | 4 | (Drensvæske) ESBL | G-s-nT | | | Positive |
| 34 | E.Coli 1059771-1 M1 | 24 | 3 | 8 | 1,5 | 32 | 8 | > 32 | > 4 | >16 | 4 | ESBL | G---NT | | | Positive |
| 35 | E.Coli 1062251-1 M1 | 128 | 12 | 6 | 1,5 | 8 | 12 | > 32 | > 4 | >16 | 8 | ESBL | Gk--NT | | | Positive |
| 36 | E.Coli 1064602-1 M1 | 48 | 32 | 6 | 4 | 16 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positive | aac(6`)-Ib-cr | Positive |
| 37 | Citro 1066265-1 M1 | 6 | 48 | 32 | 2 | 1,5 | 12 | 8 | <0,25 | >16 | >16 | AmpC | gKs--T | | | |
| 38 | E.coli 1070356-1 M1 | 32 | 48 | 16 | 8 | 24 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positive | aac(6`)-Ib-cr | Positive |
| 40 | E.Coli 1084014-1 M1 | 48 | 4 | 3 | 0,5 | 6 | 6 | > 32 | > 4 | >16 | <1 | ESBL | G---nt | | | Positive |
| 41 | E.Coli 1090743-1 M1 | 48 | 48 | 6 | 8 | 12 | 32 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positiv | aac(6`)-Ib-cr | Positive |
| 42 | E.Coli 1093843-1 M2 | 48 | 8 | 2 | 1 | 6 | 6 | > 32 | > 4 | >16 | 8 | K-res TEM1/TEM2 | G---nt | | | Positive |
| 43 | E.Coli 1105024-1 M1 | 96 | 8 | 8 | 0,75 | 8 | 8 | > 32 | > 4 | >16 | 8 | AmpC | G---NT | | | Positive |
| 44 | E.Coli 1105220-1 M1 | 24 | 32 | 4 | 3 | 8 | 32 | > 32 | > 4 | >16 | >16 | Plasmid AmpC | GK--NT | Positive | aac(6`)-Ib-cr | Positive |

# APPENDIX 2: ESBL

| ECOFF/R | | ≤2/≥8 | ≤8/≥32 | ≤16/≥64 | ≤8/≥32 | ≤2/≥8 | ≤2/≥8 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GM | KM | SM | AK | NC | TM | NAL | CIP | GEN | TOB | Extra comment | Res.kode | Screening aac(6`)-Ib | Sequence | aac(3`)-II |
| 45 | E.Coli 1106482-1 M1 | 48 | 32 | 4 | 2 | 12 | 48 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 46 | E.Coli 1107015-1 M1 | 48 | 8 | 128 | 1,5 | 12 | 24 | > 32 | > 4 | >16 | 8 | AmpC | G-S-NT | | | Positiv |
| 47 | KPNPN 1107136-1 M1 | 64 | >256 | 512 | 2 | 6 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GKS-nT | Positiv | aac(6`)-Ib-cr | Positiv |
| 49 | E.Coli 1106570-1 M2 | 32 | 16 | 128 | 1 | 16 | 12 | > 32 | > 4 | >16 | 8 | AmpC | Gk-S-NT | | | Positiv |
| 50 | E.Coli 1118926-1 M1 | 32 | 32 | 96 | 3 | 8 | 24 | > 32 | > 4 | >16 | >16 | (Secretion from abdomon) ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 51 | Cfreu 1120730-1 M1 | 96 | 24 | 96 | 2 | 8 | 16 | > 32 | > 4 | >16 | >16 | ESBL | GkS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 52 | E.Coli 1123006-1 M1 | 6 | 16 | 8 | 2 | 6 | 16 | > 32 | > 4 | >16 | >16 | ESBL | Gk--nT | Positiv | aac(6`)-Ib-cr | Positiv |
| 53 | E.Coli 1123537-1 M1 | 48 | 16 | 768 | 1,5 | 4 | 6 | > 32 | 1 | >16 | 4 | ESBL | GkS-nt | | | Positiv |
| 54 | E.Coli 1123537-1 M2 | 256 | 16 | 768 | 1,5 | 24 | 24 | > 32 | 1 | >16 | 8 | ESBL | GkS-NT | | | Positiv |
| 55 | E.Coli 1129329-1 M1 | 32 | 3 | 4 | 0,75 | 4 | 8 | > 32 | > 4 | >16 | 2 | ESBL | G---nT | | | Positiv |
| 56 | KPNPN 1137803-1 M1 | 96 | >256 | 192 | 2 | 8 | 16 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 57 | E.Coli 1136540-1 M1 | 48 | 6 | 3 | 0,75 | 8 | 6 | > 32 | > 4 | >16 | 8 | K-res Plasmid AmpC | G---Nt | | | Positiv |
| 58 | E.Coli 1137606-1 M1 | 64 | 48 | 96 | 3 | 12 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 59 | E.Coli 1138489-1 M1 | 24 | 24 | 6 | 3 | 8 | 16 | > 32 | >4 | >16 | 8 | ESBL | Gk--NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 60 | E.Coli 1139965-1 M1 | 24 | 4 | 64 | 0,5 | 16 | 8 | > 32 | >4 | >16 | 4 | ESBL | G-S-NT | | | Positiv |
| 61 | E.Coli 1143686-1 M1 | 48 | 32 | 16 | 3 | 8 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 62 | E.Coli 1144282-1 M1 | 24 | 3 | 4 | 0,5 | 2 | 3 | <2 | <0,25 | >16 | 4 | ESBL | G----t | | | Positiv |
| 63 | E.coli 1140723-1 M1 | 128 | 32 | 128 | 4 | 12 | 32 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 64 | KPNPN 1149305-1 M1 | 0,38 | 32 | 96 | 4 | 4 | 8 | > 32 | > 4 | >16 | >16 | ESBL | -KS-nT | Positiv | aac(6`)-Ib-cr | |
| 65 | E.Coli 1150540-1 M1 | 24 | 3 | 2 | 0,5 | 4 | 4 | > 32 | > 4 | >16 | 4 | ESBL | G---nt | | | Positiv |
| 66 | E.Coli 1152126-1 M1 | 96 | 12 | 512 | 0,75 | 12 | 8 | > 32 | > 4 | >16 | 8 | K-res AmpC | GkS-NT | | | Positiv |
| 67 | E.Coli 1151427-1 M1 | 64 | >256 | 256 | 0,5 | 8 | 8 | > 32 | > 4 | >16 | 8 | K-res Plasmid AmpC | GKS-NT | | | Positiv |
| 68 | E.Coli 1150060-1 M1 | 24 | >256 | 64 | 1,5 | 4 | 12 | > 32 | > 4 | >16 | 8 | ESBL | GKS-nT | Positiv | aac(6`)-Ib-cr | Positiv |
| 69 | P.Mir 1157115-1 M1 | 48 | >256 | 32 | 1 | 8 | 6 | > 32 | > 4 | >16 | 8 | Selective flush ESBL | GKs-Nt | | | Positiv |
| 70 | E.Coli 1159470-1 M1 | 256 | >256 | 568 | 2 | 16 | 32 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 71 | E.Coli 1173485-1 M1 | 96 | 12 | 128 | 1 | 12 | 16 | <2 | <0,25 | >16 | 8 | | GkS-NT | | | Positiv |
| 72 | E.Coli 1174970-1 M1 | 48 | 6 | 12 | 2 | 8 | 8 | > 32 | 0,5 | >16 | 8 | ESBL | G---NT | | | Positiv |
| 73 | E.Coli 1174600-1 M1 | 96 | 128 | 768 | 4 | 12 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 74 | E.Coli 1178447-1 M1 | 12 | 16 | 32 | 0,5 | 1 | 1,5 | > 32 | 1 | >16 | 2 | ESBL | Gks--- | | | |
| 75 | E.Coli 1177688-1 M1 | 96 | 12 | 6 | 1 | 16 | 16 | > 32 | > 4 | >16 | 8 | K-res AmpC | Gk--NT | | | Positiv |
| 76 | E.Coli 1179813-1 M1 | 48 | 48 | 4 | 6 | 16 | 48 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 77 | E.Coli 1181265-1 M1 | 64 | 48 | 96 | 4 | 24 | 32 | > 32 | > 4 | >16 | >16 | ESBL | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 78 | E.Coli 1186295-1 M1 | 48 | 12 | 192 | 1,5 | 6 | 8 | > 32 | 0,5 | >16 | 8 | ESBL | GkS-nT | | | Positiv |
| 79 | E.Coli 1188193-1 M1 | 48 | 48 | 8 | 3 | 8 | 24 | > 32 | > 4 | >16 | >16 | ESBL | GK--NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 80 | Styph 1187496-1 M1 | 48 | 3 | 256 | 0,38 | 0,38 | 3 | 4 | <0,25 | >16 | 8 | (Fæces) ESBL | G-S--t | | | |
| 81 | E.Coli 1189057-1 M1 | 128 | 12 | 1,5 | 4 | 8 | 16 | > 32 | > 4 | >16 | 8 | ESBL | Gk--NT | | | Positiv |
| 82 | Mmorg 1021313-1 M2 | 24 | >256 | 4 | 1 | 4 | 4 | > 32 | >4 | >16 | 8 | ESBL | GK--nt | Positiv | aac(6`)-Ib-cr | Positiv |
| 84 | E.Coli 10068181 M1 | 0,5 | 48 | 32 | 12 | 8 | 32 | >32 | >4 | | | ESBL | -KsaNT | Positiv | aac(6`)-Ib-cr | |
| 89 | E.Coli 1089888-1 M1 | 0,38 | 1,5 | 96 | 1,5 | 0,38 | 0,75 | >32 | >4 | s | | (blood) ESBL | --S--- | | | |
| 92 | Sop 1150060-1 M2 | 32 | >256 | 96 | 1,5 | 6 | 16 | >32 | >4 | >16 | | K-res ESBL | GKS-nT | Positiv | aac(6`)-Ib-cr | Positiv |

## APPENDIX 3: BLOOD CULTURE

| ECOFF/R | | ≤2/≥8 | ≤8/≥32 | ≤16/≥64 | ≤8/≥32 | ≤2/≥8 | ≤2/≥8 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GM | KM | SM | AK | NC | TM | GEN | NET | CIP | Res.code | screening aac(6`)-Ib | Sequence | aac(3`)-II |
| 1 | Klebs 1029336-4 | 256 | 16 | 6 | 2 | 24 | 16 | | R | S | Gk-- NT | | | Positiv |
| 2 | E.Coli 1132947-2 | 12 | 4 | 16 | 2 | 16 | 24 | | I | S | G---NT | | | |
| 3 | E.Coli 1197276-2 | 1 | 3 | >384 | 3 | 1 | 1 | | I | S | --S--- | | | |
| 4 | E.Coli 1079101-1 | 64 | 256 | >1024 | 12 | 32 | 64 | | I | R | GKSaNT | Positiv | aac(6`)-Ib-cr | Positiv |
| 5 | E.Coli 1089946-1 | 1,5 | 4 | 12 | 4 | 1 | 1,5 | I | | S | ------ | | | |
| 7 | E.Coli 1096614-1 | 64 | 12 | >1024 | 4 | 48 | 256 | R | | S | GkS-NT | | | |
| 8 | E.Coli 1106372-3 | 256 | 16 | 96 | 2 | 8 | 12 | R | | R | GkS-NT | | | Positiv |
| 10 | E.Coli 1192505-4 | 256 | 32 | 32 | 3 | 48 | 16 | R | | I | GKs-NT | | | Positiv |
| 11 | G.st 1021892-2 | 256 | 256 | 16 | 12 | 32 | 96 | R | | R | GK-aNT | Positiv | aac(6`)-Ib-cr | Positiv |
| 12 | E.Coli 1035306-1 | 256 | 24 | 128 | 3 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 13 | E.Coli 1046926-4 | 256 | 24 | 384 | 3 | 16 | 12 | R | | R | GkS-NT | | | Positiv |
| 14 | Klebs-pn sp.pn 106334-1 | 48 | 256 | 96 | 3 | 12 | 256 | R | | S | GKS-NT | Positiv | aac(6`)-Ib | |
| 15 | E.Coli 1077578-1 | 96 | 6 | 16 | 2 | 32 | 12 | R | | R | G---NT | | | Positiv |
| 16 | E.Coli 1113212-4 | 96 | 8 | 16 | 3 | 16 | 6 | R | | R | G---Nt | | | Positiv |
| 17 | E.Coli 1155560-1 | 256 | 16 | 128 | 3 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 18 | E.Coli 1015406-1 | >256 | 12 | 256 | 3 | 16 | 12 | R | | R | GkS-NT | | | Positiv |
| 19 | Klebs.pneum spp.pneum 1023065-3 | 64 | 48 | 128 | 4 | 8 | 24 | R | | R | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 20 | E.Coli 1057601-2 | 1 | 3 | 192 | 2 | 0,75 | 0,75 | R | | S | --S--- | | | |
| 21 | E.Coli 1070048-1 | 96 | 8 | 12 | 2 | 6 | 6 | R | | I | G---nt | | | Positiv |
| 22 | E.Coli 1070152-1 | 96 | 128 | 96 | 8 | 16 | 32-48 | R | | R | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 23 | E.Coli 1096687-1 | 256 | 96 | 16 | 16 | 24 | 32 | R | | R | GK-aNT | Positiv | aac(6`)-Ib-cr | Positiv |
| 24 | E.Coli 1097110-1 | >256 | 24 | 512 | 3 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 26 | E.Coli 1131676-1 | >256 | >256 | >1024 | 4 | 16 | 24 | R | | R | GKS-NT | Positiv | aac(6`)-Ib | Positiv |
| 27 | E.Coli 1192781-4 | >256 | 12 | 6 | 2 | 8 | 8 | R | | R | Gk—NT | | | Positiv |
| 28 | E.Coli 1016115-1 | >256 | 24 | 384 | 3-4 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 29 | E.Coli 1025243-2 | 256 | 12 | >1024 | 3 | 8 | 8 | R | | R | GkS-NT | | | Positiv |
| 30 | E.Coli 1034974-1 | 256 | 16 | 8 | 3 | 12 | 8 | R | | S | Gk—NT | | | Positiv |
| 31 | E.Coli 1041884-1 | 256 | 12 | 128 | 2 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 32 | E.Coli 1058151-1 | >256 | 16 | 8 | 4 | 12 | 12 | R | | R | Gk—NT | | | Positiv |
| 33 | E.Coli 1066678-1 | 96 | 96 | 12 | 6 | 12 | 32 | R | | R | GK—NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 34 | E.Coli 1104063-1 | >256 | 16 | 24 | 3 | 12 | 24 | R | | R | Gks-NT | | | Positiv |
| 35 | E.Coli 1175173-1 | >256 | 16 | 192 | 3 | 12 | 16 | R | | R | GkS-NT | | | Positiv |
| 36 | E.Coli 1008758-1 | 192 | 16 | 8 | 3 | 6 | 8 | R | | R | Gk—nT | | | Positiv |
| 37 | E.Coli 1060028-1 | 8 | 32 | 32 | 3 | 1 | 16 | I | | R | GKs—T | | | |
| 38 | E.Coli 1060264-1 | 8 | 2 | 12 | 2 | 0,75 | 0,75 | I | | S | G----- | | | |
| 39 | E.Coli 1085238-3 | >256 | 12 | 16 | 3 | 12 | 8 | R | | R | Gk—NT | | | Positiv |
| 40 | E.Coli 1095499-3 | >256 | 192 | 384 | 3 | 24 | 16 | R | | R | GKS-NT | Positiv | aac(6`)-Ib | Positiv |

## APPENDIX 4: BLOOD CULTURE

| ECOFF/R | | ≤2/≥8 | ≤8/≥32 | ≤16/≥64 | ≤8/≥32 | ≤2/≥8 | ≤2/≥8 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | GM | KM | SM | AK | NC | TM | GEN | NET | CIP | Res.code | screening aac(6`)-Ib | Sequence | aac(3`)-II |
| 41 | E.Coli 1100411-1 | >256 | 12 | 96 | 3 | 12 | 12 | R | | R | GkS-NT | | | Positiv |
| 42 | E.Coli 1104939-1 | >256 | 24 | 384 | 3 | 16 | 12 | R | | R | GkS-NT | | | Positiv |
| 43 | KPNPN 1126222-2 | 48 | 64 | 6 | 12 | 12 | 12 | R | | R | GK-aNT | | | Positiv |
| 44 | Klebs 1140399-4 | 32 | 64 | 8 | 12 | 24 | 12 | R | | R | Gk-aNT | | | Positiv |
| 45 | KPNPN 1147792-1 | 6 | 3 | 12 | 2 | 1,5 | 2 | I | | S | g----- | | | |
| 46 | E.Coli 1156514-1 | >256 | 32 | 256 | 3 | 16 | 16 | R | | R | GKS-NT | | | Positiv |
| 47 | E.coli 1167080-1 | >256 | 16 | 384 | 3 | 6 | 12 | R | | R | GkS-nT | | | Positiv |
| 48 | E.coli 1188153-1 | 64 | 64 | 16 | 6 | 8 | 32 | R | | R | GK—NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 49 | KPNPN 1190037-1 | >256 | >256 | 512 | 8 | 24 | 32 | R | | R | GKS-NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 50 | E.coli 1018391-1 | >256 | 16 | 256 | 2 | 12 | 8 | R | | R | GkS-NT | | | Positiv |
| 51 | E.coli 1020811-1 | 128 | 128 | 6 | 8 | 16 | 48 | R | | R | GK—NT | Positiv | aac(6`)-Ib-cr | Positiv |
| 52 | E.coli 1028320-1 | >256 | 16 | 12 | 3 | 8 | 12 | R | | R | Gk—NT | | | Positiv |
| 53 | Ekstra utenom liste E.coli 1182217-1 | 0,5 | 2 | 128 | 1,5 | 0,75 | 1 | | | | --S--- | | | |

**APPENDIX 5:**

Sequences found matching the genes *aac(6`)-Ib* and *aac(6`)-Ib-cr* when the SNP`s responsible for the amino acid substitutions Trp102Arg and Asp179Tyr.

| ***aac(6`)-Ib*** | **GenBank number** |
|---|---|
| Escherichia coli strain IncA/C2 plasmid | GQ293500.1 |
| Escherichia coli strain IncA/C2 plasmid | GQ293499.1 |
| Escherichia coli class I integron integrase (intI) gene | AY152821.1 |
| Escherichia coli strain nb018 | GQ214316.1 |
| Escherichia coli plasmid pKO97 | AY878717.1 |
| Escherichia coli strain 2098EC | FJ619242.1 |
| Escherichia coli strain 2039EC | FJ619241.1 |
| Escherichia coli strain 1387 plasmid pHS1387 class 1 integron | EU675686.2 |

| ***aac(6`)-Ib-cr*** | **GenBank number / NCBI reference sequnece** |
|---|---|
| Escherichia coli plasmid pEK516, complete sequence | NC_013121.1 |
| Escherichia coli strain D plasmid pEK516 | EU935738.1 |
| Escherichia coli plasmid pHSH2 encoded class 1 integron In37 | AY259086.1 |
| Escherichia coli strain 2073EC | FJ829034.1 |
| Escherichia coli strain MS12 | EU279426.1 |
| Escherichia coli strain CQ4 | EF571008.1 |

**APPENX 6**

**Map over variations:**

The Query sequence used was the FASTA sequence from Escherichia coli strain 2098EC (GenBank number: FJ619242). The search was performed in the nucleotide selection among *Enterobacteriaceae* species to find highly similar matches:

**Figure** :

ATGACTGAGCATGACCTTGCGATGCTCTATGAGTGGCTAAATCGATCTCATAT
CGTCGAGTGGTGGGGCGGAGAAGAAGCACGCCCGACACTTGCTGACGTACAGG
AACAGTACTTGCCAAGCGTTTTAGCGCAAGAGTCCGTCACTCCATACATTGCA
ATGCTGAATGGAGAGCCGATTGGGTATGCCCAGTCGTACGTTGCTCTTGGAAG
CGGGGACGGATGGTGGGAAGAAGAAACCGATCCAGGAGTACGCGGAATAGACC
AGTTACTGGCGAATGCATCACAACTGGGCAAAGGCTTGGGAACCAAGCTGGTT
CAAGCTCTGGTTGAGTTGCTGTTCAATGATCCCGAGGTCACCAAGATCCAAAC
GGACCCGTCGCCGAGCAACTTGCGAGCGATCCGATGCTACGAGAAAGCGGGGT
TTGAGAGGCAAGGTACCGTAACCACCCCAGATGGTCCAGCCGTGTACATGGTT
CAAACACGCCAGGCATTCGAGCGAACACGCAGTGATGCCTAA

*The yellow areas are variations in bases detected, manually marked in the sequence. The green illustrate the SNP`s in focus that cause this sequence to encode* AAC(6`)-Ib. *If T was C and G was T it would have been a sequence encoding* AAC(6`)-Ib-cr. *The underlined areas illustrate the primer and probe sequences used. One variation was detected in one of the primers, with the consequent risk losing its specificity. This was solved by using an ambiguous base able to anneal to all variants detected.*

| | |
|---|---|
| Filnavn: | Innledning til HOVEDFAG |
| Katalog: | C:\Users\Kine\Documents |
| Mal: | C:\Users\Kine\AppData\Roaming\Microsoft\Maler\Normal.dotm |
| Tittel: | |
| Emne: | |
| Forfatter: | Kine Risberg |
| Nøkkelord: | |
| Merknader: | |
| Opprettelsesdato: | 30.04.2010 15:06:00 |
| Versjonsnummer: | 209 |
| Sist lagret: | 20.05.2010 23:24:00 |
| Sist lagret av: | Kine Risberg |
| Samlet redigeringstid: | 7 924 minutter |
| Sist skrevet ut: | 20.05.2010 23:31:00 |
| Ved siste fullstendige utskrift | |
| Antall sider: | 70 |
| Antall ord: | 35 199 (ca.) |
| Antall tegn: | 186 559 (ca.) |