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Molecular characterization of Norwegian clinical isolates of *Escherichia coli* hyperproducing the chromosomal AmpC β-lactamase; a regional spread of an IS*911*-mediated *bla*$_{\mathrm{AmpC}}$-hyperexpressing ST131 clone

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Summary

The worldwide dissemination of antimicrobial resistance is a growing problem causing increased morbidity, mortality, and financial costs. β-lactams are an important family of antimicrobial agents and accounts for ~46% of the total antibiotic use for systemic infections in Norway. Resistance to β-lactams can be caused by several factors where the production of enzymes, β-lactamases, is the major mechanism.

Escherichia coli naturally produce small amounts of the chromosomally encoded AmpC β-lactamase. The expression bla_{AmpC} is noninducible and regulated by a weak promoter and an attenuator. Insertion sequence (IS) elements inserted into the promoter region have been described as one reason for the hyperexpression of bla_{AmpC} conferring resistance to β-lactams such as penicillins and cephalosporins, but not 4th generation cephalosporins and carbapenems.

In this study 111 *E. coli* isolates with a hyperexpressed chromosomal AmpC profile were submitted to the Reference Center for Detection of Antimicrobial Resistance (K-res) from Haukeland University Hospital during 2006-2010 and a control group representing the same years from other Norwegian clinical microbiological laboratories (*n*=100) were included. The isolates were initially screened for an insertion of an element in the bla_{AmpC}. A subset of isolates with an insertion was further molecularly characterized by sequencing of the region and linkage to IS*911*. Molecular typing was performed using multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Multi-resistance profiles were identified by antimicrobial susceptibility testing and further investigated by PCR and sequencing methods.

The results from the study shows a regional clonal spread of ST131 *E. coli* bla_{AmpC}-IS*911* isolates in the Bergen region of Norway. The spread of these isolates were identified both in isolates from Hospital 1 and 2 but also from other medical institutions such as nursing homes and general practitioners. In contrast, no isolates from the control group from other Norwegian hospitals harbored the bla_{AmpC}-IS*911* linkage. In the control group only three isolates from two Norwegian counties, Vestfold and Rogaland were identified with an

insertion in the *bla*$_{AmpC}$ region. However, in these isolates another IS-element, IS*10* was identified. In the ST131 isolates multi-resistance was observed towards important antibiotics such as ciprofloxacin, gentamicin, tobramycin, and trimethoprim-sulphamethoxazole. Resistance to ciprofloxacin was caused by mutations in the quinolone-resistance determining region of the *parC* and *gyrA* genes. The resistance mechanism to the aminoglycosides gentamicin and tobramycin were not identified, but the isolates were negative for the aminoglycoside modifying enzyme AAC(6')-Ib.

1. Introduction

1.1 The era of antibiotics

Names like Paul Ehrlich and Alexander Fleming are both important to the beginning of the modern “antibiotic era”. Paul Ehrlich hoped to cure syphilis by finding a drug targeted directly at the source of disease, the spirochete *Treponema palladium*. He executed a large-scale screening resulting in the discovery of an effective drug, named Salvarsan, in 1909. This was for many years to come the most frequently prescribed drug, until replaced by penicillin in the 1940s. The screening process used by Ehrlich inspired others and resulted in the discovery of, among other, sulfa drugs, namely sulfonamidochrysoidine (Prontosil). This was a non-patented drug easy to mass produce which early led us to the problem of sulfa drug resistance. Still, probably the most known antibiotic, penicillin, was in 1928 discovered by Alexander Fleming. His observations on the antimicrobial effects of the fungus *Penicillum chrysogenum* against Staphylococci strains revolutionized the treatment of infectious diseases. Howard Florey and Ernst Chain, both part of an Oxford team, were first in 1940 able to purify penicillin for clinical testing [1].

The discovery of Salvarsan, Prontosil and penicillin were followed by decades of new antimicrobial agents identified and introduced into clinical use, as illustrated in the timeline (figure 1). The period from the 1950s throughout the 1970s are reckoned as the golden era of discovering new classes of antimicrobial agents [1].

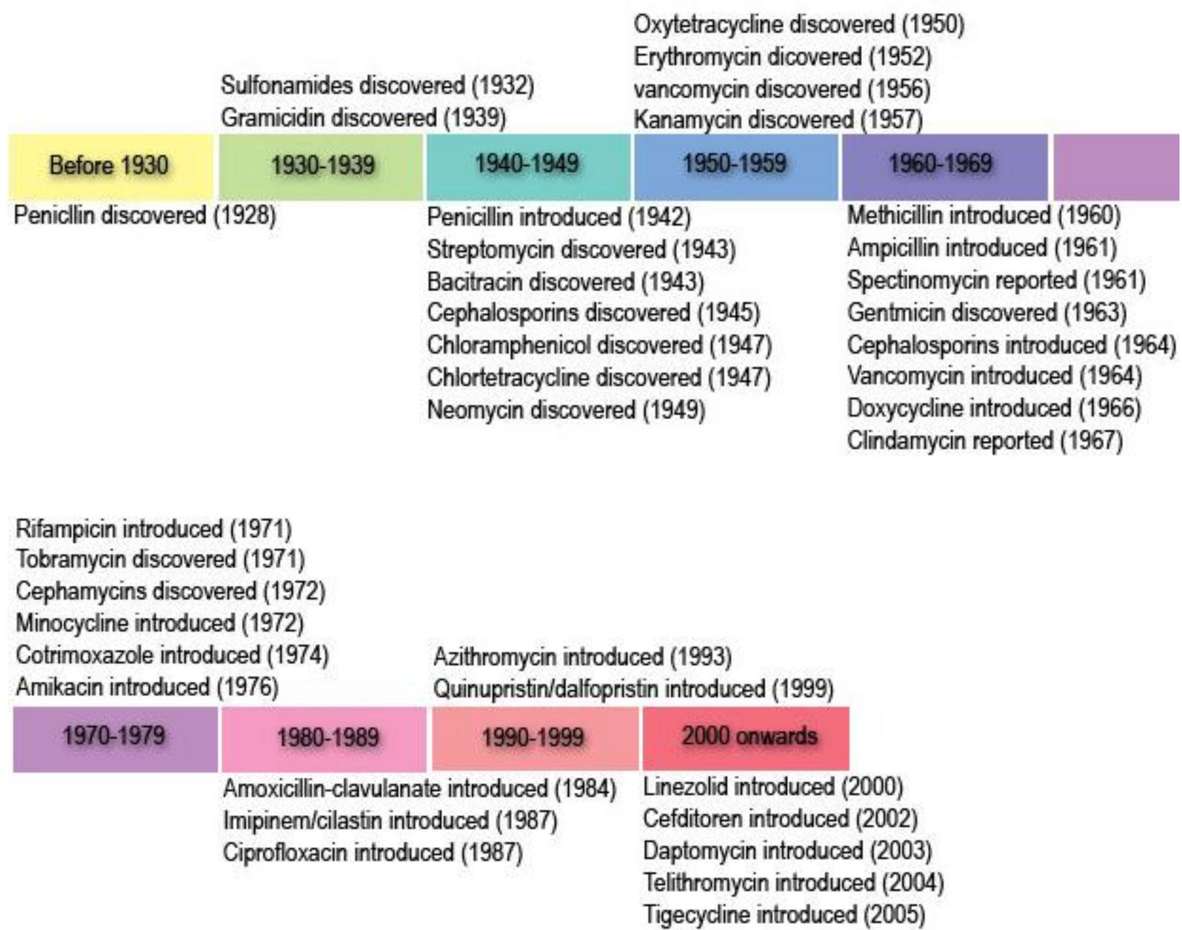

Figure 1. Timeline of the historical development of antimicrobial agents. (http://amrls.cvm.msu.edu/pharmacology/historical-perspectives/the-golden-age-of-antibacterials)

In early years the term “antibiotics” was most commonly used. This referred to natural metabolic products aimed to inhibit or kill microorganisms produced by fungi, actinomycetes, and other types of bacteria. The production of antibiotics was beneficial to the organisms as a competitional factor when claiming habitat and nutrients. The antibacterial agents used today are derivatives of natural products chemically modified (i.e. semi-synthetic) to enhance their properties. Therefore the term “antimicrobial” agent is a more descriptive term. Some of the newer antimicrobial agents, like quinolones, are even fully synthetic [2].

Antibacterial agents can be classified in three ways:

1. According to whether they are bactericidal or bacteriostatic.
2. By target site.
3. By chemical structure.

The most used classification is by target site as classification by chemical structure alone is not practical due to the diversity of antimicrobial agents. The ability of an antimicrobial agent to inhibit or kill a microorganism also varies depending on the organism. For example chloramphenicol can inhibit the growth (bacteriostatic) of *Escherichia coli* while it kills (bacteriocidal) *Haemophilus influenzae*. The five main target sites of antimicrobial agents are; (i) cell wall synthesis, (ii) protein synthesis, (iii) nucleic acid synthesis, (iv) metabolic pathways, and (v) cell membrane functions, all illustrated below in figure 2 [2].

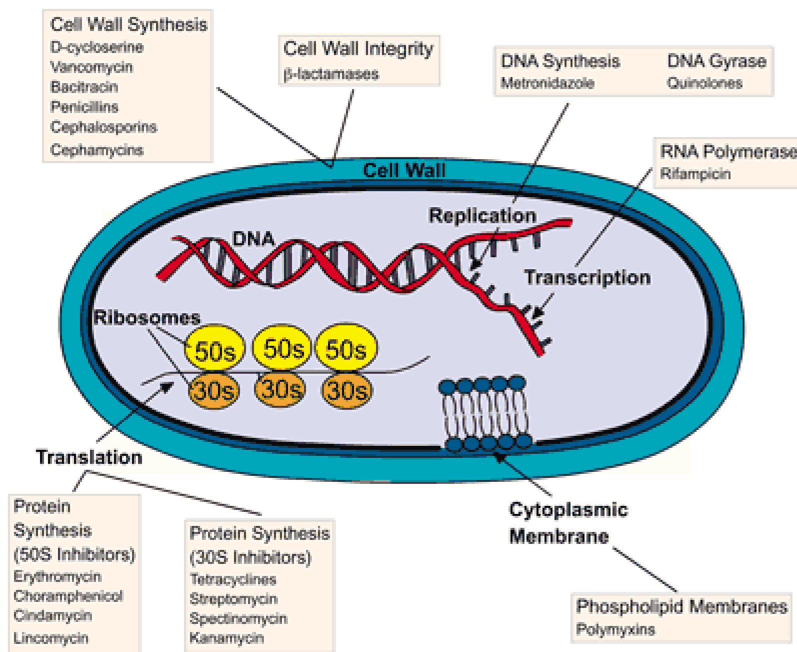

Figure 2. Antimicrobial target sites on bacterial cells with examples of antimicrobial agents listed.
(http://www.wiley.com/college/pratt/0471393878/student/activities/bacterial_drug_resistance/index.html)

1.2 β-lactam antimicrobial agents

β-lactam antimicrobial agents are a large family of bactericidal agents. The family consists of penicillins, cephalosporins, cephamycins, carbapenems, and monobactams (table 1). The structure of the β-lactams varies around the β-lactam ring, a consistent structure of the class. Penicillins, for example, have a five-membered ring attached to the β-lactam ring, while cephalosporins have a six-membered ring in addition to differing side chains attached to the rings [2]. β-lactams have for years been the most important and the most used antibiotic. In Norway β-lactams represent ~46% of the total antibiotic use for systemic infections [3]. The last developed group of β-lactams, the carbapenems is used as a last resort against bacterial infections. They are preferred when treating serious infections caused by pathogens with a multi-drug resistance profile [4].

Table 1. Classification and antimicrobial spectrum of β-lactams [5]

		Antimicrobial spectrum	
β-lactam group	**Example**	**Gram-negative**	**Gram-positive**
Narrow spectrum penicillins	Penicillin G Penicillin V	-	+
Broad spectrum penicillins	Ampicillin Piperacillin	(+)	+
1st generation cephalosporins	Cephalotin Cephalexin	(+)	+
2nd generation cephalosporins	Cefuroxime	+	+
3rd generation cephalosporins	Cefotaxime Ceftazidime	+	+
4th generation cephalosporins	Cefepime Cefpirome	+	+
Monobactams	Aztreonam	+	-
Carbapenems	Meropenem Iminopenem Ertapenem	+	+

* -: no activity, (+): limited activity, +: active

1.2.1 Mechanism of action

Carboxypeptidases, transglycosylases and transpetidases are all membrane proteins in the bacterial cell wall. These enzymes are important in the final stages of synthesizing new cell wall. More precisely, the enzymes function in the cross-linking of two glycan-linked peptide chains. Without this cross-linking the cell wall loses its stability and strength and will therefore result in cell lysis. β-lactams have the ability to bind to these membrane enzymes, hence the name “penicillin binding proteins” or PBPs. When penicillin binds to the PBPs they inhibit the cross-linking reaction leading to an accumulation of precursor cell wall units and to an activation of the autolytic system, resulting in cell lysis. Several PBPs are known and both intra- and interspecies variations are found, which explains the varying antimicrobial spectrum of β-lactams [2, 5, 6].

1.3 Antimicrobial resistance

The increase of bacterial resistance to antimicrobial agents in our society is a problem caused by many factors. From the bacterial point of view, resistance in many cases means survival and is highly valued. Bacterial strains have evolved numerous strategies to avoid antimicrobial agents like producing enzymes that inactivate or modify antimicrobial agents, have an altered binding site for the antimicrobial agent, or restrict access to the target by reduced permeability or efflux mechanisms [2].

Clinical bacterial resistance is classified according to the “SIR” classification using the following categories; susceptible (S), intermediate (I), or resistant (R).

Susceptible: A bacterial strain is said to be susceptible to a given antibiotic when it is inhibited *in vitro* by a concentration of the drug that is associated with a high likelihood of therapeutic success [7].

Intermediate: The sensitivity of a bacterial strain to a given antibiotic is said to be intermediate when it is inhibited *in vitro* by a concentration of this drug that is associated with an uncertain therapeutic effect [7].

Resistant: A bacterial strain is said to be resistant to a given antibiotic when it is inhibited *in vitro* by a concentration of this drug that is associated with a high likelihood of therapeutic failure [7].

A noteworthy dilemma is that our extensive use of antibiotics results in us selecting for resistant bacteria. We need to implement ways to quickly identify and effectively prevent the further spread of resistant strains. Factors like the increased air travel also add to the spread of resistance. We are able to travel around the world in much less than 80 days, bringing bacterial souvenirs back with us. Another important fact and a highly increasing phenomenon is the term "Medical Tourism". Due to long waiting lists and high priced insurances people choose to travel abroad for cheaper surgeries. India is one of the most visited countries offering a wide range of medical procedures like dental work and corrective surgery [8]. The problem is that India is also a country highly associated with antimicrobial resistance, exemplified by the recent spread of the carbapenemase NDM-1 [9].

Still, the most dramatic consequences of resistance are the increased morbidity and mortality. In addition to this, resistance causes an added cost to the society in the form of extended hospital stays, blocking of hospital beds for new patients, and repeated visits from the physician resulting in a lowering of the productive time. In the European region for 2007 it was estimated as many as 5503 excess deaths caused by blood stream infections (BSIs) due to methicillin resistant *Staphylococcus aureus* (MRSA) and 2712 deaths by BSIs caused by third generation cephalosporin-resistant *E. coli.* Numbers as high as 255,683 (BSIs by MRSA) and 120,065 (BSIs by third generation cephalosporin-resistant *E. coli*) were registered for excess hospital bed-days. For 2007 the costs of excess hospital stay due to BSIs caused by MRSA or third generation cephalosporin-resistant *E. coli* were estimated to 62 million Euros [10].

In general resistance in Gram-negative bacteria is faster growing compared to Gram-positives. This confers a global problem as there are fewer new and developmental antibiotics targeted at infections caused by Gram-negative bacteria [10].

1.3.1 Mechanisms of resistance

Resistance mechanisms can either be an intrinsic property of a bacterial specie or an acquired trait. Acquired resistance occurs as a result of chromosomal mutations (point mutations, deletions, inversions, insertions, etc.) or by the acquisition of genetic elements. Some species of bacteria are naturally resistant towards some types of antimicrobial agents. Intrinsic resistance covers a whole bacterial specie and provides resistance without the addition of genetic elements or mutations. Bacteria that lack a cell wall, e.g. mycoplasma, will as a consequence be intrinsic resistant to β-lactams [11].

1.3.2 Mutations

During DNA replication a randomly occurrence of incorrect nucleotides are incorporated. Mutations can lead to alterations in the drug target, drug-inactivation, the up- or down-regulation of efflux systems, as well as the loss or activation of porins and active transporters affecting the uptake pathway. In general a mutation will cause resistance to one class of antimicrobial agents, but changes affecting impermeability and efflux may result in a multiple resistance towards several classes of antimicrobial agents [12].

1.3.3 Horizontal gene transfer

Resistance genes are able to spread from one bacterium to another by the means of processes like conjugation, transformation, and transduction. The exchange of genetic material can occur between strains of the same specie and between species or genera [6].

Conjugation is the transfer of genes from one bacterium to another, requiring cell-to-cell contact. The process involves a donor that contain a transferrable element and a recipient that does not. The donor produces a pilus that attaches the two cells. The outer membrane of the two cells fuse and DNA can be transferred from the donor to the recipient. Both plasmids and chromosomal parts can be moved. The recipient is referred to as a transconjugant and can act as a donor for other recipient cells [6].

Transformation is the uptake of naked DNA from the environment. Cell lysis will release fragmented DNA that naturally competent bacteria can take up. Typically only short DNA fragments are exchanged [6].

Transduction involves the transfer of host genes from one bacterium to another by bacterial viruses. In generalized transduction, virus particles randomly incorporate fragments of the bacterial cells chromosomal DNA, but the efficiency is low. In specialized transduction, the DNA of a temperate virus excises incorrectly and takes adjacent host genes along with it, also leaving some phage genes behind. The transducing efficiency here may be very high [6].

1.4 Resistance elements

Plasmids are genetic elements that replicate independently of the host chromosome. They do not have an extracellular form and exist inside cells as free, often circular, double stranded DNA. Plasmids consist of plasmid backbones necessary for their own propagation and survival (e.g. replication, maintenance, and morbidity) as well as accessory DNA. Examples of plasmid-encoded genes are resistance genes and virulence factors. Plasmids range in size from a few thousands to hundreds of thousands of base pairs (bp) and can exist in multiple copies within a bacterium. Not all plasmids can coexist in the same cell, they are incompatible as they then often share the same replication- and partitioning systems. Based on this a classification scheme was deduced where all plasmids belong to an Inc group (incompatibility group). Plasmids belonging to the same group cannot coexist stably in a cell, while plasmids of different groups have this ability. As most plasmids are small, have a relatively high copy number, carry easily selectable traits, and have one or few sites for restriction enzymes they are highly suitable as cloning vectors [2, 13].

Transposons are elements of DNA able to move from one place to another within the genome. These elements are known to be found in all organisms, including humans. The movement of DNA is promoted by enzymes called transposases. The transposons vary in length from about 1000 bp, only carrying the genes for the transposases, to larger elements also harboring other genes, including resistance encoding genes. Common for transposons are

the inverted terminal repeats and short direct repeats for the target DNA. Transposons generally have low specificity for the target enabling it to be inserted at several places in the DNA [13].

Insertion sequence (IS) elements are the smallest type of transposons found in bacterial cells. They range in size from about 750 to 2000 bp and generally do not encode much more than the transposases required for transposition [13]. As IS elements do not encode resistance genes their mere insertion into the genome of bacteria cause alterations that may result in resistance to antimicrobial agents. Canadian hospitals studying *E. coli* isolates resistant to cefoxitin found IS*10* and IS*911* incorporated into the promoter region of the bla_{AmpC} gene, leading to an overproduction of the enzyme [14].

IS*911* belongs to the largest family of IS elements, the IS3 family. It is composed of a transposase, OrfAB, a regulatory protein, OrfA, and right and left inverted repeats (IRR and IRL) at the ends. The element is 1250 bp in size and its transposition occurs in several steps, involving two promoters (P_{IRL} and P_{junc}). OrfA regulates the transposition and also facilitates the random insertion of IS*911* into the genome of bacterial cells [15, 16].

1.5 β-lactam resistance mechanisms

Resistance to β-lactams is generally the result of one or more of the three following mechanisms [2, 4]:

1. Resistance by alteration in target site.
2. Resistance by alteration in access to the target site.
3. Resistance by production of β-lactamases.

Some bacteria produce alternative PBPs that have a lower affinity for β-lactams than the normal PBPs. The cell wall synthesis is not interrupted as these PBPs are able to continue the work while the normal PBPs are inhibited. This resistance mechanism is found in methicillin-

resistant staphylococci (*Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE)) where the *mecA* gene encodes the additional PBPs [2].

Gram-negative bacteria are naturally less susceptible to β-lactams compared to Gram-positive bacteria due to a more difficult-to-reach target. For the β-lactams to reach the PBPs they need to be able to diffuse through protein channels in the outer membrane. Mutations in these porin channels result in resistant bacteria as the access to the target site is blocked. If other types of antimicrobial agents use the same porins cross resistance may be seen [2].

Efflux pumps are transport proteins involved in the extrusion of toxic substrates found in both Gram-negative and Gram-positive bacteria. These pumps also play a part in β-lactam resistance as the antimicrobial agents are actively pumped out of the bacterial cell. Some efflux pumps are drug specific while others may act on multiple antimicrobial agents increasing the risk of multidrug resistant bacteria. High-level resistance due to efflux alone is not of great concern, but it makes the bacteria better equipped to survive in the presence of antimicrobial agents and possibly develop further mutations in genes encoding the target site of antibiotics [17].

1.5.1 β-lactamases

An enzyme able to hydrolyze penicillin was identified in *E. coli* as early as in 1940, before penicillin was taken into clinical use, proving a natural origin [4]. The β-lactamases execute their effect in hindering the work of β-lactams by hydrolyzing the β-lactam ring structure. The enzymes can be encoded on both plasmids and chromosomes and are highly selected for by the extensive use of β-lactams in our society. The β-lactamases are located differently in Gram-positive and Gram-negative bacteria. The enzymes are mostly extracellular in Gram-positive bacteria, while periplasmic in Gram-negative species. There has been indentified over 890 β-lactamases targeting various β-lactam antibiotics [2, 4].

Classification of β-lactamases can be done according to the functional properties (Bush-Jacoby-Medeiros scheme) or by the molecular class (Ambler classification scheme). The molecular classification is based on the sequence similarities and catalytic mechanisms. Class A, C, and D all have a serine at their active site, while class B has a zinc based active site (table 2). The functional classification groups are based on the substrate profile of the enzyme and its inhibitory profile. Group 1 is cephalosporinases, group 2 consist of broad-spectrum, inhibitor-resistant, and extended-spectrum beta-lactamases and serine carbapenemases. The last group, group 3, holds the metallo-β-lactamases (table 3) [18, 19].

Table 2. Ambler classification schemes for bacterial β-lactamases[19].

Ambler classification scheme	
Class	
A	Serine Based Penicillinases
B	Zinc Based Metallo-β-lactamases
C	Serine Based Cephalosporinases
D	Serine Based Oxacillinases

Table 3. Bush-Jacoby-Medeiros classification scheme for bacterial β-lactamases [18].

Bush-Jacoby-Medeiros			Inhibited by	
Group	**Substrate(s)**	**Characteristic(s)**	**CA or TZB**	**EDTA**
1	Cephalosporins	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	No	No
1e	Cephalosporins	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	No	No
2a	Penicillins	Greater hydrolysis of bezylpenicillin than cephalosporins	Yes	No
2b	Penicillins, early cephalosporins	Similar hydrolysis of benzylpenicillin and cephalosporins	Yes	No
2be	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	Yes	No
2br	Penicillins	Resistance to clauvulanic acid, sulbactam, and tazobactam	No	No
2ber	Extended-spectrum cephalosporins, monobactams	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	No	No
2c	Carbenicillin	Increased hydrolysis of carbenicillin	Yes	No
2ce	Carbenicillin, cefepime	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	Yes	No
2d	Cloaxillin	Increased hydrolysis of cloaxcillin or oxacillin	Variable	No
2de	Extended-spectrum cephalosporins	Hydrolyses cloaxcillin or oxacillin and oxyimino-β-lactams	Variable	No
2df	Carbapenems	Hydrolyses cloaxicillin or oxacillin and carbapenems	Variable	No
2e	Extended-spectrum cephalosporins	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	Yes	No
2f	Carbapenems	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	Variable	No
3a	Carbapenems	Broad-spectrum hydrolysis including carbapenems but not monobactams	No	Yes
3b	Carbapenems	Preferential hydrolysis of carbapenems	No	Yes

* CA: clavulanic acid, TZB: tazobactam

1.5.2 AmpC β-lactamases

The early discovery of a bacterial enzyme able to destroy penicillin was an important discovery for the future. Later on the enzyme was named AmpC β-lactamase and it was first seen in *E. coli*. As penicillin resistance was a spreading problem scientists started to look closer into the genetics of the resistance mechanisms. The structural gene for the production of the β-lactamase was named bla_{AmpC} and the sequence of the gene from *E. coli* was reported in 1981. The AmpC enzymes are classified as class C according to the Ambler structural classification and group 1 based on the functional classification scheme of Bush et al. [18, 20].

The molecular masses of typical AmpC enzymes range from 34 to 40 kDa. They have isoelectric points of > 8.0 and are generally located in the bacterial periplasm. Although AmpC enzymes are active against penicillins, their activity is even greater towards cephalosporins, with the exception of 4th generation cephalosporins (cefepime and cefpirome). They also hydrolyze cephamycins, like cefoxitin and cefotetan, and monobactams like aztreonam. AmpC enzymes are poorly inhibited by clavulanic acid, sulbactam, and tazobactam, and not at all by EDTA. While cloaxillin, oxacillin, and boronic acid are considered good inhibitors [20].

The structures of the enzymes are similar to the β-lactamases of class A. The distinguishing factor is the more open binding site of the class C enzymes, enabling them to better accommodate the side chains of the cephalosporins. The molecular structure of the enzymes consists of an α-helical domain on one side and an α/β domain on the opposite site. The active site is located in the center of the enzyme, divided into an R1- and R2- site, each targeted at a side chain of the antimicrobial agent [20].

In many genera of *Enterobacteriaceae,* including *Citrobacter*, *Serratia*, and *Enterobacter*, the expression of AmpC is normally low, but inducible by exposure to β-lactams. Both *Shigella* and *E. coli* are exceptions that by lacking the gene *ampR* are noninducible but rather regulated by promoter and attenuator mechanisms [20]. Still, high-level producers of *E. coli* have been

found in clinical specimens where resistance to cephalosporins is achieved by mutation or insertion elements creating a stronger promoter [21, 22].

Studies of the AmpC promoter region in *E. coli* K-12 have shown that the gene is preceded by an *frd* operon (figure 3). This operon consists of four genes of varying size; *frdA*, *frdB*, *frdC*, and *frdD*, encoding a fumarate reductase enzyme (by the two largest subunits), and an enzyme anchor to the cytoplasmic membrane (by the two smallest subunits). There is an overlapping region of the *frdD* and the *AmpC* where the promoter of AmpC is part of the preceding gene [23]. The AmpC attenuator mediates the growth rate control of the AmpC gene product as well as being a transcriptional terminator for the *frdD* operon. As the two operons are tightly connected mutations in the overlapping region will affect both regions [24-26].

Figure 3. Overview of the *E. coli* chromosomal *AmpC* and *frd* regions. Figure modified from Bergstrøm *et al.* [26].

Plasmid-mediated AmpC genes have been isolated from both nosocomial and non-nosocomial isolates from all over the globe. They have been identified since 1989 being more easily detected in species not expected to produce an AmpC β-lactamase. Several families of enzymes are known like CMY, FOX, ACC, LAT, MIR, ACT, and MOX, with only minor amino acid variations separating them. The plasmid-encoded β-lactamases are related to the chromosomally-encoded enzymes. CMY-2, the most common plasmid-mediated AmpC β-lactamase from a global perspective, shows a relatedness of 96% to chromosomal AmpC β-lactamases from *Citrobacter freundii*. Other resistance genes conferring resistance to aminoglycosides, chloramphenicol, quinolones, sulfonamide, tetracycline, and trimethoprim are often accompanying the AmpC β-lactamases on the plasmids. Other β-lactamase encoding genes can also be found including $bla_{\text{TEM-1}}$, $bla_{\text{PSE-1}}$, $bla_{\text{CTX-M-3}}$, bla_{SHV} variants, and $bla_{\text{VIM-1}}$. In order to mobilize a gene from the chromosome onto a plasmid, transposable elements are needed. The insertion sequence IS*Ecp1* is associated with several variants of CMY and ACC and holds the ability to mobilize chromosomal β-lactamases onto plasmids as well as provide

a highly efficient promoter for neighboring genes. Also the IS*CR1*, an insertion sequence common region, is known to be involved in the transfer of other AmpC β-lactamase genes into complex class 1 integrons [20].

1.6 Multi-resistance mechanisms; aminoglycosides and fluoroquinolones

1.6.1 Aminoglycoside resistance by the AAC(6`)-Ib-cr enzyme

Gentamicin, amikacin, and tobramycin are all aminoglycosides, antimicrobial agents targeted to inhibit the protein synthesis of bacteria. The most important mechanism of acquired resistance to aminoglycosides is the production of enzymes capable of modifying and inactivating the antimicrobial agents. The resistance genes are often carried on plasmids and they are transferrable between different bacterial species [2]. Recently a variant of a common aminoglycoside N-acetyltransferase, AAC(6`)-Ib, was discovered to also provide resistance to the fluoroquinolones ciprofloxacin and norfloxacin. Two amino acid mutations enable the naturally evolved enzyme to confer resistance to synthetic antimicrobial agents. By itself the degree of resistance by AAC(6`)-Ib-cr is low, but when accompanied by other quinolone resistance genes the level is raised close to the clinical breakpoint for susceptibility [27].

1.6.2 Fluoroquinolone resistance by mutations in the genes *parC* and *gyrA*

Resistance to fluoroquinolones in strains of *E. coli* was found to be caused by mutations in the genes encoding subunits of DNA gyrase and topoisomerase IV (*gyrA* and *parC*, respectively), the targets of quinolones. The described mutations for GyrA are generally found between position 67 and 106, a region referred to as the quinolone-resistance determining region (QRDR). Mutations in *parC* mostly occur at codons 80 and 84 (table 4). A single *gyrA* mutation may lead to resistance to nalidixic acid, but additional mutations in *gyrA* and/or in *parC* are required for high-level resistance to fluoroquinolones [28].

Table 4. Mutations described in GyrA and ParC of quinolone-resistant strains of *E. coli* [28]

	Codon	Wild type amino acid	Mutations described
GyrA	51[a]	Ala	Val
	67[a]	Ala	Ser
	81	Gly	Cys, Asp
	82[a]	Asp	Gly
	83	Ser	Leu, Trp, Ala, Val
	84	Ala	Pro, Val
	87	Asp	Asn, Gly, Val, Tyr, His
	106[a]	Gln	Arg, His
ParC	78	Gly	Asp
	80	Ser	Ile, Arg
	84	Glu	Lys, Val, Gly

[a] Only described in mutants obtained *in vitro*.

*Ala: Alanine, Arg: Arginine, Asn: Asparagine, Asp: Aspartic acid, Cys: Cysteine, Gln: Glutamine, Gly: Glycine, Glu: Glutamic acid, His: Histidine, Ile: Isoleucine, Leu: Leucine, Lys: Lysine, Pro: Proline, Ser: Serine, Trp: Tryptophan, Val: Valine, Tyr: Tyrosine.

1.7 *Enterobacteriaceae*

The *Enterobacteriaceae* consists of several genera and species, where some of the more common ones from a clinical perspective are *E. coli*, *Klebsiella pneumonia*, *Shigella*, *Salmonella* and *Enterobacter*. Members of the family can be found in a wide range of habitats like on plants and in soil, water, and the intestines of humans and animals. Properties shared by the *Enterobacteriaceae* family include that they are Gram-negative rods, do not form spores, are motile with peritrichous flagella or are non motile, grow both aerobically and anaerobically, ferment D-glucose and other sugars, often with gas production, are catalase positive and oxidase negative [29].

1.7.1 *Escherichia*

The genus consists of six species; *Escherichia albertii, Escherichia blattae, E. coli, Escherichia fergusonii, Escherichia hermannii*, and *Escherichia vulneris*. Apart from *E. blattae,* a commensal organism of cockroaches, all species have been isolated from human specimens [29].

1.7.2 *Escherichia coli*

E. coli is the species of *Escherichia* usually isolated from human specimens. It is a motile bacterium, facultative anaerobe with or without a capsule. *E. coli* is associated with urinary tract infections, diarrheal diseases, bacteremia, and meningitis, caused primarily by a limited number of pathogenic clones. The normal habitat of *E. coli* is the gut of humans and animals and the common route of infection is by contact and ingestion (oral-fecal). It possesses several antigens that are used for serotyping; O (somatic), H (flagellar), K (capsular), and F (fimbral) [2, 29].

During recent years the *E. coli* clone O25:H4-ST131 associated with urinary tract and blood stream infections has been observed worldwide. The clone has been isolates from both hospital settings and long-term care facilities as well as from the community. *E. coli* ST131 clones are known to be diverse and may harbor a variety of β-lactamase genes like bla_{TEM}, bla_{OXA}, and $bla_{\text{CTX-M}}$, but are especially linked to the worldwide dissemination of CTX-M-15, an extended spectrum β-lactamase (ESBL). The clones usually belong to phylogenetic group B2 and are of heterogeneous PFGE-types [30-32]

2. Aims and hypothesis for the study

In a previous Norwegian study by Igeltjørn five *E. coli* isolates showing an increased resistance to cephalosporins were found to hold IS*911* in the AmpC region. Four of the isolates were clonally related by PFGE, of sequence type 131, and from the Bergen region. The last isolate was isolated from Vestfold hospital, sequence type 550, and not related to the others by PFGE [33]. This study aims to continue the work and discoveries started by Igeltjørn and continued by the Reference Center for Detection of Antimicrobial Resistance (K-res).

The aims for this study was (i) To investigate a putative increase in β-lactam-resistant clinical *E. coli* isolates submitted to K-res from the Department of Microbiology at Haukeland University Hospital with a phenotype and genotype indicating hyperexpression of the chromosomal bla_{AmpC} gene, (ii) Molecular characterization of the isolates and compare with isolates from other Norwegian microbiology laboratories to reveal the mechanism of resistance and if there is dissemination of a successful clone in the Bergen region.

Hypothesis:

1. There is a clonal outbreak of β-lactam-resistant *E. coli* isolates in the western part of Norway (Bergen region).
2. The dissemination of *E. coli* isolates in the Bergen region is caused by a successful clone hyperexpressing the chromosomal bla_{AmpC} gene.

3. Materials and methods

3.1 Strain collections

Three strain collections for this study was selected by an extensive data mining search through the paper copies of the requisitions and reports from K-res during the period 2006-2010. The data was typed into a Microsoft Excel database.

Collection 1: *E. coli* isolates (*n*=111) from Department of Microbiology at Haukeland University Hospital, representing all *E.coli* isolates received at K-res from 2006 until 2010 and assigned as "Chromosomal AmpC with or without impermeability". In order to be assigned this profile an isolate has to have a phenotypic resistance profile consistent with AmpC production and negative plasmid-mediated AmpC PCR.

Collection 2: *E. coli* isolates (*n*=100) selected from isolates received at K-res from 2006 until 2010 characterized as "Chromosomal AmpC with or without impermeability" representing a range of other diagnostic microbiological laboratories in Norway. The isolates were used as a control group for this study. In the process of selecting isolates, emphasis was given to that, if possible, all parts of the country should be included and that the isolates used should have a time range throughout the given year.

Collection 3: A selection of 12 PCR positive for an insertion in the bla_{AmpC} region *E. coli* isolates from both Haukeland (*n*=9) and other laboratories (Vestfold *n*=2 and Haugesund *n*=1) were chosen for an extended characterization. The following studies were made for these isolates: multi-locus sequence typing (MLST), sequencing of the bla_{AmpC} region, extended antimicrobial susceptibility testing (ciprofloxacin, mecillinam, nitrofurantoin, fosfomycin, tobramycin, gentamicin, amikacin, and trimethoprim- sulfamethoxazole) and PCR/sequencing experiments for *qnr*-, *aac(6´)-Ib-cr*- and bla_{TEM} genes.

3.2 Phenotypic methods

3.2.1 Etest susceptibility testing

The antimicrobial susceptibility of a microorganism can be determined by using a gradient test such as Etest. This is a quantitative method developed to establish the minimum inhibitory concentration (MIC) of an agent, the lowest concentration that inhibits visible microbial growth (i.e. colonies on an agar plate or turbidity in broth culture). An Etest is a plastic strip with a predefined and immobilized gradient of an antimicrobial agent. When the strip is placed onto an inoculated agar plate, the antimicrobial agent diffuses into the agar. After an overnight incubation the MIC can be read on the strip at the point where the edge of the microbial growth meets the Etest strip [6]. The results were interpreted according to the clinical breakpoints set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (table 5).

In this study the following Etests were used:

1. For collection 3 (*n*=12): ciprofloxacin, mecillinam, nitrofurantoin, fosfomycin, tobramycin, gentamicin, amikacin, and trimethoprim- sulfamethoxazole.
2. Complementing Etests for the *E. coli* isolates positive for an insertion in the bla_{AmpC} region where they were lacking: ceftazidime, cefuroxime, aztreonam, and the combination test CN/CNI.

Table 5. MIC interpretive standards (mg/ml) determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST v.2.0, 01.01.2012, http://www.eucast.org/clinical_breakpoints/ and NordicAST v.2.0, 01.01.2012)

Antimicrobial agent	**S≤**	**R>**
Ciprofloxacin	0.5	1
Mecillinam	8	8
Nitrofurantoin	64	64
Fosfomycin	32	32
Tobramycin	2	4
Gentamicin	2	4
Amikacin	8	16
Trimethoprim- Sulfamethoxazole	2	4
Ceftazidime	1	4
Cefuroxime	8	8
Aztreonam	1	4
Ampicillin	8	8
Amoxicillin-Clavulanate	8	8
Piperacillin-Tazobactam	8	16
Cefoxitin	8	8
Cefotaxime	1	2
Meropenem	2	8

S: susceptible, R: resistant

3.3 Molecular methods

3.3.1 DNA isolation from bacteria

Bacterial DNA needed for this study was isolated using the QIAGEN GenoM-48 BioRobot (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. The robot executes all the steps of the isolation and all the reagents used are supplied by MagAttract DNA M48 Mini Kit (QIAGEN). The bacterial cells are lysed and the DNA binds to magnetic particles. The DNA is then washed several times before eventually eluted in water [34].

Procedure:

1. Lactose agar plates with ampicillin were streaked and incubated over night at 37°C.
2. 0.5 McFarland suspensions were made in 0.85% NaCl and 200µl was transferred to sample tubes.
3. The sample tubes were placed in the robot and the program was started.
4. After isolation, the DNA was stored at 2-8°C.

3.3.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method comparable to a copy machine, where specific regions of DNA are amplified *in vitro*. A heat stable DNA polymerase is necessary along with a pair of oligonucleotide primers for the DNA region to be amplified, as well as a supply of deoxynucleotide triphosphates (dNTPs). The method is rapid and simple in execution and is commonly used in a wide range of endeavors, from paleontology to criminology [35].

In general the PCR reaction follows three steps [35]:

1. Denaturation: The reaction is heated to a temperature, approximately 95ºC, where the hydrogen bonds of the double helix are broken creating single strands of template DNA.
2. Annealing: The temperature of the reaction is lowered to about 50-60ºC, enabling the primers to attach to the complementary regions on the single-stranded template DNA. This creates short segments of double-stranded DNA where the DNA polymerase is able to attach.
3. Extension/elongation: The temperature is raised to 72ºC, the optimal temperature for the *Taq* polymerase used in this study, and the nucleotides are added to the 3´end of the primers by the polymerase. New DNA strands complementary to the DNA template are synthesized.

This process is repeated for 20-30 cycles which yields an amplification of from a millionfold to over a billionfold copies of the template DNA (2^{20}-2^{30}). As each step in the process is rather quick, the amplification can be done in a few hours. The amplification of the template DNA is verified by using agarose gel electrophoresis [35].

For this study hot-start PCRs were performed using JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma Aldrich, St. Louis, USA). This type of PCR has a higher specificity by lowering the non-specific primer annealing. The DNA polymerase provided in the reaction mix before mentioned, is inactivated by antibodies until the reaction reaches a given temperature. The reaction mix also includes deoxynucleotides, buffer, and an inert red dye (http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/p0982bul.Par.0001.File.tmp/p0982bul.pdf).

General procedure:

1. For each reaction 2.5µl template DNA was added to 22.5µl PCR mastermix (Appendix B, table 1).
2. Positive and negative controls were included, as well as a mastermix control containing ddH_2O (Appendix B, table 5).
3. The PCR was run on a PCR thermocycler (Applied Biosystems, Foster City, Ca, USA) according to the appropriate program depending on the annealing temperature of the primers (Appendix B, table 3).

3.3.3 Agarose gel electrophoresis

Gel electrophoresis is a method used to separate, identify and purify DNA and RNA fragments through an electrical field. The gel acts as a sieve where small molecules migrate faster through than larger molecules. The rate of movement for a molecule is determined by their charge-to-mass ratio. Gel electrophoresis can therefore be used in determining the unknown size of DNA or RNA by comparing to a molecular ladder with molecules of known sizes. As nucleic acid molecules are naturally negatively charged they will migrate towards the positively charged electrode of the electrical field [36].

The location of the DNA in the gel is determined by using low concentrations of fluorescent intercalating dyes, like ethidium bromide or GelRed, to be visualized under UV light. It is possible to retrieve the bands of DNA from the gel, which can be used for a variety of other laboratory methods [37].

Procedure:

1. 1% agarose (Seakem LE agarose, Lonza, Rochland, ME, USA) was dissolved in 0.75X TBE (Appendix B, table 7) by boiling the solution in a microwave oven.
2. 0.5µg/ml EtBr (Sigma-Aldrich) or GelRed (Biotium) was added for staining of the DNA molecules.
3. The solution was poured into a gel tray of the electrophoresis apparatus containing the combs. The gel was left to solidify for about 30 minutes.
4. After removing the combs, 5µl of each PCR product was loaded into the gel wells.
5. 5µl of 1 Kb Plus DNA molecular marker (Invitrogen, Carlsbad, CA, USA) was loaded into the flanking wells (Appendix B, figure 1).
6. The electrophoresis was run at 120 V for about 90 minutes.
7. The DNA bands were visualized using GelDoc System (BioRad).

3.3.4 PCR-based detection of 16S rDNA

As a control of a successful DNA isolation universal primers were used to amplify a region of about 1500bp of the 16S rDNA followed by agarose gel electrophoresis. This shows the quality of the DNA extraction and is beneficial to check before starting further studies with the DNA. Clear 16S rDNA bands on a gel indicate a strong product and a high quality of the DNA template. If the amplification is poor or unsuccessful this indicates failed DNA isolation, DNA degradation or the presence of PCR inhibitors [38].

Mastermix, primers, and PCR conditions for detection of 16S rDNA are described in Appendix B, table 1, 3-4.

3.3.5 PCR-based screening for an insertion into the bla_{AmpC} region

All the isolates of the strain collections were screened for possible insertion into the bla_{AmpC} region using one primer located in the actual bla_{AmpC} gene and one primer located in *frdD* in front of the AmpC promoter region. The screening was performed using PCR and agarose gel electrophoresis, as previously described. Mastermix, primers, and PCR conditions are described in Appendix B, table 1, 3-4.

3.3.6 PCR-based linkage of IS*911* with *bla*$_{\text{AmpC}}$

The AmpC with insert-positive isolates intended for PFGE study (*n*=28) were investigated for the linkage between *bla*$_{\text{AmpC}}$ and the IS-element IS*911* (table 3, Appendix A), only excluding the following isolates; K34-08, K36-03, K61-40, K67-10. The IS*911* specific primer IS911-1F and the *bla*$_{\text{AmpC}}$-binding primer IS911-4R were used (table 4, Appendix B).

The screening was performed using PCR and agarose gel electrophoresis as previously described. Mastermix, primers (IS911-1F and IS911-4R), and PCR conditions are described in Appendix B, table 1, 3-4.

3.3.7 PCR-based screening for *bla*$_{\text{TEM}}$

Strain collection 3 was screened for the precence of β-lactamase TEM. TEM-1 was the first plasmid mediated β-lactamase described in Gram-negative bacteria and is still commonly found. It is responsible for up to 90% of ampicillin resistance in *E. coli*. A large number of TEM variants have been described (>130) where single amino acid substitutions occur at a limited number of positions [39]. To confirm the variant of TEM present sequencing of the region is necessary.

The screening was performed according to the previously described procedure for PCR and gel electrophoresis. Mastermix, primers, and PCR conditions are described in Appendix B, table 1, 3-4.

3.3.8 PCR-based screening for aac(6´)-Ib-cr

Strain collection 3 was screened for the presence of the gene encoding the aminoglycoside modifying enzyme AAC(6´)-Ib-cr. This variant of AAC(6´)- Ib encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin. The *cr* variant has amino acid changes, Trp102Arg and Asp179Tyr, compared to the AAC(6´)-Ib enzyme [40].

The screening was performed using the PCR- and gel electrophoresis protocols previously described. Mastermix, primers, and PCR conditions are described in Appendix B, table 1, 3-4

3.3.9 Sequencing of the Quinolone resistance determining regions

Quinolones, including fluoroquinolones, consist of a large family of bactericidal synthetic agents that interfere with the replication of the bacterial chromosome. Mutations in the target area where the quinolones attach confers resistance [2]. These mutations are often seen within conserved regions of two genes, *gyrA* and *parC*. The genes encode, respectively, the A subunit of DNA gyrase and the homologous A subunit of topoisomerase IV. The conserved regions within the genes were named "Quinolone Resistance Determining Regions", shortened to QRDR [27].

Collection 3 was screened for *parC* and *gyrA*. The screening was performed using two sets of primers (Appendix B, table 4) according to the general protocol for PCR and gel electrophoresis described earlier. Mastermix and PCR conditions are described in Appendix B, table 1 and 3. The PCR products were purified and sequenced as described below.

3.3.10. PCR-based screening for sequence type 131 (ST131)

Based on a previous study it was seen that *E. coli* isolates (*n*=6) from Haukeland University Hospital all contained IS*911* and were typed by multi-locus sequence typing (MLST) to be ST131 [33]. A rapid PCR-based method for the detection of the O25b-ST131 clone was developed by Olivier Clermont and colleagues [41]. Primers designed to target the 375 bp fragment of the *pabB* gene specifically found in this clone were used. As a positive control of the amplification an additional gene, *trpA*, was included.

The PCR program was performed according to the publication, and also additional modifications were made for the second run (Appendix B, table 3). A random sample of isolates from collection 1 were screened (*n*=11) as well as the three AmpC with insertion region positive isolates that were from Vestfold (*n*=2) and Haugesund (*n*=1). The screening was executed according to previously described methods for PCR and gel electrophoresis.

PCR conditions and primers are described in Appendix B, table 3-4. The mastermix prescription is found in table 8 in Appendix B.

3.3.11 Real- time PCR for the detection of the O25b-ST131 clone

Real-time PCR both amplifies and quantifies DNA in the same reaction. The PCR machine monitors the amplification of a PCR product at every cycle and presents the data in the form of an amplification plot. Real-time PCR yields an immediate visual result and no further detection methods like agarose gel electrophoresis are required [6]. In this study the dye SYBR Green I was used. It fluoresces when bound to double-stranded DNA, which is then detected by the PCR machine. After the amplification of the template a melt curve analysis was made. When the melting temperature (Tm) is reached, SYBR Green is released and the fluorescence decreases.

In a publication by Hiran Dhanji *et al*. another method for rapid detection of the O25b-ST131 clone was presented [42]. A specific assay for the allelic variants of the *pabB* gene is used to detect two single nucleotide polymorphisms (SNPs); thymine-144 and adenine-450. For an isolate to be characterized as ST131 both of these SNPs need to be present. According to the publication melting temperatures of 82.82°C for the "T" assay and 80.69°C for the "A" assay are expected [42].

The same sample of isolates from the PCR-based detection of ST131 was used for this study. The primer sequences are listed in Appendix B table 4 (ST131AF, ST131AR, ST131TF and ST131TR). The mastermix prescription and the PCR conditions are listed in Appendix B table 9 and 10, respectively.

Procedure:

1. The real-time PCR machine, ABI 7500 Fast Real-Time PCR Systems, was switched on. The controls and the samples were plotted in and the PCR program parameters were set.
2. To the wells of the plate 22.5µl mastermix and 2.5µl template were added. The plate was covered with optic tape.
3. The plate was given a quick spin before added to the machine.

3.4 Pulsed- field gel electrophoresis (PFGE)

One of the limitations of a standard agarose gel electrophoresis is that it is not able to properly separate DNA molecules exceeding 40kb with its constant electrical field. By applying multiple electrical fields that alternate, the DNA molecules will move in different directions allowing for a separation of up to ~5 Mb in length. The first electrical field starts the process by stretching out the coiled DNA molecules. The second field will interrupt the first electrical field and the DNA molecules will migrate in a "zigzag" path downwards in the gel. The smaller the molecule, the longer the migration in the gel [37].

In order to protect the chromosomes from being fragmented the bacteria are embedded in agarose plugs. The cells, while inside the plugs, are treated with lysosome to lyse the cells and expose the DNA. The plugs are washed before treated with protinase K, removing DNA degrading enzymes naturally present in the cells. The restriction enzyme used, *XbaI* (New England BioLabs, Ipswitch, Ma, USA), will only have a few recognition sites in the genome resulting in few fragments. XbaI recognizes the site shown below in figure 4 and creates "sticky" ends.

Figure 4. The recognition sequence for restriction enzyme *Xba*I (http://www.neb.com/nebecomm/products/productr0145.asp).

The digested fragments, as well as an appropriate marker, will then be separated on an agarose gel by the changing direction of the electric fields applied.

Isolates included in the PFGE are listed in Appendix A table 3.

Procedure:

A. Preparation of PFGE plugs:

1. Lactose agar plates with ampicillin were streaked and incubated over night at 37ºC.
2. A single bacterial colony was transferred into Falcon tubes with 5ml BHI-media (Appendix B, table 6) and incubated overnight at 37ºC with shaking.
3. 50µl of the culture was transferred into Falcon tubes with 5ml BHI-media and incubated at 37ºC for 4 hours with shaking.
4. The tubes were centrifuged at 3500 rpm for 10 min.
5. After removing the supernatant, the cells were resuspended in 1ml of cold PIV- buffer (Appendix B, table 7)
6. 495µl of the suspension were transferred to an eppendorf tube where 5µl lysosyme (100mg/ml) (Sigma-Aldrich) were added (Appendix B, table 7). The suspension was mixed by vortexing and then equilibrated to 50ºC in a water bath.
7. To each bacterial suspension, 500µl 2% agarose (BioRad) dissolved in PIV-buffer and equilibrated to 50ºC (Appendix B, table 7) was added. The suspensions were mixed by vortexing before transferred to the plug mould. The plugs were left to solidify for 15 min at 4ºC.
8. The plugs were transferred to new tubes containing 2ml lysis buffer (Appendix B, table 7) and incubated at 37ºC for 2 hours with slow shaking.
9. After removing the lysis buffer, the plugs were washed in 1 ml ddH_2O for 15 min with slow shaking.
10. After removing the ddH_2O, 1ml of ESP buffer containing 50µl/ml proteinase K-solution (20mg/ml, VWR, West Chester, PA, USA) were added (Appendix B, table 7). Incubated at 50ºC in a water bath overnight.
11. The ESP-solution was removed and the plugs were washed 2x30 min in 1ml TE-buffer with slow shaking (Appendix B, table 7).

12. The plugs were stored overnight at 4ºC in a thiurea and TE buffer solution (500µl thiurea stock solution (Table 7 Appendix B) pr 10ml TE buffer), or until needed for use.

B. Restriction enzyme digestion of DNA in agarose plugs

1. Thin slices of the plugs were cut and transferred to eppendorf tubes.
2. The slices were washed 2x30 min in TE buffer (table 7 Appendix B), 1x30 min in ddH_2O, and 1x30 min in 125µl restriction enzyme buffer (Buffer 4, New England BioLabs, Ipswitch, Ma, USA) containing bovine serum albumin (BSA) (Appendix B, table 7) with slow shaking.
3. The restriction enzyme mix was prepared. 20U *Xba*I (New England BioLabs) pr 125µl enzyme mix was used (Appendix B, table 7).
4. The restriction enzyme buffer was removed and 125µl of the restriction enzyme mix was added. The slices were incubated at 37ºC overnight with slow shaking.
5. The plugs were washed 2x30 min in TE buffer (table 7 Appendix B).

C. Gel electrophoresis

1. Agarose was prepared using 1% Seakem LE agarose (Lonza, Rochland, ME, USA) dissolved in 0.5X TBE, boiled in a microwave oven and equilibrated to 50ºC in a water bath.
2. The gel equipment was prepared and the slices were positioned on the gel comb and left for some minutes to fully stick to it. The comb was placed and the agarose poured into the chamber, a small amount of agarose was kept. The gel was left to solidify for 30 min at room temperature.
3. The electrophoresis chamber was filled with 1.8-2 l of 0.5X TBE and the circulation was started to cool the system down to 12°C.
4. After the gel was solidified the comb, as well as access gel, was removed.
5. A low range PFG marker (New England BioLabs) was included (Appendix B, figure 2). Slices of the marker were cut and placed directly into the appropriate empty wells in the gel.

6. The gel was placed in the electrophoresis chamber, the program parameters set (table 6) and the electrophoresis started.

Table 6. PFGE program parameters

Program parameters for PFGE	
Pulstime	1-20 sec
Total runtime	21 hours
Voltage	6.0 V/cm = 200V
Angle	120ºC
Temperature	12 ºC
Gel running buffer	0.5X TBE

7. The gel was stained in a GelRed solution (Appendix B, table 7) for 60 min after the electrophoresis.
8. The gel bands were visualized using the GelDoc system (BioRad, USA)

D. Interpretations of DNA relatedness

1. Analysis were made computationally in BioNumerics v6.01 (Applied Maths, Sint-Martens-Latens, Belgium) using the band-based DICE similarity coefficient and the Unweighted Pairs Geometric Matched Analysis (UPGMA). The position tolerance was set to 0.5% for optimization and the band comparison was set to 1.0%.

3.5 DNA sequencing

In this study the Sanger method for DNA sequencing by dideoxy-mediated chain termination was used. The method is based on the addition of dideoxynucleotides (ddNTP's) as well as deoxynucleotides (dNTP's) to the DNA synthesizing reaction. Each ddNTP is labeled with a different fluorescent dye that can be detected by a laser beam. During the PCR- program the DNA will denature, the sequencing primers will anneal and nucleotides will be incorporated. By incorporating a ddNTP instead of a dNTP the extension of the sequence terminates. The reason for this being that the absence of a 3´-OH group prevents the formation of a phosphodiester bond with the succeeding dNTP. As a result the PCR produces DNA of different lengths. The PCR products are separated using capillary electrophoreses and the last

ddNTP is detected by recording the different colors from the dyes. Based on this the DNA sequence can be determined. The method is quick and simple to use, but the machine has problems reading the nucleotides in close range to the sequencing primer. Vague signals can be obtained for these regions and there is a need for manual conformation [43].

For an optimal sequencing reaction it is important to in advance remove access dNTPs and primers from the template after the PCR reaction. The purification is achieved using the two hydrolytic enzymes Exonuclease I (Exo) and Shrimp Alkaline Phosphatase (SAP) which remove remaining primers and dNTPs, respectively.

In this study DNA sequencing was used for determining the sequences of:

a. bla_{AmpC} with insertion.
b. bla_{TEM}.
c. Quinolone resistance determining regions of *parC* and *gyrA*.
d. MLST: *adk, fumC, gyrB, icd, mdh, purA,* and *recA*.

Procedure:

A. Purification of the PCR product.

1. 1µl of Exo-SAP (USB, Cleveland, Ohio) was added to each PCR product. The tubes were placed in the thermocycler (Applied Biosystems) and the program was started.
2. Program
   37°C 1 min
   37°C 90 min
   85°C 15 min
   4°C ∞
3. The purified PCR product was stored at -20°C.

B. Sequencing PCR

1. The tubes were loaded according to Appendix B table 2.
2. The PCR thermocycler (Applied Biosystems) was programmed and started (Appendix B, table 3)
3. Nucleotide sequencing was performed at the Sequencing core facility at the University of Tromsø using a ABI Prism 3130XL Genetic Analyzer (Applied Biosystems).

C. Sequence analysis

1. The software package SeqMan II (DNAStar Inc., Madison, WI, USA) was used to edit and align the sequences.
2. BLAST was used in the search of nucleotide sequence homology. BLAST is available at the website of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.6 Multilocus sequence typing (MLST)

MLST is a PCR and sequencing based method developed to determine the clonality of bacterial strains. Seven housekeeping gene loci are used to characterize the strains. Each loci sequence is given an allelic number and combined they determine the sequence type (ST) of the strain. The determination is done by submitting the sequences online to the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli) and a ST is generated. The *E. coli* MLST scheme uses internal fragments of the following seven house-keeping genes:

1. *adk* : adenylate kinase
2. *fumC*: fumarate hydratase
3. *gyrB*: DNA gyrase
4. *icd*: isocitrate/isopropylmalate dehydrogenase
5. *mdh*: malate dehydrogenase
6. *purA*: adenylosuccinat dehydrogenase
7. *recA*: ATP/GTP binding motif

In this study the sequence types were determined for collection 3. The PCR and sequencing reaction were performed according to previously described methods. Mastermix, primers and PCR programs are described in Appendix B, table 1-4. Sequence analysis were performed using SeqMan II (DNAStar Inc., Madison, WI, USA).

3.7 Designing primers using primerBlast

For the sequencing of the inserts in the *bla*$_{AmpC}$ region the primers available (AmpCF and AmpCintR) were not sufficient for covering the whole region. Additional internal primers were designed using primerBLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). These were named IS911 1F, IS911 5F, IS911 2R and IS911 4R and are listed in Appendix B, table 4.

4. Results

4.1 General analysis

The study by Igeltjørn showed that IS*911* elements linked to bla_{AmpC} were found in 5 isolates of *E. coli* submitted to K-res from the time period 2005-2007 [33]. Of these, 4 isolates were clonally related and received at K-res from Haukeland University Hospital indicating a local spread of these isolates. To investigate this further an extensive data mining process was performed on all isolates submitted to K-res covering the years 2006-2010 from Norwegian clinical microbiology laboratories. As the assumption was that the IS*911*-bla_{AmpC} linkage was specific for isolates from the Bergen region a control group of isolates from other clinical microbiology laboratories in Norway was required. All isolates with the profile "Chromosomal AmpC with or without impermeability" were registered. In total, 360 isolates were registered for the study. The laboratory distribution of isolates over the time period indicated that the majority of isolates received at K-res were from Haukeland (*n*=111) (figure 5), followed by Østfold Hospital (*n*=50), Vestfold Hospital (*n*=37), and Sørlandet Hospital (*n*=38). Two hospitals only had one isolate each; Drammen Hospital and Ålesund Hospital.

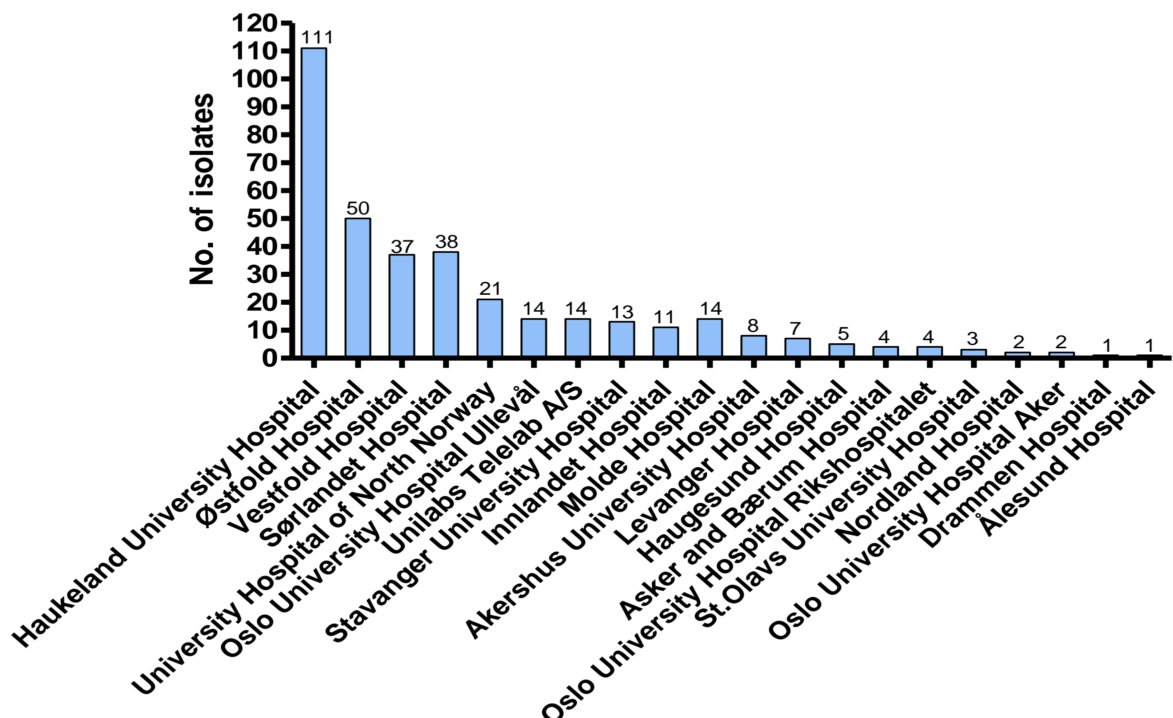

Figure 5. Graphical overview of the laboratory distribution of *E. coli* isolates with the profile "Chromosomal AmpC with or without impermeability" received at K-res from 2006-2010.

4.1.2 Distribution analysis

All the 111 isolates from Haukeland were screened for insertion in the AmpC region using the primers AmpCF and AmpCintR listed in table 4, appendix B. In total, 46 of the isolates screened positive for an insertion into the AmpC region while 64 were negative (table 7, figure 6). Overall, an increase in the ratio of positive isolates compared to negative isolates over the years was observed. The lowest ratio was observed in 2007 with only 3 positive isolates compared to 40 negative (~7%), while the highest ratio was seen in 2009 with 14 positive isolates versus 1 negative (~93%). The total number of isolates ranged from 6 to 43, with 43 isolates registered in 2007 compared to only 6 in 2010. One isolate did not give a PCR product and the screening was repeated with the same result.

Table 7. Chromosomal AmpC hyperexpressing *E. coli* isolates from Haukeland University Hospital ranged according to year received at K-res and whether they are positive or negative for an insertion into the AmpC region.

	Haukeland hospital					
	2006	**2007**	**2008**	**2009**	**2010**	**Total**
Insertion Positive	8	3	17	14	4	**46**
Insertion Negative	16	40	5	1	2	**64**
No PCR product	1	-	-	-	-	**1**
Total	**25**	**43**	**22**	**15**	**6**	**111**

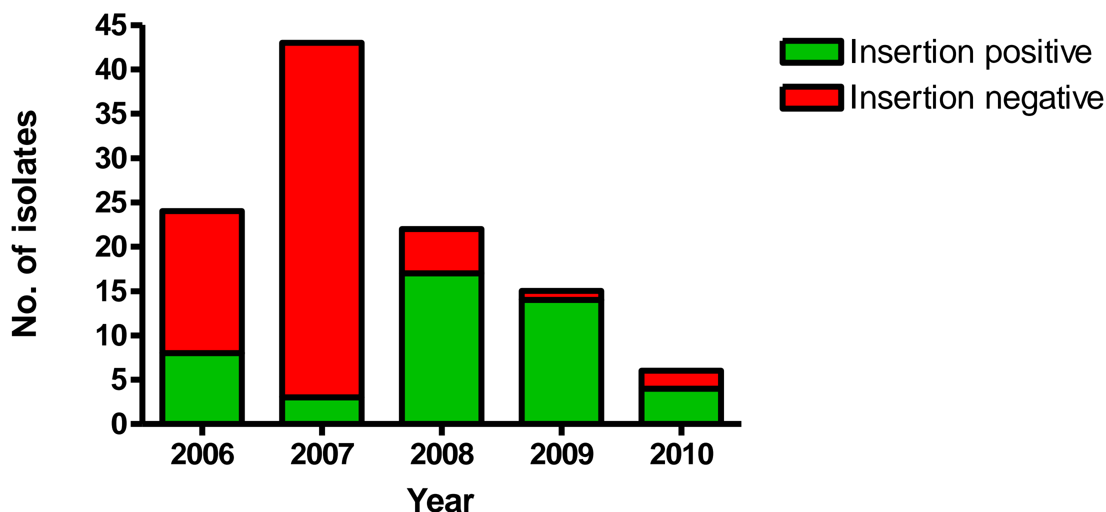

Figure 6. Graphical overview of the distribution among the Haukeland isolates of insertion positive (green) and insertion negative (red) isolates.

To investigate if whether an insertion into the AmpC region was also found in other parts of the country a control group of 100 isolates from a variety of Norwegian clinical microbiology laboratories were screened using the same primers, AmpCF and AmpCintR (Table 4, Appendix B). In contrast to the Haukeland screening only 3 isolates were AmpC with an insertion region-positive, while 97 screened negative (table 8). These isolates were from Vestfold Hospital (*n*=2) and Haugesund Hospital (*n*=1).

Table 8. Chromosomal AmpC hyperexpressing *E. coli* isolates from various Norwegian laboratories ranged according to year received at K-res and whether they are positive or negative for an insert into the AmpC region.

Various Norwegian laboratories (control group)						
	2006	**2007**	**2008**	**2009**	**2010**	**Total**
Insertion Positive	0	0	1	2	0	**3**
Insertion Negative	20	20	19	18	20	**97**
Total	**20**	**20**	**20**	**20**	**20**	**100**

Most of the bacterial isolates were collected from urine samples (n=185) followed by blood culture (n=11), abscess (n=4), pus (n=3), and secretion (n=3). Three specimen categories; bronchoalveolar lavage (BAL), drain, and expectorate, only had one isolate each (figure 7).

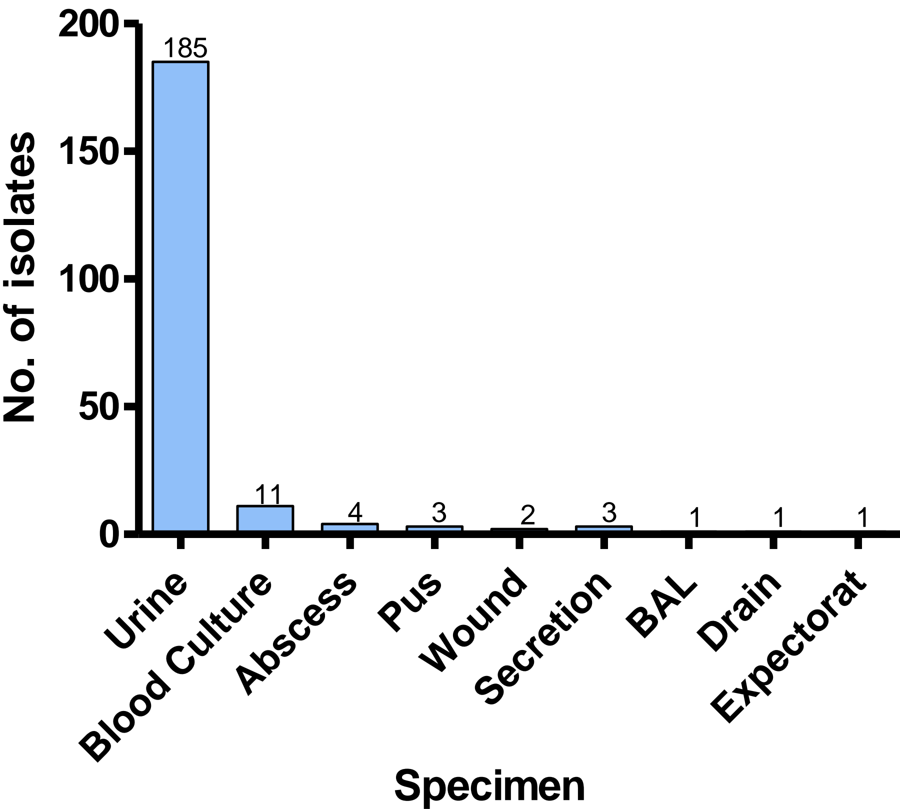

Figure 7. Graphical overview of the distribution of materials the *E. coli* isolates from collection 1 and 2 (n=211) were derived from.

The isolates received at K-res from the Department of Microbiology at Haukeland University Hospital can originate from a range of locations. Some are from inpatients at different departments within the hospital while others originate from outpatients with samples taken at a general practice or in a nursing home. For the 46 positive isolates the main location of origin was Nursing Home 1 (n=14) followed by Nursing Home 2 (n=7) (figure 8). The 46 positive isolates were divided into 13 locations, where the two hospitals, Hospital 1 and Hospital 2, had several listed locations within the same institution.

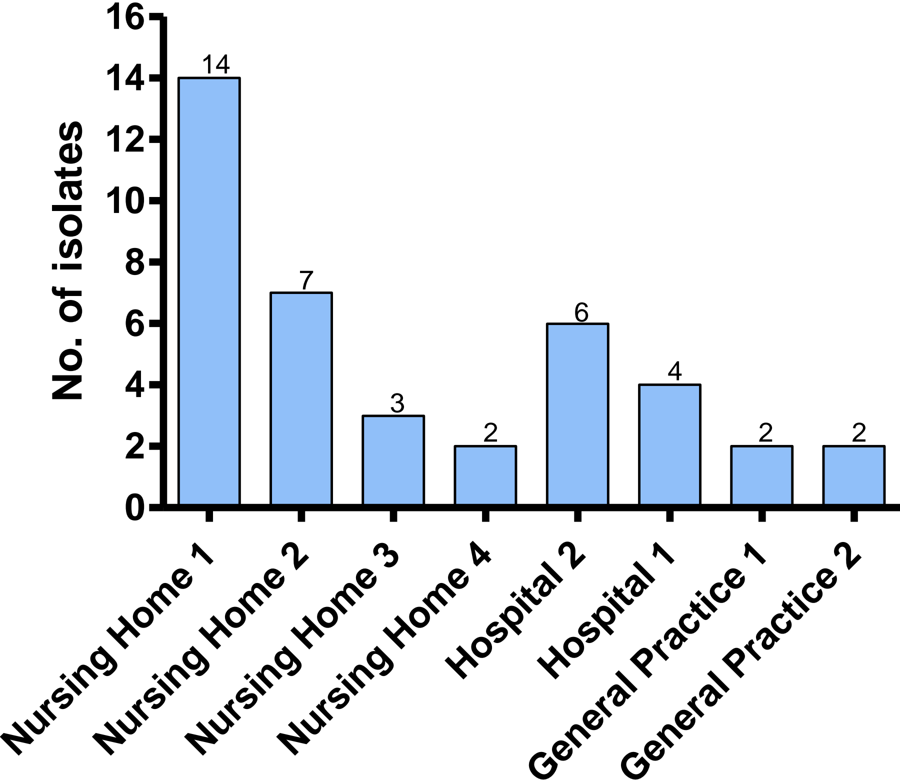

Figure 8. Graphical display of the distribution of originating locations of the isolates positive for an insertion into the AmpC region received at K-res from Haukeland University Hospital. Included in the graph are only the locations with two or more isolates ($n \geq 2$).

As the study progressed it was discovered that some bacterial isolates were derived from the same patient (table 9). A complete investigation was not performed on the strain collections.

Table 9. Overview of the patients with two- or more isolates, their location and date received at K-res. Marked in green are the isolates with an insertion into the AmpC region, while in red are the isolates with no insertion in the AmpC region.

Patient	Isolate	Location	Received (d-m-y)
A	K29-77 K29-80 K30-44	Hospital 2 Nursing Home 2 Nursing Home 4	31-01-06 03-02-06 15-03-06
B	K30-36 K36-03	General Practice 3 Nursing Home 5	03-03-06 06-11-06
C	K44-51 K45-29	General Practice 7 General Practice 7	06-06-07 10-07-07
D	K44-54 K44-60	Nursing Home 6 General Practice 4	06-06-07 12-06-07
E	K45-41 K45-48	General Practice 5 General Practice 5	16-07-07 18-07-07
F	K46-48 K46-58	Hospital 2, Surgical ward Hospital 2, Surgical ward	04-10-07 15-10-07
G	K48-48 K54-42	General Practice 2 General Practice 2	06-03-08 06-02-09
H	K49-14 K53-65	Nursing Home 1 Nursing Home 1	07-05-08 12-08-08
I	K52-78 K53-43	Hospital 1, Heart post Hospital 1, Heart post	10-11-08 11-11-08
J	K49-70 K58-06	General Practice 2 General Practice 2	15-07-08 12-06-09

4.2 Molecular results

4.2.1 PFGE analysis

32 isolates were selected for PFGE analysis, including collection 3 (*n*=12) and a random selection of isolates with an insertion in the AmpC region from Haukeland University Hospital (*n*=20), listed in table 3, Appendix A. Two cases of one patient with multiple isolates were included in the study to investigate for changes over the time period (Patient A: K29-77, K29-80, and K30-44, Patient B: K30-36 and K36-03).

All the Haukeland isolates cluster together in one large cluster with an overall similarity of 61%, and are divided into several sub clusters (figure 9). The three non-Haukeland isolates cluster together in a separate cluster with a similarity of 54%, the two Vestfold isolates being the most similar (74%).

A total of six clusters were discovered with 85% similarity all harboring isolates from a variety of locations and years of isolation. A larger cluster of isolates from 2006 was observed including the isolates; K29-80, K34-08, K30-24, K29-77, K30-36, and K36-03, marked "C3" in figure 9. The two isolates in cluster 4 are from the same location, Nursing Home 1, but from different years (2007 and 2008). Cluster 6 contains 3 isolates from the same year, 2009, from two locations; Hospital 2 and Nursing Home 2.

Overall, the Haukeland isolates are distributed more or less evenly throughout the sub clusters both considering the year of isolation and the location of origin. When looking into the originating locations of the Haukeland isolates the majority was from Nursing Home 1 (*n*=10), also seen as the major contributor on an overall basis (figure 8), followed by Hospital 2 and Nursing Home 2 with 5 isolates each. Two isolates K29-80 and K34-08 had identical PFGE profiles. They were both from the same year, 2006, but from different locations; Nursing Home 2 and Nursing Home 1, respectively. During the study it was discovered that one patient might have several samples registered, and two of these patients were included in the PFGE (table 9). Patient A with the isolates K29-77, K29-80 and K30-44 were not identical. K29-77 and K29-80 clustered closer together in cluster 3 and have an extra band

compared to K30-44 (cluster 5). They were all from 2006, but from distinct locations; Hospital 2, Nursing Home 2, and Nursing Home 4. The isolates from patient B, K30-36 and K36-03, cluster closely together in cluster 3, only separating them by one band. They are from different locations; General Practice 3and Nursing Home 5, but from the same year, 2006.

K29-77 was used as a control for the second PFGE run and the two runs of the isolate were identical.

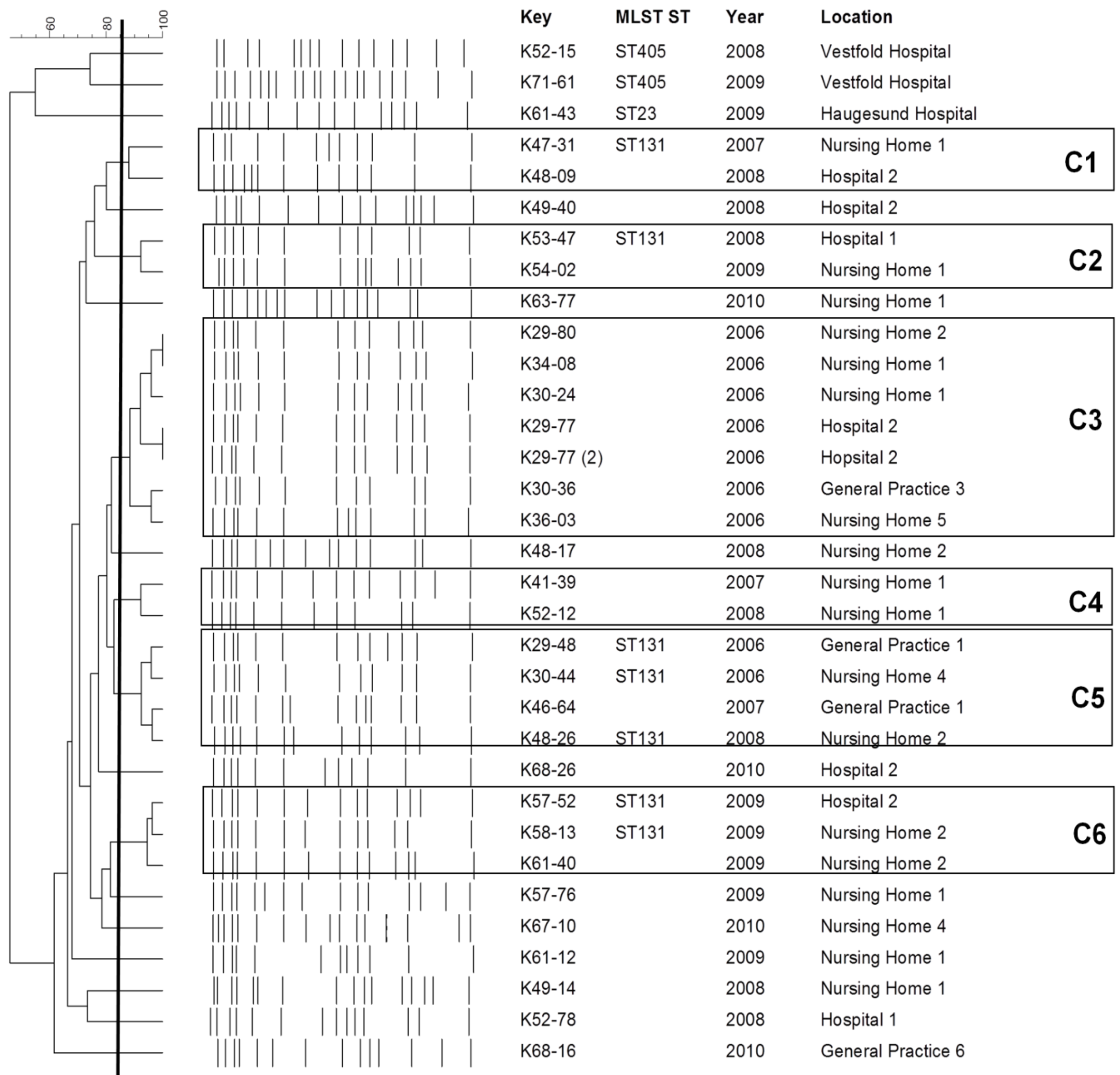

Figure 9. Dendogram illustrating the genetic relatedness of 32 isolates with an insertion in the AmpC region examined by pulsed-field gel electrophoresis (PFGE). Analysis was made using BioNumerics v6.01 (Applied Maths, Sint-Martens-Latens, Belgium). K29-77 was used as a control for the second PFGE run and is therefore listed twice. Major clusters defined by PFGE profiles with ≥85% similarity (black vertical line) are indicated.

4.2.2 Sequence type analysis; PCR, Real-time PCR, and MLST

Two previously described rapid methods; one PCR-based method and one Real-time PCR method directed at identifying the O25b-ST131 clone were investigated.

The PCR-based screening for ST131, described by Olivier Clermont and colleagues, targeted the *pabB* gene of 375bp and a control gene of the amplification, *trpA* of 427bp (primers are listed in table 4, Appendix B) [41]. For an isolate to be ST131-positive two distinct bands, one for each gene mentioned above, would be seen on an agarose gel after electrophoresis. In this study the bands visible on the gel were not as clearly defined as described in the publication. The negative control strain, *E. coli A*-81 (ATCC 25922), also had a band for the ST131-determining gene. This band was not as strong and clear as for the positive control. The study was repeated including isolates with known sequence types as negative controls (K34-04: ST117, K20-72: ST127, K05-20: ST359, K26-35: ST636, and K33-69: ST976) [44] and increasing the annealing temperature with 2°C (67°C). However, a band for *pabB* was observed in all these isolates.

The Real-time PCR screening method was directed at two single nucleotide polymorphisms (SNPs), thymine-144 and adenine-450, described in a publication by Hiran Dhanji *et al.*[42]. For an isolate to be of ST131 both the SNPs need to be present. ST131-positive isolates are determined based on melt curve analysis where the melt temperatures of 82.82°C ("T"-assay) and 80.69°C ("A"-assay) are stated in the paper. In this study the melting temperatures of the positive and negative isolates were too close to determine the sequence types of the samples. None of the temperatures came close to the expected values of 82.82°C ("T"-assay) and 80.69°C ("A"-assay), instead they were ranging from about 75°C-78°C for both assays. The study was repeated with the same results.

Collection 3 was therefore typed by the standard multi-locus sequence typing (MLST) scheme as described by Achtman *et al.* [45]. The allelic profiles of the isolates as well as the sequence types are listed in table 10.

Table 10. Sequence types determined for collection 3. The allelic numbers for each gene of the isolates are listed in the table.

		MLST genes							
Hospital	**Isolate**	***adk***	***fumC***	***gyrB***	***icd***	***mdh***	***purA***	***recA***	**Sequence type (ST)**
Haukeland	K29-48	53	40	47	13	36	28	29	**ST131**
	K30-44	53	40	47	13	36	28	29	**ST131**
	K47-31	53	40	47	13	36	28	29	**ST131**
	K48-26	53	40	47	13	36	28	29	**ST131**
	K53-47	53	40	47	13	36	28	29	**ST131**
	K57-52	53	40	47	13	36	28	29	**ST131**
	K58-13	53	40	47	13	36	28	29	**ST131**
	K63-77	53	-	47	13	36	28	29	**-**
	K68-26	53	40	47	13	36	28	29	**ST131**
Haugesund	K61-43	6	4	12	1	20	13	7	**ST23**
Vestfold	K52-15	35	37	29	25	4	5	73	**ST405**
Vestfold	K71-61	35	37	29	25	4	5	73	**ST405**

All but one of the isolates from Haukeland University Hospital belonged to sequence type 131. One of the Haukeland isolates, K63-77, did not give a PCR-product of the gene *fumC*. The PCR was repeated without any results. An additional reverse primer was used, fumCR1 (Appendix B, table 4), as mutations can occur in the primer-binding region. Still no product was obtained. Although the allelic variants of the other genes were identical to ST131 the sequence type for this isolate could not be determined. The two isolates from Vestfold both belonged to ST405, while the isolate from Haugesund belonged to ST23.

The ST131 determined isolates from Haukeland University Hospital can be found in all sub clusters of the PFGE dendogram (figure 7), apart from cluster 3 and 4. This indicates a large diversity of the ST131 isolates.

4.2.3 Sequencing and analysis of the *bla*$_{AmpC}$ with an insertion region

The AmpC with an insertion region was sequenced and analyzed for all isolates in collection 3 using the primers AmpCF and AmpCintR for the screening process and also the *IS911* specific primers IS*911* 1F, 2R, and 5F, and the IS*911* primer 4R (which binds in the AmpC region), (Table 4, Appendix B) for the sequencing of the region.

The nucleotide sequences from the nine isolates derived from Haukeland University Hospital were aligned against each other and were 100% identical. They were also aligned against reference sequences for IS*911* (AY555729.1) and AmpC (AY899338.1) in the ClustalW program (Figure 1 Appendix C) with 100% nucleotide identity to the reference sequences.

Analysis of the sequence showed that IS*911* was inserted 11 bp upstream of the wild type -10 box between position -25 and -24 (figure 10). The changes resulted in a 17 bp distance between the -10 box and the IS*911* specific sequence TTGACC, thought to act as a -35 box. The changes in the promoter region are likely driving the hyperexpression of the *bla*$_{AmpC}$ gene. The insertion of the IS-element created duplications at position -22 to –24. The *IS911* element's insertion into the chromosome was at the same place, and its structure and length identical to what described in a Canadian study [14].

Sequencing of the three isolates from other locations than Haukeland, Vestfold and Haugesund, did not result in a complete sequence with the selected primers. The AmpC primers, AmpCF and AmpCintR, gave a sequencing product, but resulted in unclosed poor sequences. Out of the four primers designed for this study all but one, IS911 4R, failed to give a sequencing product. This primer was located in the *bla*$_{AmpC}$-region and not in the IS*911* region. BLAST searches with the sequences available all indicated the presence of *IS10* and not *IS911* in these isolates. The IS*10* element is of about the same size as IS*911* (1329 bp versus 1250 bp, respectively). The PCR product using the primers AmpCF and AmpCintR in the initial screening were of the same size as for the Haukeland isolates on agarose gel electrophoresis.

To verify that bla_{AmpC} was linked to IS*911* in the Haukeland isolates, 28 isolates, both from Haukeland (*n*=25) and non-Haukeland (*n*=3), isolates were screened for the linkage of IS*911* with bla_{AmpC}. The primers IS911-1F and IS911-4R were used (Appendix B, table 4). The three non-Haukeland isolates (K52-15, K71-61, and K61-43) gave no PCR product as expected, while the isolates from Haukeland all gave a positive PCR product linking IS*911* with bla_{AmpC}.

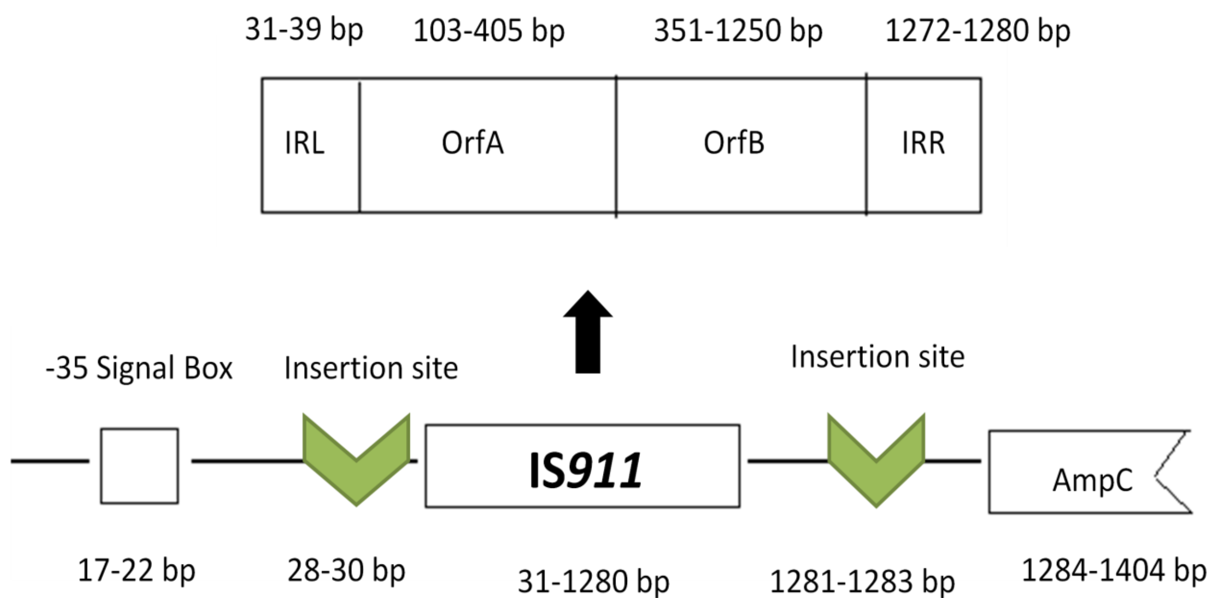

Figure 10. Illustration of the structure and insertion of IS*911* into the *E. coli* bla_{AmpC} promoter region.

4.2.4 Antimicrobial susceptibility profile

An extended antimicrobial susceptibility profile of collection 3 was determined. To investigate possible co-resistance a fluoroquinolone, ciprofloxacin, and aminoglycosides; tobramycin, gentamicin, and amikacin were included (table 11). The other MIC values were collected from previous analysis by K-res during the data mining process. The list was supplemented with antimicrobial susceptibility testing were results were lacking.

All of the isolates are I and/or R to the β-lactam antimicrobial agents with the exception of the carbapenems. Among the penicillins all isolates showed high-level resistance towards ampicillin, as well as resistance to amoxicillin-clavulanate. Eight isolates were susceptible to piperacillin-tazobactam while remaining isolates were intermediate susceptible. For the cephalosporins, high-level resistance was observed against cefoxitin, an indicator for AmpC-hyperexpression. All the Haukeland isolates were resistant to cefotaxime and ceftazidime with the exception of K48-26 that was intermediate susceptible to cefotaxime and K57-52 that were intermediate susceptible to both cefotaxime and ceftazidime. In contrast, only the isolate from Haugesund was resistant to ceftazidime out of the three non-Haukeland isolates. For the monobactam aztreonam the MIC value range was from 4-32 mg/L with four intermediate susceptible isolates, K29-48, K52-15, K71-61, and K63-77, and the rest being resistant. MIC values for the carbapenem meropenem were low and all isolates were susceptible. With respect to non-β-lactams all isolates were resistant to ciprofloxacin, tobramycin, and gentamicin, but susceptible to amikacin. For Trimethoprim-Sulfamethoxazole the MIC range was from 0,0625 to >32 mg/L, with 9/12 isolates with a MIC >32 mg/L. With respect to the antimicrobial agents specifically used for urinary tract infections all isolates were susceptible to mecillinam and two isolates, K71-61 and K68-26, were resistant to nitrofurantoin and fosfomycin, respectively.

Table 11. Antimicrobial susceptibility profile of collection 3 isolates.

AM: Ampicillin

XL: Amoxicillin-clauvulanate

PTc: Piperacillin-Tazobactam

FX: Cefoxitin

CTL: Cefotaxime

TZ: Ceftazidime

AT: Aztreonam

MP: Meropenem

CI: Ciprofloxacin

MM: Mecillinam

NI: Nitrofurantoin

FM: Fosfomycin

TM Tobramycin

GM: Gentamicin

AK: Amikacin

TS: Trimethoprim- Sulfamethoxazole

K-res No.	Received	Location	Specimen	*Species*	AM	XL	PTc	FX	CTL	TZ	AT	MP	CI	M M	NI	FM	TM	GM	AK	TS
K29-48	291205	Haukeland	Urin	*E.coli*	> 256	64	8	128	4	8	4	0,0625	>32	1	8	0,5	8	32	2	>32
K30-44	150306	Haukeland	Urin	*E.coli*	> 256	32	8	256	4	8	8	0,0312	>32	1	8	0,125	8	32	2	>32
K47-31	301107	Haukeland	Urin	*E. coli*	> 256	32	8	> 256	4	8	8	0,0625	>32	1	4	0,5	8	32	2	0,0625
K48-26	130208	Haukeland	Urin	*E. coli*	> 256	16	8	256	2	8	32	0,0625	>32	2	4	0,5	8	32	2	>32
K53-47	101208	Haukeland	Blood culture	*E. coli*	> 256	32	16	> 256	4	8	16	0,125	>32	2	8	0,5	8	32	2	>32
K52-15	120808	Vestfold	Urin	*E.coli*	>256	16	4	256	2	2	4	0,0312	>32	1	8	0,25	8	32	2	>32
K57-52	150409	Haukeland	Urin	*E. coli*	> 256	32	4	64	2	4	8	0,0312	>32	1	4	0,5	8	32	2	>32
K58-13	290609	Haukeland	Urin	*E. coli*	> 256	32	16	> 256	8	8	16	0,0625	>32	1	8	0,5	8	32	2	>32
K61-43	061009	Haugesund	Urin	*E.coli*	>256	16	4	128	2	8	8	0,0156	>32	1	4	0,25	8	32	2	>32
K71-61	101209	Vestfold	Urin	*E.coli*	>256	64	2	128	1	2	4	0,125	>32	8	>512	0,5	8	32	2	0,125
K63-77	290310	Haukeland	Urin	*E.coli*	>256	64	16	256	8	16	4	0,125	>32	8	32	0,5	8	64	2	1
K68-2[illegible]	131010	Haukeland	Urin	*E.coli*	>256	32	16	256	4	16	8	0,0312	>32	1	4	64	8	64	2	>32

4.2.5 *bla*$_{TEM}$

All isolates of collection 3 were screened for *bla*$_{TEM}$ using the primers *bla*$_{TEM}$ –F$_{TW}$ and *bla*$_{TEM}$ –R mod $_{TW}$ listed in table 4, Appendix B. All isolates were positive for TEM and were therefore sequenced using the same primers. BLAST searches showed that all isolates harbored *bla*$_{TEM-1}$.

4.3 Multi-resistance analysis

As the isolates showed co-resistance to ciprofloxacin and the aminoglycosides tobramycin and gentamicin they were investigated for the presence of *aac(6')-Ib-cr* and mutations in the QRDR.

4.3.1 AAC(6`)-Ib-cr analysis

The isolates were screened for the gene encoding the aminoglycoside modifying enzyme AAC(6`)-Ib-cr using the primers aac(6`)-Ib-F and aac(6`)-Ib-R (table 4, Appendix B). All isolates screened negative for the gene.

4.3.2 Quinolone resistance determining region (QRDR) analysis

The Quinolone resistance determining regions *parC* and *gyrA* were sequenced using the primers parCF, parCR, gyrA6, and gyrA631R (table 4, Appendix B).

The DNA sequences of the QRDR were translated into protein sequence and aligned with reference sequences for ParC (NP_417491) and (GyrA NP_416734) using the ClustalW program to investigate for mutations known to confer ciprofloxacin resistance. The ClustalW alignments are found in Appendix C, figure 2 for *gyrA* and figure 3 for *parC*. All isolates harbored one or two mutations for both of the genes (table 12). The isolates therefore have from three to four mutations all together, where the amino acid changes are all previously described in the literature [27]. All of the Haukeland isolates have the same mutations for both of the genes. In the isolate from Haugesund only one mutations is found in *parC,* but has apart from this identical mutations compared to the Haukeland isolates. The isolates from

Vestfold both harbored two mutations in both of the genes. Only one mutation, Y87D in *gyrA* differ these isolates from Haukeland.

Table 12. Mutations in the Quinolone resistance determining region (QRDR) for *parC* and *gyrA* determined after a ClustalW alignment against reference sequences (ParC NP_417491 and GyrA NP_416734).

		QRDR mutations	
Hospital	**Isolate**	***parC***	***gyrA***
Haukeland	K29-48	I80S, V84E	L83S, N87D
	K30-44	I80S, V84E	L83S, N87D
	K47-31	I80S, V84E	L83S, N87D
	K48-26	I80S, V84E	L83S, N87D
	K53-47	I80S, V84E	L83S, N87D
	K57-52	I80S, V84E	L83S, N87D
	K58-13	I80S, V84E	L83S, N87D
	K63-77	I80S, V84E	L83S, N87D
	K68-26	I80S, V84E	L83S, N87D
Haugesund	K61-43	I80S	L83S, N87D
Vestfold	K52-15	R80S, V84E	L83S, Y87D
Vestfold	K71-61	R80S, V84E	L83S, Y87D

* Arg/R: Arginine, Asn/N: Asparagine, Asp/D: Aspartic acid, Glu/E: Glutamic acid, Ile/I: Isoleucine, Leu/L: Leucine, Ser/S: Serine, Val/V: Valine, Tyr/Y: Tyrosine.

5. Discussion

E. coli naturally produce small amounts of the chromosomally encoded β-lactamase AmpC. The expression of bla_{AmpC} is noninducible and regulated by a weak promoter and an attenuator. Insertion sequence (IS) elements inserted into the promoter region have been described as one reason for the hyperexpression of bla_{AmpC} conferring resistance to penicillins and cephalosporins, except 4th generation cephalosporins.

5.1 General discussion

Overall, there is a higher number of *E. coli* isolates with a profile of hyperproduction of the chromosomal AmpC received at K-res from Haukeland University Hospital (n=111) from 2006-2010 than any other Norwegian clinical microbiology laboratory. This could indicate higher incidents of chromosomal AmpC from the Bergen area or differences in the number of isolates referred to K-res. This could reflect differences in their routines of referring isolates to K-res or internal methods for detection of isolates with an AmpC profile. When looking into the yearly distribution of the chromosomal AmpC *E. coli* isolates received from Haukeland the numbers each year show great variability. In 2007 43 isolates were registered while only 6 in 2010. This could be a result of changing procedures for the reporting of questionable isolates to K-res and does not necessarily mean a reduction in cases. The criteria for referring isolates to K-res also changed during the period to exclude isolates without co-resistance to other antimicrobial agents such as aminoglycosides, ciprofloxacin, trimethoprim-sulphametoxazole, and nitrofurantoin (www.unn.no/kres).

Nursing Home 1 was indicated as the main location of origin of the Haukeland AmpC with an insertion region positive isolates (figure 6). As the numbers of duplicate isolates are not taken into consideration for the study, the distribution of originating locations might alter. The routines of the differing locations regarding the reporting of questionable isolates to Haukeland University Hospital are also unknown and important factors. How many samples each nursing home or general practice sent in during the time period 2006-2010 is not known. The occurrence of the clone might be more evenly distributed among the location than this study suggests.

As previously discovered by Igeltjørn *E. coli* isolates from Haukeland University hospital harbored an IS*911* element inserted into the AmpC region [33]. After a screening of the AmpC region of the 111 Haukeland isolates a total of 46 isolates proved to have an insertion in this region. Compared to the control group of non-Haukeland isolates only three had an insertion region in the AmpC gene. The numbers highly indicate a region-specific bacterial feature. A sample of the positive Haukeland isolates was screened specifically for IS*911* insertion in the AmpC region, which they all were positive for. This indicates that this element is present in all the Haukeland isolates with an insert. The insertion of an element in the promoter region of the AmpC gene has been observed in several cases leading to the hyperexpression of the β-lactamase. Insertion elements like IS*10,* IS*Ec10,* IS*2,* and IS*911* have all been observed executing the same function [14, 21, 46].

In this study it was discovered that IS*911* was inserted between the -35 box and the -10 box in the promoter region, hence creating an alternative and stronger promoter compared to the wild type. The IS*911* in the Haukeland isolates were inserted between position -24 and -25 also described in cefoxitin resistant isolates from *E. coli* in a Canadian study [14]. The hyperproduction of bla_{AmpC} is reflected on the MIC values for the isolates particularly for cefoxitin which is an indicator for hyperproduction.

The three non-Haukeland isolates (Vestfold n=2, Haugesund n=1) were also positive for an insertion region in the AmpC promoter region. Optimal primers for this region were not available as the insertion region proved to be different compared to the Haukeland isolates. BLAST searches revealed another IS-element, IS*10.* This element is also described in *E. coli* from a Canadian study [14]. The finding of IS*10* in the non-Haukeland isolates also support the hypothesis that there is a regional spread of IS*911*-bla_{AmpC} isolates in the Bergen region. IS*10* belongs to a different IS family than *IS911,* family 4, and forms a part of the composite tetracycline resistance transposon Tn*10* [47].

During the study 10 cases were discovered where one patient had several bacterial samples registered. This was not investigated closer into so the possibility of more duplicate or triplicate isolates is likely, and its occurrence was not taken into consideration for the study. A

precaution is therefore needed when interpreting the results. A total accuracy in the numbers of isolates can only be achieved after a thorough look into the patient registers at Haukeland University Hospital. Patient transfer between institutions also complicates the analysis as for some patients samples have been submitted from different institutions (table 9). However, the findings of the same clone in the same patient indicate long term colonization that could be important with respect to reinfection and further spread.

MLST analysis confirmed that the isolates derived from Haukeland University Hospital belonged to ST131. One of the isolates, K63-77, did not give a PCR product for one of the genes, *fumC*, and the sequence type could not be determined. Nevertheless, this isolate resembles the other isolates from Haukeland for the six other genes used for the MLST. Depending on the degree of mutations in the *fumC* gene, one can assume that K63-77 is of the same ST as the rest of the Haukeland isolates or a single locus variant.

ST131 is a globally disseminated multi-drug resistant *E. coli* clone associated with urinary tract and blood stream infections [30]. The specimen distribution for this study reflects this with the highest prevalence of urine samples (n=185) followed by blood culture (n=11).

The ST131 clone has been described in a wide range of locations including hospitals, long-term facilities and in the community [30]. The locations of the isolates for this study were found to include both several departments within two hospitals, Hospital 1 and Hospital 2, several general practices and nursing homes. The clone is not limited to one location making its spread more worrisome.

ST131 is associated worldwide with the spread of ESBLs of the CTX-M family, especially CTX-M-15 [32, 48]. It has been found to be associated with ciprofloxacin resistance in eight European countries [49] and CMY-2 production in Norwegian isolates [44]. The clone has not only been identified in patients but also from stool samples from healthy individuals in France [50]. ST131 is as well identified in companion animals and poultry, including the possibility of transmission through animal contact and food consumption [51].

The regional spread of the ST131 clone in the Bergen region is to date mainly associated with the hyperexpression of chomosomal *bla*$_{AmpC}$. As the worldwide disseminated ST131 clone is often found to hold ESBLs, particularly CTX-M-15, there is a high likelihood for the transfer of resistance mechanisms by horizontal gene transfer, increasing the resistance profile of the Bergen clone.

XbaI-PFGE-types of ST131 isolates have previously been shown to be diverse, as also discovered in this study [32]. All isolates from Haukeland University Hospital clustered together with an overall similarity of 61%. This indicates a genetic diversity within the sequence type and cause difficulties identifying the clones. This problem was also recognized by Olivier Clermont et al. describing the difficulty of recognizing members of the O25b-ST131 clone as PFGE patterns are varying within a similarity of ≥60% [41].

The three non-Haukeland isolates were unrelated to ST131 and belonged to ST405 (Vestfold) and ST23 (Haugesund). ST405 is part of a larger sequence type complex (STC405), and also a sequence type associated with the global dissemination of CTX-M-15 [52]. It has been identified in a Norwegian study harboring other CTX-M variant such as CTX-M-3 and CTX-M-14 [32]. Naseer *et al.* characterized a novel single locus variant of ST405, namely ST964, a sequence type also associated with CTX-M-15 [32].

ST23 belongs to a larger complex, STC23, also including ST90, ST88, and ST650. In a Spanish study several of the sequence types of STC23 were associated with the dispersal of CTX-M variants [53].

Two cases of patients with more than one bacterial isolate were included in the PFGE study. For both patients changes in the PFGE profiles were observed. Patient A had three isolates K29-77, K29-80, and K30-44, where all were sampled with about a month apart. The isolates K29-77 and K29-80 cluster together in cluster 3, while K30-44 belongs to cluster 5. K30-44 was isolated last and is lacking one gel band compared to the other two isolates. Patient B had two isolates, K30-36 and K36-03, isolated eight months apart. Both isolates cluster together in

cluster 3, but a one band change is seen. Isolate K36-03 has one extra gel band and was isolated last of the two. The small changes observed of either loosing or acquiring a gel band indicate that the bacteria have evolved over time.

The bla_{TEM-1} was identified in all isolates of collection 3. This is a plasmid encoded enzyme commonly found in *Enterobacteriaceae*. The enzyme confers resistance to penicillins like ampicillin, ticarcillin, and piperacillin, and narrow-spectrum cephalosporins, like cephalothin or cefazolin. For this study the TEM-1 enzyme was not a great contributor to the antimicrobial resistance profile as the isolates would be resistant to the same and more antimicrobial agents by the hyperexpression of AmpC. ESBLs derive from amino acid substitutions of, among others, TEM-1 resulting in resistance to oxyimino-beta-lactam substrates, like ceftazidime, ceftriaxone, and cefepime. If the isolates of the study had harbored other TEM-types, ESBLs, resistance against also 4th generation cephalosporins may have been observed [54].

5.2 Multi-resistance

Quinolones have a wide spectrum of activity and have been extensively used and misused in both human and veterinary medicine, leading to the emergence and spread of resistant strains. The fluoroquinolone ciprofloxacin has been pointed out as the most consumed antimicrobial agent worldwide [55], and the ST131 clone is associated with the spread of fluoroquinolone resistance [32, 56]. Alterations in the quinolone targets, DNA gyrase and topoisomerase IV, is one of the mechanisms of resistance described in the literature. Single mutations in *gyrA* have been described to result in high-level resistance to nalidixic acid. In order to obtain high-level resistance to fluoroquinolones additional mutations are needed in the same gene and/or in another target such as *parC* [28].

Mutations in both *parC* and *gyrA* were observed for all strains of collection 3. The Haukeland isolates had the following mutations for *parC*; I80S and V84E, and for *gyrA*; L83S and N87D. The isolate from Haugesund had one mutation in *parC*, I80S, and two in *gyrA,* L83S and N87D. The two isolates from Vestfold had two mutations for both of the genes, *parC*; R80S and V84E, and for *gyrA*; L83S and Y87D. All the above mentioned mutations are

described by Ruiz as quinolone resistance conferring changes. As described, the most frequent mutations seen for *E. coil* in *gyrA* are, as discovered by this study, at the codons 83 and 87. For *parC* the most commonly observed mutations are at codons 80 and 84 [28]. This is in accordance to the mutations observed in this study, explaining the high-level resistance to ciprofloxacin.

Resistance to the fluoroquinolone ciprofloxacin may also be conferred by the aminoglycoside modifying enzyme AAC(6`)-Ib-cr. Screening of collection 3 proved that none of the isolates harbor this enzyme. The high-level resistance observed to ciprofloxacin may therefore be caused solely by the multiple mutations in the target regions, DNA gyrase and topoisomerase IV, or the presence of *qnr*-genes. As the isolates also were resistant to the aminoglycosides tobramycin and gentamicin, this resistance may be caused by other aminoglycoside modifying enzymes, efflux, or by alterations of the target or the cell wall permeability [2].

6. Concluding remarks

The results indicate that there is a successful spread of an *E. coli* ST131 clone with an IS*911*-associated hyperexpressing *bla*$_{AmpC}$ in the Bergen region. As this specific clone or the *bla*$_{AmpC}$-IS*911* linkage was not identified in other Norwegian isolates further supports the regional location of this spread and that this is an unusual profile among Norwegian isolates. The only isolates with an insertion in the chromosomal *bla*$_{AmpC}$ region harboring IS*10* instead of IS*911* were identified in two different Norwegian counties, Vestfold and Rogaland.

The clonal spread of this clone in the Bergen region should be monitored and investigated further. As a start an investigation into the patient registers at Haukeland University Hospital should be executed in order to get an overview of the number of isolates from the same patient. As Nursing Home 1 was pointed out as the main originating location of this clone it would be interesting visiting the location for a closer look into its hygiene routines and its connections to general practices, hospitals and other nursing homes. The route of transmission could then more easily be determined in order to control the spread.

The finding that the *bla*$_{AmpC}$-IS*911* linkage isolates belonged to ST131 should raise concerns considering its widespread occurrence and multi-resistance highly virulent uropathogenic profile. The co-resistance profile observed in the Haukeland isolates with resistance to ciprofloxacin, gentamicin, tobramycin, and trimethoprim-sulfamethoxazole is worrying in this aspect. Further studies are needed in order to determine the aminoglycoside resistance mechanism(s) of these isolates.

7. References

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Appendix A

Table 1. Bacterial isolates in strain collection 1 (Haukeland University Hospital n=111) and 2 (other hospitals n=100).

	2006					
Number	**K-res number**	**Received**	**Hospital**	**Material**	***Specie***	**Comments**
1	K29-48	291205	Haukeland	Urine	*E.coli*	
2	K29-53	301205	Haukeland	Urine	*E.coli*	
3	K29-74	270106	Haukeland	Urine	*E.coli*	
4	K29-77	310106	Haukeland	Blood culture	*E.coli*	Same patient as K29-80 and K30-44
5	K29-80	030206	Haukeland	Urine	*E.coli*	Same patient as K29-77 and K30-44
6	K29-81	030206	Haukeland	Urine	*E.coli*	
7	K30-1	030206	Haukeland	Urine	*E.coli*	
8	K30-20	200206	Haukeland	Urine	*E.coli*	
9	K30-23	270206	Haukeland	Urine	*E.coli*	
10	K30-24	270206	Haukeland	Urine	*E.coli*	
11	K30-25	270206	Haukeland	Urine	*E.coli*	
12	K30-35	030306	Haukeland	Urine	*E.coli*	
13	K30-36	030306	Haukeland	Urine	*E.coli*	Same patient as K36-3
14	K30-44	150306	Haukeland	Urine	*E.coli*	Same patient as K29-77 and K29-80
15	K30-58	040406	Haukeland	Urine	*E.coli*	
16	K30-60	070406	Haukeland	Urine	*E.coli*	
17	K33-03	090506	Haukeland	Urine	*E.coli*	
18	K33-14	230506	Haukeland	Urine	*E.coli*	
19	K33-19	290506	Haukeland	Urine	*E.coli*	
20	K34-8	230806	Haukeland	Urine	*E.coli*	
21	K34-25	060906	Haukeland	Urine	*E.coli*	
22	K34-34	140906	Haukeland	Urine	*E.coli*	
23	K34-70	201006	Haukeland	Urine	*E.coli*	
24	K36-3	061106	Haukeland	Urine	*E.coli*	Same patient as K30-36
25	K36-17	201106	Haukeland	Urine	*E.coli*	
26	K29-27	151205	Stavanger Universitetssykehus	Urine	*E.coli*	
27	K30-18	200206	Sykehuset i Vestfold	Urine	*E.coli*	
28	K30-29	200206	Sykehuset Østfold	Urine	*E.coli*	
29	K30-57	300306	Sykehuset Østfold	Urine	*E.coli*	
30	K30-81	020506	Rikshospitalet	Urine	*E.coli*	
31	K33-01	080506	Sykehuset Østfold	Urine	*E.coli*	
32	K33-09	180506	Rikshospitalet	Urine	*E.coli*	
33	K33-16	230506	Sykehuset i Vestfold	Urine	*E.coli*	
34	K33-71	250706	UNN	Urine	*E.coli*	
35	K34-10	230806	Sykehuset i Vestfold	Puss	*E.coli*	
36	K34-15	290806	UNN	Pressure wound	*E.coli*	
37	K34-23	061106	Sykehuset Innlandet	Urine	*E.coli*	

38	K34-32	121106	Sykehuset Østfold	Urine	*E.coli*	
39	K34-36	180906	Sørlandet Sykehus	Urine	*E.coli*	
40	K34-60	091006	Stavanger Universitetssykehus	Urine	*E.coli*	
41	K36-01	061106	Sykehuset i Vestfold	Urine	*E.coli*	
42	K36-05	101106	UNN	Urine	*E.coli*	
43	K36-09	161106	Sykehuset Østfold	Urine	*E.coli*	
44	K36-10	161106	Sykehuset Innlandet	Urine	*E.coli*	
45	K36-13	161106	Ullevål	BAL	*E.coli*	

2007

Number	K-res number	Received	Hospital	Material	*Specie*	Comments
46	K36-40	221206	Haukeland	Urine	*E. coli*	
47	K41-11	070207	Haukeland	Urine	*E. coli*	
48	K41-22	020307	Haukeland	Blood	*E. coli*	
49	K41-33	160307	Haukeland	Urine	*E. coli*	
50	K41-34	190307	Haukeland	Blood	*E. coli*	
51	K41-39	220307	Haukeland	Urine	*E. coli*	
52	K41-46	280307	Haukeland	Urine	*E. coli*	
53	K41-52	300307	Haukeland	Urine	*E. coli*	
54	K41-61	120407	Haukeland	Urine	*E. coli*	
55	K41-81	260407	Haukeland	Urine	*E. coli*	
56	K44-26	210507	Haukeland	Urine	*E. coli*	
57	K44-50	060607	Haukeland	Urine	*E. coli*	
58	K44-51	060607	Haukeland	Urine	*E. coli*	Same patient as K45-29
59	K44-52	060607	Haukeland	Urine	*E. coli*	
60	K44-53	060607	Haukeland	Urine	*E. coli*	
61	k44-54	060607	Haukeland	Urine	*E. coli*	Same patient as K44-60
62	K44-60	120607	Haukeland	Urine	*E. coli*	Same patient as K44-54
63	K44-63	140607	Haukeland	Urine	*E. coli*	
64	K44-64	140607	Haukeland	Urine	*E. coli*	
65	K44-70	220607	Haukeland	Urine	*E. coli*	
66	K44-77	270607	Haukeland	Urine	*E. coli*	
67	K44-78	270607	Haukeland	Urine	*E. coli*	
68	K45-23	020707	Haukeland	Urine	*E. coli*	
69	K45-24	020707	Haukeland	Urine	*E. coli*	
70	K45-29	100707	Haukeland	Urine	*E. coli*	Same patient as K44-51
71	K45-40	160707	Haukeland	Urine	*E. coli*	
72	K45-41	160707	Haukeland	Urine	*E. coli*	Same patient as K45-48
73	K45-48	180707	Haukeland	Urine	*E. coli*	Same patient as K45-41
74	K45-59	020807	Haukeland	Urine	*E. coli*	
75	K45-60	020807	Haukeland	Urine	*E.coli*	
76	K45-69	200807	Haukeland	Urine	*E. coli*	
77	K46-10	100907	Haukeland	Urine	*E. coli*	
78	K46-21	140907	Haukeland	Urine	*E. coli*	
79	K46-32	200907	Haukeland	Urine	*E. coli*	
80	K46-48	041007	Haukeland	Operation wound	*E. coli*	Same patient as K46-58
81	K46-49	051007	Haukeland	Urine	*E. coli*	

82	K46-58	151007	Haukeland	Drain	*E. coli*	Same patient as K46-48
83	K46-64	191007	Haukeland	Urine	*E. coli*	
84	K47-20	161107	Haukeland	Urine	*E.coli*	
85	K47-21	161107	Haukeland	Urine	*E. coli*	
86	K47-24	231107	Haukeland	Urine	*E. coli*	
87	K47-31	301107	Haukeland	Urine	*E. coli*	
88	K47-33	301107	Haukeland	Urine	*E. coli*	
89	K36-55	050107	Sykehuset i Vestfold	Abdominal abscess	*E.coli*	
90	K36-72	170107	Sykehuset Østfold	Urine	*E.coli*	
91	K36-76	190107	Sørlandet Sykehus	Urine	*E.coli*	
92	K41-06	310107	Rikshospitalet	Urine	*E.coli*	
93	K41-19	230207	UNN	Urine	*E.coli*	
94	K41-32	160307	Haugesund	Urine	*E.coli*	
95	K41-55	030407	Sykehuset i Vestfold	Urine	*E.coli*	
96	K44-05	020507	Ullevål	Urine	*E.coli*	
97	K44-22	110507	UNN	Urine	*E.coli*	
98	K44-32	230507	Sykehuset Østfold	Urine	*E.coli*	
99	K45-58	010807	Sørlandet Sykehus	Urine	*E.coli*	
100	K45-66	130807	Sykehuset Østfold	Urine	*E.coli*	
101	K46-19	130907	St.Olavs Hospital	Blood culture	*E.coli*	
102	K46-20	130907	Sykehuset i Vestfold	Urine	*E.coli*	
103	K46-28	190907	Sørlandet Sykehus	Urine	*E.coli*	
104	K46-34	250907	Haugesund	Urine	*E.coli*	
105	K46-35	250907	Haugesund	Urine	*E.coli*	
106	K46-46	041007	Helse Nord-Trøndelag	Blood culture	*E.coli*	
107	K46-66	241007	St.Olavs Hospital	Blood culture	*E.coli*	
108	K46-81	291007	UNN	Urine	*E.coli*	

2008

Number	K-res number	Received	Hospital	Material	*Specie*	Comments
109	K47-78	241207	Haukeland	Urine	*E. coli*	
110	K47-79	241207	Haukeland	Urine	*E. coli*	
111	K48-3	020108	Haukeland	Urine	*E. coli*	
112	K48-9	080108	Haukeland	Urine	*E. coli*	
113	K48-12	110108	Haukeland	Urine	*E. coli*	
114	K48-17	300108	Haukeland	Urine	*E. coli*	
115	K48-26	130208	Haukeland	Urine	*E. coli*	
116	K48-48	060308	Haukeland	Urine	*E. coli*	Same patient as K54-52
117	K49-14	070508	Haukeland	Urine	*E. coli*	Same patient as K53-65
118	K49-31	280508	Haukeland	Urine	*E. coli*	
119	K49-40	050608	Haukeland	Urine	*E. coli*	
120	K49-63	020708	Haukeland	Urine	*E. coli*	
121	K49-66	070708	Haukeland	Urine	*E. coli*	
122	K49-70	150708	Haukeland	Urine	*E. coli*	
123	K52-12	110808	Haukeland	Urine	*E. coli*	
124	K52-40	40908	Haukeland	Urine	*E. coli*	
125	K52-46	150908	Haukeland	Urine	*E. coli*	

126	K52-78	101108	Haukeland	Urine	*E. coli*	Same patient as K53-43
127	K53-43	111108	Haukeland	Urine	*E. coli*	Same patient as K52-78
128	K53-47	101208	Haukeland	Blood culture	*E. coli*	
129	K53-58	11208	Haukeland	Urine	*E. coli*	
130	K53-65	120808	Haukeland	Urine	*E. coli*	Same patient as K49-14
131	K48-1	030108	Sykehuset i Vestfold	Abdominal abscess	*E.coli*	
132	K48-2	020108	Sykehuset Østfold	Urine	*E.coli*	
133	K48-38	200208	Sykehuset Østfold	Urine	*E.coli*	
134	K48-57	270308	Sørlandet Sykehus	Urine	*E.coli*	
135	K49-5	050508	Sørlandet Sykehus	Urine	*E.coli*	
136	K49-25	270508	Sykehuset i Vestfold	Urine	*E.coli*	
137	K49-29	230508	Sykehuset Østfold	Urine	*E.coli*	
138	K49-34	300508	Molde Sjukehus	Urine	*E.coli*	
139	K49-75	160708	Sørlandet Sykehus	-	*E.coli*	
140	K49-78	230708	Helse Nord-Trøndelag	Secretion from gallbladder	*E.coli*	
141	K52-3	290708	Stavanger Universistetssykehus	Urine	*E.coli*	
142	K52-4	240708	Sykehuset i Vestfold	Urine	*E.coli*	
143	K52-15	120808	Sykehuset i Vestfold	Urine	*E.coli*	
144	K52-24	190808	Sykehuset Innlandet	Abdominal pus	*E.coli*	
145	K52-28	210808	Sørlandet Sykehus	Urine	*E.coli*	
146	K52-44	110908	Stavanger Universistetssykehus	Pelvic abscess	*E.coli*	
147	K52-48	160908	Haugesund	Urine	*E.coli*	
148	K52-62	021008	Sykehuset Østfold	Urine	*E.coli*	
149	K53-45	131108	Sørlandet Sykehus	Urine	*E.coli*	
150	K53-68	121208	Helse Nord-Trøndelag	Urine	*E.coli*	

2009

Number	K-res number	Received	Hospital	Material	*Specie*	Comments
151	K54-02	210109	Haukeland	Urine	*E. coli*	
152	K54-42	060209	Haukeland	Urine	*E. coli*	Same patient as K48-48
153	K54-60	090209	Haukeland	Urine	*E. coli*	
154	K55-66	040309	Haukeland	Urine	*E. coli*	
155	K57-52	150409	Haukeland	Urine	*E. coli*	
156	K57-76	120509	Haukeland	Urine	*E. coli*	
157	K58-06	120609	Haukeland	Urine	*E. coli*	Same patient as K49-70
158	K58-11	220609	Haukeland	Urine	*E. coli*	
159	K58-12	260609	Haukeland	Urine	*E. coli*	
160	K58-13	290609	Haukeland	Urine	*E. coli*	
161	K61-02	040809	Haukeland	Urine	*E. coli*	
162	K61-12	010909	Haukeland	Urine	*E. coli*	
163	K61-13	020909	Haukeland	Urine	*E. coli*	
164	K61-17	150909	Haukeland	Urine	*E. coli*	
165	K61-40	051009	Haukeland	Urine	*E. coli*	
166	K58-05	040609	Sørlandet Sykehus	Urine	*E.coli*	
167	K58-08	180609	Ullevål	Urine	*E.coli*	
168	K58-24	230709	Stavanger Universitetssykehus	Urine	*E.coli*	
169	K58-32	280709	Sykehuset Østfold	Urine	*E.coli*	

170	K53-75	050109	UNN	Urine	*E.coli*	
171	K53-80	160109	Sørlandet Sykehus	Urine	*E.coli*	
172	K55-04	160209	Sørlandet Sykehus	Urine	*E.coli*	
173	K55-55	260209	Sørlandet Sykehus	Urine	*E.coli*	
174	K57-43	060409	Sørlandet Sykehus	Urine	*E.coli*	
175	K57-44	070409	Sørlandet Sykehus	Urine	*E.coli*	
176	K57-53	150409	Sørlandet Sykehus	Urine	*E.coli*	
177	K58-23	200709	Sørlandet Sykehus	Urine	*E.coli*	
178	K61-43	061009	Haugesund Sjukehus	Urine	*E.coli*	
179	K61-76	061109	Ullevål	Urine	*E.coli*	
180	K61-78	171109	Molde Sjukehus	Urine	*E.coli*	
181	K61-81	241109	Stavanger Universitetssykehus	Urine	*E.coli*	
182	K71-39	301109	Ullevål	Tracheal secretion	*E.coli*	
183	K71-40	011209	Ullevål	Urine	*E.coli*	
184	K71-58	071209	Ullevål	Urine	*E.coli*	
185	K71-61	101209	Sykehuset Vestfold	Urine	*E.coli*	

2010

Number	K-res number	Received	Hospital	Material	*Specie*	Comments
186	K63-77	290310	Haukeland	Urine	*E.coli*	
187	K66-56	260410	Haukeland	Urine	*E.coli*	
188	K67-10	280610	Haukeland	Urine	*E.coli*	
189	K68-16	151210	Haukeland	Urine	*E.coli*	
190	K68-26	131010	Haukeland	Urine	*E.coli*	
191	K68-76	081210	Haukeland	Blood culture	*E.coli*	
192	K63-47	120210	Sykehuset Østfold HF	Urine	*E.coli*	
193	K63-70	120310	Sykehuset Østfold HF	Blood culture	*E.coli*	
194	K63-73	160310	Ullevål	Urine	*E.coli*	
195	K63-75	180310	Ullevål	Urine	*E.coli*	
196	K66-47	140410	St.Olavs Hospital	Urine	*E.coli*	
197	K66-50	190410	Rikshospitalet	Urine	*E.coli*	
198	K67-03	070610	Sykehuset i Vestfold	Urine	*E.coli*	
199	K67-18	020710	Sykehuset Vestfold	Urine	*E.coli*	
200	K67-29	120710	Sørlandet Sykehus	Blood culture	*E.coli*	
201	K67-45	020810	Sykehuset i Vestfold	Urine	*E.coli*	
202	K67-51	090810	Sørlandet Sykehus	Urine	*E.coli*	
203	K67-58	160810	Sykehuset Østfold HF	Abdominal pus	*E.coli*	
204	K68-07	210910	Sykehuset Innlandet HF	Urine	*E.coli*	
205	K68-14	011010	Stavanger Universitetssykehus	Urine	*E.coli*	
206	K68-15	011010	Sørlandet Sykehus	Urine	*E.coli*	
207	K68-29	191010	Molde Sjukehus	Wound secretion	*E.coli*	
208	K68-54	061110	Helse Nord-Trøndelag	Vaginal abscess	*E.coli*	
209	K68-57	171110	Stavanger Universitetssykehus	Urine	*E.coli*	
210	K68-68	241110	Nordlandssykehuset	Blood culture	*E.coli*	
211	K68-78	101210	Sykehuset Innlandet HF	Expectorate	*E.coli*	

Table 2. Collection 3 (*n*=12).

Collection 3

Number	K-res Number	Received	Hospital	Material	Specie
1	K29-48	291205	Haukeland	Urine	*E.coli*
2	K30-44	150306	Haukeland	Urine	*E.coli*
3	K47-31	301107	Haukeland	Urine	*E. coli*
4	K48-26	130208	Haukeland	Urine	*E. coli*
5	K53-47	101208	Haukeland	Blood culture	*E. coli*
6	K52-15	120808	Vestfold	Urine	*E.coli*
7	K57-52	150409	Haukeland	Urine	*E. coli*
8	K58-13	290609	Haukeland	Urine	*E. coli*
9	K61-43	61009	Haugesund	Urine	*E.coli*
10	K71-61	101209	Vestfold	Urine	*E.coli*
11	K63-77	290310	Haukeland	Urine	*E.coli*
12	K68-26	131010	Haukeland	Urine	*E.coli*

Table 3. 32 AmpC with insert -positive isolates (IS*911* and IS*10)* included in the PFGE; Haukeland (*n*=29), Vestfold (*n*=2), and Haugesund (*n*=1).

PFGE isolates

Number	K-res Number	Received	Hospital	Material	Specie
1	K29-48	291205	Haukeland	Urine	*E.coli*
2	K29-77	310106	Haukeland	Blood culture	*E.coli*
3	K29-80	030206	Haukeland	Urine	*E.coli*
4	K30-24	270206	Haukeland	Urine	*E.coli*
5	K30-36	030306	Haukeland	Urine	*E.coli*
6	K30-44	150306	Haukeland	Urine	*E.coli*
7	K34-8	230806	Haukeland	Urine	*E.coli*
8	K36-3	061106	Haukeland	Urine	*E.coli*
9	K41-39	220307	Haukeland	Urine	*E. coli*
10	K46-64	191007	Haukeland	Urine	*E. coli*
11	K47-31	301107	Haukeland	Urine	*E. coli*
12	K48-9	080108	Haukeland	Urine	*E. coli*
13	K48-17	300108	Haukeland	Urine	*E. coli*
14	K48-26	130208	Haukeland	Urine	*E. coli*
15	K49-14	070508	Haukeland	Urine	*E. coli*
16	K49-40	050608	Haukeland	Urine	*E. coli*
17	K52-12	110808	Haukeland	Urine	*E. coli*
18	K52-15	120808	Vestfold	Urine	*E.coli*
19	K52-78	101108	Haukeland	Urine	*E. coli*
20	K53-47	101208	Haukeland	Blood culture	*E. coli*
21	K54-02	210109	Haukeland	Urine	*E. coli*
22	K57-52	150409	Haukeland	Urine	*E. coli*

23	K57-76	120509	Haukeland	Urine	*E. coli*
24	K58-13	290609	Haukeland	Urine	*E. coli*
25	K61-12	010909	Haukeland	Urine	*E. coli*
26	K61-40	051009	Haukeland	Urine	*E. coli*
27	K61-43	61009	Haugesund	Urine	*E.coli*
28	K71-61	101209	Vestfold	Urine	*E.coli*
29	K63-77	290310	Haukeland	Urine	*E.coli*
30	K67-10	280610	Haukeland	Urine	*E.coli*
31	K68-16	151210	Haukeland	Urine	*E.coli*
32	K68-26	131010	Haukeland	Urine	*E.coli*

Appendix B

Table 1. General prescription for mastermix for PCR-based methods.

General Mastermix	**Amount**
JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich)	500µl
Primer F (50pmole/µl)	8µl
Primer R (50pmole/µl)	8µl
ddH_2O	384µl
Total	**900µl**

Table 2. General prescription for sequencing mastermix.

Sequencing Mastermix (pr.reaction)	**Amount**
Big Dye v 3.1 (Applied Biosystems)	1µl
5x Sequencing buffer (Applied Biosystems)	3µl
Primer 3.2 pmole/µl (Eurogentec S.A)	0.5µl
ddH_2O	15µl
DNA template	0.5µl
Total	**20µl**

Table 3. PCR program parameters used in this study.

PCR	**Initial denaturation**	**Cycles**	**Denaturation**	**Primer annealing**	**Elongation**	**Final elongation**
16S rDNA	95°C/1min	30	95°C/30sec	55°C/30sec	72°C/1min	72°C/7min (then 4°C→∞)
AmpC with insert	95°C/5min	30	95°C/30sec	55°C/30sec	72°C/2min	72°C/5min (then 4°C→∞)
IS*911*	95°C/5min	30	95°C/30sec	55°C/30sec	72°C/1min	72°C/7min (then 4°C→∞)
bla_{TEM}	94°C/1min	25	95°C/30sec	50°C/30sec	72°C/1min	72°C/7min (then 4°C→∞)
aac(6´)-Ib-cr	95°C/5min	30	95°C/15sec	58°C/15sec	72°C/40sec	72°C/4min (then 4°C→∞)
QRDR (qyrA/parC)	94°C/5min	30	94°C/15sec	55°C/15sec	72°C/40sec	72°C/4min (then 4°C→∞)
ST131 PCR	95°C/5min	30	95°C/30sec	65°C/30sec	72°C/1min	72°C/5min (then 4°C→∞)
ST131 PCR - modified	95°C/5min	30	95°C/30sec	67°C/30sec	72°C/1min	72°C/5min (then 4°C→∞)
Sequencing	96°C/5min	25	96°C/10sec	50°C/10sec	60°C/4min	4°C→∞
MLST *E. coli*	95°C/2min	30	95°C/1min	54°-60° C/1min	72°C/2min	72°C/5min (4°C→∞)

Table 4. Primers used in this study.

Name	DNA sequence 5'-3'	Target site	Amplicon size (bp)	Reference
16SrDNA-F	AGAGTTTGATCMTGGCTCAG	16SrDNA	~1500 bp	Modified from fD1 and fD [38]
16SrDNA-R	ACGGHTACCTTGTTACGACTT			Modified from rP1 and rP2, rP3 [38]
AmpCF	TTCCTGATGATCGTTCTGCC	*frdD*	-	[57]
AmpCintR	GCAAGTCGCTTGAGGATTTC	*bla*$_{AmpC}$		[33]
*bla*TEM- F $_{TW}$	ATGAGTATTCAACATTTCCG	*bla*$_{TEM}$	858 bp	[58]
*bla*TEM- R mod $_{TW}$	CCAATGCTTAATCAGTGAGG			
aac(6´)-Ib-F	TTGCGATGCTCTATGAGTGGCTA	aac(6')-Ib	482 bp	[40]
aac(6´)-Ib-R	CTCGAATGCCTGGCGTGTTT			
gyrA6	CGACCTTGCGAGAGAAAT	QRDR of gyrA	620 bp	[59]
gyrA631R	GTTCCATCAGCCCTTCAA			
parCF	TGAATTTAGGGAAAACGCCTA	QRDR of parC	559 bp	[60]
parCR	GCCACTTCACGCAGGTTATG			
O25pabBspe.F	TCCAGCAGGTGCTGGATCGT	pabB specific for O25b-ST131	347 bp	[41]
O25pabBspe.R	GCGAAATTTTTCGCCGTACTGT			
trpA.F	GCTACGAATCTCTGTTTGCC	trpA	427 bp	[41]
trpA2.R	GCAACGCGGCCTGGCGGAAG			
ST131TF	GGTGCTCCAGCAGGTG	Thymine-144 SNP	40 bp	[42]
ST131TR	TGGGCGAATGTCTGC			
ST131AF	GGCAATCCAATATGACCC	Adenine-450 SNP	49 bp	[42]
ST131AR	ACCTGGCGAAATTTTTCG			
IS911 1F	CGGGAAACTCAGGGCGCGTT	IS*911*	-	This study
IS911 5F	TACCAGATGGGGCGCTGGCT	IS*911*	-	
IS911 2R	CGCCGGCACCCATTCGTTCT	IS*911*	-	
IS911 4R	TAGCGTCGCCACCAAGCACG	*bla*$_{AmpC}$	-	
adkF	ATT CTG CTT GGC GCT CCG GG	adk	583 bp	http://mlst.ucc.ie/mlst/dbs/Ecoli/documents/primersColi_html
adkR	CCG TCA ACT TTC GCG TAT TT			
fumCF	TCA CAG GTC GCC AGC GCT TC	fumC	806 bp	
fumCR	GTA CGC AGC GAA AAA GAT TC			
fumCR1	**TCC CGG CAG ATA AGC TGT GG**			
gyrBF	TCG GCG ACA CGG ATG ACG GC	gyrB	911 bp	
gyrBR	ATC AGG CCT TCA CGC GCA TC			
icdF	ATGGAAAGTAAAGTAGTTGTTC CGGCACA	icd	878 bp	
icdR	GGA CGC AGC AGG ATC TGT T			
mdhF	ATG AAA GTC GCA GTC CTC GGC GCT GCT GGC GG	mdh	932 bp	
mdhR	TTA ACG AAC TCC TGC CCC AGA GCG TAT CTT TCT T			

purAF	CGC GCT GAT GAA AGA GAT GA	purA	816 bp	
purAR	CAT ACG GTA AGC CAC GCA GA			
recAF	CGC ATT CGC TTT ACC CTG ACC	recA	780 bp	
recAR	TCG TCG AAA TCT ACG GAC CGGA			

Table 5. Positive and negative PCR controls used in this study.

PCR	Positive control	Negative control
16S rDNA	*Enterococcus faecalis* A2-32, (ATCC 29212) *E.coli* A-81 (ATCC 25922)	ddH_2O
AmpC IS911	*E.coli K63*-77	*E.coli* A-81 (ATCC 25922)
*bla*TEM	*E.coli* A-44 J62 TEM-3	*E.coli* A-81 (ATCC 25922)
AAC(6´)-Ib-cr	*E.coli* K64-02 K64-11	
ST131 PCR	K5-56	*E.coli* A-81 (ATCC 25922)
ST131 Real-time PCR	K5-56	*E.coli* A-81 (ATCC 25922)

Table6. Bacterial growth media used in this study.

Lactose agar plates	**Brain Heart Infusion (BHI) broth**
Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromtymolblue solution 0.2% (Merck) ddH_2O	BHI broth base (Oxoid) ddH_2O
Lactose agar plates w/ 100mg/ml ampicillin	**Mueller-Hinton agar (MH)**
Tryptose Blood Agar Base (Oxoid) Lactose (Oxoid) Bromtymolblue solution 0.2% (Merck) ddH_2O Ampicillin (Bristol-Myers Squibb)	MH II agar base (Becton, Dickinson and Company, USA) ddH_2O

Table 7. Reagents and solutions

<table>
<tr><td><u>PIV-buffer</u>
1M NaCl (Merck)
10mM Tris-HCl pH 7.6 (Sigma-Aldrich)</td><td><u>TE-buffer</u>
10 mM Tris-HCl pH 8 (Sigma-Aldrich)
0.1 mM EDTA (Sigma-Aldrich)
ddH_2O</td></tr>
<tr><td><u>Lysis buffer (for 5 isolates)</u>
10ml basic buffer
0.02g Sodium deoxycholate (Sigma-Aldrich)
0.5g N-laurolsylsarcosine (Sigma-Aldrich)
0.01g lysosyme (Sigma-Aldrich)
1µl RNase One (10U/µl) (Promega)</td><td><u>ESP-buffer</u>
0.5 M EDTA (pH 9.0-9.5)
1% N-laurolsylsarcosine (Sigma-Aldrich)</td></tr>
<tr><td><u>Restriction enzyme buffer with bovine serum albumin (BSA)</u>
Buffer 4 (10x) (New England BioLabs)
BSA (100x) (New England BioLabs)
ddH_2O</td><td><u>Xba I mixture</u>
Buffer 4 (10x) (New England BioLabs)
BSA (100x) (New England BioLabs)
Xba I (20U/µl) (New England BioLabs)
ddH_2O</td></tr>
<tr><td><u>GelRed staining solution</u>
45µl 10000X GelRed
15 ml 1M $NaCl_2$
135 ml ddH_2O</td><td><u>10X TBE buffer</u>
108 g Tris Base (Sigma-Aldrich)
55 g Boric acid (Sigma-Aldrich)
40 ml 0.5M EDTA pH 8.0 (Sigma-Aldrich)
ddH_2O to 1 litre</td></tr>
<tr><td><u>Basic buffer</u>
0,5 % Brij 58 (Merck)
1 M Tris-HCl pH 8 (Sigma-Aldrich)
0.5 M EDTA pH 8 (Sigma-Aldrich)
NaCl (Merck)
ddH2O</td><td><u>0.85% Saline</u>
8.5 g NaCl (Merck)
ddH_2O to 1 litre</td></tr>
<tr><td><u>Thiurea Stock 5mM</u>
0,389 g
1 litre ddH_20</td><td></td></tr>
</table>

Table 8. Mastermix used for PCR-based detection of ST131

Mastermix	**Amount**
JumpStart REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich)	250µl
O25pabBspe.F (50pmole/µl)	8µl
O25pabBspe.R (50pmole/µl)	8µl
trpA.F (50pmole/µl)	4.8µl
trpA2.R (50pmole/µl)	4.8µl
ddH_2O	174.4µl
Total	**450µl**

Table 9. Mastermixes used for real-time PCR detection of ST131

Mastermix "T" assay	**Amount**
SYBR Green	250µl
ST131 TF (50pmole/µl)	8µl
ST131 TR (50pmole/µl)	8µl
ddH_2O	195µl
Total	**450µl**

Mastermix "A" assay	**Amount**
SYBR Green	250µl
ST131 AF (50pmole/µl)	8µl
ST131 AR (50pmole/µl)	8µl
ddH_2O	195µl
Total	**450µl**

Table 10. PCR programs used for real-time PCR detection of ST131

Cycle	**Program 1**	**Program 2**
Hold 1	95°C/5min	95°C/15min
Cycling (40 repeats)	95°C/5sec	94°C/20sec
	58°C/30sec	65°C/20sec
		72°C/20sec
Hold 2		65°C/30sec
Melt curve analysis	95°C/15sec	95°C/30sec
	60°C/1min	60°C/1min
	95°C/30sec	95°C/30sec

Molecular markers

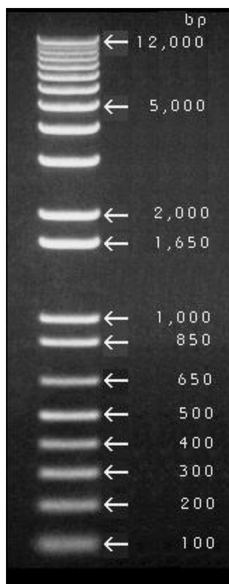

Figure 1. Band sizes of the 1 Kb Plus DNA Ladder, Invitrogen (http://tools.invitrogen.com/content/sfs/manuals/10787018.pdf)

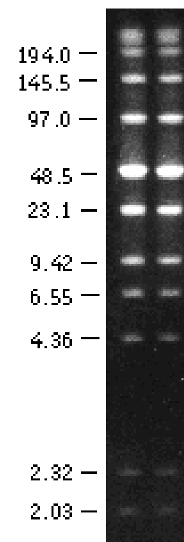

Figure 2. Low range PFG marker, New England BioLabs, (http://www.neb.com/nebecomm/products/productN0350.asp)

Appendix C

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AmpC            ------------------------------------------------------------
K68-26          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K63-77          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K58-13          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K57-52          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K53-47          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K48-26          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K47-31          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K30-44          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
K29-48          TCAGAACAACACAGGTGCCATAATGAAAAAAAGAAATTTCAGCGCAGAGTTTAAACGCGA 60
IS911           ------------------------------AAGAAATTTCAGCGCAGAGTTTAAACGCGA 30

AmpC            ------------------------------------------------------------
K68-26          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K63-77          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K58-13          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K57-52          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K53-47          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K48-26          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K47-31          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K30-44          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
K29-48          ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 120
IS911           ATCCGCTCAACTGGTCGTTGACCAGAATTACACCGTGGCAGATGCAGCCAGCGCTATGGA 90

AmpC            ------------------------------------------------------------
K68-26          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K63-77          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K58-13          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K57-52          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K53-47          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K48-26          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K47-31          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K30-44          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
K29-48          TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 180
IS911           TGTCGGCCTTTCCACAATGACGCGATGGGTGAAACAATTACGTGATGAGCGGCAGGGAAA 150

AmpC            ------------------------------------------------------------
K68-26          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K63-77          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K58-13          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K57-52          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K53-47          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K48-26          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K47-31          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K30-44          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
K29-48          AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 240
IS911           AACACCAAAAGCCTCCCCTATTACCCCGGAACAAATTGAAATCCGTGAGCTCAGGAAAAA 210

AmpC            ------------------------------------------------------------
K68-26          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K63-77          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K58-13          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K57-52          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K53-47          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K48-26          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K47-31          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K30-44          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
K29-48          GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 300
IS911           GCTACAACGTATTGAAATGGAAAATGAAATATTAAAAAAGGCTACCGCGCTCTTGATGTC 270
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AmpC            ------------------------------------------------------------
K68-26          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K63-77          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K58-13          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K57-52          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K53-47          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K48-26          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K47-31          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K30-44          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
K29-48          AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 360
IS911           AGACTCCCTGAACAGTTCTCGATAATCGGGAAACTCAGGGCGCGTTATCCTGTGGCCACT 330

AmpC            ------------------------------------------------------------
K68-26          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K63-77          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K58-13          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K57-52          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K53-47          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K48-26          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K47-31          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K30-44          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
K29-48          CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 420
IS911           CTCTGCCATGTGTTCGGGGTCCATCGCAGCAGCTACAAATACTGGAAAAACCGTCCTGAA 390

AmpC            ------------------------------------------------------------
K68-26          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K63-77          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K58-13          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K57-52          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K53-47          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K48-26          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K47-31          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K30-44          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
K29-48          AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 480
IS911           AAGCCAGACGGCAGACGGGCTGTATTACGCAGCCAGGTACTTGAACTGCATGGCATCAGC 450

AmpC            ------------------------------------------------------------
K68-26          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K63-77          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K58-13          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K57-52          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K53-47          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K48-26          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K47-31          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K30-44          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
K29-48          CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 540
IS911           CACGGCTCTGCCGGAGCAAGAAGCATCGCCACAATGGCAACCCAGAGAGGCTACCAGATG 510

AmpC            ------------------------------------------------------------
K68-26          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K63-77          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K58-13          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K57-52          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K53-47          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K48-26          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K47-31          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K30-44          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
K29-48          GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 600
IS911           GGGCGCTGGCTTGCTGGCAGACTCATGAAAGAGCTGGGGCTGGTCAGCTGTCAGCAGCCG 570
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AmpC      ------------------------------------------------------------
K68-26    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K63-77    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K58-13    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K57-52    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K53-47    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K48-26    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K47-31    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K30-44    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
K29-48    ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 660
IS911     ACTTACCGGTATAAACGTGGTGGTCATGAACATGTTGCTATCCCTAACTACCTTGAACGG 630

AmpC      ------------------------------------------------------------
K68-26    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K63-77    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K58-13    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K57-52    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K53-47    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K48-26    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K47-31    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K30-44    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
K29-48    CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 720
IS911     CAGTTCGCCGTGACCGAGCCAAATCAGGTGTGGTGCGGTGATGTGACCTATATCTGGACG 690

AmpC      ------------------------------------------------------------
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K63-77    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K58-13    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K57-52    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K53-47    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K48-26    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K47-31    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K30-44    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
K29-48    GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 780
IS911     GGTAAGCGCTGGGCGTACCTCGCCGTTGTTCTCGACCTGTTCGCAAGAAAACCAGTGGGC 750

AmpC      ------------------------------------------------------------
K68-26    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K63-77    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K58-13    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K57-52    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K53-47    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K48-26    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K47-31    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K30-44    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
K29-48    TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 840
IS911     TGGGCCATGTCGTTCTCGCCGGACAGCAGGCTTACCATGAAAGCACTGGAAATGGCATGG 810

AmpC      ------------------------------------------------------------
K68-26    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K63-77    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K58-13    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K57-52    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K53-47    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K48-26    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K47-31    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K30-44    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
K29-48    GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 900
IS911     GAAACCCGTGGTAAGCCCGTCGGGGTGATGTTCCACAGCGATCAAGGCAGTCATTATACG 870

AmpC      ------------------------------------------------------------
K68-26    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K63-77    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K58-13    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K57-52    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K53-47    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K48-26    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K47-31    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K30-44    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
K29-48    AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 960
IS911     AGCAGGCAGTTCCGGCAGTTACTGTGGAGATACCGGATCAGGCAGAGTATGAGTCGGCGT 930
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AmpC       ------------------------------------------------------------
K68-26     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K63-77     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K58-13     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K57-52     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K53-47     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K48-26     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K47-31     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K30-44     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
K29-48     GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 1020
IS911      GGAAACTGCTGGGATAACAGCCCAATGGAGCGCTTCTTCAGGAGTCTGAAGAACGAATGG 990

AmpC       ------------------------------------------------------------
K68-26     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K63-77     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K58-13     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K57-52     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K53-47     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K48-26     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K47-31     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K30-44     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
K29-48     GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1080
IS911      GTGCCGGCGACGGGCTATGTAAGCTTCAGCGATGCAGCTCACGCAATAACGGACTATATC 1050

AmpC       ------------------------------------------------------------
K68-26     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K63-77     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K58-13     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K57-52     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K53-47     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K48-26     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K47-31     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K30-44     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
K29-48     GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1140
IS911      GTTGGATATTACAGCGCACTAAGACCGCACGAATATAATGGTGGGTTACCACCAAACGAA 1110

AmpC       ------------------------------------------------------------
K68-26     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K63-77     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K58-13     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K57-52     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K53-47     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K48-26     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K47-31     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K30-44     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
K29-48     TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1200
IS911      TCGGAAAATCGATACTGGAAAAAACTCTAACTCGGTGGCCAGTTTTTGTTGACCACTTCA 1170

AmpC       ------------------------------------------------------------
K68-26     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K63-77     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K58-13     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K57-52     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K53-47     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K48-26     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K47-31     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K30-44     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
K29-48     ATTGGTGTCGTTACAATCTAACGCATCGCCAATGTAAATCCGGCCCGCCTATGGCGGGCC 1260
IS911      ------------------------------------------------------------

AmpC       ------------------------------------------------------------
K68-26     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K63-77     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K58-13     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K57-52     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K53-47     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K48-26     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K47-31     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K30-44     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
K29-48     GTTTTGTATGGAAACCAGACCCTATGTTCAAAACGACGCTCTGCACCTTATTAATTACCG 1320
IS911      ------------------------------------------------------------
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AmpC            -------------------------------------------TGTGCATCGCACAATTA 17
K68-26          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K63-77          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K58-13          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K57-52          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K53-47          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K48-26          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K47-31          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K30-44          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
K29-48          CCTCTTGCTCCACATTTGCCGCCCCTCAACAAATCAACGATATTGTGCATCGCACAATTA 1380
IS911           ------------------------------------------------------------

AmpC            CCCCGCTTATAGAGCAACAAAAGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 77
K68-26          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K63-77          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K58-13          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K57-52          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K53-47          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K48-26          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K47-31          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K30-44          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
K29-48          CCCCGCTTATAGAGCAACAAAGGATCCCCGGTATGGCGGTAGCGGTAATTTATCAGGGTA 1440
IS911           ------------------------------------------------------------

AmpC            AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 137
K68-26          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K63-77          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K58-13          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K57-52          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K53-47          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K48-26          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K47-31          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K30-44          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
K29-48          AACCTTATTACTTTACCTGGGGCTATGCGGACATCGCCAAAAAGCAGCCCGTCACACAGC 1500
IS911           ------------------------------------------------------------

AmpC            AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 197
K68-26          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K63-77          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K58-13          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K57-52          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K53-47          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K48-26          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K47-31          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K30-44          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
K29-48          AAACGTTGTTTGAGTTAGGTTCGGTCAGCAAAACATTTACGGGCGTGCTTGGTGGCGACG 1560
IS911           ------------------------------------------------------------

AmpC            CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 253
K68-26          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K63-77          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K58-13          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K57-52          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K53-47          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K48-26          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K47-31          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K30-44          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
K29-48          CTATTGCTCGAGGGGAAATCAAGTTAAGCGATCCCACAACAAAATACTGGCCTGAA 1616
IS911           --------------------------------------------------------
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Figure 1. ClustalW alignment of the nucleotide sequence of the AmpC IS*911* region in colletion 3 isolates from Haukeland University Hospital, aligned against IS*911* AY555729.1 and bla_{AmpC} AY899338.1.

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CLUSTAL 2.1 multiple sequence alignment


Haukeland       EITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKS 60
Haugesund       EITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKS 60
Vestfold        EITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKS 60
ref             EITPVNIEEELKSSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKS 60
                ************************************************************

Haukeland       ARVVGDVIGKYHPHGDLAVYNTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAMRYTEIR 120
Haugesund       ARVVGDVIGKYHPHGDLAVYNTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAMRYTEIR 120
Vestfold        ARVVGDVIGKYHPHGDLAVYYTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAMRYTEIR 120
ref             ARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQGNFGSIDGDSAAAMRYTEIR 120
                **************** *** ***************************************

Haukeland       LAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHN 180
Haugesund       LAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHN 180
Vestfold        LAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHN 180
ref             LAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHN 180
                ************************************************************

Haukeland       LTEVINGCLAYIDDEDISIE 200
Haugesund       LTEVINGCLAYIDDEDISIE 200
Vestfold        LTEVINGCLAYIDDEDISIE 200
ref             LTEVINGCLAYIDDEDISIE 200
                ********************
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Figure 2. ClustalW alignment of the QRDR of *gyrA* from collection 3 (divided into hospital of origin). Reference sequence used was GyrA NP_416743.

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CLUSTAL 2.1 multiple sequence alignment


ref             NAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNASAKFKKSARTVGDVLGKYH 60
Haugesund       NAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNASAKFKKSARTVGDVLGKYH 60
Haukeland       NAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNASAKFKKSARTVGDVLGKYH 60
Vestfold        NAYLNYSMYVIMDRALPFIGDGLKPVQRRIVYAMSELGLNASAKFKKSARTVGDVLGKYH 60
                ************************************************************

ref             PHGDSACYEAMVLMAQPFSYRYPLVDGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSE 120
Haugesund       PHGDIACYEAMVLMAQPFSYRYPLVDGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSE 120
Haukeland       PHGDIACYVAMVLMAQPFSYRYPLVDGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSE 120
Vestfold        PHGDRACYVAMVLMAQPFSYRYPLVDGQGNWGAPDDPKSFAAMRYTESRLSKYSELLLSE 120
                **** *** ***************************************************

ref             LGQGTADWVPNFDGTLQEPKMLPARLPNILLNGTTGIAVGMATDIPPHNL 170
Haugesund       LGQGTADWVPNFDGTLQEPKMLPARLPNILLNGTTGIAVGMATDIPPHNL 170
Haukeland       LGQGTADWVPNFDGTLQEPKMLPARLPNILLNGTTGIAVGMATDIPPHNL 170
Vestfold        LGQGTADWVPNFDGTLQEPKMLPARLPNILLNGTTGIAVGMATDIPPHNL 170
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Figure 3. ClustalW alignment of the QRDR of *parC* from collection 3 (divided into hospital of origin). Reference sequence used was ParC NP_417491.