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UNIVERSITY OF STAVANGER

Molecular characterization of extended-spectrum β-lactamase producing *Klebsiella pneumoniae* from the Stavanger region between 2003 and 2012

By

Håkon Kaspersen

A thesis submitted in fulfilment for the

degree of Master of science

at the

Faculty of Science and Technology

Department of Mathematics and Natural Sciences

"Science is built up of facts, as a house is built of stones; but an accumulation of facts is no more a science than a heap of stones is a house"

- *Henri Poincaré, Science and Hypothesis, 1905*

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Håkon Kaspersen, Stavanger 2015

Abstract

Klebsiella pneumoniae is a Gram-negative rod in the Enterobacteriaceae family, known to cause both community- and hospital-acquired infections, especially in immunocompromised patients. In the hospital setting, *K. pneumoniae* strains resistant to antibiotics are increasingly reported to cause infections and outbreaks. *K. pneumoniae* is commonly found to produce extended-spectrum β-lactamases (ESBLs), which inactivate the most important antibiotics: penicillins, third generation cephalosporins and monobactams. The most widespread ESBL enzyme among Enterobacteriaceae today is CTX-M-15, which is encoded and spread between strains by plasmids.

In this project, all (n = 49) $ESBL_A$- producing *K. pneumoniae* isolates isolated from clinical samples at Stavanger University Hospital between 2003 and 2012 were characterized at a molecular level. Species identification and antibiotic resistance profile was confirmed by mass spectrometry and the Vitek 2 system, respectively. $ESBL_A$-encoding genes were identified by PCR and gene sequencing, and clonal relatedness was investigated by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Plasmids were identified and characterized by *S1*-PFGE, PCR-based replicon typing and Southern blot hybridization with bla_{CTX-M}, bla_{SHV} and $IncFII_K$ probes.

All isolates were confirmed $ESBL_A$ - producing *K. pneumoniae*. Identified $ESBL_A$ encoding genes were typed as $bla_{CTX-M-15}$ (n = 20), $bla_{CTX-M-3}$ (n = 1), bla_{SHV-2} (n = 1), bla_{SHV-2A} (n = 3), bla_{SHV-5} (n = 13), and bla_{SHV-12} (n = 5). The $bla_{CTX-M-15}$ positive isolates were mainly clonally unrelated, and a common plasmid encoding $bla_{CTX-M-15}$ could not be identified among these isolates. Two $bla_{CTX-M-15}$ positive isolates were found to be clonally related to a strain which caused a neonatal intensive care unit outbreak at Stavanger University Hospital in 2008 – 2009. Twelve bla_{SHV-5} positive isolates were found to be closely related by PFGE and were typed as sequence type (ST) 29 by MLST. A ~230 kbp plasmid was found to bear bla_{SHV-5} in all bla_{SHV-5} positive ST29 isolates.

The most prevalent $ESBL_A$-encoding genes among clinical *K. pneumoniae* isolates from the Stavanger region were $bla_{CTX-M-15}$ and bla_{SHV-5}. bla_{SHV-5} positive ST29 isolates have caused sporadic infections between 2003 and 2012, and may represent an endemic clone in the Stavanger region and hospital setting. In contrast, the bla_{CTX-M} positive isolates were associated with diverse clones and plasmids.

Abbreviations

AST	*Antimicrobial susceptibility testing*
CTX-M	*Cefotaximase Munich*
ddNTP	*Dideoxy nucleotide triphosphate*
DIG	*Digoxigenin*
DNA	*Deoxyribonucleic acid*
dUTP	*Deoxyuracil triphosphate*
ECDC	*European Centre for Disease Prevention and Control*
ESBL	*Extended-spectrum β-lactamase*
EUCAST	*European Committee on Antimicrobial Susceptibility Testing*
Inc-type	*Incompatibility type*
Kbp	*Kilobase pairs*
Kp	*Klebsiella pneumoniae*
MALDI-TOF	*Matrix-assisted laser desorption ionization - time of flight*
MDR	*Multidrug resistant*
MIC	*Minimum inhibitory concentration*
MLST	*Multilocus sequence typing*
PBP	*Penicillin binding proteins*
PBRT	*PCR-based replicon typing*
PCR	*Polymerase chain reaction*
PFGE	*Pulsed-field gel electrophoresis*
SHV	*Sulfhydryl variable*
ST	*Sequence type*
TEM	*Temoneira*
UPGMA	*Unweighted pair group method with arithmetic mean*

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1. Introduction

1.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative rod in the Enterobacteriaceae family. The bacterium is found indigenously in soil and waters, but also on mucosal surfaces in mammals, including humans [69]. *K. pneumoniae* is part of the gut microbiota in humans, and is usually not causing disease. However, it can cause a variety of infections in immunocompromised patients. It can also cause infections when present elsewhere in the body, causing urinary tract infection, respiratory tract infection, and bloodstream infection [37]. *K. pneumoniae* is often associated with nosocomial infections, which are infections acquired during hospitalization [80]. *K. pneumoniae* is often found to be resistant to many commonly used antibiotics, making infections caused by this bacterium difficult to treat.

Different taxonomic systems are used to classify the genus *Klebsiella.* In this thesis, the Ørskov classification is used, which divides the species *K. pneumoniae* into three subspecies; *pneumoniae*, *ozaenae* and *rhinoscleromatis*, due to the disease they were found to cause [60]. *K. pneumoniae* subsp. *pneumoniae* is the subspecies of interest in this study, and is referred to when *K. pneumoniae* is written.

A bacterias ability to cause disease is known as pathogenicity, while virulence refers to the severity of the disease [85], although these terms are used interchangeably. Virulence in *K. pneumoniae* is based on many factors, but the capsule is regarded as the most important [13]. The capsule impairs phagocytosis, which increases bacterial survivability. *K. pneumoniae* is known to have 78 capsule (K) types, of which K1, K2, K4 and K5 are considered the most virulent. Many virulence genes have been identified, some of which are associated with capsule polysaccharide production and a mucoid phenotype, such as *rmp*A and *rmp*A2 [25, 63] . *Klebsiella* species harbouring the *rmp*A gene have been linked to pyogenic liver abscesses [89]. Hypervirulent and hypermucoviscous *Klebsiella* has emerged in the last few years. Hypermucoviscosity is a phenotype characterized by highly viscous and sticky colonies, and the term is often used in conjunction with hypervirulence [77]. Hypervirulent *K. pneumoniae* are not, or have not yet, been associated with antibiotic resistance [10].

1.2 β-lactam antibiotics

Antibiotics are antimicrobial compounds that either kill or inhibit bacterial growth. These compounds have different targets in the bacterial cell, depending on their mode of action. One of the most commonly used types of antibiotics is the β-lactams. The β-lactam antibiotics have the β-lactam ring in common, while side chains are variable. There are four groups of β-lactam antibiotics: penicillins, monobactams, cephalosporins and carbapenems (figure 1.1) [59]. Examples include ampicillin, aztreonam, cefotaxime, and meropenem, respectively.

Mainly, β-lactams inhibit cell wall synthesis by binding to penicillin binding proteins (PBPs), which are crucial enzymes in the cross-linking of peptidoglycan. Peptidoglycan is part of the cell wall of prokaryotes, and inhibition of peptidoglycan cross-linking will, as the cell grows, weaken cell integrity and eventually lyse the cell. Some β-lactam antibiotics have other modes of action in addition to the one described above. For example, cephalosporins might trigger autolytic enzymes in the cell envelope, causing cell lysis [59]. Additionally, β-lactams have a different spectrum of activity, as some are broad-spectrum antibiotics with activity against both Gram-positive and –negative bacteria, while others have a narrow spectrum of activity, like the monobactams (which only affects aerobic Gram-negatives) [59]. Third and later generation cephalosporins have an extended spectrum, which means they have an even broader range of activity.

Testing for susceptibility to antibiotics is called antimicrobial susceptibility testing (AST). An isolate that has low susceptibility to an antibiotic is said to be resistant. There is a variety of AST procedures available today. Commonly used automated AST-systems, such as Vitek 2, measure the minimum inhibitory concentration (MIC), which is the lowest concentration (mg/L) of an antibacterial that is needed to inhibit bacterial growth [51]. MIC values are set for each drug-bug combination by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and are used as a standard in most European countries [46]. The gold standard for determining MIC values is the broth culture method, where a bacterium is suspended in broth with increasing concentration of an antimicrobial agent. No visible growth in a tube means that the concentration of the antimicrobial agent in that tube is the minimum inhibitory concentration. AST can also be determined manually by the disk-diffusion method, where agar plates and tablets with antimicrobial agents are used. Susceptibility is determined by the zone inhibition around each tablet [51].

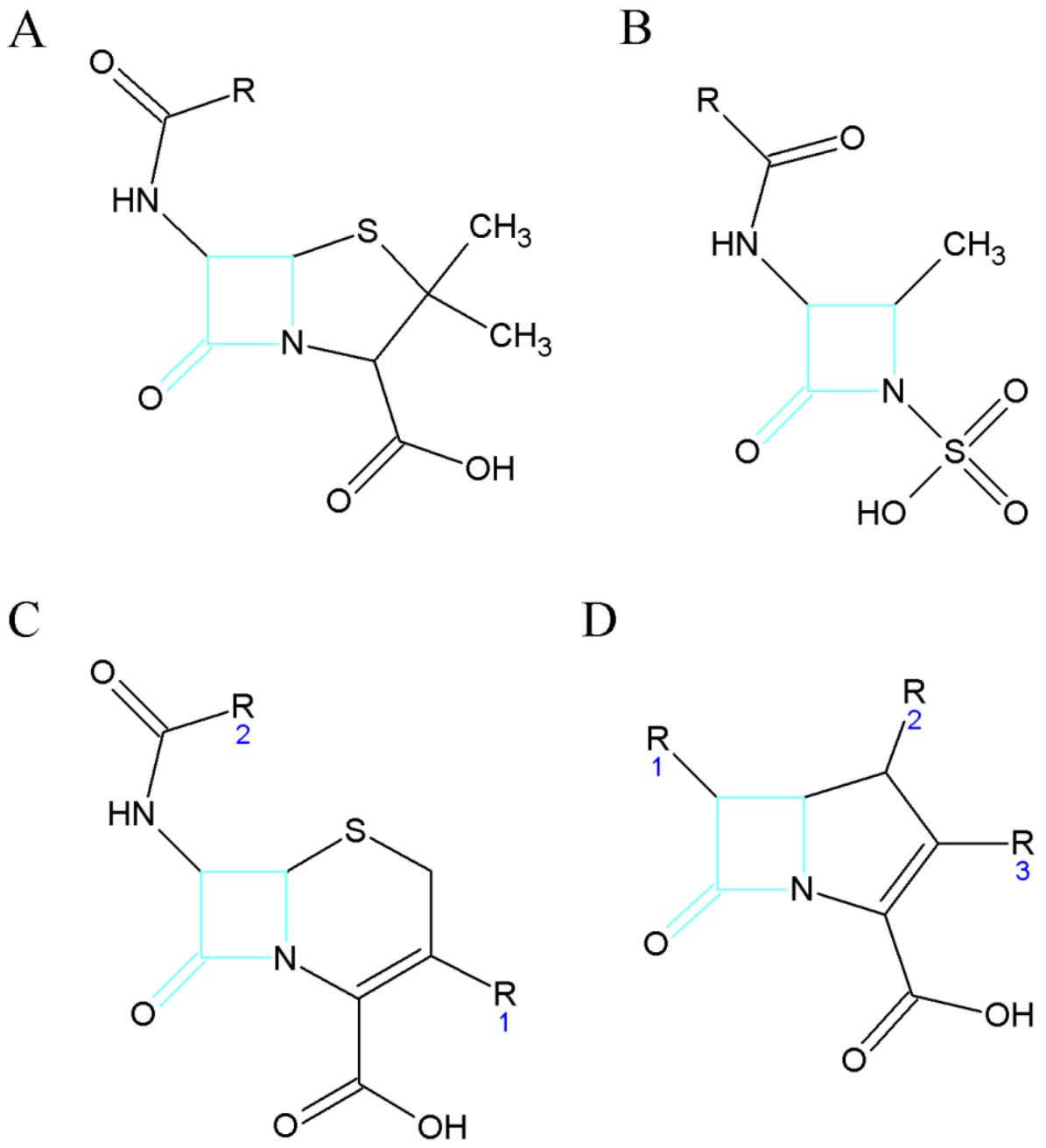


Figure 1.1: General structures of the four groups of β-lactam antibiotics. A: penicillins, B: monobactams, C: cephalosporins, and D: carbapenems. The main structure, the β-lactam ring (marked in teal), is central in each molecule. Variable areas are marked with R.

1.3 β-lactamases: definition and classification

The β-lactamases are a class of enzymes that hydrolyse the β-lactam ring of penicillins, cephalosporins, and other β-lactam antibiotics [58]. This inactivates the drug, giving it a slightly different structure (figure 1.2). This structure difference makes it unable to bind to the PBP active site. β-lactamases are classified into narrow-spectrum (penicillinases), broad-spectrum (ampicillinases), extended-spectrum β-lactamases (ESBLs), and carbapenemases [50], based on range of activity.

The classical definition of ESBLs is a β-lactamase with hydrolytic activity against penicillins, extended-spectrum cephalosporins and monobactams, and is inhibited by clavulanic acid [39].

Different classification schemes are available for β-lactamase classification. The most commonly used are the Bush-Jacoby-Medeiros [17] and the Ambler [4] schemes, which classify β-lactamases by their functional characteristics and primary structure, respectively. Based on these classification systems, an enzyme is classified as an ESBL if it is a molecular class A, and a functional class 2be enzyme. However, these classification schemes are not convenient for the apparent complexity and quantity of enzymes which have been discovered during recent years. In light of this, a new classification scheme was suggested by Giske *et al.* [39]. Briefly, ESBLs are categorized into three classes, $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$. The $ESBL_A$ class contains the former "classical" ESBLs, the CTX-M, TEM, and SHV enzymes, along with the less prevalent VEB and PER variants. The $ESBL_M$ class is divided into $ESBL_{M\text{-}C}$ (plasmid-mediated AmpC) and $ESBL_{M\text{-}D}$ (OXA-ESBL). The $ESBL_{CARBA}$ class include enzymes with the broadest spectrum that confer resistance to extended-spectrum cephalosporins and at least one carbapenem. This class is further divided into three subclasses; $ESBL_{CARBA\text{-}A}$, $ESBL_{CARBA\text{-}B}$ and $ESBL_{CARBA\text{-}D}$. The $ESBL_A$ enzymes CTX-M, TEM and SHV are of interest in this study.

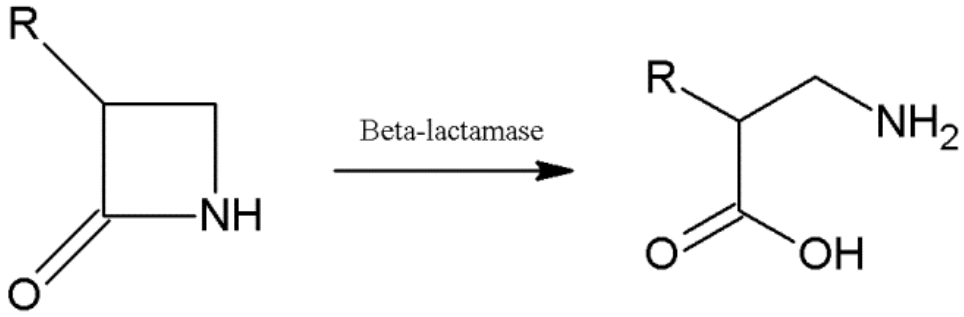


Figure 1.2: Hydrolysis of the β-lactam ring by action of a β-lactamase.

1.4 The $ESBL_A$ enzymes

During the last decades, hundreds of new $ESBL_A$ enzymes have been identified. The Lahey database (www.lahey.org/Studies) contains hundreds of $ESBL_A$ protein sequences of most known CTX-M, TEM and SHV types. The SHV and TEM enzymes differ from one another only by a few amino acid substitutions, while the CTX-M family is widely different [45].

The TEM variant was one of the first ESBLs to be detected. First found in 1965, the TEM type β-lactamase was named after the *E. coli* infected patient, Temoneira, in Athens, Greece [31]. To date, over 220 variants of the TEM enzyme has been identified [18], and the bla_{TEM} gene (encoding the enzyme) is usually found on plasmids [67]. TEM-1 has almost no hydrolytic activity against extended-spectrum cephalosporins, and is therefore not an ESBL enzyme. However, TEM-1 confers resistance towards ampicillin and other penicillins. A single amino acid substitution led to the formation of TEM-3, which was also capable of hydrolysing cefotaxime (a third generation cephalosporin), and is therefore per definition an ESBL [79].

The SHV – enzymes were thought to be substrate variable, and were therefore given its name, which is short for sulfhydryl variable [67]. There are approximately 193 SHV variants identified to date [18]. The non-ESBL enzymes SHV-1 and SHV-11 are chromosomally encoded by most *K. pneumoniae* strains, but can also be plasmid mediated [6, 41]. One amino acid substitution in the SHV-1 sequence was discovered in 1983. The new enzyme could effectively hydrolyse third generation cephalosporins, such as ceftazidime, and was designated SHV-2 [67].

In 1990, Bauernfeind *et al.* detected an *E. coli* strain from a German patient, producing a new ESBL enzyme. The new enzyme had higher substrate affinity for cefotaxime compared to the SHV-enzymes, and was designated CTX-M-1 (Cefotaximase-Munich) [8]. Roughly 160 variants of CTX-M enzymes are described to date [18], and the $bla_{CTX\text{-}M}$ gene is usually found on plasmids. Whereas the SHV and TEM enzymes are related, it seems that the CTX-M enzymes have a different origin. The CTX-M variant has a high sequence homology to a chromosomal β-lactamase found in *Kluyvera spp*. The CTX-M enzymes are categorized into five groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (figure 1.3), based on sequence homology [12].

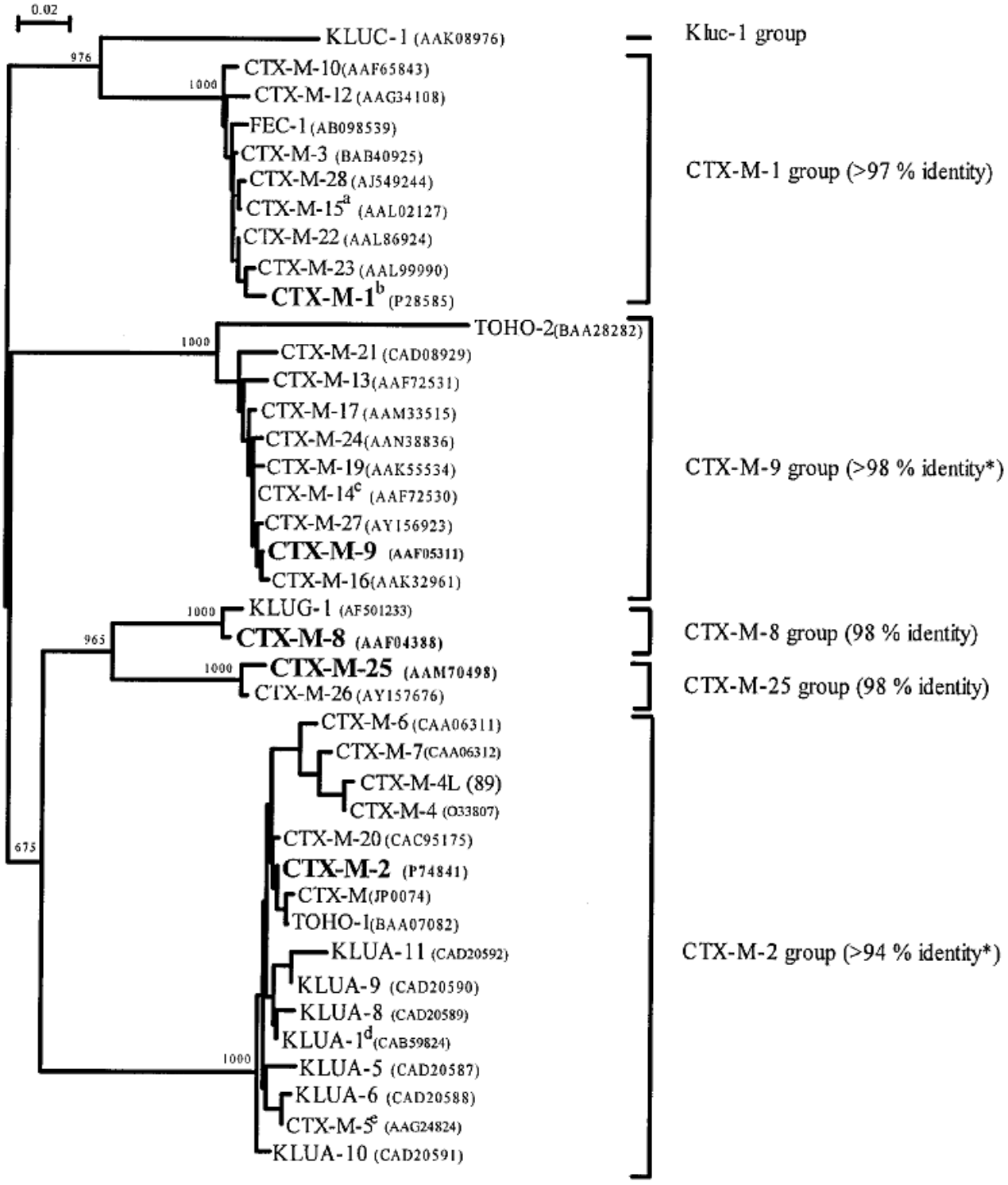


Figure 1.3: A dendrogram representing groups of the CTX-M family. Branch length is corresponding to amino acid changes. Source: Bonnet R., 2004 [12].

1.5 Spread of antibiotic resistant *K. pneumoniae*

Antimicrobial resistance is emerging worldwide, also in Europe – and in Norway. Antimicrobial resistance, especially the emergence of ESBL-producing Gram-negative bacteria such as *K. pneumoniae*, is of great concern due to their limited treatment options. The European Centre for Disease Prevention and Control (ECDC) reports a very high amount of *K. pneumoniae* resistant to third generation cephalosporins in southern and eastern Europe in 2013 (figure 1.4). The prevalence of ESBL – producing *K. pneumoniae* varies geographically in Europe. However, even if a low prevalence is reported in the Scandinavian countries, NORM (Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway) reports an increasing amount of resistant *K. pneumoniae* from 2003 – 2013 (figure 1.5).

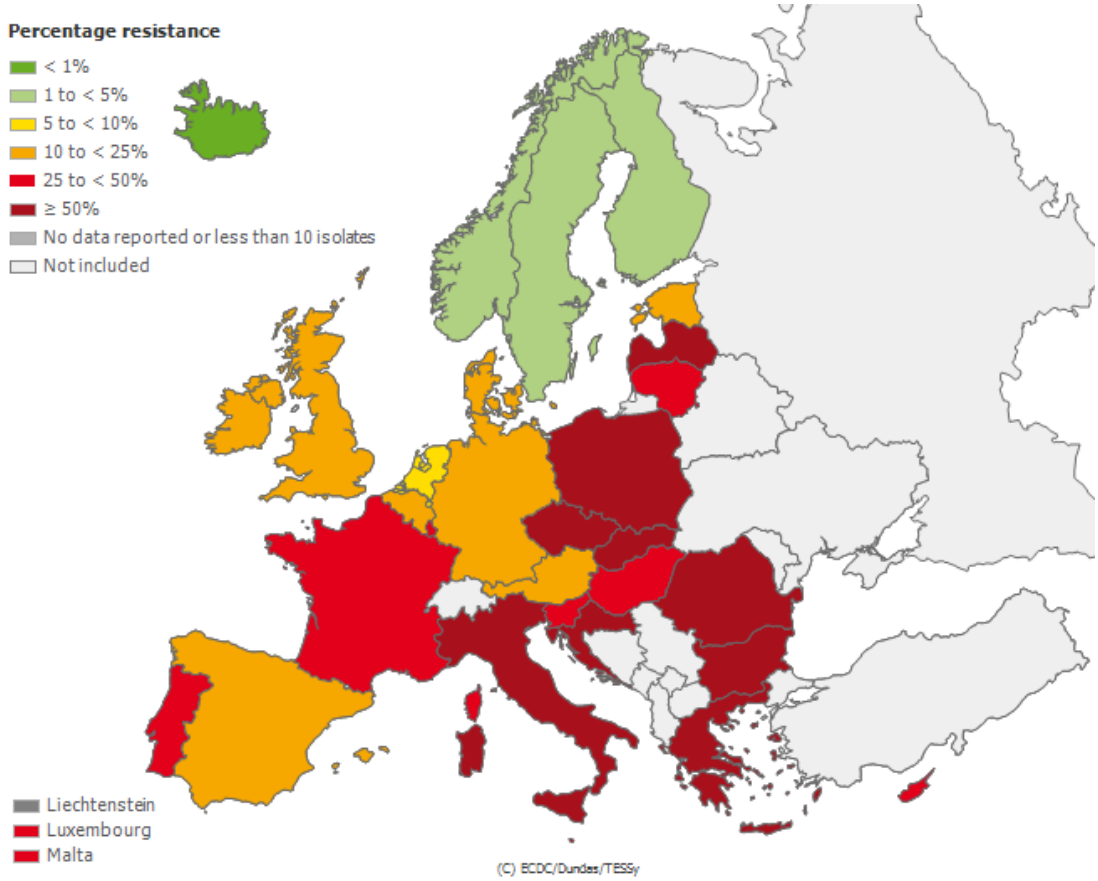


Figure 1.4: Proportion of 3rd. generation cephalosporin resistant *K. pneumoniae* isolates in participating countries in 2013. Source: ECDC/EARS-Net (www.ecdc.europa.eu).

Low prevalence does not mean that outbreaks do not occur. A clonal outbreak occurred at Stavanger University Hospital in 2008 – 2009, where an ESBL-producing *K. pneumoniae* spread in a neonatal intensive care unit, infecting a total of 58 infants [73]. Contaminated breast milk was found to be the main source of the CTX-M-15 producing *K. pneumoniae,* and the outbreak ended after screening procedures and isolation of the affected patients.

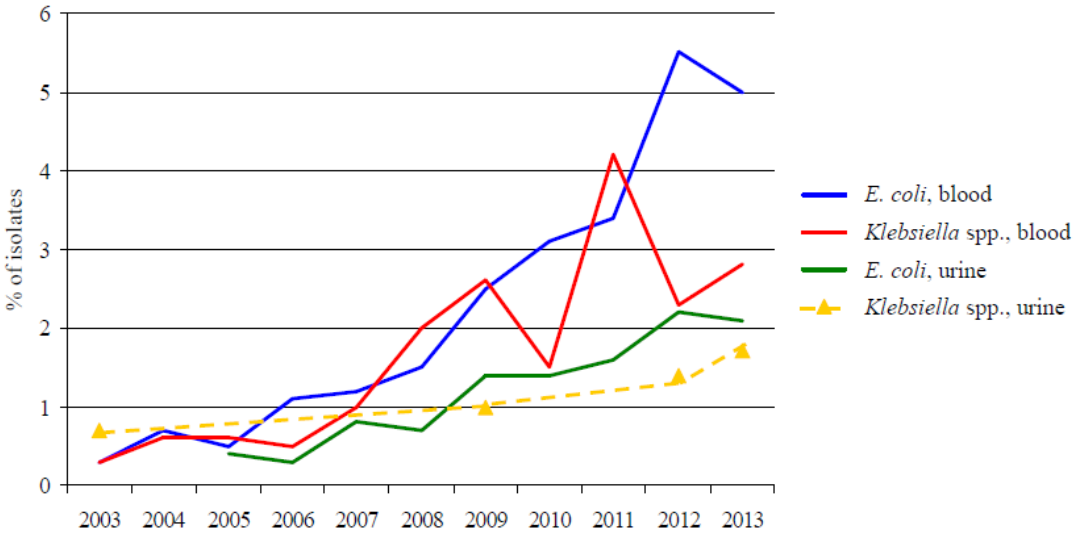


Figure 1.5: Prevalence of ESBL-production among *E. coli* and *Klebsiella spp*. isolates from blood and urine 2003 – 2013. Source: NORM/NORM-VET 2013 (www.vetinst.no).

1.6 Dissemination of ESBL

1.6.1 Clonal dissemination

Some bacteria may be more successful than others, depending on many different factors, including pathogenicity, virulence, and antibiotic resistance. These are called successful clones, and may be present in the environment for a very long time [50]. They spread through normal cell division (vertically) and may cause infections or even outbreaks. On a global scale, the *E. coli* sequence type (ST) 131 is spreading clonally, and is mainly associated with CTX-M-15 [65]. Also, the *K. pneumoniae* multiresistant ST11 clone is regarded as internationally prevalent [88]. Specific clones are sometimes associated with specific diseases. For example, *K. pneumoniae* ST23 is known to cause pyogenic liver abscesses [40].

A bacterial clone is endemic if it persists in a specific geographical area, and is more frequently detected in that area compared to other areas [1].

1.6.2 Plasmid mediated dissemination

Plasmids are small, independent genetic elements (figure 1.6) which may be transferred horizontally between bacteria by conjugation [52]. Some plasmids have their own origin of replication, which make them able to replicate independently from the chromosome, although they do rely on enzymes from the host cell to replicate. Usually, plasmids harbour accessory genes (encoding virulence or antibiotic resistance traits) which may result in increased bacterial survival in a given environment.

Some plasmids are present in high numbers in the host cell, i.e. high copy number plasmids, while others may have only a few copies present, i.e. low copy number plasmids [52].

There are many ways to group plasmids. One method is to type the plasmid replicon (origin of replication, figure 1.6) using PCR, determining the *incompatibility type* (Inc-type), or replicon type, of the plasmid. One bacterial host cell may contain several different plasmids. However, if two

genetically similar plasmids are present in the same cell, only one of them can be maintained. This is because the plasmids will compete for the same replication machinery already present in the host cell [28]. Genetically similar plasmids are therefore *incompatible*, and only one of them will be maintained after cell division. Two plasmids can only be maintained by one cell if they are of different replicon types. One plasmid may also have multiple replicon types. To date, there are 27 known replicon types [20]. The ESBL-enzyme CTX-M-15 is frequently associated with low copy-number plasmids of the IncF replicon types (e.g. FII_K, FIB, and FII) [21]. Earlier studies have linked CTX-M-15 to plasmids of 90 and 180 kilobase pairs (kbp) [49, 90].

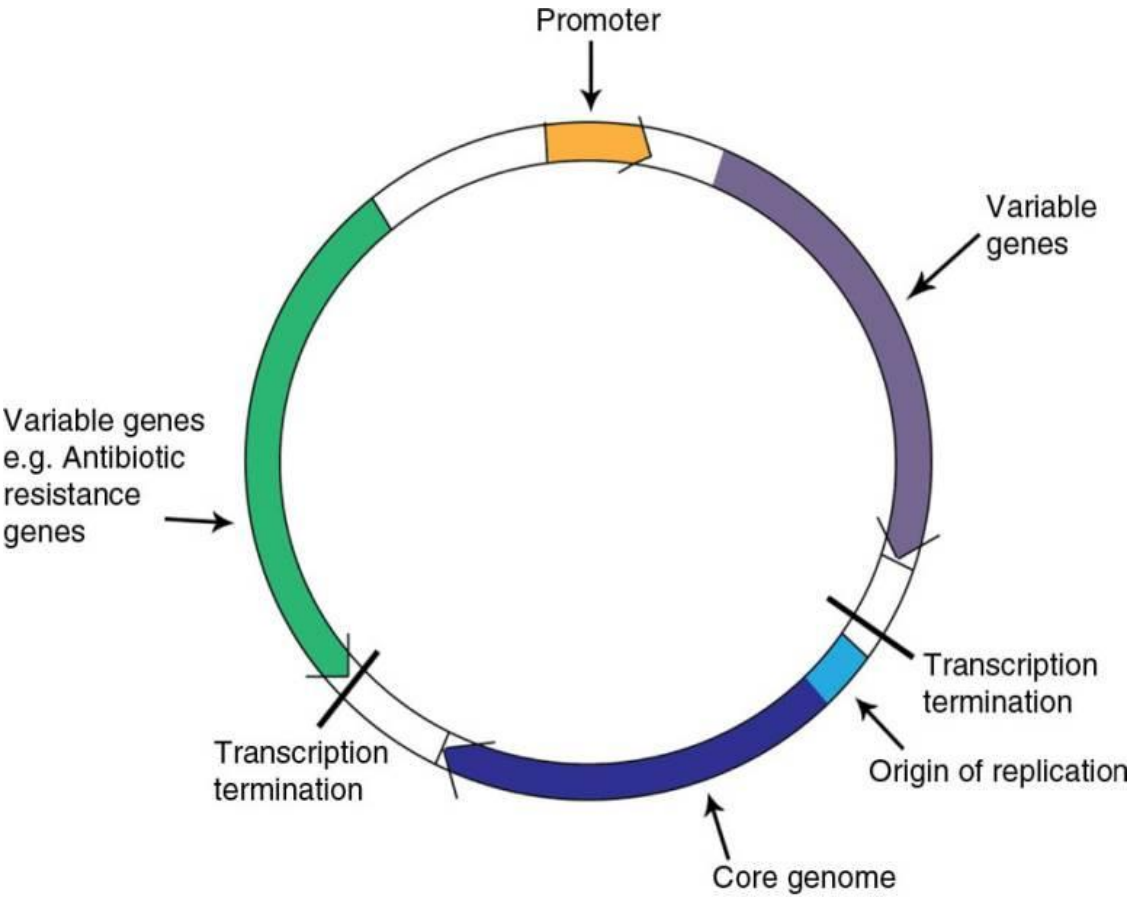


Figure 1.6: A schematic diagram of a typical antibiotic resistance plasmid. ESBL-encoding genes and genes conferring resistance to other antibiotics are usually found in the variable regions. Source: Brolund, A. 2014 [15].

Plasmids may harbour ESBL-encoding genes. Along with these, the plasmids may also carry genes that confer resistance to other antibiotics, making treatment options even narrower. This is called co-resistance, and these genes are often carried in conjunction with the ESBL-gene. Bacteria that harbour genes that confer resistance to three or more different types of antibiotics are called multidrug resistant (MDR) [53].

ESBL-encoding genes have been linked to other mobile genetic elements, such as transposons and insertion sequences [70]. The genetic environment of various $bla_{CTX\text{-}M}$ genes have previously been mapped [34], and IS*Ecp*-1-like insertion sequences, along with tnpA-1 to 3 transposons have been associated with the mobilization of the $bla_{CTX\text{-}M}$ genes [19].

2. Aims of the study

The main aim of the study was to characterize all clinical $ESBL_A$-producing *K. pneumoniae* isolates isolated from clinical samples at Stavanger University Hospital between 2003 and 2012 at a molecular level.

Specific aims:

1. Determine the types of $ESBL_A$ – encoding genes present in clinical *K. pneumoniae* isolates in the Stavanger region
2. Identify *K. pneumoniae* clones associated with clinical infection, that may have contributed to the spread of $ESBL_A$ –encoding genes in the Stavanger region
3. Identify $ESBL_A$-encoding plasmids that may have contributed to the spread of $ESBL_A$ – encoding genes among *K. pneumoniae* in the Stavanger region

3. Materials and methods

3.1 Bacterial isolate collection

At the Department of Medical Microbiology, Stavanger University Hospital, all Enterobacteriaceae isolates from clinical samples have been screened for $ESBL_A$-production since 2003. ESBL-producing isolates were stored on freezing-beads at -70 °C. In this study, all clinical $ESBL_A$-producing *K. pneumoniae* isolates identified between 2003 and 2012 were included (n = 55). In addition, three isolates associated with an outbreak at the neonatal intensive care unit in 2008 – 2009 were included. These isolates were recovered from faecal- and breast milk screening samples. Nine clinical isolates were excluded, as they were found to be either non-ESBL producers (n = 2), falsely identified as *K. pneumoniae* (n = 1), not viable after storage (n = 1), or not available (n = 5).

Of the remaining 49 isolates, 30 were isolated from hospitalized patients (including the three screening samples), and 19 from out – patients. The clinical isolates were isolated from urine- (n = 25), respiratory- (n = 12), wound- (n = 7), central venous catheter- (n = 1), and blood- (n = 1) samples. The screening samples were from faeces (n = 2) and breast milk (n = 1).

For clarification, all $bla_{CTX\text{-}M\text{-}15}$ positive isolates are henceforth called "CTX-M-15 group" isolates and the clonally related $bla_{SHV\text{-}5}$ positive isolates are called "SHV-5 group" isolates. Isolate IDs marked with "a" and "b" originated from the same patient, but were isolated separately due to phenotypical differences. Figure 3.1 illustrates which methods were performed on which isolates.

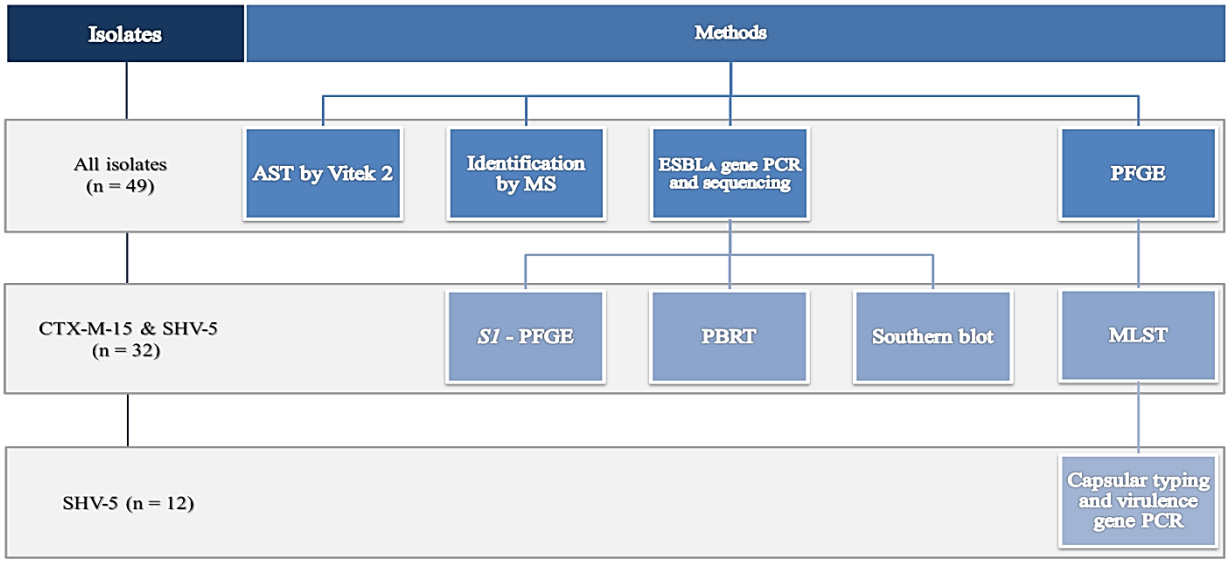


Figure 3.1: An overview of methods used in this study on which isolates. Isolates are marked in grey, and methods are marked in blue. AST = antimicrobial susceptibility testing, MS = mass spectrometry, PFGE = pulsed field gel electrophoresis, PBRT = PCR-based replicon typing, MLST = multilocus sequence typing.

3.2 $ESBL_A$ – screening

All isolates (n = 49) were confirmed to be $ESBL_A$ – producing *K. pneumoniae* by growth on selective ESBL – agar and subsequent species identification.

All isolates were inoculated on two agar plates. Plate one, a selective chromogenic ESBL agar (bioMérieux, Marcy l'Étoile, France), was used to confirm $ESBL_A$-production. Plate two was a modified MacConkey agar (see appendix A for recipe) with one 30 µg cefotaxime tablet (Rosco Diagnostics AS, Taastrup, Denmark). The agar plates were incubated at 35 °C overnight. On plate one, growth of blue-green colonies suggested ESBL-producing *K. pneumoniae*, while brown colonies suggested ESBL-producing *E. coli* (according to the manufacturer). On plate two, cefotaxime susceptibility was evaluated by roughly measuring the zone around the cefotaxime disk.

3.3 Species identification

All isolates were previously identified to the species level. In this study, these results were confirmed by mass spectrometry.

Matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry is a quick and reliable method for bacterial species identification and is presently used in many laboratories around the world. Identification of bacterial species by mass spectrometry is based on the molecular fingerprint (mass spectrum) of the bacterium. Each fingerprint is highly distinctive, and by comparing the fingerprint of the bacterium in question with reference spectra from different species of bacteria, the computer can identify the isolate based on similarities between these spectra. The spectrum is based on the glycoproteins, lipoproteins and other membrane-bound molecules, which the high-energy UV laser irradiates [72]. When these molecules are ionized by the laser, they travel through the TOF (time of flight) separator, which separates them by charge and mass. Finally, they reach a detector which will send the signal to the computer. The Microflex MALDI-TOF (Bruker Daltonik, Bremen, Germany) is presently used for bacterial identification at Stavanger University Hospital. The MALDI biotyper CA software is used to analyse MALDI-TOF data. Each isolate is given a score based on the similarity between the reference and sample spectrum. The score value is from 0 to 3, where 2.3 – 3 is regarded as highly probable species identification [29]. Protocol used is presented in box 1.

MALDI-TOF analysis

1. *Inoculate isolates on blood agar (see recipe in appendix) and incubate overnight at 35 °C.*
2. *Pick a single colony with a toothpick, and smear onto the target plate.*
3. *Add matrix (1.0 µl) to each sample on the plate.*
4. *Let plate dry before MALDI-TOF analysis.*

Box 1: MALDI-TOF analysis protocol, as given by the manufacturer (Bruker Daltonik).

3.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was automatically performed on all isolates by the Vitek 2 system (bioMérieux, Marcy l'Étoile, France) to determine the antimicrobial susceptibility pattern.

Vitek 2 utilizes cards that determine the antimicrobial susceptibility pattern of a bacterial isolate. The card "AST-N209" was used in this study as it is specifically made for resistance determination for Gram-negative rods. The card test susceptibility to the following antibiotics: ampicillin, trimethoprim, nitrofurantoin, trimethoprim/sulfamethoxazole, ciprofloxacin, cefuroxime, cefotaxime, ceftriaxone, ceftazidime, gentamicin, meropenem, piperacillin/tazobactam, cefoxitin, amoxicillin/clavulanic acid, cefuroxime axetil, aztreonam, nalidixic acid, and tobramycin. The samples are drawn into the cards by vacuum, and the tube on the card is hermetically sealed. The card contains dehydrated antibiotics, and colorimetry is used to detect susceptibility. Colour changes are registered by the computer every 15 minutes, under a controlled environment [68]. MIC values are calculated and interpreted as R (resistant), I (intermediate), or S (sensitive) depending on the MIC value. The classification of MIC values for each drug/bug combination has been determined according to EUCAST clinical breakpoints [46]. Protocol used is presented in box 2.

Vitek 2 analysis for Gram-negative rods

1. *Inoculate isolates on blood agar and incubate at 35 °C overnight.*
2. *Suspend fresh colonies in 0.45 % saline solution (3.0 ml) in plastic tubes to a concentration of 0.5 – 0.63 McFarland[1].*
3. *Place bacterial suspension in a Vitek 2 rack, and place the AST-N209 card in an empty tube in the adjacent slot. Use one card for each isolate.*
4. *Put the whole rack inside the Vitek 2 instrument for analysis.*

[1]Measured on a DEN-1 McFarland densitometer (Montebello Diagnostics, Oslo, Norway)

Box 2: Vitek 2 analysis protocol for Gram-negative rods, as given by the manufacturer (bioMérieux).

Manual AST was performed by the disk diffusion method on two samples due to uncertain Vitek 2 results. Fresh colonies were suspended in a 0.45 % saline solution as described for the Vitek 2 sample preparation. A sterile cotton swab was used to spread the bacterial suspension in an even layer on a Mueller-Hinton (MH) agar plate (see recipe in appendix A). Three plates were prepared for each sample. Antibiotic disks were then applied to the inoculated MH-plates. These antibiotic disk panels are listed in table 3.1, and relative positions of the compounds are presented in figure 3.2.

Table 3.1: Antibiotic disk panels used in manual AST of Enterobacteriaceae, routinely used in the Medical Microbiology Laboratory at Stavanger University Hospital.

Panel	*Enterobacteriaceae (C)*	*Enterobacteriaceae extended (D)*	*ESBL*
Antibiotic compounds	Ampicillin 10 µg	Cefuroxime 30 µg	Ceftazidime/Clavulanic acid 30 µg / 10 µg
	Trimethoprim/Sulfamethoxazole 1.25µg / 23.75 µg	Gentamicin 10 µg	Cefotaxime/Clavulanic acid 30 µg / 10 µg
	Ciprofloxacin 5 µg	Meropenem 10 µg	Ceftazidime 30 µg
	Ceftazidime 10 µg	Piperacillin / Tazobactam 30 µg / 6 µg	Cefotaxime 30 µg
	Cefotaxime 5 µg		Cefoxitin 30 µg
	Amoxicillin/Clavulanic acid 20 µg / 10 µg		Ertapenem 10 µg

An ESBL-phenotype and co-resistance to other relevant antibiotics were determined from the AST results. The following antibiotics were considered to define whether an $ESBL_A$ positive isolate was resistant to other relevant antibiotic groups (i.e. co-resistant): nitrofurantoin, trimethoprim /sulfamethoxazole, ciprofloxacin, gentamicin, and tobramycin.

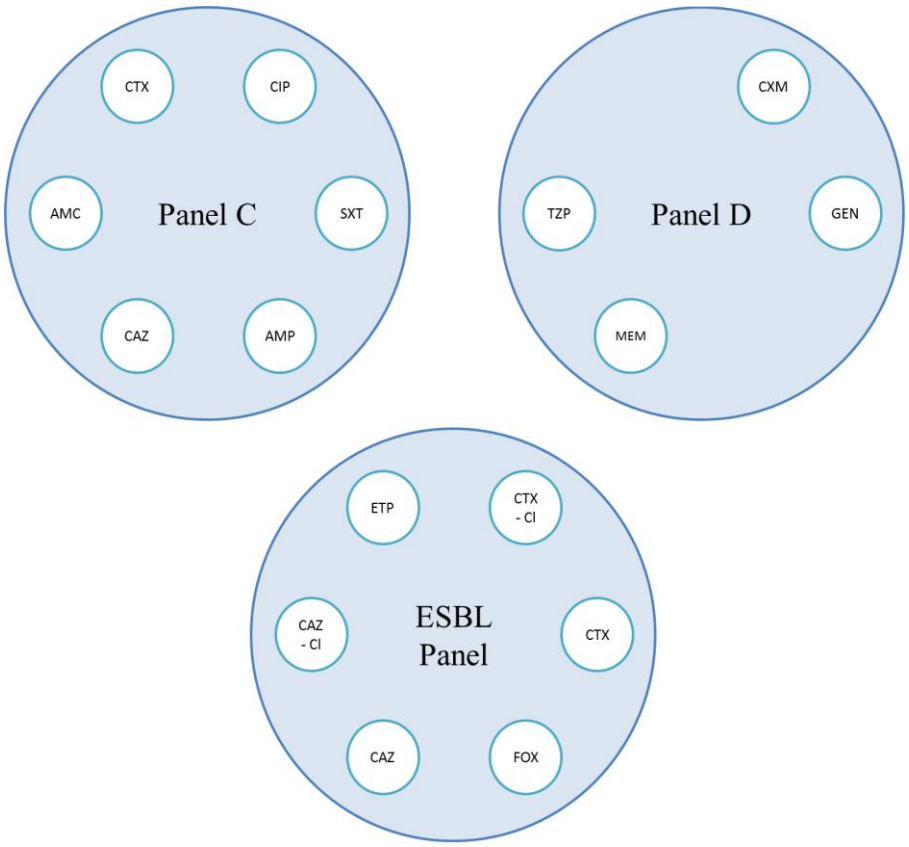


Figure 3.2: Manual AST setup. The three blue circles each represent one Mueller-Hinton agar-plate, and the white circles inside each represent antibiotic tablets. CTX = cefotaxime, CTX-Cl = cefotaxime / clavulanic acid, CIP = ciprofloxacin, SXT = trimethoprim / sulfamethoxazole, AMP = ampicillin, CAZ = ceftazidime, CAZ-Cl = ceftazidime / clavulanic acid, AMC = amoxicillin / clavulanic acid, CXM = cefuroxime, GEN = gentamicin, MEM = meropenem, TZP = piperacillin / tazobactam, FOX = cefoxitin, ETP = ertapenem.

3.5 DNA extraction

Bacterial DNA was extracted by transferring a few colonies to 500 µl of sterile water in an Eppendorf tube. The suspension was then mixed well, and boiled at 100 °C for 10 minutes. After boiling, the suspensions were centrifuged at 13.000 x g for five minutes. The supernatant, containing bacterial DNA, was then transferred to a new tube and stored at 4 °C.

3.6 Conventional PCR and gel electrophoresis

Polymerase chain reaction (PCR) is a method which is used to amplify a specific gene of interest by the use of a thermocycler. It is based on three main steps, denaturation, annealing, and elongation of DNA. The denaturation step makes the DNA single stranded. Primers anneal to the single stranded DNA, and finally the polymerase incorporate new nucleotides that are complementary to the reference sequence. The cycle is repeated many times, which will make millions of copies of the original sequence. The enzyme *Thermus aquaticus* (Taq) polymerase is commonly used, as it tolerates high temperatures.

One-directional gel electrophoresis is normally used to separate DNA fragments amplified by conventional PCR. DNA fragments move toward the positive pole of the electrophoresis chamber because of its negative charge, and is separated based on their size. Agarose is used to create the matrix needed for separation. Small pores in the gel are formed which allows small fragments to move fast, while larger fragments move slower. The size of the pores is varying depending on agarose concentration.

3.6.1 16S rRNA PCR

To confirm the presence of bacterial DNA, a PCR targeting the 16S rRNA gene was performed on all extracts, as previously described [55]. Primers, along with amplicon sizes, are listed in appendix A. Reagents used are listed in table 3.2. The PCR program is described in table 3.3. PCR products were visualized on an agarose gel (1.0 %, 110 V, 30 minutes). Positive controls are listed in appendix A. The "HotStarTaq Master Mix kit" (Qiagen, Hilden, Germany) was used.

Table 3.2: 16S rRNA PCR reagents.

Reagent	***Volume (per reaction)***
H_2O	7.20 µl
2x HotStarTaq Master Mix (Qiagen)	10.0 µl
16S primer F (10 µM)	0.40 µl
16S primer R (10 µM)	0.40 µl
Template	2.0 µl
Total	20.0 µl

Table 3.3: 16S rRNA PCR program [55].

Cycles	***Temperature (°C)***	***Time***
-	95	15 m
32	95	40 s
	58	60 s
	72	60 s
-	72	10 m

3.6.2 ESBL$_A$ – PCRs

All isolates were screened for the presence of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ genes by a PCR specifically targeting these genes, as previously described [81]. Primers are listed in table 3.7, PCR reagents in table 3.4, and PCR program in table 3.5. Detailed primer information is listed in appendix A. A subsequent gel electrophoresis was run to visualize the PCR products (1.0 % agarose gel, 110 V, 30 minutes). Positive controls are listed in appendix A.

Table 3.4: bla$_{CTX-M,}$ bla$_{TEM}$ and bla$_{SHV}$ PCR reagents.

	Reagent	***Volume (per reaction)***
bla$_{CTX-M}$	H_2O	6.0 µl
	2x HotStarTaq Master Mix (Qiagen)	10.0 µl
	bla$_{CTX-M}$ F (10 µM)	1.0 µl
	bla$_{CTX-M}$ R (10 µM)	1.0 µl
	Template	2.0 µl
	Total	20.0 µl
bla$_{SHV}$ & *bla*$_{TEM}$	H_2O	7.20 µl
	2x HotStarTaq Master Mix (Qiagen)	10.0 µl
	bla$_{Tem}$ F/bla$_{SHV}$ F (10 µM)	0.40 µl
	bla$_{Tem}$ R/bla$_{SHV}$ R (10 µM)	0.40 µl
	Template	2.0 µl
	Total	20.0 µl

Table 3.5: bla$_{CTX-M}$, bla$_{TEM}$ and bla$_{SHV}$ PCR programs [81].

	Cycles	***Temperature (°C)***	***Time***
bla$_{CTX-M}$ & *bla*$_{TEM}$	-	95	15 m
	35	94	30 s
		50	30 s
		72	60 s
	-	72	10 m
bla$_{SHV}$	-	94	5 m
	35	94	60 s
		58	30 s
		72	60 s
	-	72	5 m

CTX-M group 1 real-time PCR (RT-PCR) (see 3.8) positive isolates were run on *bla*$_{CTX-M\ group\ 1}$ specific PCRs. Primers used are listed in table 3.7, detailed primer information is listed in appendix A. Two PCR reactions were run to cover the whole sequence, with subsequent sequencing. PCR programs were the same as for the *bla*$_{SHV}$ PCR described in table 3.5. Reagents are listed in table 3.6. Positive controls are listed in appendix A.

Table 3.6: bla$_{CTX-M\ group\ 1}$ specific PCR reagents.

Reagent	***Volume (per reaction)***
H_2O	6.80 µl
2 x HotStarTaq MM (Qiagen)	10.0 µl
F primer	0.60 µl
R primer	0.60 µl
Template	2.0 µl
Total	20.0 µl

Table 3.7: Primers used for PCR of bla_{CTX-M}, $bla_{CTX-M\ group\ 1}$, bla_{SHV} and bla_{TEM} genes. Detailed primer information is listed in appendix A.

Gene	***bla*$_{CTX-M}$**	***CTX-M group 1***	***bla*$_{SHV}$**	***bla*$_{TEM}$**
PCR primers	*bla*$_{CTX-M}$ F *bla*$_{CTX-M}$ R	CTX-M-1 F Orf477 R IS*Ecp*-tnpA1 F IS*Ecp*-tnpA2 F IS*Ecp*-tnpA3 F CTX-M-1 R	*bla*$_{SHV}$ F *bla*$_{SHV}$ R	*bla*$_{TEM}$ F *bla*$_{TEM}$ R

3.6.3 PCR-based replicon typing

The CTX-M-15 and SHV-5 group isolates were analysed by PCR-based replicon typing (PBRT) to determine the replicon types of CTX-M-15 and SHV-5 encoding plasmids.

A commercially available PBRT kit was used (Diatheva, Fano PU, Italy) [22].

The kit is based on eight multiplex PCR reactions. In total, 25 primer pairs are used to detect the most common inc-types in Enterobacteriaceae (inc HI1, HI2, I1, I2, X1, X2, L/M, N, FIA, FIB, FIC, FII, FII_K, W, Y, P, A/C, T, K, U, R, B/O, HIB-M and FIB-M). The kit contains pre-made master mix for each multiplex PCR reaction, DNA polymerase, and positive controls.

For each reaction, pre-made master mix (23.80 µl) was mixed with DNA polymerase (0.20 µl), after which the template was added (1.0 µl). Each PCR reaction was run under the same conditions (table 3.8).

Table 3.8: PRBT PCR-program, as given by the producer (Diatheva).

Cycles	*Temperature (°C)*	*Time*
-	95	10m
25	95	60s
	60	30s
	72	60s
-	72	5m

Gel electrophoresis was run to visualize the amplicons. Loading dye (5.0 µl) was added directly to the PCR product, which was loaded into a 2.5 % agarose gel, along with a 100 bp plus ladder (Qiagen). The gel was run on 120 volt for 70 minutes. Visible bands were compared with the positive control and identified.

3.6.4 Capsular typing and virulence gene PCR

Capsular typing and virulence gene detection was performed on the SHV-5 group isolates to detect the presence of selected capsule types or virulence genes.

A multiplex PCR covering the K – types K1, K2, K5, K20, K54, and K57, as well as the virulence genes *rmp*A and *wca*G have previously been described [83]. The PCR also contains a *K. pneumoniae* 16S rRNA (*Kpn* 16S) primer set.

A master mix was made with all the primers (listed in appendix A). PCR reagents are presented in table 3.9, and PCR program is described in table 3.10. The kit "Qiagen multiplex PCR kit" was used. PCR products were run on an agarose gel (1.5 %, 110 V for ~80 minutes). PCR controls are listed in appendix A. Negative controls included a non-virulent *K. pneumoniae,* and a standard contamination control with water.

Table 3.9: Capsular typing and virulence gene multiplex PCR reagents [83].

Reagent	***Volume (per reaction)***
2 x Multiplex PCR master mix (Qiagen)	12.5 µl
Primer (18) (10.0 µM)	9.0 µl
H_2O	1.5 µl
Template	2.0 µl
Total	25.0 µl

Table 3.10: Capsular typing and virulence gene multiplex PCR program [83].

Cycles	***Temperature (°C)***	***Time***
-	95	15 m
35	94	30 s
	58	90 s
	72	90 s
-	72	10 m

3.6.5 Multilocus sequence typing

The sequence types of the CTX-M-15 and SHV-5 group isolates were determined through multilocus sequence typing (MLST).

MLST is a method developed for typing of many bacterial species. MLST for *K. pneumoniae* was developed by Diancourt *et al.*, and is currently used the world over to determine the ST of isolates [32]. This is done by obtaining the DNA sequences of seven genes and by plotting the sequences into a database for comparison (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). The database will report allele numbers for each gene, and the combination of the allele numbers gives the sequence type.

Seven genes are PCR-amplified and sequenced; all of which are housekeeping genes in *K. pneumoniae*: *rpoB* (β-subunit of RNA polymerase B), *gapA* (glyceraldehyde-3-phosphate dehydrogenase), *mdh* (malate dehydrogenase), *pgi* (phosphoglucose isomerase), *phoE* (phosphoporine E), *infB* (translation initiation factor 2), and *tonB* (periplasmic energy transducer).

A Hot Star Taq Master Mix kit was used (Qiagen) with the same primer concentrations as the $bla_{\text{CTX-M}}$ PCR described under section 3.6.2. PCR program is listed in table 3.11, and primers are listed in appendix A. All genes were amplified at an annealing temperature of 50 °C, except *gapA* (60 °C) and *tonB* (45 °C).

Table 3.11: MLST PCR program as given by the Pasteur website. The original PCR program settings are listed in parenthesis (changed due to different polymerase chemistry).

Cycles	***Temperature (°C)***	***Time***
-	95 (94)	15m (2m)
35	94	20s
	45, 50, 60	30s
	72	30s
-	72	5m

Sequencing was performed as described in section 3.7. The sequences were manually inspected by using SeqScape version 2.5 (Applied Biosystems, Foster City, CA, USA) software. Reference sequences were given at the Pasteur website (link above).

3.7 Sanger sequencing

The *bla*$_{\text{CTX-M group 1}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes were typed by sequencing of amplified PCR products to determine the ESBL$_{\text{A}}$ –encoding gene type.

Sanger sequencing is commonly used when sequencing smaller DNA fragments, for example genes or gene cassettes. The technique is based on the use of dideoxynucleotides (ddNTP's), which have no hydroxyl group on the 3' carbon atom in the sugar. Because the hydroxyl group is lacking, the polymerase cannot elongate the DNA fragment further, effectively terminating the polymerization. Consequently, the DNA fragments will become different sizes, depending on where the terminator base is incorporated. These ddNTP's are added in the reaction solution, along with the template DNA and radioactively labelled primers. The reaction is run on a thermal cycler, and after completion, the DNA fragments are released from the template by denaturing at 95 °C. The sample tube now holds a collection of many different labelled DNA fragments, along with the single-strands of the template DNA [76]. The fragments are then analysed in a capillary electrophoresis instrument to determine the correct nucleotide sequence. The instrument uses gel electrophoresis to separate the fragments by size and thus read the correct nucleotide sequence by laser detection.

The bla$_{\text{CTX-M group 1}}$ genes were sequenced first, then the *bla*$_{\text{SHV}}$. If no ESBL-encoding variants were found, *bla*$_{\text{TEM}}$ was sequenced (if the isolate was positive for *bla*$_{\text{TEM}}$). Primers used for sequencing are listed in table 3.12. Detailed primer information is listed in appendix A. The BigDye Terminator v1.1 sequencing kit (Applied Biosystems) was used. PCR products from the *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ PCRs, described under section 3.6.2, were sequenced.

Table 3.12: Primers used for sequencing of bla$_{\text{CTX-M group 1}}$, bla$_{\text{SHV}}$ and bla$_{\text{TEM}}$ genes.

Gene	***bla*$_{\text{CTX-M group 1}}$**	***bla*$_{\text{SHV}}$**	***bla*$_{\text{TEM}}$**
Sequencing primers	CTX-M-1 F Orf477 R IS*Ecp*-tnpA2 F	*bla*$_{\text{SHV}}$ F *bla*$_{\text{SHV}}$ R Core SHV F Core SHV R	*bla*$_{\text{TEM}}$ F *bla*$_{\text{TEM}}$ R TEM F extra TEM R extra

Prior to sequencing, residual primers and nucleotides were removed from the PCR product by treatment with ExoSAP-IT (Affymetrix, Mercury Park, UK). Protocol used is presented in box 3. The sequencing reaction reagents are listed in table 3.13, and PCR program is listed in table 3.14, taken from the "BigDye Terminator v1.1 Cycle Sequencing Kit Protocol" (found at lifetechnologies.com).

ExoSAP-IT

1. *Add ExoSAP-IT (2.0 µl) to an aliquot of the PCR product (5.0 µl).*
2. *Incubate at 37 °C for 15 minutes.*
3. *Incubate at 80 °C for 15 minutes.*
4. *Add purified PCR product (2.0 µl) to a sequencing master mix (18.0 µl) (table 3.13).*

Box 3: ExoSAP-IT protocol, as given by the manufacturer (Affymetrix).

Table 3.13: Sequencing reaction reagents, concentrations and volumes. This setup was used for all sequencing reactions in this study, regardless of gene analyzed. Source: lifetechnologies.com

Reagent	***Stock concentration***	***Volume(per reaction)***
Water		12.68 µl
BigDye v. 1.1 (Applied Biosystems)	2.5 x	2.0 µl
BigDye buffer (Applied Biosystems)	5 x	3.0 µl
Primer	10 pmol/µl	0.32 µl
PCR product		2.0 µl
Total		20.0 µl

Table 3.14: Sequencing reaction PCR program used for all genes sequenced in this study. Source: lifetechnologies.com.

Cycles	***Temperature***	***Time***
1	95	60 s
25	95	10 s
	54	5 s
	60	4 m

The sequences were cleaned by the ZR DNA Sequencing Clean-up Kit (Zymo Research, Irvine, USA). Protocol used is presented in box 4.

ZR DNA Sequencing Clean-up

1. *Add sequencing binding buffer (240.0 µl) to the sequencing product and transfer to a Zymo-Spin column placed in a collection tube.*
2. *Centrifuge at 13.000 rpm for 30 seconds, discard flow-through.*
3. *Add sequencing wash buffer (300.0 µl) to the column.*
4. *Centrifuge at 13.000 rpm for 30 seconds, discard flow-through.*
5. *Place column in a 1.5 ml Eppendorf tube.*
6. *Add PCR-grade water (20.0 µl) directly to the filter in the column.*
7. *Centrifuge at 13.000 rpm for 30 seconds to elute sequencing product.*
8. *Sequencing product now ready to be analysed.*

Box 4: ZR DNA sequencing clean-up kit protocol, as given by the manufacturer (Zymo Research).

The sequences were analysed on a 3130 Genetic analyser (Applied Biosystems).

The $bla_{CTX\text{-}M\ group\ 1}$, bla_{TEM} and bla_{SHV} sequences obtained were converted to protein sequences using the ExPASy translate tool (http://web.expasy.org/translate/). Possible amino acid changes were detected by comparing the protein sequences with sequences published in the Lahey database, and type of enzyme was determined.

MLST DNA sequences were directly plotted into the Pasteur database for identification of allele numbers.

3.8 Real-Time PCR

To determine the CTX-M group, a group-specific RT – PCR assay was performed on all *bla*$_{CTX-M}$ positive isolates, as previously described [11].

Real-time PCR (RT-PCR) is a method where the amplification of PCR product is visualized in real time, by using a fluorescent probe. There are many probe systems in use, like TaqMan and FRET probes. In this study, TaqMan probes were used. These probes are fitted with one reporter, which is the fluorescent molecule, and one quencher, which inhibits the fluorescence. The probe is designed to anneal somewhere between the primers. When the polymerase polymerize along the DNA strand, the probe will break apart and release the quencher from the reporter, allowing fluorescence. Fluorescence increase when more PCR product is produced, resulting in a sigmoid graph in the software [42].

The RT – PCR was carried out on the Viia7 system (Applied Biosystems). The primers and probes used for this RT-PCR were specifically designed for the CTX-M-1 and CTX-M-9 groups. RT-PCR reagents are listed in table 3.15. Probes are listed in table 3.16, and primers are listed in appendix A. PCR program is presented in table 3.17. Positive controls are listed in appendix A.

Table 3.15: RT-PCR reagents for CTX-M group specific analysis. Modified from Birkett *et al.* 2006 [11].

Reagent	***Volume (per reaction)***
H_2O	9.56 µl
4x TaqMan Fast Virus Master Mix (Qiagen)	5.0 µl
bla$_{CTX-M}$ F (100 µM)	0.18 µl
bla$_{CTX-M}$ R (100 µM)	0.18 µl
CTX-M group specific probe	0.08 µl
Template	5.0 µl
Total	20.0 µl

Table 3.16: RT-PCR probes for the detection of CTX-M groups. Modified from Birkett *et al*. 2006 [11].

Group	***Probe sequence***	***Reporter/Quencher***
CTX-M-1	5'- CCC GAC AGC TGG GAG ACG AAA CGT - 3'	FAM-TAMRA
CTX-M-9	5'- CTG GAT CGC ACT GAA CCT ACG CTG A - 3'	VIC-TAMRA

Table 3.17: RT-PCR program for CTX-M group identification on the Viia7 system.

Stage	***Cycles***	***Temperature (°C)***	***Time***
Pre-read	-	50	30 s
Hold	-	50	5 m
		95	20 s
PCR	40	95	3 s
		60	30 s
Post-read	-	50	30 s

3.9 Pulsed field gel electrophoresis

All isolates were subjected to pulsed field gel electrophoresis (PFGE) for genotyping.

PFGE is a method used in many microbiology labs to determine whether the isolates in question are clonally related. The method is derived from the classic gel electrophoresis, which separates DNA-fragments on the basis of their size and therefore also their charge. However, in conventional gel electrophoresis there is only one source of current, and the flow is unidirectional. In PFGE, the current usually flows from three different sources, with an angle of 120 degrees between each electrode. These electrodes are switched on and off based on timers which are specified by the user. However, the unidirectional current is always flowing [23].

PFGE produces better separation compared to conventional gel electrophoresis. Plugs with suspended, lysed cells are treated with a restriction enzyme that cut DNA at specific recognition sites unique to the enzyme. This generates DNA fragments of various sizes, depending on the genome of the microbe analysed. Each isolate will thus produce a specific band pattern on the gel.

An enzyme commonly used for *K. pneumoniae* PFGE is *XbaI*, which has the following recognition site [2]:

```
      ▼
5’ – T CTAGA - 3’
3’ – AGATC T – 5’
           ▲
```

Protocol used is presented in box 5. The isolates were analysed using the standard operating procedure for PulseNet PFGE of various Enterobacteriaceae [24]. All solutions used in the PFGE protocol are listed in section 3.12, table 3.26.

XbaI – PFGE

1. *Inoculate 10 isolates on blood agar and incubate for 20-24 hours at 35 °C.*
2. *Suspend bacterial colonies in cell suspension buffer (2.0 ml) at a concentration of 3-4 McFarland.*
3. *Mix the cell suspension (200.0 µl) with 20 mg/ml Proteinase K (10.0 µl), then add 1% SeaKem Gold agarose[1] (200.0 µl), mix briefly and transfer to PFGE plug moulds.*
4. *After solidification, lyse the plugs at 55 °C for 1.5 – 2.0 hours with agitation in a cell lysis buffer (5.0 ml) with added Proteinase K (25.0 µl).*
5. *Wash plugs twice with pre-heated dH_2O (55 °C, 10 - 15 ml) for 10-15 minutes at 55 °C, then four times with pre-heated TE-buffer (55 °C, 10-15 ml) for 10 – 15 minutes at 55 °C.*
6. *Store washed plugs in TE-buffer at 4 °C.*
7. *Wash plugs twice with pre-heated TE-buffer after storage, before use.*
8. *Cut 1.5 mm pieces of the plugs and incubate at room temperature for 10 – 15 minutes in 1:10 diluted XbaI[2] restriction buffer (200.0 µl).*
9. *Incubate plugs at 37 °C for 1.5 – 2.0 hours in XbaI restriction enzyme[2] mix (table 3.18).*
10. *Prepare a 1.0 % SeaKem Gold agarose gel and equilibrate at 55 °C.*
11. *Fill the electrophoresis chamber with 0.5 x TBE buffer (2.0 – 2.2 L). Calibrate pump to pump one litre per minute, and set temperature to 14 °C.*
12. *Incubate the plugs in 0.5 x TBE buffer (200.0 µl) for five minutes at room temperature.*
13. *Mount the plugs on the gel comb and fix with agarose. Pour the gel, remove the comb and add lambda ladder[2].*
14. *Seal wells with agarose and run the gel with the settings presented in table 3.19.*
15. *Dye gel with ethidium bromide solution for 20-30 minutes, and then de-stain with dH_2O for 60 – 90 minutes with water change every 20 minutes.*
16. *Depict gel.*

[1] *BioRad, Oslo, Norway*
[2] *New England Biolabs, Ipswich, UK*

Box 5: *XbaI* PFGE protocol [24].

The gel was depicted in ChemiDoc XRS+ (BioRad), and the image was analysed in Bionumerics (Applied Maths, St-Martens-Latem, Belgium). A phylogenetic tree was devised from the band relations by using the Dice coefficient with 1.5 % position tolerance. Clustering analysis was performed by the unweighted pair group method with arithmetic mean (UPGMA). Isolates above the 80 % similarity mark were considered closely related.

Table 3.18: *XbaI* restriction enzyme mix used in PFGE of *K. pneumoniae*, in accordance with the PulseNet central Standard Operating Procedure for PulseNet PFGE of various Enterobacteriaceae. BSA = bovine serum albumin.

Reagent	***Volume (per sample)***
dH_2O	173.0 µl
Restriction enzyme buffer (10x)	20.0 µl
BSA (10 mg/ml)	2.0 µl
XbaI (10 U/µl)	5.0 µl
Total	200 µl

Table 3.19: PFGE program parameters for *K. pneumoniae* used at Stavanger University Hospital, provided by PulseNet Central for various Enterobacteriaceae.

Parameter	***Value***
Pulse Time	1 – 20 s
Total run time	21 H
Voltage	6.0 v/cm (200 V)
Angle	120°
Buffer temperature	14 °C
Buffer	0.5 x TBE

Some isolates produced smears on the PFGE gels. An addition of thiourea (2.20 ml of a 300 mM solution, with a final concentration of 300 µM) into the running buffer (2.20 L) prompted the isolates to produce visible bands, as previously described [78].

3.10 Plasmid detection

The CTX-M-15 and SHV-5 group isolates were treated with the enzyme *S1*-nuclease and subsequently subjected to PFGE to detect plasmids present in these isolates.

The detection of plasmids and their size using *S1*-PFGE has previously been described [7].The method is based on the enzyme S1-nuclease, which was isolated from the mould *Aspergillus orzyae*. The enzyme attacks single-stranded regions, and for plasmids that are negatively supercoiled, this will lead to a linearization of the plasmid due to the nicking action of the enzyme and loss of supercoiling. By linearizing plasmids, one can separate them by size with PFGE, and thus find out how many plasmids an isolate carry and how big they are.

Protocol used is presented in box 6. All solutions used in the *S1*-PFGE protocol are listed in section 3.12, table 3.27.

S1-PFGE

1. *Cut 1.5 mm slices from the plugs and wash twice in TE-buffer (1.0 ml) at room temperature for 30 minutes.*
2. *Incubate plugs with Tris-HCl (1.0 ml) for 30 minutes at room temperature.*
3. *Wash plugs with 1:10 diluted S1-nuclease[1] buffer (125.0 µl) for 30 minutes.*
4. *Incubate plugs with S1-nuclease[1] mix (table 3.20) at 37 °C for 25 minutes.*
5. *Inactivate the enzyme with 20mM EDTA (100.0 µl) and incubate for 2-5 minutes.*
6. *Wash plugs twice with cold TE-buffer (1.0 ml) for 30 minutes.*
7. *Prepare a 1.0 % agarose gel with Pulsed Field certified agarose[2] and 0.5 x TBE buffer and equilibrate to 55 °C.*
8. *Fix the plugs and pour the gel in a similar manner as described in box 5.*
9. *Add low-range PFG marker[3] as ladder.*
10. *Run the gel with the parameters listed in table 3.21.*

[1]Takara, Saint-Germain-en-Laye, France
[2]BioRad
[3]New England Biolabs

Box 6: S1-nuclease PFGE protocol, modified from Naseer *et al.* 2009 [62]. Additional references: [7, 56, 74].

Table 3.20: S1-nuclease enzyme mix used for *S1*-PFGE. The volume of enzyme depends on the unit amount per µl of the stock solution.

Reagent	***Volume***
S1-nuclease buffer (10x) (*Takara*)	12.5 µl
S1-nuclease (180 U/µl) (*Takara*)	0.23 µl
dH_2O	112.26 µl
Total	125 µl

Table 3.21: PFGE parameters used for *S1*-PFGE. The parameters are designed to effectively separate smaller DNA fragments. Modified protocol from Naseer *et al*. 2009 [62].

Parameter	***Value***
Pulse Time	1-20 s
Run Time	15 H
Voltage	6.0 v/cm (200 V)
Angle	120°
Buffer temperature	15 °C
Buffer	0.5 x TBE

The plasmids were sized using the ChemiDoc XRS+ Image Lab software (BioRad).

The isolates that produced smears in PFGE also did in *S1*-PFGE. The addition of thiourea as described in section 3.9 solved the issue.

3.11 Southern blot hybridization

The CTX-M-15 and SHV-5 group isolates were analysed by Southern blot and hybridization to localize the $ESBL_A$ –encoding gene and detect the replicon type of the plasmid.

Southern blot is a classic molecular technique that is based on the transfer of DNA from a gel to a membrane by capillary forces, vacuum transfer or other methods. Southern blot was developed by Edwin Southern in 1975, as a way to detect specific nucleotide sequences in separated and digested DNA fragments [16]. The gel is pre-treated with HCl to depurinate the DNA, then with NaOH to denature. This is done to ease transfer of DNA to the membrane. The membrane is then hybridized with a probe, which can be visualized in a variety of ways, including colorimetry, fluorescence, or radioactive labelling. Various applications of Southern blot have been developed. Specific probes can easily be made by PCR, which makes this method especially useful.

Probes for Southern blot hybridization are quick and easy to synthesize to date, due to effective PCR methods. The probe is a DNA sequence that complements the gene sequence of interest, labelled with DIG-dUTP (digoxigenin-11-2'-deoxy-uridine-5'-triphosphate), which is incorporated in the PCR reaction (figure 3.3) [43].

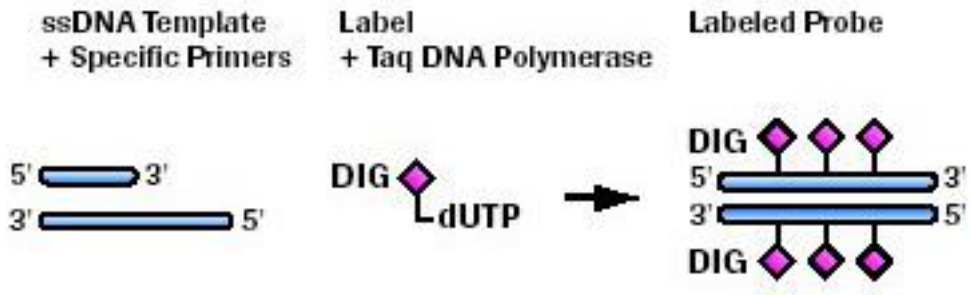


Figure 3.3: DIG-dUTP PCR labeling reaction. The DIG-dUTP is incorporated by the polymerase due to the deoxy-uridine-triphosphate end of the molecule, which is used as a nucleotide by the polymerase. Source: lifescience.roche.com, used with permission from Roche Diagnostics Norway.

The DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany), utilizes colorimetric detection to visualize bands on the membrane. Antibodies, specifically fragments of polyclonal anti-digoxigenin, will bind to the DIG-labelled probe which has been hybridized to the fixated nucleic acids on the membrane. A blocking solution blocks any unwanted bonding of these antibodies. The antibodies are coupled with an alkaline phosphatase, which will catalyse the colour reaction needed for colorimetric detection. The colour substrate solution contains BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate / nitroblue tetrazolium chloride) which will be dephosphorylated by the alkaline phosphatase added previously. The following redox reaction that takes place will produce a purple precipitate at the site of enzyme activity, and will form the bands on the membrane [43].

In this study, Southern blot was used to link plasmids identified with S1-PFGE with the replicon type and $ESBL_A$-gene previously found, by the use of a specific probe which targets the $bla_{CTX\text{-}M}$ and bla_{SHV}-genes. Also, an FII_K-probe was made to determine if the plasmid was carrying an FII_K-replicon along with the $ESBL_A$-gene. The DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics) was used to make the probes and the post-transfer and -hybridization steps. All solutions used in the Southern blot protocol are listed in section 3.12, table 3.28.

3.11.1 Synthesis of hybridization probes

PCR and cleanup

One *bla*$_{CTX-M}$, one *bla*$_{SHV}$ and one IncFII$_K$ probe was made. A PCR was performed on these genes as described under section 3.6.2. (The IncFII$_K$ primers (appendix A) and PCR program (table 3.22) was found in Villa *et al.* 2010 [86]). The IncFII$_K$ PCR used the same reagents and concentrations as the *bla*$_{CTX-M}$ PCR listed in section 3.6.2.

Table 3.22: IncFII$_K$ PCR program. The original PCR program settings are in parentheses [86].

Cycles	*Temperature (°C)*	*Time*
-	95 (94)	15 m (5 m)
30	94	60 s
	60	30 s
	72	60 s
-	72	5 m

The PCR product was then purified by the QIAquick PCR purification kit (Quiagen), protocol presented in box 7.

QIAquick PCR purification kit

1. *Add five volumes buffer PB to one volume PCR product and transfer to a QIAquick spin column with a collection tube.*
2. *Centrifuge at 13.000 rpm for 30-60 seconds, and discard flow-through.*
3. *Add buffer PE (750.0 µl) to the spin column.*
4. *Centrifuge at 13.000 rpm for 30-60 seconds.*
5. *Discard flow-through and centrifuge at 13.000 rpm for 30-60 seconds to remove residual ethanol.*
6. *Place column in clean 1.5 ml tube and add buffer EB (30.0 µl) directly onto the filter.*
7. *Incubate one minute, then centrifuge at 13.000 rpm for 30-60 seconds, to elute the DNA.*

Box 7: QIAquick PCR purification kit protocol, as given by the manufacturer (Qiagen).

Following the purification, the concentration of the PCR product needed to be ascertained. This was done with the Qubit assay (Invitrogen, Thermo Scientific, Massachusetts, USA), presented in box 8.

Qubit assay

1. *Prepare one assay tube per sample and two tubes for the standards.*
2. *Dilute the Qubit reagent 1:200 in Qubit buffer. Prepare approximately 200 µl per tube.*
3. *Dilute each standard (broad range) (10.0 µl) in the working solution (190 µl).*
4. *Dilute samples (2.0 µl) in the working solution (198 µl).*
5. *Vortex all tubes and incubate for two minutes at room temperature.*
6. *Analyse on the Qubit 2.0 fluorometer, following machine instructions.*

Box 8: Qubit assay protocol, as given by the manufacturer (Invitrogen).

The DNA was now ready to be labelled with DIG-dNTP, protocol presented in box 9.

Template labelling

1. *Dilute one microgram of template with water, to a total volume of 16.0 µl.*
2. *Denature the template by boiling at 100 °C for 10 minutes, then rapidly cool.*
3. *Add DIG-High Prime (4.0 µl) to the template, mix gently, and incubate at 37 °C for 20 hours.*
4. *Heat the sample to 65 °C for 10 minutes to stop the reaction.*
5. *Calculate reaction yield from table 3.23.*

Box 9: Template labelling protocol, from the DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche).

Table 3.23: DIG-High Prime labelling reaction yield after one and 20 hours. Optimal template amount was deemed to start at 1000 ng, with a total yield of 2300 ng after 20 hours (as stated by the manufacturer).

Template DNA	***1 Hour***	***20 Hours***
10 ng	45 ng	600 ng
30 ng	130 ng	1050 ng
100 ng	270 ng	1500 ng
300 ng	450 ng	2000 ng
1000 ng	850 ng	2300 ng
3000 ng	1350 ng	2650 ng

Prior to utilization, the final concentration of the probes had to be calculated, and their effectiveness had to be ascertained. The probes and a DIG-labelled control were diluted to a concentration of 1 ng/µl, and a series of dilutions were made (table 3.24).

Table 3.24: Dilution series for testing of probes made for Southern blot. The column «DNA (µl)» describes how many µl of the probe and the control that are used, and the column «from tube...» refers to which tube the volume is taken from.

ID	*DNA (µl)*	*From tube…*	*DNA dilution buffer (µl)*	*Dilution*	*End concentration*
1	-	Diluted original	-	-	1 ng/µl
2	2	1	198	1:100	10 pg/µl
3	15	2	35	1:3.3	3 pg/µl
4	5	2	45	1:10	1 pg/µl
5	5	3	45	1:10	0.3 pg/µl
6	5	4	45	1:10	0.1 pg/µl
7	5	5	45	1:10	0.03 pg/µl
8	5	6	45	1:10	0.01 pg/µl
9	0	-	50	-	0

The serially diluted probes were then fixated to a membrane and detected, protocol presented in box 10.

Probe fixation and detection

1. *Add diluted probes (1.0 µl) to a marked, positively charged nylon membrane[1] and fixate at 100 °C for two hours.*
2. *Incubate membrane in maleic acid buffer (30.0 ml) at room temperature for two minutes.*
3. *Incubate membrane in blocking solution (10.0 ml) for 30 minutes with agitation.*
4. *Incubate membrane in antibody solution (10.0 ml) for 30 minutes with agitation.*
5. *Wash membrane twice with washing buffer (10.0 ml) for 15 minutes.*
6. *Incubate membrane in detection buffer (10.0 ml) for 2-5 minutes.*
7. *Incubate in the dark with colour substrate solution (2.0 ml) for approximately 30 minutes, or until desired colour intensity has been reached.*
8. *Wash membrane with dH_2O to stop the reaction.*

[1]Hybond N+, Amersham

Box 10: Probe fixation and detection protocol, from the DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche).

The colour intensity of the probes was compared to the control DNA, and the approximate concentration was calculated (table 3.25).

Table 3.25: Calculated probe concentrations for all three probes used in the southern blot analysis. The concentrations are approximate values.

Probe	*Concentration*
$bla_{\text{CTX-M}}$	37.5 ng/ml
bla_{SHV}	7.6 ng/ml
IncFII_{K}	25 ng/ml

3.11.2 Transfer and detection

Pre-treatment and transfer

A vacuum blotting system was used for the transfer of *S1*-PFGE gel to a positively charged membrane by the use of a vacuum Blotter model 785 (Bio Rad), protocol presented in box 11.

Pre-treatment and transfer

1. *Wash gel in pure water, then depurinate with 0.25 N HCl for 30 minutes with agitation.*
2. *Wash gel twice in pure water, then denature with 0.5 N NaOH for 40 minutes.*
3. *Neutralize with neutralizing buffer for 30 minutes.*
4. *Wash gel with 10 x SSC buffer.*
5. *Moisten positively charged nylon membrane and filter paper in dH_2O and 10 x SSC buffer.*
6. *Place filter paper and membrane on vacuum membrane. Assemble the rest of the vacuum blotter.*
7. *Place gel on top of membrane, remove any bubbles.*
8. *Calibrate vacuum pump to 10 in Hg. Check system for leaks.*
9. *Add 10 x SSC buffers (1.0 – 1.5 L) to the transfer chamber.*
10. *Transfer for 90 minutes.*
11. *Wash membrane in 2 x SSC buffer, then air dry.*
12. *Fixate overnight at 100 °C.*

Box 11: Gel pre-treatment and transfer protocol, as given by the Vacuum blotter manufacturer (BioRad).

Hybridization and immunological detection

The membrane was now ready for hybridization and subsequent detection. Protocol presented in box 12.

Hybridization and immunological detection

1. *Put fixated membrane inside a hybridization tube with the nucleic acids facing the middle of the tube.*
2. *Add pre-heated (40 °C) DIG Easy Hyb buffer (40.0 ml) to the tube and pre-incubate membrane for 30 minutes with rotation.*
3. *Denature DIG-labelled probe by boiling for five minutes, then cool. Add denatured probe to pre-heated (40 °C) DIG Easy Hyb buffer to a final concentration of 25 ng/ml (concentration depends on the effectivity of the probe).*
4. *Add probe mix to the tube and incubate at 40 °C overnight with rotation.*
5. *The DIG Easy Hyb buffers may be re-used several times. Simply freeze the solutions between each use, and denature the probe at 68 °C for 10 minutes prior to hybridization.*
6. *Wash membrane in stringency wash buffer twice at room temperature for five minutes with agitation. Wash again with pre-heated (68 °C) stringency wash buffer twice at 68 °C for 15 minutes with agitation.*
7. *Wash membrane in washing buffer for 1 – 5 minutes.*
8. *Incubate membrane with blocking solution (200.0 ml) for 30 minutes.*
9. *Incubate membrane with antibody solution (60.0 ml) for 30 minutes.*
10. *Wash membrane twice with washing buffer (200.0 ml) for 15 minutes.*
11. *Stabilize with detection buffer (60.0 ml) for 2 – 5 minutes.*
12. *Incubate in the dark with colour substrate solution (10.0 – 20.0 ml) until desired colour intensity is reached (30 minutes – 16 hours).*
13. *Stop the reaction by washing with dH_2O.*

Box 12: Hybridization and immunological detection protocol, from the DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche).

Bands were detected and compared with the *S1*-gel for identification.

3.12 Solutions

Table 3.26: Reagents used for *XbaI*-PFGE.

Solution	***Contents***	***Origin***
TE-buffer	10 mM Tris, 1 mM EDTA, pH 8.0	In-house
Cell suspension buffer	100 mM Tris, 100 mM EDTA, pH 8.0	In-house
Cell lysis buffer	50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl	In-house
Ethidium bromide solution	1.0 ng / ml Ethidium bromide	In-house

Table 3.27: Reagents used for *S1*-PFGE.

Solution	***Contents***	***Origin***
TE-buffer	10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0	In-house
Tris-HCl	1.0 M Tris-HCl, pH 7.5	In-house
EDTA	20 mM EDTA	In-house

Table 3.28: Reagents used for Southern blot and hybridization.

Solution	***Contents***	***Origin***
Pure water	Filtered dH_2O	In-house
Depurination solution	0.25 N HCl	In-house
Denaturation solution	0.5 N NaOH	In-house
Neutralizing buffer	1 M Tris-HCl, 1.5 M NaCl	Sigma Aldrich, Oslo, Norway
20x SSC buffer	3 M NaCl, 300 mM trisodium citrate, pH 7.0	Sigma Aldrich
Stringency wash	2x SSC (Sigma Aldrich), 0.1 % SDS (Sigma Aldrich)	In-house
10 x Washing buffer	0.1 M maleic acid, 0.15 M NaCl, 0.3 % (v/v) Tween 20, pH 7.5	DIG Wash and Block buffer set
Detection buffer	0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5	In-house
Maleic acid buffer	0.1 M Maleic acid, 0.15 M NaCl, pH 7.5	DIG Wash and Block buffer set
Dig Easy Hyb buffer	Urea*	DIG High Prime DNA Labelling and Detection Starter Kit I
Blocking solution	Pre-treated casein*	DIG High Prime DNA Labelling and Detection Starter Kit I
Antibody solution	Polyclonal sheep anti-DIG antibodies + alkaline phosphatase*	DIG High Prime DNA Labelling and Detection Starter Kit I
Colour substrate solution	BCIP/NBT*	DIG High Prime DNA Labelling and Detection Starter Kit I
DNA dilution buffer	50 µg/ml fish sperm DNA in 10 mM Tris-HCl, 1 mM EDTA, pH 8	DIG High Prime DNA Labelling and Detection Starter Kit I

**Concentrations not given by the manufacturer*

4. Results

4.1 Species identification and phenotypical analysis

To confirm species identification and $ESBL_A$ – production selected isolates (n = 58) were inoculated on chromogenic ESBL medium and modified MacConkey medium with a cefotaxime disk. In total, 49 isolates were putative $ESBL_A$ – producing *K. pneumoniae* and included for further analysis.

The 49 isolates were identified by MALDI-TOF as *K. pneumoniae* subsp. *pneumoniae,* with an average score of 2.29 ± 0.066. AST by Vitek 2 was successful on all isolates, except two, where manual susceptibility testing was performed. Detailed results are listed in appendix B. Co-resistance profiles are presented in figure 4.4.

4.2 Enzyme characterization

The included isolates were screened for the $ESBL_A$-genes bla_{CTX-M}, bla_{TEM} and bla_{SHV}. Twenty-six were positive for bla_{CTX-M}, 44 positive for bla_{SHV}, and 35 positive for bla_{TEM} (table 4.1). Of the 26 bla_{CTX-M} positive isolates, 22 were categorized as $bla_{CTX-M\ group\ 1}$, and two as $bla_{CTX-M\ group\ 9}$. Two isolates could not be typed into a specific CTX-M group.

$ESBL_A$ – encoding genes were further typed through sequencing. The ESBL genes found are as follows: $bla_{CTX-M-15}$ (n = 20), $bla_{CTX-M-3}$ (n = 1), bla_{SHV-12} (n = 5), bla_{SHV-2} (n = 1), bla_{SHV-27} (n = 1), bla_{SHV-2A} (n = 3) and bla_{SHV-5} (n = 13). The CTX-M – encoding gene could not be typed in five isolates. $ESBL_A$ – encoding genes for each isolate are listed in table 4.1. One TEM – encoding gene was sequenced, and was found to be a non-$ESBL_A$ – encoding gene.

Table 4.1: Screening for $ESBL_A$ – encoding genes by PCR and sequencing. $ESBL_A$ – encoding genes are listed in red (as found in the Lahey database).

ID	*PCR*			*Sequencing*		
	CTX-M	*TEM*	*SHV*	*CTX-M*	*TEM*	*SHV*
1a	-	+	+	-	*	SHV-5
1b	-	+	+	-	*	SHV-5
2	+	-	+	UT	-	SHV-11
3	-	+	+	-	*	SHV-12
4	-	+	+	-	*	SHV-5
5	+	-	-	UT	-	-
6	-	-	+	-	-	SHV-5
7	-	-	+	-	-	SHV-5
8	+	+	-	UT	*	-
9	-	-	+	-	-	SHV-11
10	+	+	+	CTX-M-15	*	SHV-1
11	-	+	+	-	*	SHV-5
12	-	+	+	-	*	SHV-5
13	+	+	+	CTX-M-15	*	SHV-52
14	+	+	+	CTX-M-15	*	SHV-11
15	-	+	+	-	*	SHV-5
16	+	-	+	CTX-M-15	-	SHV-1
17	+	+	+	CTX-M-15	*	SHV-11
18	+	+	+	CTX-M-15	*	SHV-11
19	+	+	+	CTX-M-15	*	SHV-52
20a	+	+	+	CTX-M-15	*	SHV-11
20b	+	+	+	CTX-M-15	*	SHV-11
22	+	+	+	CTX-M-15	*	SHV-11
23	-	-	+	-	-	SHV-2A
25	+	+	+	CTX-M-15	*	SHV-27
28	+	-	+	UT	-	SHV-11
29	+	+	+	CTX-M-15	*	SHV-11
32	+	+	+	CTX-M-15	*	SHV-11
33	+	-	+	UT	-	SHV-1
34	-	-	+	-	-	SHV-5
35	+	+	+	CTX-M-15	*	SHV-2A
36	-	+	+	-	*	SHV-5
37	+	+	+	CTX-M-15	*	SHV-1
38	-	-	+	-	-	SHV-12
39	-	+	+	-	*	SHV-2
40	+	+	+	CTX-M-15	*	SHV-1
42	-	+	+	-	*	SHV-5
43	-	+	+	-	TEM-57	SHV-33
44	-	-	+	-	-	SHV-12
45	+	+	-	CTX-M-15	*	-
46		+	+	-	*	SHV-5
47	+	+	+	CTX-M-15	*	SHV-11
48	+	+	+	CTX-M-15	*	SHV-32
51	-	-	+	-	-	SHV-12
53	-	+	+	-	*	SHV-12
54	-	+	+	-	*	SHV-5
55	+	+	-	CTX-M-15	*	-
56	+	+	-	CTX-M-3	*	-
57	-	-	+	-	-	SHV-2A

*UT = untypeable. * = Not sequenced.*

4.3 Determination of clonal relatedness

4.3.1 PFGE

All isolates were subjected to *XbaI* – PFGE for detection of clonal relatedness. Band patterns were analysed in Bionumerics, and a phylogenetic tree was devised from the band relations (figure 4.4). In general, two clones were identified. Some similarity between some of the isolates was detected. Six nodes were found above the 80 % similarity mark. All SHV-5 group isolates grouped at around 88 %. Isolate 29 and 34 grouped at 89 %, 20a and 20b at 90 %, 17 and 18 at 100 %, 13 and 19 at 92 %, and 51 and 57 at 84 % (figure 4.4).

4.3.2 MLST

To further characterize the isolates, the sequence types of the CTX-M-15 group and the SHV-5 group isolates were identified by MLST. All isolates were successfully typed. The following STs were identified: ST4 (1), ST15 (1), ST17 (4), ST25 (1), ST29 (13), ST48 (2), ST111 (1), ST186 (1), ST277 (1), ST298 (1), ST307 (1), ST348 (1), and ST485 (2). Additionally, two potentially new allele combinations were found. Detailed results are listed in table 4.2.

Table 4.2: MLST results. Isolate ID, allele combinations and sequence type is given for each isolate. All allele combinations were found at the Pasteur database, with the exception of the two possibly new combinations (ID 32 and 48).

Group	*ID*	*rpoB*	*gapA*	*mdh*	*pgi*	*phoE*	*infB*	*tonB*	*ST*
CTX-M-15 group	10	4	2	2	2	6	3	4	29
	13	1	2	1	1	7	1	12	485
	14	3	3	1	1	3	1	1	4
	16	4	2	1	37	45	1	9	186
	17	4	2	1	1	4	1	4	17
	18	4	2	1	1	4	1	4	17
	19	1	2	1	1	7	1	12	485
	20a	4	2	1	1	4	1	4	17
	20b	4	2	1	1	4	1	4	17
	22	4	2	1	1	10	1	13	25
	25	1	3	1	1	1	1	43	277
	29	4	2	5	1	17	1	42	111
	32	4	4	2	1	9	1	12	New
	35	15	2	20	1	12	1	16	348
	37	1	1	1	1	1	1	1	15
	40	1	4	2	52	1	1	7	307
	45	1	2	2	1	1	1	7	298
	47	1	2	2	2	7	5	10	48
	48	4	2	17	1	20	6	25	New
	55	1	2	2	2	7	5	10	48
SHV-5 group	1a	4	2	2	2	6	3	4	29
	1b	4	2	2	2	6	3	4	29
	4	4	2	2	2	6	3	4	29
	6	4	2	2	2	6	3	4	29
	7	4	2	2	2	6	3	4	29
	11	4	2	2	2	6	3	4	29
	12	4	2	2	2	6	3	4	29
	15	4	2	2	2	6	3	4	29
	36	4	2	2	2	6	3	4	29
	42	4	2	2	2	6	3	4	29
	46	4	2	2	2	6	3	4	29
	54	4	2	2	2	6	3	4	29

4.4 Detection of plasmid replicon types

PBRT was performed on the CTX-M-15 group isolates to detect 27 different plasmid replicon types. Additionally, the M6 PCR (Inc U, X1, R, and FII_K) was performed on the SHV-5 group isolates to screen for the presence of $IncFII_K$ plasmids. Replicon types detected are listed in table 4.3. In general, the most dominant replicon types were $IncFII_K$ and IncFIB. The CTX-M-15 group isolates contained 1-3 replicon types. All SHV-5 group isolates were positive for $IncFII_K$.

Table 4.3: PCR-based replicon typing results.

ESBL	*ID*	*Replicon type*	*ID*	*Replicon type*
CTX-M-15 group	10	R, FII, FII_K	25	FIB, FII_K
	13	I1, FII_K	29	FIB, FII_K
	14	I1, FII_K	32	FIB, FII_K
	16	FIB, R	35	FIB, FII_K
	17	R, FII_K	37	FII_K
	18	R, FII_K	40	FIB
	19	I1, FIB, FII_K	45	FIB, FII_K
	20a	R, FII_K	47	FII_K
	20b	R, FII_K	48	FIB, FII_K
	22	FIB, FII_K	55	R, FII_K
SHV-5 group	1a	FII_K	12	FII_K
	1b	FII_K	15	FII_K
	4	FII_K	36	FII_K
	6	FII_K	42	FII_K
	7	FII_K	46	FII_K
	11	FII_K	54	FII_K

4.5 Detection of plasmid number and sizes

The CTX-M-15 group and SHV-5 group isolates were subjected to *S1* – PFGE to detect any similar plasmids. A ~230 kbp plasmid was detected in all SHV-5 group isolates. Several plasmids of different sizes were detected among the CTX-M-15 group isolates (figure 4.1).

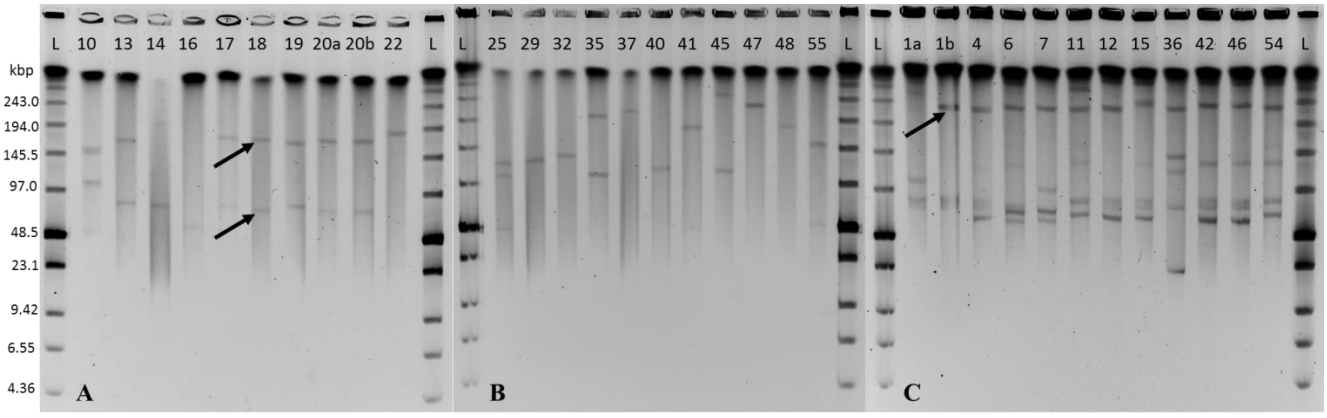


Figure 4.1: *S1*-PFGE of the CTX-M-15 group isolates (A and B) and the SHV-5 group isolates (C). First and last lanes in each picture are the low-range ladders (New England Biolabs). Each band of the low-range ladder is sized to the far left (kbp).In the sample lanes one band represents one plasmid. Similarly sized plasmids are visible on image C (~ 230 kbp), see arrow. Plasmids across five isolates in the first image are of the same approximate size (~ 170 kbp and 70 kbp), as indicated by the arrows.

4.6 Location of $ESBL_A$ – encoding genes on specific plasmids

Southern blot was used to link the $ESBL_A$ – encoding gene in the CTX-M-15 and SHV-5 group isolates to a specific plasmid, and furthermore to determine the size and replicon type of the plasmid carrying the $ESBL_A$ – encoding gene. Southern blot hybridization was successful on two of six membranes, one membrane applying the $bla_{CTX\text{-}M}$ probe and one membrane applying the bla_{SHV} probe. Hybridization with $IncFII_K$ probe on three membranes and one membrane with $bla_{CTX\text{-}M}$ probe gave no colorimetric signals. In the SHV-5 group isolates the $ESBL_A$ – encoding gene was carried by a ~230 kbp plasmid (figure 4.2). Plasmids bearing the $bla_{CTX\text{-}M\text{-}15}$ gene were found to be of diverse sizes.

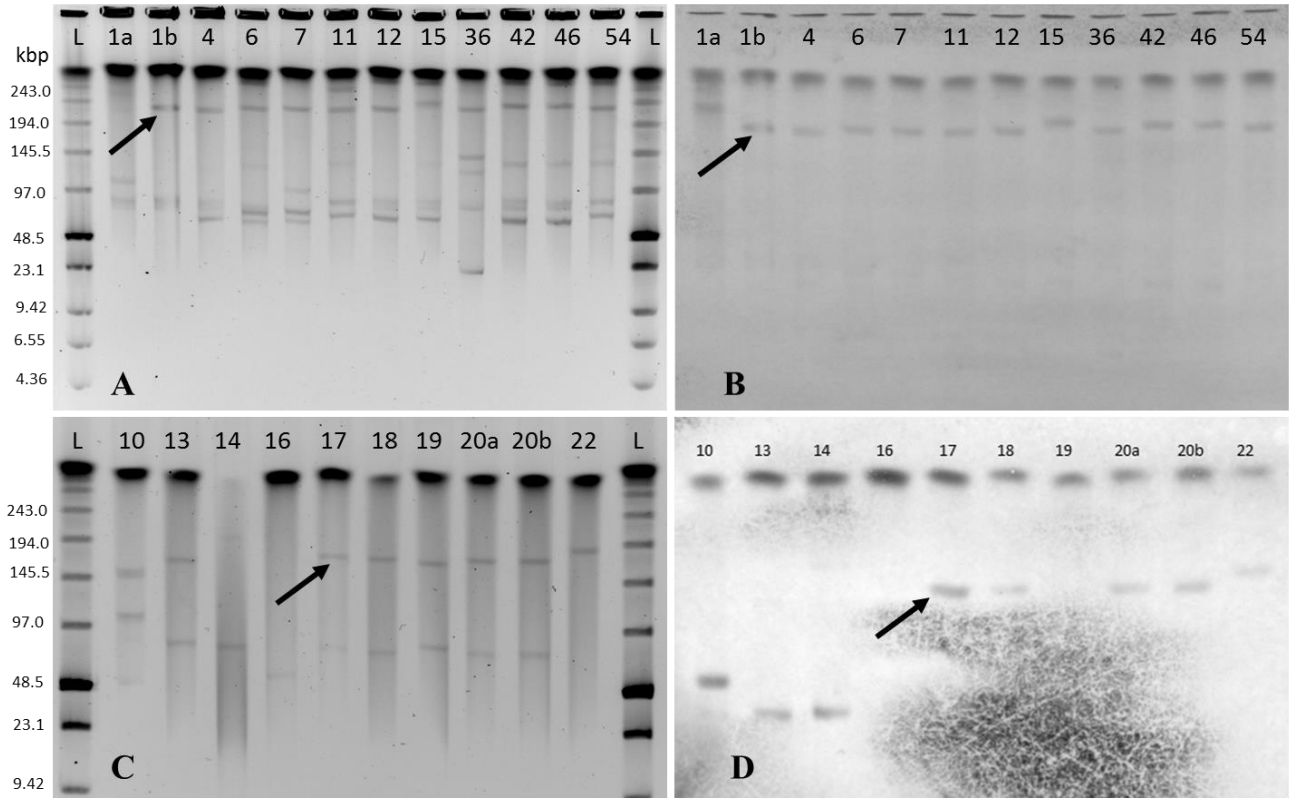


Figure 4.2: *S1*-PFGE of SHV-5 (A) and CTX-M-15 (C) group isolates and Southern blot hybridization with bla_{SHV} probe (B) and $bla_{CTX\text{-}M}$ probe (D). Low range PFG marker (New England Biolabs) band sizes are listed on the left (kbp). The bla_{SHV}-hybridized bands were approximately 230 kbp, indicated by the arrow in A and B. The $bla_{CTX\text{-}M}$ hybridized bands ranged from 80 to 170 (arrow in C and D) kbp.

4.7 Capsular typing and virulence gene screening

The SHV-5 group isolates were screened for capsular types and virulence genes commonly present in hypervirulent *K. pneumoniae* strains by PCR and gel electrophoresis (figure 4.3). None of the capsular types or virulence genes screened for was detected.

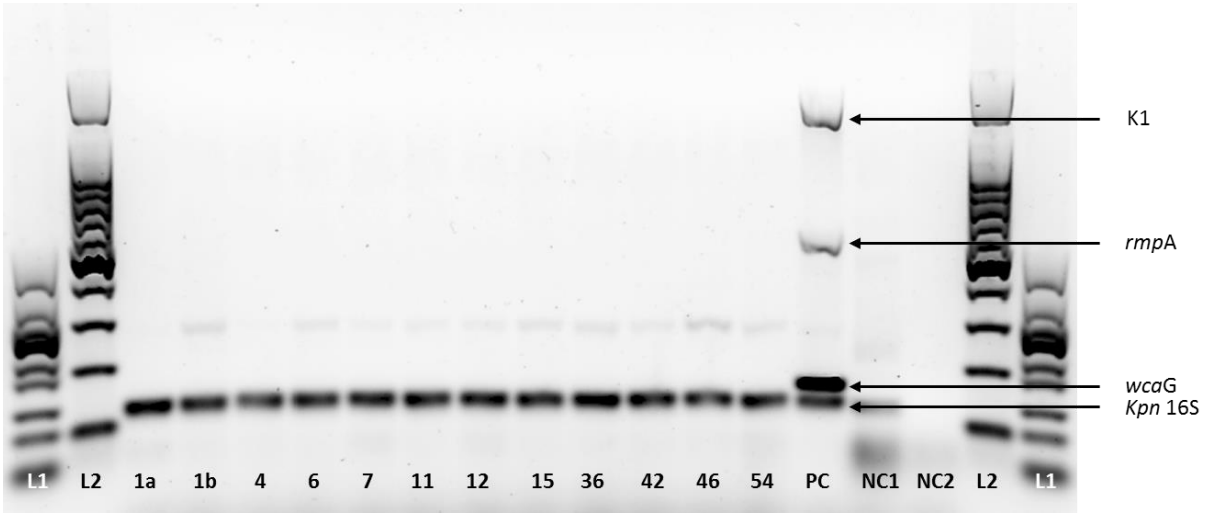
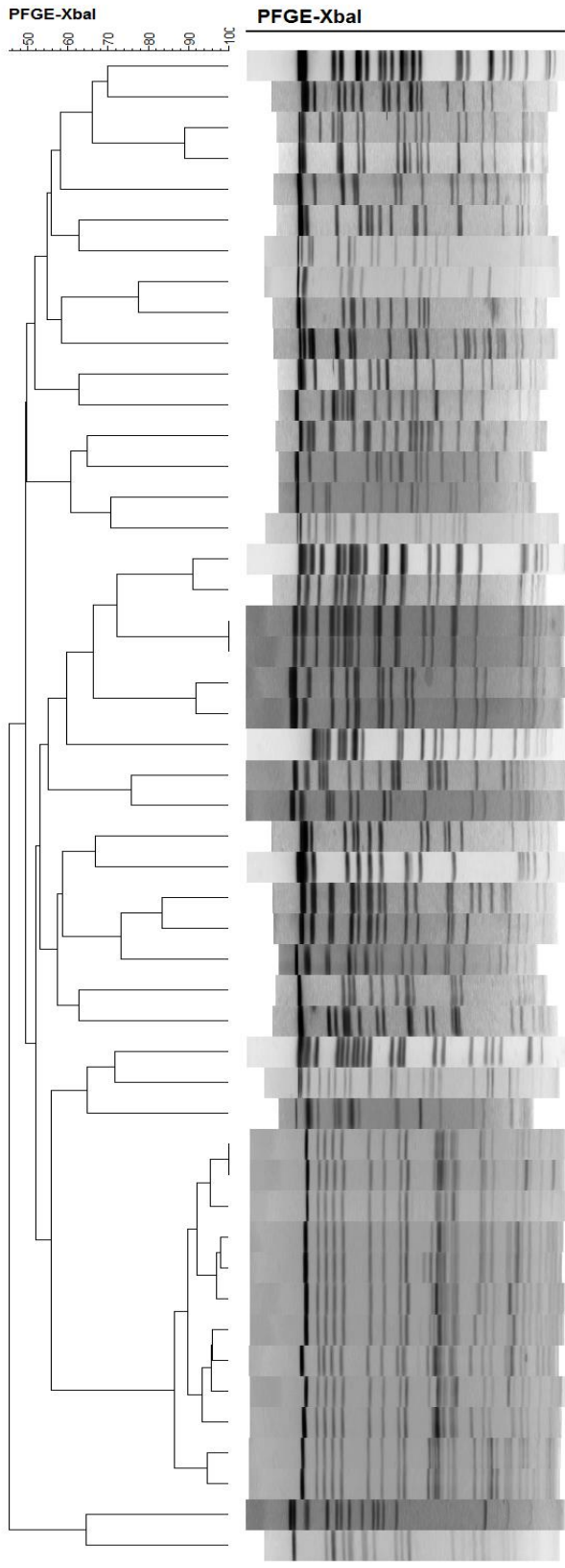


Figure 4.3: Results for capsule typing and virulence gene detection of the SHV-5 group isolates. L1 and L2 are 50 bp and 100 bp plus ladders (Qiagen), respectively. NC1 = negative control 1, a *K. pneumoniae* isolate negative for all targeted genes except Kpn 16S rRNA. NC2 is a contamination control with water. All isolates were positive for the Kpn 16S rRNA, but no capsule type or virulence genes were detected. Genes with positive controls are marked with arrows. Ghost bands are present on almost all isolates, approximately 280 bp.

4.8 Summary of results

The most relevant results are summarized in figure 4.4, a phylogenetic tree devised from the band relations detected by *XbaI* – PFGE.



PFGE-XbaI	ID	Year	ST	Enzyme	Plasmid	Co-res
	45	2011	298	CTX-M-15	*	ST, G, T
	48	2012	New	CTX-M-15	*	ST, C, G, T
	29	2010	111	CTX-M-15		N, ST, C ,T
	34	2011		SHV-5		
	23	2009		SHV-2a		
	32	2011	New	CTX-M-15		N, ST, C ,G ,T
	8	2007		CTX-M		
	2	2003		CTX-M		
	22	2009	25	CTX-M-15	180	ST
	56	2012		CTX-M-3		
	33	2011		CTX-M		
	35	2011	348	CTX-M-15, SHV-2a		N, ST, G, T
	25	2009	277	CTX-M-15, SHV-27		ST, G, T
	44	2011		SHV-12		
	40	2011	307	CTX-M-15	*	N, ST, C, G, T
	9	2007				
	20a	2009	17	CTX-M-15	170	N, ST, G, T
	20b	2009	17	CTX-M-15	170	N, ST, G, T
	17	2008	17	CTX-M-15	170	N, ST, G, T
	18	2008	17	CTX-M-15	170	N, ST, G, T
	13	2008	485	CTX-M-15	80	N, ST
	19	2009	485	CTX-M-15	*	ST
	39	2011		SHV-2		
	10	2007	29	CTX-M-15	100	ST, C, G, T
	14	2008	4	CTX-M-15	80	ST, G, T
	47	2012	48	CTX-M-15	*	ST, G, T
	55	2012	48	CTX-M-15	*	ST
	51	2012		SHV-12		
	57	2012		SHV-2a		
	38	2011		SHV-12		
	28	2010		CTX-M		
	53	2012		SHV-12		
	37	2011	15	CTX-M-15	*	ST, C, T
	5	2006		CTX-M		
	43	2011				
	1b	2003	29	SHV-5	230	N, C, G, T
	4	2005	29	SHV-5	220	G, T
	1a	2003	29	SHV-5	240	G, T
	12	2007	29	SHV-5	230	G, T
	15	2008	29	SHV-5	240	N, G, T
	6	2006	29	SHV-5	230	T
	11	2007	29	SHV-5	220	N, G, T
	54	2012	29	SHV-5	230	N, G, T
	7	2007	29	SHV-5	220	T
	36	2011	29	SHV-5	220	N, C, G, T
	42	2011	29	SHV-5	230	G, T
	46	2012	29	SHV-5	230	G, T
	16	2008	186	CTX-M-15	*	ST, G, T
	3	2005		SHV-12		

Figure 4.4: An overview of PFGE patterns, STs, $ESBL_A$ – encoding genes, $ESBL_A$ – bearing plasmids and co-resistance to relevant antibiotics. PFGE band patterns are compared by the Dice coefficient and 1.5 % positional tolerance. Clustering analysis was performed by UPGMA. Percentage similarity reference is placed at the upper left. N = nitrofurantoin, ST = trimethoprim/sulfamethoxazole, C = ciprofloxacin, G = gentamicin, T = tobramycin.*ESBL-bearing plasmid not identified.

5. Discussion

In this study, all clinical $ESBL_A$-producing *K. pneumoniae* isolates isolated at the Department of Medical Microbiology at Stavanger University Hospital between 2003 and 2012 were characterized to get an overview of the most common $ESBL_A$ – encoding genes in this collection, and also to identify possible clones and/or plasmids which may have contributed to the dissemination of $ESBL_A$ among *K. pneumoniae* in the Stavanger region and -hospital.

5.1 Methodical discussion

The CTX-M-15 enzymes are the most widespread of the $ESBL_A$-enzymes. Thus, in this study, we intended to select all $bla_{CTX\text{-}M\text{-}15}$ positive isolates for more detailed molecular characterization, i.e. MLST and plasmid analysis. As we disclosed clonal relatedness between several $bla_{SHV\text{-}5}$ positive isolates, it was decided to also include these isolates for more detailed analysis.

Three of the isolates included in this study (ID 17, 18 and 19) had been isolated during an outbreak in Stavanger University Hospital in 2008 – 2009. These isolates were included to investigate if other isolates in this collection were related to the outbreak strains.

5.1.1 PCR

$ESBL_A$ gene PCR

The following $ESBL_A$ genes were identified: $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}3}$, $bla_{SHV\text{-}2}$, $bla_{SHV\text{-}2A}$, $bla_{SHV\text{-}5}$, and $bla_{SHV\text{-}12}$. The CTX-M group of two isolates could not be defined because no amplification was detected in the RT-PCR. There are many groups of $bla_{CTX\text{-}M}$ genes. It is possible that the untypeable $bla_{CTX\text{-}M}$ genes were from another group, and was not detected by the $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ group specific RT-PCRs. Three isolates, which had their CTX-M group defined, could not be identified through sequencing. Two of the three isolates were of the CTX-M-9 group. These were not sequenced because of the main interest in the CTX-M-1 group. The last of the three was a group 1 CTX-M, but was not amplified in the $bla_{CTX\text{-}M\ group\ 1}$ specific PCR and was therefore not sequenced. The reason for this might be that the gene in this isolate is a variant that is not detected by the primers, or the genetic environment around the gene itself might be different, which means the primers might not anneal to the target.

The bla_{SHV} gene was not detected in all isolates. This may be due to isolates simply being bla_{SHV} negative. Similar results were presented by Alibi *et al.* in 2015, where bla_{SHV} genes were detected in 89 % of the isolates analysed [3]. Only 14.28 % of ESBL-positive *K. pneumoniae* were found to be *bla*SHV positive by Gholipour *et al*. in 2014 [38], suggesting that the results found in this study are as expected.

Some PCR programs had to be adjusted to fit the chemistry of the HotStarTaq master mix kit polymerase (Qiagen), specifically MLST and IncFII$_K$ PCRs. The HotStarTaq polymerase needs a 15 minute initiation phase at 95 °C to activate, and the PCR programs were adjusted accordingly.

MLST

The MLST PCR running conditions were described at the Pasteur website, along with the primers. The website states the *tonB* gene PCR needs a 50 mM $MgCl_2$ concentration, while all the other genes need 25 mM $MgCl_2$ for primers to work. However, in this study PCR products were amplified on all genes with the use of HotStarTaq master mix kit, which contains 3 mM $MgCl_2$.

5.1.2 Sanger sequencing

Two different ESBL variants of *bla*$_{\text{CTX-M group 1}}$ genes were identified through sequencing. Additionally, six different ESBL variants of *bla*$_{\text{SHV}}$ genes were identified. No ESBL variants of *bla*$_{\text{TEM}}$ were identified.

Sequencing *bla*$_{\text{SHV}}$ genes was not as simple as sequencing the *bla*$_{\text{CTX-M}}$, because of the chromosomal non-ESBL *bla*$_{\text{SHV}}$ gene present in almost all *K. pneumoniae* isolates, which leads to a mixed signal, or double peaks, when sequencing. Chromosomal genes are present in only one copy, while a plasmid-borne *bla*$_{\text{SHV}}$ would have a higher copy number (depending on the plasmid being a low-copy or high-copy number plasmid). Thus, the plasmid-borne *bla*$_{\text{SHV}}$ genes produce a higher signal than chromosomal *bla*$_{\text{SHV}}$, and the sequences could be differentiated. In this study, double peaks were present in almost all isolates carrying an ESBL type *bla*$_{\text{SHV}}$, suggesting the presence of chromosomal *bla*$_{\text{SHV}}$ genes. To completely resolve this problem, one could isolate the plasmid DNA and run a PCR targeting the *bla*$_{\text{SHV}}$ gene. With no part of the chromosome present, no double peaks should be seen.

Haanperä *et al.* has previously described another solution to this problem [41]. The article describes the use of pyrosequencing at positions 238 and 240, which are the known amino acid positions for substitutions that lead to an ESBL phenotype (Ambler numbering scheme, [5]). This area is also the area where double peaks were observed in this study. Pyrosequencing is therefore a technique that might be useful to define SHV type ESBL genes in *K. pneumoniae.*

Additional sequencing primers were used to cover the whole gene sequence of the *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes, as the initial PCR primers were insufficient. The bla$_{\text{CTX-M-1}}$ gene was sequenced using three primers. One *bla*$_{\text{CTX-M}}$ positive isolate was not amplified by the *bla*$_{\text{CTX-M group 1}}$ specific PCR without changing the forward primer to *IS*Ecp-tnpA3-F, due to the different genetic environment of that isolate.

5.1.3 PFGE – *XbaI* and *S1*-nuclease

PFGE was performed on all isolates, and two clones were detected. A phylogenetic tree was devised from the PFGE results. Band relations were calculated by the Dice coefficient with 1.5 % positional tolerance, and cluster analysis was done by UPGMA. These are the most commonly used criteria used for *Klebsiella* PFGE. Bands were marked manually in Bionumerics on the PFGE gels, which might affect the final phylogenetic tree. Care was therefore taken to place bands correctly, with a slightly lenient positional tolerance (1.5 %).

Twelve isolates had to be run with thiourea in the running buffer for bands to appear, both on PFGE and *S1*-PFGE. This has been previously described as an effective solution for some bacterial species [87]. Reactive tris radicals from the running- and gel buffer seemingly have a degrading effect on DNA molecules over time, which may have been the cause for the smears appearing on the gel. The

addition of thiourea, either in the running buffer or the gel buffer, will increase the stability of the DNA molecules because thiourea scavenges the excess reactive tris radicals [78].

Originally, the *S1*-PFGE procedure describes the use of plugs made of low melting point agarose. However, plugs for PFGE had previously been made with SeaKem gold agarose. Therefore, the *S1*-PFGE protocol was tested on the PFGE plugs, with success. The 12 isolates that produced smears on PFGE also did in the *S1*-PFGE. This was resolved by using new plugs with a higher bacterial concentration (~5 McFarland). New plugs with higher bacterial concentration were made for all *S1*-gels used in Southern blot, which had a positive effect on the signal strength after detection.

5.1.4 Southern blot hybridization

Southern blot hybridization was successful on two of six membranes, one membrane applying the $bla_{CTX\text{-}M}$ probe and one membrane applying the bla_{SHV} probe. Hybridization with $IncFII_K$ probe on three membranes and one membrane with $bla_{CTX\text{-}M}$ probe gave no colorimetric signals.

Labelling

Three probes were made using the DIG-high prime DNA labelling and detection starter kit I. The labelled probes were fixed to a membrane for determination of sensitivity. If the 0.1 pg/µl dilution was visible, then the probe had the desired sensitivity for southern blot hybridization. The bla_{SHV} probe was calculated to be very low in concentration (7.6 ng/ml). In spite of this, the bla_{SHV} probe produced the clearest bands with just 30 minutes exposure to the colour substrate solution. Since the calculation of labelled probe concentration was partially based on visual input (colour forming on membrane), it is possible that calculations were slightly incorrect.

Detection

The $bla_{CTX\text{-}M}$ probe was not as sensitive as the bla_{SHV} probe, as it seemed to need longer time for visible bands to appear. A lot more background was seen on the membrane hybridized with $bla_{CTX\text{-}M}$ probe. This is probably because the membrane had to be exposed to the colour substrate solution for a longer period of time, which might have visualized unspecific binding of antibodies. The addition of more probe mix to the hybridization solution for a higher concentration might have made bands appear faster, with less background.

The $IncFII_K$ probe did not give any colorimetric signal after hybridization and detection. The general temperature of 40 °C was used for all probes when hybridizing overnight. However, this may not be the optimal temperature for the $IncFII_K$ probe, and may be one of many reasons why no bands appeared on the hybridized membrane. According to the manufacturer instructions for the DIG-high prime DNA labelling and detection starter kit I, the optimal hybridization temperature is calculated from GC content and percent homology of probe to target sequence, which is described by the following equations:

$T_M = 49.82 + 0.41\ (\%\ G + C) - (600 / I)$, where I = length of hybrid in base pairs

$T_{Opt} = T_M - 20$ to 25 °C

The $IncFII_K$ probe hybridization temperature was calculated to be 38.80 – 43.80 °C. A slightly increased hybridization temperature than 40 °C would have led to more specific bonding which may have led to hybridization of the probe. Probe sequence differences may result in a different optimal

hybridization temperature, which may be the reason for no bands appearing. In general, a lower temperature may lead to less specific bonding, and may also have been a solution for the problem.

Transfer

The vacuum transfer protocol had to be adjusted for complete transfer. The original protocol stated that 5 in Hg for 90 minutes was sufficient for complete transfer for generally all gels, but gel thickness, percentage, agarose type, and also template DNA size are factors that can affect the transfer. In this study, it was found that 5 in Hg was not enough pressure to successfully transfer plasmid DNA to the nylon membrane. The gel was dyed with ethidium bromide before and after transfer to see if the transfer was successful. By increasing the pressure to 10 in Hg, transfer of plasmid DNA was successful.

5.2 Results discussion

Several distinct $ESBL_A$ genes were identified by sequencing. Of the most abundant were the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}5}$ genes. Additionally, SHV-11 was identified in 11 isolates and SHV-1 in five. Since double peaks were found in many isolates, it is highly probable that SHV-1 or SHV-11 is present in these isolates as well. *K. pneumoniae* normally carry a chromosomal SHV-1 or SHV-11 encoding gene [6, 41]. Haanperä *et al.* detected two bla_{SHV} genes in 95.3 % of the isolates analysed, where the SHV-1 and SHV-11 encoding genes were regarded as chromosomal [41]. Similar results were found in this study, as most isolates positive for an SHV-type $ESBL_A$ encoding gene had double peaks. However, the $ESBL_A$-encoding genes were of main interest in this study.

Two $ESBL_A$-encoding genes were identified in two isolates (ID 25 and 35), namely $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}27}$ and $bla_{SHV\text{-}2A}$, respectively. No $ESBL_A$ encoding gene was detected in two isolates (ID 9 and 43). However, both isolates were given an ESBL phenotype from the Vitek 2 instrument. Isolate 9 had the non-ESBL $bla_{SHV\text{-}11}$ gene, with resistance towards ceftazidime, but not cefotaxime. Isolate 43 was positive for the non-ESBL genes $bla_{SHV\text{-}33}$ and $bla_{TEM\text{-}57}$, and was resistant to both cefotaxime and ceftazidime. Since no $ESBL_A$ – encoding gene was identified, it is possible that these two isolates harbour the rare VEB or PER variants of $ESBL_A$, which have not been tested for.

Most CTX-M-15 group isolates were resistant to cefotaxime whereas most of the SHV-5 group isolates were resistant to ceftazidime. This is expected, as CTX-M- and SHV – enzymes are known to have the highest substrate affinity for cefotaxime and ceftazidime, respectively. A Gram-negative strain is regarded as multidrug resistant when it is resistant to three or more different groups of antibiotics [53]. The ST17 clone had identical co-resistance profiles (resistant to trimethoprim/sulfamethoxazole, nitrofurantoin, gentamicin and tobramycin). These four isolates were therefore regarded as multidrug resistant. This is probably attributed to the same ESBL-bearing plasmid in all four isolates. However, only six of the SHV – group isolates were multidrug resistant. This may be due to the presence of many different plasmids in these isolates, as seen on the *S1*-PFGE. The SHV-5 group isolates were isolated over a time period of nine years, during which the clone may have lost or gained new plasmids, depending on the environment of the clone. Different resistance genes may be present on the varying plasmids, thus giving different co-resistance profiles.

5.2.1 SHV-5 – group isolates

The SHV – group isolates, collected between 2003 and 2012, were all found to be closely related by *XbaI* – PFGE. The nine fragments, ranging from ~480 – 730 kbp were identical in all 12 isolates, whereas the smaller fragments were of varying numbers and sizes. Depending on the mutational rate of a bacterial strain, the DNA sequence and also the PFGE-pattern of a clone is expected to change over the years. The SHV – group isolates were also of the same sequence type (ST29), which also suggests that these isolates may represent a SHV-5 producing *K. pneumoniae* clone, which has been present in the region and in the hospital environment for a long time, sporadically causing clinical infections.

Hypermucoviscous *K. pneumoniae* ST29, of capsule type K54, has previously been described to cause mycotic aneurysm. This strain seemed to be highly virulent, but not particularly resistant to antibiotics [26]. As the SHV-5 producing strain detected in this study was highly mucoid and of ST29, screening of these isolates for the presence of virulence genes and K-types (including K54) known to be associated with hypermucoviscosity and/or hypervirulence was performed. None of the virulence genes or K-types screened for were detected. However, more than 70 capsule types are defined for *K. pneumoniae*, but only six were screened for. To determine the capsule type of this clone, one could perform a PCR targeting the capsule encoding operon and sequence the product [14]. None of the SHV-5 group isolates were positive for the virulence gene *rmp*A, which has been linked to a hypermucoviscous phenotype. The *rmp*A gene can be located either in the chromosome (c-*rmp*A) or on a plasmid (p-*rmp*A). A plasmid – borne variant of the *rmp*A gene, *rmp*A2, has also been described. It is possible that the SHV-5 group isolates carry *rmp*A2. However, the *rmp*A gene is usually related to the serotypes K1, K2, K5, K16, K20, K54, K57 and KN1, and is rarely associated with other serotypes [44].

SHV-5 producing *K. pneumoniae* has been found to cause outbreaks and sporadic infections in earlier studies [30, 54, 84]. Prodinger *et al*. detected an 80 kbp, $bla_{\text{SHV-5}}$ bearing plasmid in outbreak isolates of *K. pneumoniae* in 1996 [71]. Plasmid transfer was associated with the dissemination of the $bla_{\text{SHV-5}}$ gene, as it had also spread to other bacteria (*E. coli* and *K. oxytoca*). However, in another study, clonal spread of a $bla_{\text{SHV-5}}$ bearing plasmid was described, causing sporadic infections in different hospital wards [9]. The plasmid was found to be stable over time, and also harboured resistance genes that conferred resistance towards gentamicin and trimethoprim/sulfamethoxazole. Additionally, all isolates analysed had the capsule type K1. Since the strain was detected in a stool sample, it was suggested that the mode of transmission was from faecal contamination. An SHV-5 producing *K. pneumoniae* strain with ST20 has previously been described as the causative agent of a neonatal intensive care unit outbreak in Greece [57].

The $bla_{\text{SHV-5}}$ positive ST29 clone (SHV-5 group) seems to be a stable clone, and might be considered endemic in the Stavanger region and in the hospital environment in the period 2003 – 2012.

5.2.2 CTX-M-15 – group isolates

The phylogenetic tree devised from PFGE data suggests a high degree of diversity among the CTX-M-15 group isolates as only a few isolates had similar or closely related PFGE patterns (≥80 % similarity). As expected, the two isolates (ID number 17 and 18 and ST17) from the outbreak in the neonatal intensive care unit in 2008 – 2009 were indistinguishable by PFGE, with 100 % similarity. Additionally, isolate 20a and b (also ST17) had related PFGE patterns, but with lower similarity to one another and the outbreak isolates. Isolate 20a and b were also recovered in 2009, but from a patient admitted to another ward. It is unknown if there was any link between the two wards, or between the patient from whom isolate 20a and b were recovered and any neonatal intensive care unit patient.

The $bla_{\text{CTX-M-15}}$ gene was identified on plasmids ranging from 80 to 180 kbp. Various sizes and replicon types of $bla_{\text{CTX-M-15}}$ bearing plasmids have previously been described [27, 33, 75, 90]. In this study, the replicon type of the $bla_{\text{CTX-M-15}}$ bearing plasmids could not be determined. The $bla_{\text{CTX-M-15}}$ gene is mainly associated with IncFII plasmids in *K. pneumoniae* [47]. However, since isolate 17, 18, 20a and 20b (170 kbp plasmid bearing $bla_{\text{CTX-M-15}}$, ST17) was from the outbreak in 2008 – 2009, the $ESBL_A$ gene was located on an $IncFII_K$ plasmid, as previously described [49].

Most of the CTX-M-15 group isolates were not clonally related, and carried $bla_{\text{CTX-M-15}}$ encoding plasmids of different sizes (~70 – 230 kbp). This seems to be in accordance with the global picture of the dissemination of $bla_{\text{CTX-M-15}}$ genes, as it has been found to be connected to various clones [66] and plasmids of different sizes and replicon types.

6. Conclusions

- The most prevalent $ESBL_A$-encoding genes among clinical *K. pneumoniae* isolates from patients in the Stavanger region were $bla_{CTX\text{-}M\text{-}15}$ and $bla_{SHV\text{-}5}$.

- The ST29, $bla_{SHV\text{-}5}$ positive clone seemed to have persisted in the Stavanger region and hospital environment in the period 2003 – 2012
    - This may represent an endemic clone in the region
    - The clone has caused sporadic infections, especially in the hospital setting

- The ST17, $bla_{CTX\text{-}M\text{-}15}$ positive clone, which caused an outbreak in the hospital in 2008 – 2009, was only identified in one patient not associated with the outbreak. Thus, this clone does not seem to have become endemic, at least not among clinical isolates, in the Stavanger region

- The $bla_{CTX\text{-}M\text{-}15}$ gene was associated with diverse clones and plasmids in clinical isolates in the Stavanger region

7. Future prospective

The results from this study may have a diagnostic consequence. Knowledge of dominating $ESBL_A$ genes (*bla*$_{CTX\text{-}M\text{-}15}$ and *bla*$_{SHV\text{-}5}$) is needed if new molecular (PCR) methods are to be developed, for quick detection of common $ESBL_A$ genes in clinical samples. The development of such methods may have a therapeutic consequence, and thus of great importance for the patient.

In this study, *K. pneumoniae* clones which have caused infections were identified (for example ST29). Whether or not the clone has caused an outbreak is unknown, but it seems to have persisted in the hospital environment and caused sporadic infections. This may be useful information for future infection control measures.

For future studies, all methods may be applied to this collection of isolates for full identification of potential clones, similar plasmids and full $ESBL_A$ gene profile. Also, the inclusion of newer isolates from 2013 – 2015 may be of interest, to ascertain whether the ST29 clone is still persistent in the hospital environment.

8. References

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Appendix A – Primers, PCR-controls and recipes

Table A.1: List of all primers used in this study.

Use	*Gene*	*Primer name*	*Sequence*	*Size (bp)*	*Ref.*
Bacterial detection	*16S*	*16S F*	AGA GTT TGA TCC TGG CTC AG	512	[55]
		16S R	GTA TTA CCG CGG CTG CTG		
PCR & ESBL$_A$ - gene sequencing	*bla$_{CTX-M}$*	*bla$_{CTX-M}$ F*	SCS ATG TGC AGY ACC AGT AA	585	[81]
		bla$_{CTX-M}$ R	ACC AGA AYV AGC GGB GC		
	bla$_{CTX-M}$ group 1	*CTX-M-1 F*	GGT TAA AAA ATC ACT GCG TC	800 - 1700	[34]
		CTX-M-1 R	TTG GTG ACG ATT TTA GCC GC		
		ISEcp1-tnpA1 F	AAT ACT ACC TTG CTT TCT GA		
		ISEcp1-tnpA2 F	TCA TTT TTG GGC GAA TGA AGC		
		ISEcp1-tnpA3 F	CAG CAA ATA AAG ACC TTT CGT		
		Orf477 R	GGT GGC ATA ATT TTT GAA GT		
	bla$_{SHV}$	*bla$_{SHV}$ F*	ATG CGT TAT ATT CGC CTG TG	862	[81]
		bla$_{SHV}$ R	AGC GTT GCC AGT GCT CGA TC		
		Core SHV F	GGC CGC GTA GGC ATG ATA GA	714	[64]
		Core SHV R	CCC GGC GAT TTG CTG ATT TC		
	bla$_{TEM}$	*bla$_{TEM}$ F*	ATG AGT ATT CAA CAT TTC CG	858	[81]
		bla$_{TEM}$ R	CCA ATG CTT AAT CAG TGA GG		
		TEM F extra	TCA TGT AAC TCG CCT TGA TCG T		This study
		TEM R extra	ACG CTC GTC GTT TGG TAT GG		
Multilocus sequence typing	*rpoB*	*VIC3*	GGC GAA ATG GCW GAG AAC CA	501	[32]
		VIC2	GAG TCT TCG AAG TTG TAA CC		
	gapA	*gapA173*	TGA AAT ATG ACT CCA CTC ACG G	450	
		gapA181	CTT CAG AAG CGG CTT TGA TGG CTT		
	mdh	*mdh130*	CCC AAC TCG CTT CAG GTT CAG	477	
		mdh867	CCG TTT TTC CCC AGC AGC AG		
	pgi	*pgi1F*	GAG AAA AAC CTG CCT GTA CTG CTG GC	432	
		pgi1R	CGC GCC ACG CTT TAT AGC GGT TAA T		
		pgi2F (Seq)	CTG CTG GCG CTG ATC GGC AT		
		pgi2R (Seq)	TTA TAG CGG TTA ATC AGG CCG T		
	phoE	*phoE604.1*	ACC TAC CGC AAC ACC GAC TTC TTC GG	420	
		phoE604.2	TGA TCA GAA CTG GTA GGT GAT		
	infB	*infB1F*	CTC GCT GCT GGA CTA TAT TCG	318	
		infB1R	CGC TTT CAG CTC AAG AAC TTC		
		infB2F (Seq)	ACT AAG GTT GCC TCC GGC GAA GC		
	tonB	*tonB1F*	CTT TAT ACC TCG GTA CAT CAG GTT	414	
		tonB2R	ATT CGC CGG CTG RGC RGA GAG		
*Probe**	*FII$_K$*	*FII$_K$ FW*	TCT TCT TCA ATC TTG GCG GA	142-148	[86]
		FII$_K$ RV	GCT TAT GTT GCA CRG AAG GA		
Capsular typing	*K1*	*MagAF1*	GGTGCTCTTTACATCATTGC	1283	[35]
		MagAR1	GCAATGGCCATTTGCGTTAG		
	K2	*K2wzy-F1*	GACCCGATATTCATACTTGACAGAG	641	[82]
		K2wzy-R1	CCTGAAGTAAAATCGTAAATAGATGGC		
	K5	*K5wzxF360*	TGGTAGTGATGCTCGCGA	280	
		K5wzxR639	CCTGAACCCACCCCAATC		
	K54	*wzxK54F*	CATTAGCTCAGTGGTTGGCT	881	[36]
		wzxK54R	GCTTGACAAACACCATAGCAG		
	K57	*wzyK57F*	CTCAGGGCTAGAAGTGTCAT	1037	
		wzyK57R	CACTAACCCAGAAAGTCGAG		
	K20	*wzyK20F*	CGGTGCTACAGTGCATCATT	741	
		wzyK20R	GTTATACGATGCTCAGTCGC		
	rmpA	*rmpAF*	ACTGGGCTACCTCTGCTTCA	516	[61]
		rmpAR	CTTGCATGAGCCATCTTTCA		
	wcaG	*wcaGF*	GGTTGGKTCAGCAATCGTA	169	[83]
		wcaGR	ACTATTCCGCCAACTTTTGC		
	K. pn 16S	*K. pn Pf*	ATTTGAAGAGGTTGCAAACGAT	130	[48]
		K. pn Pr1	TTCACTCTGAAGTTTTCTTGTGTTC		

**Probe for southern blot*

Table A.2: PCR controls used in this study.

Target PCR	*Content*	*Origin*
16S rRNA	*K. pneumoniae* bacterial DNA	In-house
$bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}	*K. pneumoniae* strain positive for $bla_{\text{CTX-M,}}$ bla_{TEM} and bla_{SHV}	In-house
CTX-M-1 RT-PCR	*K. pneumoniae* strain positive for $bla_{\text{CTX-M group 1}}$	In-house
CTX-M-9 RT-PCR	*K. pneumoniae* strain positive for $bla_{\text{CTX-M group 9}}$	In-house
Capsular typing	*K. pneumoniae* positive for K1, *rmp*A and *wca*G	In-house

Table A.3: In-house recipes for media used in this study.

Agar	*Ingredients*	*Per litre*
Blood	Blood agar base no. 2 (Oxoid)	40.0 g
	Distilled water	1000 ml
	Human blood	62.5 ml (5.9 %)
Modified MacConkey	Bact Agar (Oxoid)	18.75 g
	Peptone Gelatine (Merck)	6.25 g
	Peptone Casein (Merck)	3.75 g
	Beef Extract (Lab Lemco)	6.5 g
	Sodium Chloride (VWR)	5.0 g
	5.0 M Sodium Hydroxide	2.25 ml
	Distilled water	1000 ml
	Crystal Violet solution (1.0 %)	5.0 ml
	Lactic acid concentrate (VWR)	0.75 ml
	Lactose (Merck)	10.0 g
	Sodium Thiosulfate	1.0 g
	Bromothymol Blue solution (2.5 %)	40.0 ml
Mueller Hinton	Mueller Hinton II Agar (BBL)	38.0 g
	Distilled water	1000 ml

Appendix B – Extended results

Table A.4: MALDI-TOF identification results.

ID	*Species*	*Score*	*ID*	*Species*	*Score*
1a	*K. pneumoniae*	2.479	*28*	*K. pneumoniae*	2.451
1b	*K. pneumoniae*	2.450	*29*	*K. pneumoniae*	2.200
2	*K. pneumoniae*	2.321	*32*	*K. pneumoniae*	2.237
3	*K. pneumoniae*	2.431	*33*	*K. pneumoniae*	2.364
4	*K. pneumoniae*	2.512	*34*	*K. pneumoniae*	2.094
5	*K. pneumoniae*	2.226	*35*	*K. pneumoniae*	2.242
6	*K. pneumoniae*	2.451	*36*	*K. pneumoniae*	2.263
7	*K. pneumoniae*	2.526	*37*	*K. pneumoniae*	2.326
8	*K. pneumoniae*	2.367	*38*	*K. pneumoniae*	2.294
9	*K. pneumoniae*	2.479	*39*	*K. pneumoniae*	2.325
10	*K. pneumoniae*	2.395	*40*	*K. pneumoniae*	2.249
11	*K. pneumoniae*	2.365	*42*	*K. pneumoniae*	2.300
12	*K. pneumoniae*	2.273	*43*	*K. pneumoniae*	2.151
13	*K. pneumoniae*	2.293	*44*	*K. pneumoniae*	2.421
14	*K. pneumoniae*	2.265	*45*	*K. pneumoniae*	2.373
15	*K. pneumoniae*	2.357	*46*	*K. pneumoniae*	2.357
16	*K. pneumoniae*	2.360	*47*	*K. pneumoniae*	2.094
17	*K. pneumoniae*	2.257	*48*	*K. pneumoniae*	2.300
18	*K. pneumoniae*	2.337	*51*	*K. pneumoniae*	2.151
19	*K. pneumoniae*	2.389	*53*	*K. pneumoniae*	2.344
20a	*K. pneumoniae*	2.299	*54*	*K. pneumoniae*	2.255
20b	*K. pneumoniae*	2.333	*55*	*K. pneumoniae*	2.328
22	*K. pneumoniae*	2.166	*56*	*K. pneumoniae*	2.014
23	*K. pneumoniae*	2.251	*57*	*K. pneumoniae*	2.299
25	*K. pneumoniae*	2.346			

Table A.5: VITEK 2 antimicrobial susceptibility testing results.

ID	*Phenotype*	*AMP*	*TMP*	*NIT*	*SXT*	*CIP*	*CXM*	*CTX*	*CAZ*	*GEN*	*TZP*	*FOX*	*AMC*	*CXA*	*AZT*	*NAL*	*TOB*
1a	ESBL	R	S	S	S	I	R	R	R	R	R	R	R	R	R	R	R
1b	ESBL	R	R	R	S	R	R	R	R	R	I	R	S	R	R	R	R
2	ESBL	R	S	S	S	S	R	R	S	S	S	S	S	R	I	S	S
3	ESBL	R	I	S	S	S	R	R	R	S	R	R	R	R	R	R	S
4	ESBL	R	S	S	S	I	R	I	R	R	R	S	S	R	R	R	R
5	ESBL	R	S	S	S	S	R	R	I	I	S	S	S	R	R	S	R
6	ESBL	R	S	S	S	I	R	I	R	I	I	R	S	R	R	R	R
7	ESBL	R	S	S	S	I	R	I	R	I	I	R	S	R	R	R	R
8	ESBL	R	R	S	R	S	R	R	I	R	I	S	R	R	R	S	R
9	ESBL	R	R	S	S	R	S	S	R	R	R	S	R	S	S	R	R
10	ESBL	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R
11	ESBL	R	S	R	S	I	R	R	R	R	R	S	S	R	R	R	R
12	ESBL	R	S	S	S	I	R	R	R	R	I	R	S	R	R	R	R
13	ESBL	R	R	R	R	S	R	R	R	S	S	S	R	R	R	S	S
14	ESBL	R	R	S	R	S	R	R	R	R	R	S	R	R	R	S	R
15	ESBL	R	S	R	S	I	R	R	R	R	R	S	S	R	R	R	R
16	ESBL	R	R	S	R	I	R	R	I	R	S	S	S	R	R	S	R
17	ESBL	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	R
18	ESBL	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	R
19	ESBL	R	R	S	R	S	R	R	R	S	S	S	R	R	R	S	S
20a	ESBL	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	R
20b	ESBL	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	R
22	ESBL	R	R	S	R	S	R	R	R	S	S	S	S	R	R	S	S
23	?	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S
25	ESBL	R	R	S	R	I	R	R	R	R	I	S	R	R	R	S	R
28	ESBL	R	R	S	S	S	R	R	I	S	S	S	R	R	R	S	S
29	ESBL	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R
32	ESBL	R	R	R	R	R	R	R	R	R	I	S	R	R	R	R	R
33	ESBL	R	S	S	S	S	R	R	S	S	S	S	S	R	S	S	S
34	ESBL	R	R	S	R	S	R	R	R	R	S	S	R	R	R	S	R
35	ESBL	R	R	R	R	S	R	R	R	R	R	S	R	R	R	S	R
36	ESBL	R	S	R	S	R	R	I	R	R	R	R	R	R	R	R	R
37	ESBL	R	R	S	R	R	R	R	I	S	I	S	R	R	R	R	R
38	ESBL	R	R	R	R	R	R	R	R	S	I	R	S	R	R	R	S
39	?	R	R	R	R	S	S	S	S	R	S	S	S	S	S	R	R
40	ESBL	R	R	R	R	R	R	R	R	R	I	S	R	R	R	R	R
42	ESBL	R	S	S	S	I	R	S	R	R	I	S	S	R	R	R	R
43	ESBL	R	S	R	S	I	R	R	R	S	S	S	R	R	R	S	S
44	ESBL	R	S	S	S	S	R	R	R	S	S	S	S	R	R	S	S
45	ESBL	R	R	S	R	S	R	R	R	R	S	S	S	R	R	S	R
46	ESBL	R	S	S	S	I	R	S	R	R	I	S	S	R	R	R	R
47	ESBL	R	R	S	R	S	R	R	R	R	R	S	R	R	R	S	R
48	ESBL	R	R	S	R	R	R	R	I	R	I	S	R	R	R	R	R
51	ESBL	R	R	R	R	R	R	R	R	S	S	S	S	R	R	R	S
53	ESBL	R	S	S	S	S	R	R	R	R	S	S	S	R	R	S	R
54	ESBL	R	S	R	S	I	R	S	R	R	I	R	S	R	R	R	R
55	ESBL	R	R	S	R	S	R	R	R	S	S	S	S	R	R	S	S
56	ESBL	R	S	R	S	S	R	R	I	S	I	S	R	R	R	R	S
57	ESBL	R	R	R	R	R	R	R	R	S	R	S	R	R	I	R	S

AMP = ampicillin, TMP = trimethoprim, NIT = nitrofurantoin, SXT = trimethoprim/sulfamethoxazole, CIP = ciprofloxacin, CXM = cefuroxime, CTX = cefotaxime, CAZ = ceftazidime, GEN = gentamicin, TZP = piperacillin/tazobactam, Fox = cefoxitin, AMC = amoxicillin/clavulanic acid, CXA = cefuroxime axetil, AZT = aztreonam, NAL = naldixic acid, Tob = tobramycin. R = resistant, I = intermediate, S = sensitive.

Table A.6: Manual antibiotic susceptibility testing by disk diffusion.

Panel	*Antimicrobial compound*	*23*	*39*
C	AMP	R	R
	SXT	R	R
	CIP	S	S
	CTX	R	I
	CAZ	I	R
D	GEN	S	R
	CEF	R	R
	TZP	S	S
	MER	S	S
ESBL	CAZ / Caz + Clav.	3 mm*	6 mm*
	CTX / CTX + Clav.	7 mm*	7 mm*
	FOX	S	S

** Isolates were positive for the ESBL test (in the ESBL panel) if differential diameter were above 5 mm.*

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