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# Extended-spectrum β-lactamases and carbapenemases in clinical isolates of Enterobacteriaceae in Norway.

Aspects of detection and epidemiology.

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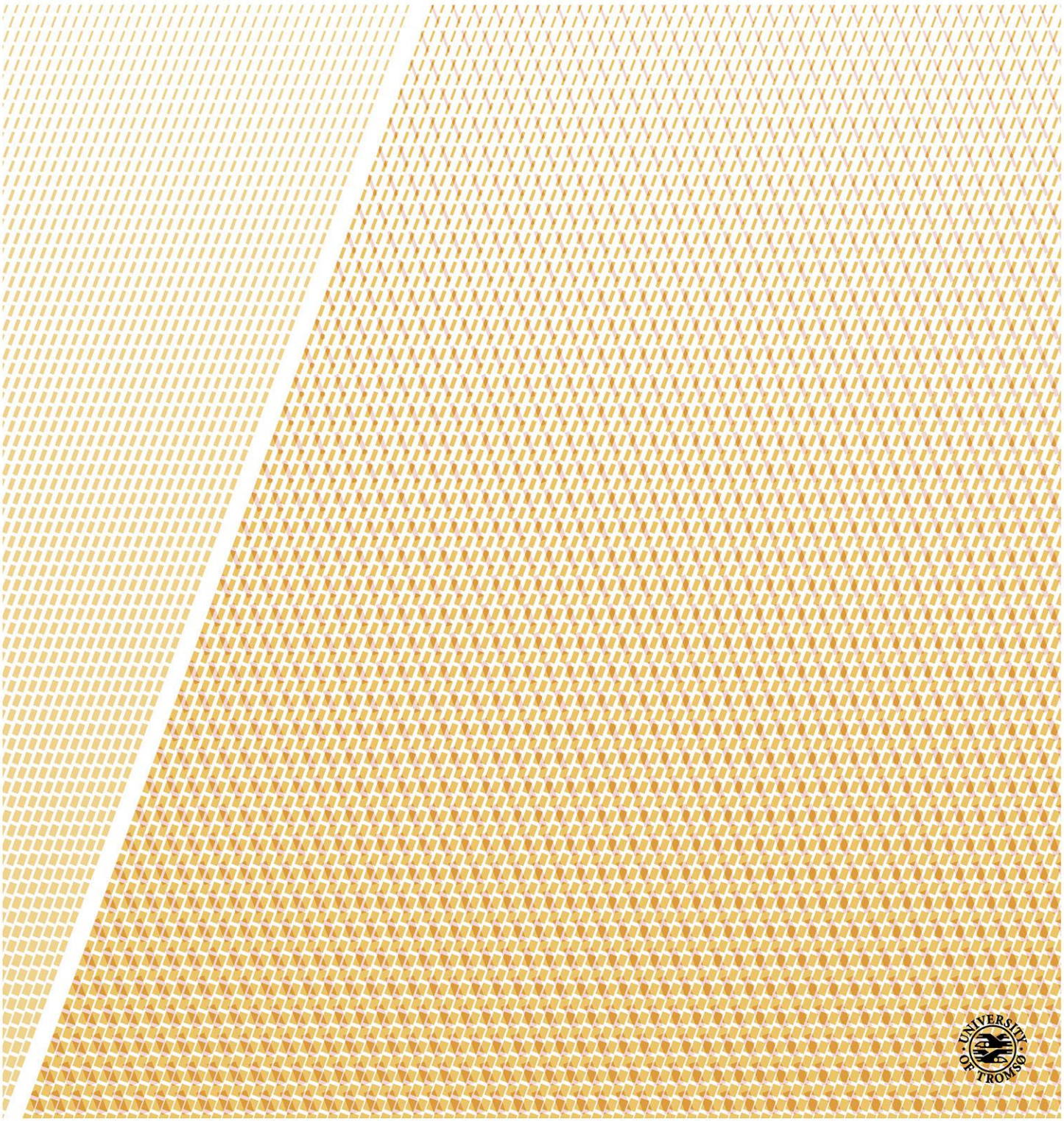
**Ståle Tofteland**

*A dissertation for the degree of Philosophiae Doctor – 2015*



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*Aspects of detection and epidemiology.*

*By*
*Ståle Tofteland.*


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Voiebyen in Kristiansand, Summer 2014.

Sincerely,

Ståle Tofteland.

# LIST OF PAPERS

## Paper 1

**Tofteland S, Haldorsen B, Dahl KH, Simonsen GS, Steinbakk M, Walsh TR, Sundsfjord A; Norwegian ESBL Study Group.** Effects of phenotype and genotype on methods for detection of extended-spectrum-beta-lactamase-producing clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Norway. J.Clin.Microbiol. 2007 Jan; 45(1):199-205.

## Paper 2

**Naseer U, Haldorsen B, Tofteland S, Hegstad K, Scheutz F, Simonsen GS, Sundsfjord A; Norwegian ESBL Study Group.** Molecular characterization of CTX-M-15-producing clinical isolates of *Escherichia coli* reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway. APMIS. 2009 Jul;117(7):526-36.

## Paper 3

**Tofteland S, Dahl KH, Aasnæs B, Sundsfjord A, Naseer U.** A nationwide study of mechanisms conferring reduced susceptibility to extended-spectrum cephalosporins in clinical *Escherichia coli* and *Klebsiella* spp. isolates. Scand J Infect Dis. 2012 Dec;44(12):927-33.

## Paper 4

**Ståle Tofteland, Umaer Naseer, Jan Helge Lislevand, Arnfinn Sundsfjord, and Ørjan Samuelsen.** A long-term low-frequent hospital outbreak of KPC-producing *Klebsiella pneumoniae* involving intergenus plasmid diffusion and a persisting environmental reservoir. PLOS1 2013 March; (8)3; e59015.

# ABBREVIATIONS

| | |
|---|---|
| AG | aminoglycoside |
| ASC | active surveillance culturing |
| AST | Antimicrobial susceptibility testing |
| BA | boronic acic |
| *bla* | gene encoding β-lactamase |
| $bla_{CTX\text{-}M}$ | gene encoding CTX-M β-lactamase |
| bp | basepair |
| BURST | based upon related sequence type |
| cAmpC | chromosomal AmpC cephalosporinase |
| CAZ | ceftazidime |
| CC | clonal complex |
| CDT | combined disk method |
| CIP | ciprofloxacin |
| CLA | clavulanic acid |
| CLOX | cloxacillin |
| CLSI | Clinical and Laboratory Standards Institute |
| CMY | cefamycin β-lactamase |
| CPD | cefpodoxime |
| CPE | carpbapenemase-producing Enterobacteriaceae |
| CRE | Carbapenem resistant Enterobacteriaceae |
| CTX | cefotaxime |
| CTX-M | cefotaximase-Munich β-lactamase |
| DDS | double-disk synergy method |
| EARS-Net, ECDC | Antimicrobial ressitance interactive database, ECDC |
| ECDC | European Centre for Disease Control and Prevention |
| EDTA | Ethylenediaminetetraacetic acid |
| ESBL | Extended-spectrum –β-lactamase |
| ESBL-E | ESBL-producing Enterobacteriaceae |
| ESC | extended-spectrum cephalosporins |
| EUCAST | European Committee on Antimicrobial Susceptibility Testing |
| FOX | cefoxitin |
| GEN | gentamicin |
| HGT | horizontal gene transfer |
| ICU | intensive care unit |
| IEF | isoelectric focusing |
| IMP | imipenemase |
| Inc | incompatibility |
| IS | insertion sequence |

| | |
|---|---|
| IS*CR* | insertion sequence common region |
| kB | kilobase |
| KPC | Klebsiella pneumoniae carbapenemase |
| K-res | Norwegian National Advisory Unit on Detection of Antimicrobial Resistance |
| LOS | length of stay |
| LTCF | long time care facilities |
| MBL | Metallo-β-lactamase |
| MDR | multi-drug resistance |
| MHT | Modified Hodge Test |
| MIC | Minimum inhibitory concentration |
| MLST | Multi-Locus Sequence Typing |
| NDM | New Dehli carbapenemase |
| N-ESBL | Norwegian ESBL -study |
| NORM | Norwegian Surveillance System for Antimicrobial Resistance |
| NPV | negative predictive value |
| OMP | outer membrane protein |
| OXA | oxacillinase |
| OXY | OXY-β-lactamase (from *K.oxytoca*) |
| pAmpC | plasmid-mediated AmpC cephalosporinase |
| PBP | penicillin binding protein |
| PBRT | PCR-based replicon typing |
| PFGE | Pulsed-Field Gel Electrophoresis |
| pI | isoelectric point |
| PK/PD | Pharmacokinetic/Pharmacodynamic -ratio |
| PPV | positive predictive value |
| RESPECT | Resistance to expanded spectrum cephalosporins in Norway-study |
| R-plasmids | plasmids encoding antibiotic resistance |
| SHA | Sørlandet Hospital Enterprise-location Arendal |
| SHK | Sørlandet Hospital Enterprise-location Kristiansand |
| SHV | sulfhydryl variable β-lactamase |
| SLVs | single locus variants |
| SSHF | Sørlandet Hospital Enterprise |
| ST | sequence type |
| SXT | trimethoprim/sulfamethoxazole |
| TEM | Temoniera β-lactamase |
| TOB | tobramycin |
| UTI | urinary tract infection |
| VIM | Verona integron-encoded metallo-β-lactamase |
| XDR | extremely-drug resistance |

# PREFACE

This study has taken place over a relative long period of time (2003-2014). As a starting point, it is thus important to put them into a national and international context and timeline. During these years important changes have occurred in the global epidemiology of clinical relevant antimicrobial resistant Gram negative bacterial pathogens. The global spread of extended spectrum β-lactamases (ESBLs) has received particular attention and also Norway is affected. Prior to the initiation of this study in 2003, essentially no knowledge existed on the presence of ESBLs in Enterobacteriaceae in Norway. Moreover, national guidelines for laboratory detection of ESBLs were lacking.

The international epidemiology of ESBLs prior to the year 2000 was mainly related to nosocomial isolates usually linked to outbreaks of TEM-/SHV-ESBLs in *K.pneumoniae*. This epidemiology of ESBL producers, for the greater part affecting hospitals, was unfamiliar to Norway, and thus not given much attention. Then, some studies observed the emergence of a CTX-M-type ESBL in clinical isolates of *E.coli* in several countries including Europe. These observations triggered an investigation to find these ESBLs in Norway, if present (**paper 1**). Subsequently, we were able to study the molecular epidemiology of the emerging CTX-M producing *E.coli* in our country (**paper 2**). As national guidelines for ESBL detection were lacking, we wanted to evaluate the most appropriate methods for ESBL detection in clinical *E.coli* and *Klebsiella spp.* isolates (**paper 1 and paper 3**) also taking advantage of the Norwegian surveillance programme for antimicrobial resistance in human pathogens (NORM). In process, the first nosocomial outbreak of KPC-producing *K.pneumoniae* in Scandinavia emerged (2007-10), presenting us with an opportunity to investigate another aspect of the epidemiology to the "newer β-lactamases" in Norway[1,2] (**paper 4**). The unusual character of this outbreak, being low frequent of prolonged duration, made us able to explore the significance of alternative reservoirs and modes of transmission in the hospital setting.

It has been an interesting journey.

# A. INTRODUCTION

## A.1 THE PATHOGENS

The Enterobacteriaceae family belong to the Gammaproteobacteria class of Gram-negative, rod-shaped, non-spore-forming and facultative anaerobic bacteria. Most Enterobacteriaceae have the ability to ferment sugars, produce catalase and reduce nitrate to nitrite, as well as being oxidase negative and motile by peritrichous flagella [3,4].

**HABITATE.** Many members of the Enterobacteriaceae family are common members of the gut microflora in humans and animals. For *Escherichia coli*, the intestine is its primary reservoir. It is recovered from the stools of almost all humans and animals, and is the most frequently isolated facultative anaerobe from the intestine of humans[4,5]. *E. coli* is less frequently encountered in the environment, and their presence in water and food sources usually indicate faecal contamination[6,7]. Comparatively, *Klebsiella pneumoniae* is more ubiquitous, and frequently found in environmental reservoirs of water and soil , as well as in stool samples and nasopharynx from healthy individuals [8]. Detection rates in stool vary (5-38 %), with significantly higher carrier rates in hospitalized patients, particularly associated to length of stay and antibiotic use [8].

**HOST-MICROBE INTERACTION AND DISEASE.** The ability to cause infection in general is related to pathogen-specific, host-specific, and sometimes device-specific factors [4]. If host immunity or mucosal barriers are compromised, *E. coli* and *K. pneumoniae* strains may cause opportunistic infections.
In Enterobacteriaceae, pathogen-specific virulence determinants include: adhesion; pili and fimbria, secretory systems; including toxins with various effects, i.e. promotion of secretion and damage of cell cytoskeleton, siderophores; promoting (co-enzymatic) iron acquisition essential to the activity of several bacterial enzymes, and antiphagocytic properties; usually involving formation of capsule. Differences in the composition of outer membrane lipopolysaccharides (LPS) may also affect virulence[3,5].

***E.COLI.*** In humans biological significant *E. coli* strains constitute commensal and a limited number of (strictly) pathogenic strains which have captured additional virulence determinants by which they may cause intestinal and extra-intestinal (ExPEC) disease, including urinary tract infections or sepsis and meningitis in healthy individuals [5,9]. Intestinal pathogenic strains are distinct from the commensal strains, and infrequently encountered in the flora of the large intestine, but seen to regularly produce disease in healthy individuals when ingested in sufficient dosages [9]. On the contrary, ExPEC strains are capable of stable colonization of the intestine in approximately one fifth of healthy individuals [9].
The *E. coli* genome is extremely versatile, with less than 50 % of DNA representing the common core genomic material [10]. Regions termed pathogenicity islands (PAIs) account for much of this

variation in the chromosomal DNA of *E. coli* and constitutes large DNA-regions (10-200 kb) associated with various virulence determinants [11]. Pathogenic *E.coli* lineages may also carry additional mobile genetic elements (transposons, plasmids, bacteriophages) compared to commensal strains encoding virulence determinants. Prominence of different virulence determinants vary in accordance with the strains clinical manifestation. Strains related to urinary tract infections are commonly associated with P type fimbriae adhesion, alfa-hemolysin invasion and scavenge of iron by aerobactin siderophores, whereas production of various entero- and verotoxin play a major role in intestinal disease[4,5].

***K. PNEUMONIAE.*** *K. pneumoniae* is a non-motile member of the Enterobacteriaceae family which is frequently associated with opportunistic infections in debilitated patients, causing urinary tract infections, pneumonia and sepsis [8]. In most countries, *K. pneumoniae* is second only to *E. coli* in causing gram-negative nosocomial sepsis[12,13]. Furthermore, nosocomial outbreaks of *K.pneumoniae* are common due to their ability of rapid dissemination among hospitalized patients, particularly in neonatal units[14,15]. *K. pneumoniae* strains originating from the environment comprise of the same set of virulence factors as clinical strains, and are equal in their abilities to colonize the intestine, thereby representing a continuous challenge to the immune-compromised host[15-18].

One of the most common K-serotypes identified in clinical isolates, K2, is however infrequently retrieved in environmental isolates [8]. Still, there is a gap in our understanding of how environmental *K. pneumoniae* contribute to disease[16,19]. Nevertheless, nosocomial spread of *K. pneumoniae* strains originating in the environment has been indicated in several reported cases[15,20-27].

Intestinal colonization usually occurs prior to *K. pneumoniae* infection[14,16]. Pathogenicity of *K. pneumoniae* strains is in general dependent upon virulence factors constituting adhesions of fimbrial type 1 or 3, antiphagocytic properties exerted by the prominent capsule and the LPS layer [8,16,28].

## A.2 BETA-LACTAM ANTIBIOTICS

The discovery of the β-lactam-antibiotic penicillin by Alexander Fleming in 1928, and its subsequent large scale use from 1944 and onwards, introduced the "era of antibiotics" [29]. Today, β-lactams are our most important and extensively used class of antibiotics constituting approximately 50% of the global antibiotic consumption [30].

The convenience of these drugs is owed to its many natural or synthetic derivatives with diverse bacterial spectrums, which include targeted narrow-spectrum ecology-friendly antibiotics and broad-spectrum alternatives targeting both gram-positive and gram-negative bacteria. Most bacterial species are covered except mycobacteria, intracellular pathogens and cell wall deficient bacteria like *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* [31]. In addition, the β-lactams in clinical use are generally associated with low toxicity credited to excellent selectivity by attacking the pencillin-binding proteins (PBPs) in the bacterial cell wall which is structurally non-paralleled in human cells.

**MODE OF ACTION.** β-lactams act by inhibiting the final step in the cell wall peptidoglycan synthesis in metabolically active and dividing bacteria (Figure 1). However, the exact mechanism remains unclear with regards to the various bactericidal interactions of autolysins especially in gram-positive bacteria[31-33].

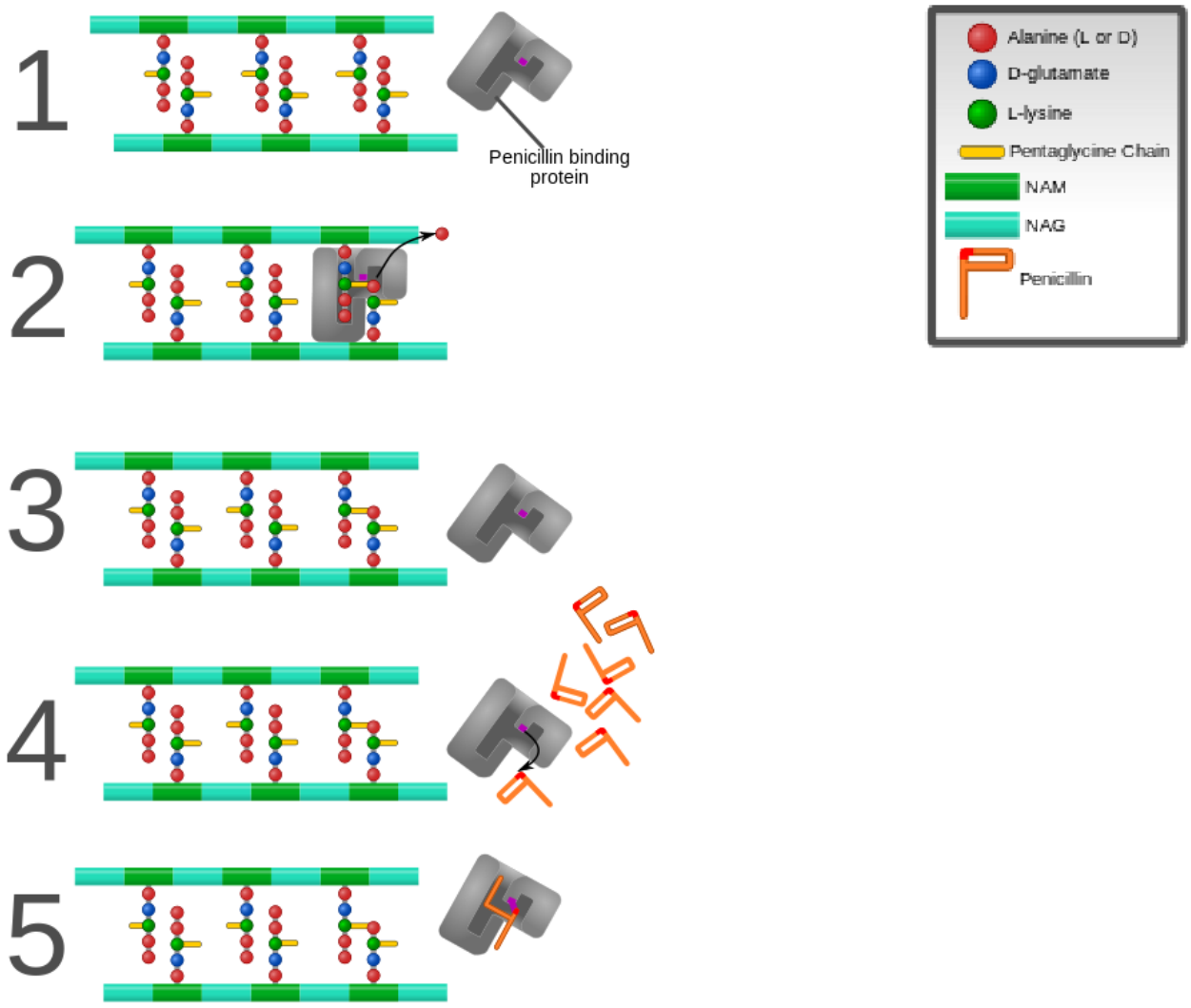



*Figure 1. Schematic presentation of the mode of action of β-lactam antibiotics: Alternating NAM and NAG subunits in the bacterial cell wall are depicted (1). The peptide side-branches of NAM subunits are crosslinked by PBP-transpeptidases (2, 3). Penicillin attacks the PBP active site, the serine hydroxyl-group (violet) (4), forming an irreversible covalent link to the PBP, thereby producing a permanent block of the active site (5) and consequently blocking cell wall synthesis. (Figure from [34]).*

**CHEMICAL STRUCTURE.** The active structure of a four-membered β-lactam ring is part of all β-lactams [31] (Figure 2). In addition to the β-lactam ring, a fused bicyclic ring structure is present in all classes of β-lactam antibiotics with the exception of monobactams [29]. Accordingly, β-lactams are classified into four different groups due to differences in the chemical structure of the fused ring; the penicillins, the cephalosporins, the carbapenems, and the monobactams (lacking the ring). Due to their different mode of action, a fifth group is often separated from the penicillins; the penicillin-inhibitors. The β-lactam ring structure introduces ring strain making these structures susceptible to hydrolysis, and the fused ring structures further increases this phenomenon. Within each class, the antimicrobial affinity to target (spectrum of activity), β-lactamase-stability and pharmacokinetic properties are balanced through different side branches [35]. Thus, in cephalosporins the R1-modifications affect spectrum of activity by changing the stability to β-lactamases and affinity to the antibiotic drug target whereas R2- modifications influence pharmacokinetic properties (Figure 2)[29,36]. Difficulties in combining an enhanced spectrum activity and simultaneous retaining β-lactamase-stability has entailed a secondary strategy in the penicillin class of antibiotics; the development of β-lactamase-inhibitors protecting a β-lactamase-labile penicillin in β-lactamase-inhibitor combinations [29]. Apart from the number and properties of porin channels in the gram-negative bacteria outer membrane, diffusion is related to physiochemical characteristics of the antibiotic. Thus, the small zwitterionic carbapenem, imipenem, traverse the outer membrane by permeation more easily than carbenicillin belonging to the carboxypenicillins that is a highly charged and large penicillin molecule [36].

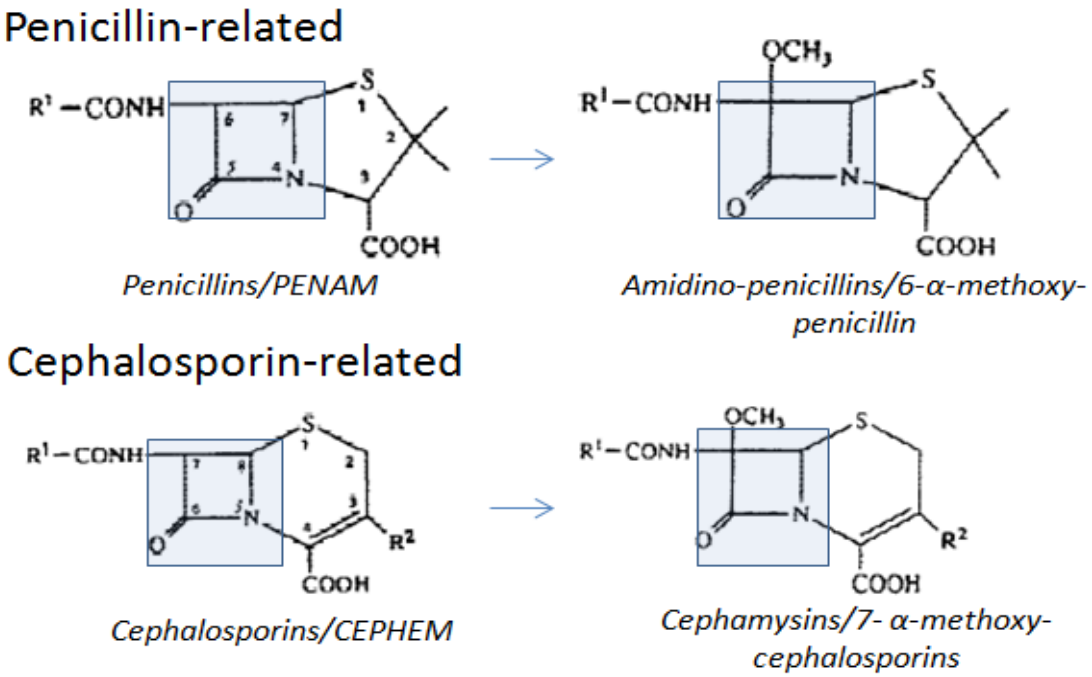
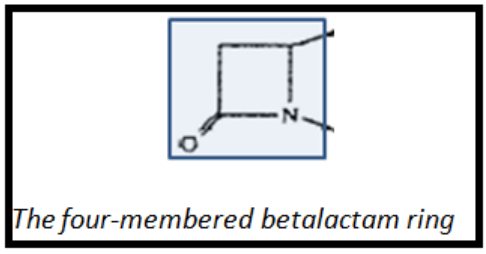



*Figure 2. The four-membered β-lactam ring (right).The chemical structure of β-lactam antibiotics. R1 and R2 constitute different acyl-substituents (above). (Figure reprint modified with permission from Am Ass of Pharm Sci. [35]).*



## SPECTRUM OF ANTIMICROBIAL ACTIVITY OF SELECTED B-LACTAM ANTIBIOTICS AND DIAGNOSTIC USE IN DETECTION OF B-LACTAMASE PRODUCING ENTEROBACTERIACAE.

*Table1: Spectrum of activity and diagnostic use of β-lactam antibiotics in Enterobacteriaceae.*

| Antibiotic class | Spectrum of activity | Diagnostic use |
|---|---|---|
| Penicillin | **Ampicillin**: not stable to inducible classC β-lactamases present in several species as well as chromosomal SHV- and TEM-1 β-lactamases.<br>**Mecillinam;** *in vitro* activity towards Enterobacteriaceae is good, including most ESBL-producing isolates. | **Temocillin:** High-level temocillin resistance has been proposed as a phenotypic marker of OXA-type carbapenemase production in Enterobacteriaceae in isolates lacking synergy to KPC/MBL inhibitors. |
| Penicillin/ β-lactamase-inbitor | **Piperacillin-tazobactam:** *In vitro* susceptibility is most often recognized in ESBL-producing *E. coli* and *K.pneumoniae* isolates. Stably derepressed ampC mutants of *Enterobacter cloacae* hydrolyze piperacillin. | **Amoxycillin-clavulanate:** is mainly used to diagnose broad-spectrum β-lactamases and ESBLs in Enterobacteriaceae. |
| Cephamycin | Not in clinical use in Norway. | **Cefoxitin** is used diagnostically to signify possible AmpC-production, as it is labile to AmpC and a prominent inducer of chromosomal AmpC-production (except in *E.coli*). |
| Cephalosporin 2nd gen | **Cefuroxime:** activity against many Enterobacteriaceae, including TEM-1 *E. coli* and *K.pneumoniae*. Activity to Enterobacteriaceae harbouring inducible classC enzymes is limited as cefuroxime is labile to hydrolysis by these enzymes, and activity thus relies on its weak ability to induce AmpC | Not used to diagnose plasmid mediated AmpCs, ESBLs or carbapenemases in Enterobacteriaceae. |
| Cephalosporin 3rd gen | **Cefotaxime and ceftazidime:** labile to stably derepressed ampC-mutants when present in species with inducible classC β-lactamases, labile to most ESBLs. Ceftazidime display anti-pseudomonal activity. | **Cefotaxime or ceftriaxon and ceftazidime:** combined use as indicator substrates for ESBL production.<br>**Cefpodoxime:** only in diagnostic use, significantly hydrolysed by the vast majority of ESBLs. |
| Monobactam | **Aztreonam:** is not substrate for hydrolyses by classB-metallo-β-lactamases. Anti-pseudomonal activity. | |
| Carbapenem | **Imipenem, meropenem, ertapenem:** Labile towards carbapenemases. Imipenem display reduced susceptibility to *Enterobacter cloacae* and *Proteus spp*. Ertapenem is not effective in treating *Pseudomonas spp.* | **Imipenem, meropenem, ertapenem:** Potential indicators of most carbapenemases in Enterobacteriaceae except OXA-type carbapenemases. |

## A.3 BACTERIAL RESISTANCE AND ANTIMICROBIAL SUSCEPTIBILITY TESTING

The process of and premises for setting clinical breakpoints and screening cut-offs greatly influence the laboratory methods to detect mechanisms of resistance and the clinical categorization of antimicrobial susceptibility. In this study, we sought to detect specific mechanisms mediating different levels of reduced susceptibility to 3rd generation cephalosporins and their significance for clinical categorization of susceptibility (**paper 1 and paper 3**), as well as the consequences of the variable and often low-level expression of carbapenemases in Enterobacteriaceae, including laboratory detection of *bla*$_{KPC}$ (**paper 4**). Understanding the limitations confounded by underlying premises and methodology is important for the correct interpretation of results from our study.

**PARAMETERS IN ANTIMICROBIAL SUSCEPTIBILITY TESTING.** Antimicrobial susceptibility testing (AST) methodology has continuously developed throughout the antibiotic era [37]. The concept of minimal inhibitory concentration (MIC) is fundamental for all developments within antimicrobial susceptibility testing and gives an estimate of the antibacterial effect *in vitro* [38]. MIC is defined as the lowest concentration of an antibiotic that will inhibit visible growth of a given microorganism *in vitro*. MIC analysis by reference standard is performed in a series of twofold concentrations by broth dilutions after overnight incubation, and serves as the gold standard to which other methods of phenotypic susceptibility testing systems, including agar dilution, gradient diffusion, disk diffusion, and various automated AST systems, have to relate and calibrate themselves [39]. However, whatever methods that are applied in performing antimicrobial susceptibility testing, they are themselves dependent on several factors in order to reliably reproduce *accurate* and *precise* results, and the ISO standards have been developed to achieve this [40,41]. The ISO standards include inoculums size and growth phase, composition of media including pH and ion content, incubation time, temperature and atmosphere, and qualified reading personnel [42]. Still, however, there are controversies whether a sufficient level of accuracy is achievable in everyday routine phenotypic testing to detect all clinically or epidemiologically significant resistance [43-45]. The **disk diffusion method (a.m. Kirby-Bauer)** originally was standardized by Bauer in the mid-1960s [46]. In recent years, standardization of wild-type (defined later) disk diffusion histograms, has been taken up by EUCAST and associated national antimicrobial susceptibility testing committees (NACs).

**IMPORTANCE OF ANTIMICROBIAL SUSCEPTIBILITY TESTING.** Antimicrobial susceptibility testing is undertaken to predict outcome of treatment in individual patients and to guide future empirical treatment [42,47]. Antimicrobial susceptibility testing is a tool for antimicrobial resistance surveillance and essential in tailoring appropriate infection control

measurements and antibiotic usage, antibiotic policy making, antibiotic legislation and regulation [42].

**CLINICAL SUSCEPTIBILITY AND RESISTANCE.** According to "The European Committee on Antimicrobial Susceptibility Testing" (EUCAST), clinically susceptible (S) and resistant (R) isolates are associated with a high likelihood of therapeutic success or failure, respectively, whereas clinically intermediate (I) isolates are associated with "*uncertain therapeutic effect"* [48]*. It implies that an infection... may appropriately be treated at body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretation*" (http://www.srga.org/Eucastwt/eucastdefinitions.htm). Clinical breakpoints (Figure 3) are MIC concentrations set by organizations and committees such as EUCAST and their North American counterpart CLSI (Clinical and Laboratory Standards Institute) to separate into these clinical categories, which may change over time with evolving microbial populations, basic understanding (on resistance mechanisms, pharmacokinetics and pharmacodynamics (PK/PD), methodological knowledge, and data from clinical studies [49].

**MICROBIOLOGICAL SUSCEPTIBILITY AND RESISTANCE.** The concept of wild type (wt) bacterial populations and epidemiological cut-offs (ECOFFs) (Figure 3) is a useful tool when determining clinical breakpoints, for detection of low-level resistance and excluding resistances (i.e. cefoxitin to exclude methicillin resistance in *Staphylococcus aureus*, nalidixin to exclude chromosomal fluoroquinolone resistance in Enterobacteriaceae), and as a possible tool in surveillance [50]. According to EUCAST; *"a microorganism is defined as wild type for a species by the absence of acquired and mutational resistance mechanisms to the drug in question"* (http://www.srga.org/Eucastwt/eucastdefinitioms.htm). ECOFFs represent the upper and lower limits of wt and resistant bacteria within a population, respectively. For methodological purposes, clinical breakpoints should avoid splitting the wild type population (Figure 3). ECOFFs may be useful in setting preliminary clinical breakpoint when PK/PD data is insufficient and clinical data suggest a given drug is useful for treatment.

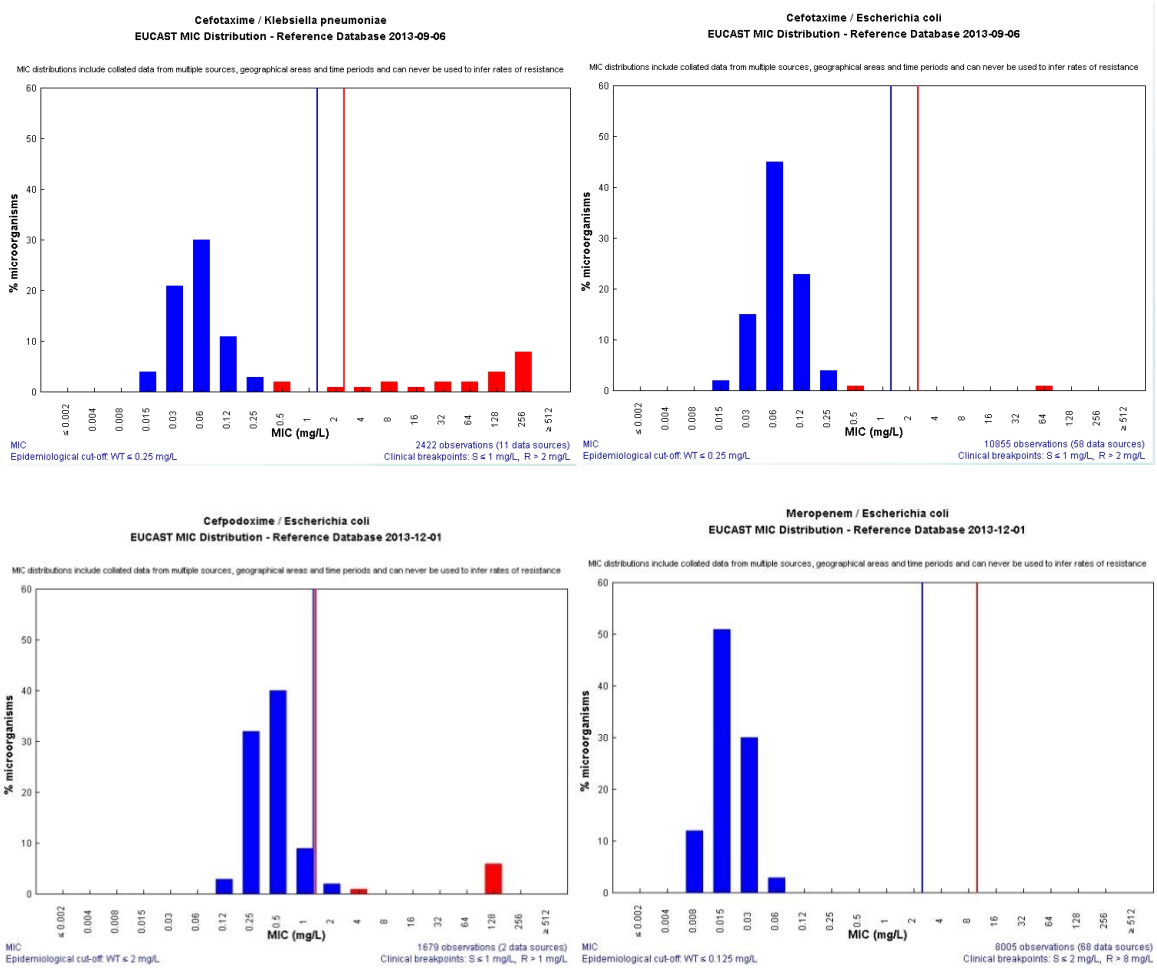



*Figure 3. MIC distributions collected by EUCAST. The ECOFF distributions are displayed in blue colours. (http://mic.eucast.org/Eucast2/)*

**SETTING CLINICAL BREAKPOINTS - THE EUCAST APPROACH.** Historically, significant differences have existed between breakpoints set by different breakpoint-setting organizations. However, during the last ten years EUCAST has worked to harmonize the process of breakpoint setting in Europe, and a strict procedure for setting breakpoints in new antibiotics and harmonizing breakpoints for existing drugs has been developed[51]. A majority of national breakpoint committees (NACs) in Europe has presently adopted EUCAST breakpoints. The EUCAST procedures for setting breakpoints were launched in 2010 [52]. The procedures take into account PK/PD – issues including dosage and formulation, results from pharmacodynamics studies in hollow fibre infection models, animal models, or, if available, human studies[53-55]. Statistical modelling (Monte Carlo simulations) is used to set tentative PK/PD- breakpoints. Further, wild type distributions and ECOFFS are evaluated to avoid methodological errors, and results from clinical trials as well as existing knowledge of the molecular mechanism relevant in

setting a certain breakpoint is accounted for. A set of expert rules are thus defined to be superimposed on the results of the AST requiring edition on that account[56].

**WHICH IS THE BEST PREDICTOR OF OUTCOME – MIC OR MECHANISM?** The significance of detecting ESBLs or carbapenemases for infection control and public health purposes is agreed upon[57]. The potential epidemiological consequences precipitated by not detecting carbapenemase-producing Enterobacteriaceae expressing low-level resistances are, however, uncertain and controversies still exists whether to report individual results according to the results obtained by phenotypic examination or to report according to inferred mechanism[58]. After adopting new and lower clinical breakpoint in 2009 in concordance with results suggested by PK/PD- cut offs (BPs), the current position of EUCAST is to "report as tested", i.e. clinical categorization according to the results obtained by phenotypic examination[56]. As from 2010, the CLSI also report as tested, although some differences, including ceftazidime and cefepime, remain between EUCAST and CLSI with regard to clinical breakpoints that may have clinical effects favoring the present clinical breakpoints decided by EUCAST[59-61]. A significant number of antimicrobial susceptibility testing experts insist to "report by mechanism" [43]. The contenders of the EUCAST view claim that in clinical studies, MICs of 1-4 mg/L to a given 3$^{rd}$ generation cephalosporins displayed by ESBL- producing Enterobacteriaceae could not reliably predict clinical success[62]. The same situation applies for the frequent identification of carbapenemases displaying low-level resistance, even if clinical studies are equivocal[63]. Further, they question if routine antimicrobial susceptibility testing is precise enough to discriminate reliably between Enterobacteriaceae displaying MICs within the 1-4 mg/L range, thereby posing a fundamental quality control challenge to antimicrobial susceptibility testing [43].

## A.4 DISSEMINATION OF ANTIBOTIC RESISTANCE

The use of antibiotics in different ecological niches is the main selector of resistant bacteria and mobile genetic elements conferring resistance. Facilitating factors (e.g. travel, contaminated food, poor sanitation in the community and lack of infection control in hospitals and long-term care facilities (LTCF), and non-antibiotic selection (e.g. heavy metals)), are superimposed and contribute to the magnitude of dissemination of resistant gene determinants by mobile elements and/or MDR-clones [64-66].

The specific role and contribution of different mobile genetic elements, strains and/or clones to the dissemination of ESBLs and carbapenemases in Norway was explored in our study (i) at a national level exploring the apparent allodemic situation of CTX-M producing *E.coli* dissemination (**paper 2**) and (ii) at a local level during an nosocomial outbreak of MDR-*K.pneumoniae* (**paper 4**) revealing the significance of both an internationally successful *K.pneumoniae* clone and a specific promiscuous plasmid [21,65,67].

**DISSEMINATION OF ANTIBIOTIC RESISTANCE BY MOBILE GENETIC ELEMENTS IN ENTEROBACTERIACEAE.** Genome plasticity is essential in the adaptability of bacteria to different environments, including antibiotic-enriched environments. This is made possible through regulation of gene functions and/or by mutation and horizontal gen transfer (HGT). Mechanisms of HGT are essential in facilitating the dissemination of β-lactamases (such as ESBLs and carbapenemases).

Genes encoding bacterial resistance are either *intrinsic* (inherent) or *acquired* of origin. The genetic information encoding a particular mechanism of resistance is (i) intrinsic when it occurs as a result of cell division (i.e. vertical transmission) and (ii) acquired when it is a result of horizontal gene transfer (HGT – horizontal transmissions) or arises from *de novo* mutations[68-70].

**Horizontal gene transfer (HGT).** HGT involve the transfer of resistance encoding genes on plasmids, (lysogenic) bacteriophages, transposons or other mobile genetic material [69]. Transfer is mediated through different genetic mechanisms; namely transduction via bacteriophages, conjugation via plasmids and conjugative transposons, and transformation [69]. The latter involve incorporation of either chromosomal DNA, plasmids, or other DNA from dying microorganisms into the chromosome [69]. Naturally competent species are capable of picking up exogenous DNA and incorporate it into their genomes. Enterobacteriaceae however, does not belong to this category of species.

**Plasmids.** Plasmids are extrachromosomal, usually circular, supercoiled, and doubled stranded DNA located within the bacterial cytoplasma. They are also recognized by their capability of

autonomous replication and copy number control during cell division. Plasmid addiction systems, such as toxin-antitoxin post-segregation killing (PSK) systems, could maintain plasmids in the host even in the absence of a selective environment [71]. Unlike chromosomes, plasmids generally do not encode functions essential to bacterial growth, but provide gene products (i.e. virulence factors, antibiotic resistance determinants, metabolic pathways) that can be of benefit and enhance fitness of the bacterium under certain conditions[72-75]. In the Datta collection it has been recognized that prior to the "antibiotic era", plasmids rarely carried antibiotic resistance determinants [76].

Through conjugation, plasmids are capable of dissemination into other strains, species, or even genus. However, transfer capabilities vary and are unequally restricted among plasmids, defining broad - and narrow-host-range plasmids, respectively. Thus, plasmids have an evolution of their own with a set of core genes that remains relatively stable for long periods of time constituting different plasmid-species[77].

Plasmids are classified into different incompatibility groups. It is possible for bacteria to maintain several types of plasmids for many cell generations [78]. However, some plasmids are said to be incompatible as they have similar replication control mechanisms or partition competing during cell division, resulting in the loss of one of the incompatible plasmid types functions [78]. Recombination events may however alter the compatibility[73,79].

The most frequently encountered or "epidemic resistance plasmids" in Enterobacteriaceae carrying $bla_{\text{ESBLs}}$ include the IncF, IncI1, Inc L/M, IncA/C, and IncHI2 plasmids [80]. Sequence-based typing schemes have been provided for these Inc-plasmids to identify the relatedness of different plasmid scaffolds in strain collections from humans, animals and the environment [80]. Plasmids and the bacterial host interplay. Certain plasmids appear to increase fitness of specific bacterial clones particularly through supply of adequate virulence factors, antibiotic resistance determinants and maintenance factors beneficial to these clones[80-82]. Plasmid content may change by loss and acquisition of insertion sequence (IS) elements, transposons, and integrons. Thus, plasmids are dynamics structures and perfect platforms for adopting new resistance determinants and transmitting multidrug resistance between different species[83].

**Transposons.** Transposons have the ability to jump, or transpose, from one place in the DNA to another by non-homologous recombination[84,85]. A transposase is, however, obligate to this process, cutting donor DNA at the ends of the transposon and then at the point of insert on the target DNA. Transposition may occur either between plasmids or into or out of the chromosome [70]. Transposons may carry different genes including genes encoding antimicrobial resistance. Structurally, transposons are characterized by short inverted terminal repeat nucleotids enabling movement toward direct repeat nucleotids that could be identified in most recipient/target-DNA. During the process of insertion the direct repeats are duplicated [86]. Transposons move either in a "copy and paste" (replicative) (e.g. Tn3) or "cut and paste"-manner [87]. IS elements are the

smallest (<2.5 kb) and simplest transposable elements and contain mostly genes encoding transposase enzymes and no selectable genes (including antibiotic resistance genes) [86]. Composite transposons are larger and consists of two IS elements of the same type embracing a central DNA sequence that is not by itself able to transpose [74]. The central DNA sequence may encode for antibiotic resistance. Both IS elements and composite transposons usually transpose in a "cut and paste" manner without replication. Conjugative transposons either carry or make use of a self-transferable apparatus with additional enzymes (integrase (unlike integrases in integrons), excinase, resolvase) and "cut and paste" the DNA involved. In contrast to conjugative plasmids, there is no replication in the donor cell [88,89].

**Integrons.** Integrons are themselves not mobile, but can be mobilized indirectly by being captured by other mobile genetic elements such as plasmids and transposons[90]. They were discovered through systematic molecular investigation of unrelated resistance plasmids and transposons [91]. Regarding their structure, the integrons have associated components, gene cassettes, which are incorporated through site-specific recombination. The integron possess an attachment site (attI), where integron encoded integrase mediate these site-specific recombination events [91]. Gene cassettes lack promoters and normally contain only a single gene and a "59 base element" constituting the specific recombination site[90,91]. In this way the gene cassettes are made movable, and represent a mechanism by which various (one or multiple) resistance genes may be captured and collected behind a single common promoter [91]. Integrons have proved to be important in the dissemination of multidrug resistance among Enterobacteriaceae, by facilitating resistance accumulation and promoting co-selection processes[67,92,93]. Class I integrons are the most commonly encountered class of integrons in Gram negative bacteria, including in Enterobacteriaceae[94-98].

**Investigation.** Diverse techniques exist to analyse plasmids, transposons and integrons for nosocomial outbreak and surveillance purposes[20,21,99]. In this study, plasmid typing was used to identify and characterize the R-plasmids (carrying $bla_{\text{CTX-Ms}}$) circulating in clinical isolates of *E.coli* in Norway (**paper 2)**. Typing of transferable genetic elements was also undertaken to support outbreak investigation in understanding patterns of transmissions (**paper 4**). Interpreting some of these analyses may be complicated by the frequent rearrangements of these structures[83,99]. Plasmid replicons are comparatively stable elements within the plasmid and thus convenient targets for exploring phylogenetic relatedness, evolution and surveillance[73]**.** Plasmids can be typed into different incompatibility families using a PCR-based replicon-typing approach, or further discriminated with a multi-locus sequence-based replicon-typing approach recently developed for the most prevalent incompatibility plasmid families of Enterobacteriaceae [80]**.**

**EXPANSION OF MDR RESISTANT CLONES IN ENTEROBACTERIACEAE.** Genome plasticity is a prerequisite for the effective formation of evolutionary fit bacterial clones conferring resistance. Such bacterial lineages appear better equipped to dominate by an increased rate of expansion.

**Clones and strains.** An exact distinction of these terms is difficult. Clones are bacterial isolates that are suggested to have the same common origin whereas a given strain are indistinguishable by "any phenotypic or genotypic" method applied[65]. Asexual bacterial reproduction results in offsprings that are identical to their ancestor, with spontaneous *de novo* mutations in the chromosomal giving rise to different clonal lineages. However, exchange of DNA takes place within different lineages of a species, or between species (less frequently) by horizontal gene transfer events e.g. conjugation, transduction, and transformation[100]. The nature of each species vary from clonal to recombinant, and thus also the level of genetic exchange within and between [101]. In "clonal species" the contribution of recombination in genetic diversity is relatively low, whereas in "non-clonal-species" the contribution is relatively higher. Even if significant differences exist, few species are located at the extremes of this scale [100]. Phage-mediated transduction is important in exchange of chromosomal DNA in Enterobacteriaceae, which are not naturally transformable organisms[5,100,102].

**Investigation by molecular typing.** By the use of evolutionary oriented multi locus sequence typing (MLST), important clones of Enterobacteriaceae have been identified in the dissemination of antimicrobial resistance. Both *Klebsiella spp*. and *E. coli* are considered comparatively clonal bacterial species. Clonality of these species is reflected by the stability and success of important ST-lineages such as the uropathogenic ST131 *E.coli* and ST258 *K.pneumoniae* [103]. Successful clones with enhanced ability to disseminate and cause disease in humans has been acknowledged with clonal structures participating in vertical transmissions that are easily missed by more discriminative typing methods such as pulse field gel electrophoresis (PFGE). Accordingly, it was recognized four years after their first discovery that the *E.coli* CTX-M-15 PFGE A-E strains in the UK all belonged to or descended from the same evolutionary lineage (ST131). Successful clones often seem to be able to acquire different (or the same) resistant determinants by several occasions[65,104,105].

Investigations of bacterial populations in nosocomial outbreaks (**paper 4**) require a different molecular typing approach than when studying the evolution of bacterial populations (**paper2)**. MLST is a typing method based on differences in housekeeping genes and therefore useful in an evolutionary context and for global epidemiology, but it may not have the sufficient discriminatory power in examining a hospital outbreak of clonal bacterial species e.g. Enterobacteriaceae (**paper 4**). In outbreak situations, a method analysing the whole genome or the variable regions of the genome is more appropriate. In addition, when interpreting different methods one must bear in mind that some level of genetic change is likely to occur as nosocomial pathogens move from one patient to another[99]. Traditionally pulsed-field gel electrophoreses

(PFGE) typing based on restriction enzyme digestion patterns has been used for this purpose in many bacteria [106]. However, even if discriminative, PFGE results are laborious an time consuming, and rapid PCR-based methods with high discriminatory power (e.g. multi-locus variable tandem repeat analysis MLVA or rep-PCR systems (DiversiLab®)) offers reliable alternatives in most local settings [107]. The discriminatory power required to infer genetic relatedness might vary in different outbreaks settings. In hospital outbreaks where the epidemiological linkage is established, the demand on the discriminatory power of the typing method is generally lower than in situations where epidemiological linkage is only hypothetical. Contrary, in natural competent bacteria that frequently take up DNA, a discriminatory technique may falsely classify an epidemic outbreak as non-epidemic [108]. More than one typing method may be necessary to investigate the chromosomal relatedness of different strains.
Whole genome sequencing (WGS) has the potential of yielding more complete and less fragmented data. This has been illustrated by Johnson et al. in the case of the evolution and dissemination of CTX-M 15 in ST131 *E.coli* subclones bridging the gap between information gained by MLST and PFGE, respectively, as well as by offering detailed data revealing unexpected modes of transmission in outbreak investigations by a higher level of certainty [109,110].

No ideal typing method is readily available to accommodate all situations within a reasonable timeframe and the choice of appropriate typing method(s) in general is dependent on a set of "performance and convenience criteria" [107]. According to van Belkum et al., these factors relate to the biology of the organism, the epidemiological context and other factors such as speed, costs, local skills and equipment, and possibilities for sharing of results[111].

## A.5 MECHANISMS AND EPIDEMIOLOGY OF RESISTANCE TO 3RD GENERATION CEPHALOSPORINS AND CARBAPENEMS IN ENTEROBACTERIACEAE

In bacteria, genes encoding antibiotic resistance in bacteria mediate different biochemical mechanisms of resistance. These mechanisms are usually divided into three classes; (i) decreased accumulation of an antibiotic through active efflux or reduced permeability of the bacterial cell, (ii) modification of the antibiotic target molecule (e.g. PBP modification), and (iii) bacterial modification of the antibiotic itself (e.g. β-lactamases) [68,112]. These mechanisms may act alone or in concert to create the resistance phenotype of the bacterial cell to a particular antibiotic[113-115].

The different mechanisms of antibiotic resistance mediating low-level or high-level resistance to 3rd generation cephalosporins and consequences for phenotypic testing were specifically addressed in this study (**paper 1 and 3**). A wide array of factors and mechanisms affect the level of resistance to carbapenems in Enterobacteriaceae, which in turn complicate correct detection of mechanism(s) in these isolates (**paper 4**).

**MECHANISMS OF B-LACTAM RESISTANCE IN ENTEROBACTERIACEAE.** Reduced susceptibility to β-lactams in Enterobacteriaceae may result from the activity of periplasmatic β-lactamases, outer membrane protein (OMP) changes, efflux or PBP changes [116] (Figure 4). In general, PBP changes play a minor role in generating resistance to β-lactams in Enterobacteriaceae, and efflux does not play any significant role in developing resistance to 3rd generation cephalosporins and carbapenems [113,114,117,118]. Production of β-lactamases is the most important mechanism of resistance to β-lactam antibiotics among clinical isolates of gram negative bacteria, including Enterobacteriaceae. However, interplay of different mechanisms may alter the MICs of some β-lactams; most importantly the combination of broad spectrum β-lactamases and OMP changes may result in reduced susceptibility to 3rd generation cephalosporins, and extended spectrum β-lactamases and OMP changes may result in reduced susceptibility to carbapenems [119,120].

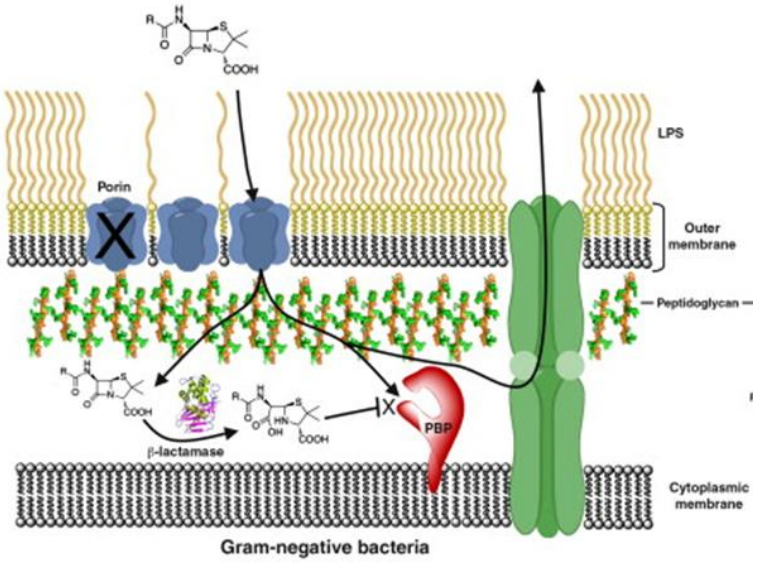



*Figure 4. The doubled layered cell wall membrane in gram-negative bacteria. The outer membrane resist large hydrophobic molecules and the OMPs are water-filled channels that serve as main entrances of hydrophilic antibiotics such as β-lactams. After diffusing through the OMP, a given β-lactam in the periplasmic space may be attacked by β-lactamases or efflux pumps (green) if it is a substrate for these structures. Traversing the periplasm the β-lactam reaches its target, the PBPs[121]. (Figure reprinted with permission from Current Opinion in Microbiology [122]).*

**B-LACTAMASES.**

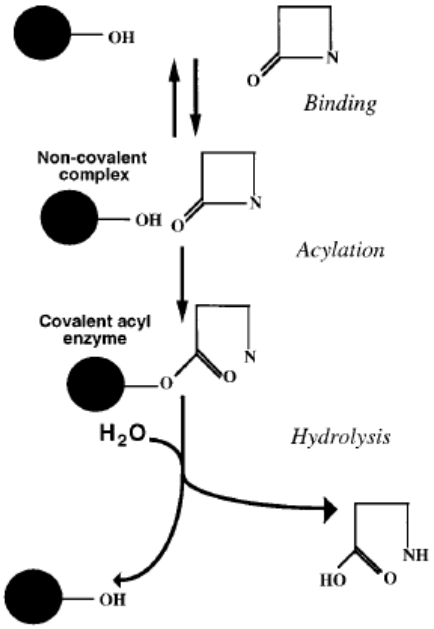



*Figure 5. Action of β-lactamases. B-lactamases inactivate β-lactams by hydrolysis before it reaches the PBP target[123]. This hydrolysis is made possible by structural similarities between the PBP target and the β-lactamase[124]. In serine β-lactamases (class A, C and D) a free –OH-group in the active site of the enzyme attacks the β-lactam-ring in a two step reaction. Finally, the active enzyme is liberated through hydrolysis. In metallo β-lactamases (class B) metal cations ($Zn^{2+}$) catalyze the hydrolysis step. (Figure reprinted with permission from Clin Microb Rev.[120]).*

**DEFINITIONS AND CLASSIFICATIONS OF B-LACTAMASES.** Two schemes are widely used for the classification of β-lactamases; the Ambler and Bush-Jacoby-Medeiros classifications [125-127] (Table 2). The sequence-based Ambler classification was proposed in 1980 and designates β-lactamases into four classes, A to D. Class A, C and D include evolutionary distinct groups of serine enzymes, whereas class B contains zinc-dependent types [120]. Jack and Richmond first proposed a functional classification of β-lactamases in 1970. This classification was expanded by Richmond and Sykes in 1973, and then by Sykes and Matthews in 1986. Bush further reorganized the classification in 1989. In 1995, Bush, Jacoby, and Medeiros revised this classification (Bush-Jacoby-Medeiros classification). More recently, Bush has performed another update[126-128]. The numbers of revisions reflect the difficulties in encompassing functional characteristics into evolving and emerging β-lactamases[129]. According to this classification, enzymes are first separated according to their inhibition characteristics to EDTA (enzymes yielding a positive result assigned to class 3). Secondly, they are grouped according to substrate profile into penicillinases or cephalosporinases. Some penicillinases are sub-grouped as oxacillin- or carbenicillin hydrolyzing, respectively. Finally, the enzymes are classified according to their inhibition of clavulanic acid [126] (Table 2).

According to these schemes traditional ESBLs are classified as Ambler class A, Bush-Medeiros-Jacoby group 2be, the "e" denoting expanded-spectrum β-lactamase. ESBL-types may include TEM-, SHV- and CTX-M-ESBLs[129]. Chromosomal OXY-type β-lactamases in *Klebsiella oxytoca* also classifies as class A group 2be enzymes, whereas AmpC enzymes, both of chromosomal and plasmid origin, classifies as class C group 1 enzymes and the carbapenemase KPC-enzymes as class A group 2f enzymes.

*Table 2. Functional grouping and corresponding molecular classification of β-lactamases[a]. (Table reprinted with permissions from Annual Rev Microbiol [2]).*

| Bush-Jacoby group | Molecular class | Defining characteristic(s) | Selected enzymes |
|---|---|---|---|
| 1 | C | Hydrolyzes cephalosporins and cephamycins, generally with higher $k_{cat}$ values than penicillins<br>Not inhibited by CLA and TZB<br>High affinity for aztreonam | *Escherichia coli* and *Pseudomonas aeruginosa* AmpC, CMY-2, FOX-1, MIR-1, P99 |
| 1e | C | Hydrolysis of penicillins, cephamycins, expanded-spectrum cephalosporins, monobactams[b]<br>Not inhibited by CLA and TZB | GC1, CMY-37 |
| 2a | A | Efficient hydrolysis of penicillins<br>Inhibited by CLA and TZB | PC1 and other staphylococcal penicillinases |
| 2b | A | Efficient hydrolysis of penicillins and early cephalosporins (cephaloridine, cefazolin, cephalothin) | SHV-1, TEM-1, TEM-2, TLE-1 (TEM-90) |

| Bush-Jacoby group | Molecular class | Defining characteristic(s) | Selected enzymes |
|---|---|---|---|
| | | Inhibited by CLA and TZB | |
| 2be | A | Hydrolysis of penicillins, expanded-spectrum cephalosporins, monobactams<br>Inhibited by CLA and TZB | ESBLs[b] CTX-M-15, CTX-M-44 (Toho-1), PER-1, SFO-1, SHV-5, TEM-10, TEM-26, VEB-1 |
| 2br | A | Efficient hydrolysis of penicillins and early cephalosporins<br>Not well inhibited by CLA | IRTs: TEM-30, TEM-76, TEM-103, SHV-10, SHV-26 |
| 2ber | A | Hydrolysis of penicillins, expanded-spectrum cephalosporins, monobactams<br>Less efficiently inhibited by CLA and TZB | CMTs: TEM-50, TEM-68, TEM-89 |
| 2c | A | Efficient hydrolysis of carbenicillin<br>Inhibited by CLA | PSE-1, CARB-3 |
| 2d | D | Efficient hydrolysis of cloxacillin or oxacillin<br>Not always inhibited by CLA | OXA-1, OXA-10 |
| 2de | D | Hydrolysis of penicillins and expanded spectrum cephalosporins<br>Not always inhibited by CLA | ESBLs: OXA-11, OXA-15 |
| 2df | D | Hydrolysis of carbapenems and cloxacillin or oxacillin<br>Not always inhibited by CLA | OXA-23, OXA-48 |
| 2e | A | Efficient hydrolysis of cephalosporins<br>Inhibited by CLA and TZB but not by aztreonam | CepA |
| 2f | A | Hydrolysis of carbapenems, cephalosporins, penicillins, and cephamycins<br>Poorly inhibited by CLA, low inhibition by TZB | IMI-1, KPC-2, KPC-3, SME-1, GES-2 |
| 3a | B | Hydrolysis of all β-lactams except monobactams<br>Inhibited by EDTA and metal ion chelators, not inhibited by CLA and TZB | IMP-1, L1, NDM-1, VIM-1 |
| 3b | B | Preferential hydrolysis of carbapenems<br>Inhibited by EDTA and metal ion chelators, not inhibited by CLA and TZB | CphA, Sfh-1 |

[a]Adapted from [126,127]. [b]Expanded-spectrum β-lactams are usually defined as those cephalosporins and monobactams that contain a side chain containing an aminothiazoleoxime moiety extending from the β-lactam ring. These include the cephalosporins cefotaxime, ceftriaxone, ceftazidime, cefepime and cefpodoxime, and the monobactam aztreonam. The enzymes in groups 1e, 2be, and 2de are known as ESBLs. Abbreviations: CLA, clavulanic acid; CMT, complex mutant TEM; ESBL, extended spectrum β-lactamase; IRT, inhibitor-resistant TEM; TZB, tazobactam

The current ESBL definition, while convenient for researchers, is less useful for broader use among clinicians, infection control specialist and the public as a whole. As the term "ESBL" is familiar to infection control workers, and has reached a level of awareness among clinicians and media, it has been proposed to use this term and broaden it to cover all clinically important

plasmid encoded β-lactamases with activity to extended-spectrum cephalosporins or carbapenems[130]. In this classification scheme carbapenems are described as "ESBLs with hydrolytical activity against carbapenems" (ESBL–CARBA) and group 2be enzymes as ESBL-A. Plasmid mediated AmpC enzymes are included in a third category (ESBL-M, where "M" denotes a miscellaneous group). It is argued that this definition would facilitate and better meet the needs of clinicians and infection control specialist to communicate appropriately in the guidance of therapy and the implementation of appropriate infection control measures.

**CHROMOSOMAL B-LACTAMASES.**

Chromosomal β-lactamases exist in all Enterobacteriaceae except for *Salmonella spp*., with various degrees of expression [120]. In some Enterobacteriaceae (i.e. *Enterobacter spp., Citrobacter freundii, Morganella morganii, Providencia stuarti* and *Proteus rettgeri), ampD* mutants are present with a frequency of $10^{-5}$-$10^{-8}$. Stably derepressed mutant that are high-level resistant to 3rd generation cephalosporins, are comparatively easily selected in the individual patient during treatment β-lactam antibiotics, particularly in high inoculums *Enterobacter spp.* and *Citrobacter freundii* infections, and by the use of β-lactam antibiotics that are less stable and poor inductors [120]. Resistance to 3rd generation cephalosporins in these species was evident and acknowledged as a clinical problem even before the emergence of the ESBLs.

***Hyperproducers of AmpC in E.coli and OXY in K.oxytoca.*** In this study (**paper 3**) we examined the prevalence of mutants of chromosomal AmpC in *E.coli* - and OXY-β-lactamases in *K.oxytoca* in Norway and their consequences for phenotypic detection of ESBL- and AmpC. In *E.coli* this mechanism usually mediate low-level resistance to 3rd generation cephalosporins, whereas high-level cefotaxime and aztreonam resistance may result from hyperproduction of OXY-β-lactamases in *K.oxytoca* while ceftazidime is moderately affected and remains within the susceptible range [120]. During therapy, resistant populations may frequently arise through *de novo* selection within the patient. Further clonal spread has sometimes been acknowledged, particularly in *K.oxytoca* [131]. Chromosomal *ampC* (class 1C) expression in *E. coli* and chromosomal classA OXY-type β-lactamase expression in *K. oxytoca* is normally constitutive at a low-level due to a weak promoter. Hyperproducing isolates may result from promoter region mutations, sometimes combined with attenuator mutations creating a stronger promoter mediating efficient transcription[131-133]. In *E.coli* even IS-elements carrying a more efficient promoter has been described[134]. Different class 1 group C –enzymes have subsequently been mobilized onto plasmids.

**PLASMID ENCODED B-LACTAMASES.**

**Broad spectrum betalactamases:** TEM-1 was the first broad-spectrum plasmid encoded β-lactamase to be detected (Greece in 1965), thus identified after the launch of the first broad-spectrum β-lactam (ampicillin) in the late 50s [135]. It is still of unknown origin. TEM-1 is mostly carried on plasmids by Tn*3*- transposons, and has rapidly disseminated and today constitute between 30-80% of all clinical *E. coli* isolates [70,136,137]. Apart from TEM-1, plasmid encoded broad -spectrum SHV-1 penicillinases originating from *K. pneumoniae* are most frequently encountered in Enterobacteriaceae. Several novel broad spectrum β-lactams were introduced in the late 1970s and the 1980s to counter the spread of these enzymes, and of these oxyimino-cephalosporins quickly became the most widely used antibiotics in therapy[70,121].

**ESBLs:** Plasmid encoded mutants of both TEM- and SHV variants were the first ESBLs[138]. TEM- and SHV-ESBLs were developed by mutations in already present *bla* genes on conjugative plasmids or transposons. Knothe described the first ESBL in 1983, a SHV-1 mutant termed SHV-2, from a *Klebsiella oxytoca* isolate from Germany [139]. Reports followed in the mid-80s of hospital outbreaks extending from the Clermont-Ferrand region to several other hospitals in France of TEM-3 producing Enterobacteriaceae [140]. Today, the numbers of variants have grown to over 216 TEM -, and 182 SHV variants [141]. Among these, SHV-2, -5, -12 and TEM-3,-10, 26,-52 and -116 are widespread in many countries [121]. Analysis of the genetic support of $bla_{\text{TEM}}$ ESBLs mostly revealed Tn*3*-transposon structures, while Tn*26* elements forming composite transposons are associated with $bla_{\text{SHV}}$ ESBLs. Thus, these mobile elements seem to be important in the acquisition of these ESBL genes. $Bla_{\text{TEM}}$- and $bla_{\text{SHV}}$-genes have never been identified inside integron structures[136]. During the 1980s and 1990s the TEM- and SHV-ESBLs were recovered from *K. pneumoniae* in nosocomial settings. Typically, intensive care units were "hot spots" for major clonal outbreaks in many hospitals, although plasmid diffusion was sometimes recognized [121,142,143]. Dissemination of these enzymes into different serotypes was attributed to the quality of *Klebsiella spp.* as a good host for large multi-resistant, low copy number plasmids [120]. Dissemination of these classical TEM- and SHV-ESBLs into *E. coli* and into the community was rare, although the $bla_{\text{TEM-ESBL}}$ progenitor TEM-1 was frequently distributed in *E. coli* [121].

Broad-spectrum TEM-1 β-lactamases in *E.coli* has affected the epidemiology of β-lactamases in Norway for years. Resistance to ampicillin in *E.coli* mainly contributed by $bla_{\text{TEM-1}}$ is presently 42.7 % in *E.coli* in blood culture isolates recovered through our national surveillance system [144]. On the contrary, TEM- and SHV-ESBLs in *K. pneumoniae* in the nosocomial settings did not affect the epidemiology of β-lactamases our country during the 1980s and 1990s. One might speculate that prudent use of antibiotics, lack of crowding and a high standard of hygiene in hospitals, limited exchange of patients between hospitals in Norway and from countries abroad may all have contributed to this situation. However, by the turn of this century, the situation dramatically changed as reports surfaced from Europe describing a new type of ESBLs in Enterobacteriaceae, the CTX-Ms, which was recovered by a significant proportion in *E.coli*.

This new situation raised the question; was Norway part of the emerging CTX-M producing *E. coli* wave (**paper 1**)? Subsequently, as the involvement of Norway was confirmed, the molecular epidemiology at clone, strain and plasmid level was explored in order to investigate patterns of dissemination and relationship to international observations (**paper 1 and 2**).

***CTX-M -producing E. coli.*** By the turn of this century the prevalence of the CTX-M enzymes increased rapidly worldwide thereby changing the epidemiology of ESBLs[121,142,145,146]. More precisely, CTX-M –enzymes were first recognized in South America some years prior to the turn of the century, in parts of Europe and Canada close to it, and then some years later they were documented also in the USA [142,146].

CTX-M enzymes have now become the most prevalent ESBL enzyme, and *E. coli* is its predominant host. Contrary to the classical ESBLs, CTX-M ESBLs are prevalent in community isolates and in extending-care facilities. The potential risk of influx of ESBLs from community into hospitals has to be accounted for in hospital infection control and outbreak investigations in institutions[147-150]. Compared to classical ESBLs a new set of risk factors are recognized for acquisition and infection by isolates carrying CTX-M enzymes. They are more likely community-onset UTIs in *E.coli* possibly affecting otherwise healthy individuals even if different risk factors currently are associated with these infections, including recent health care contact or stay in an extended care facility, recent antibiotic use, complicated recurrent UTI, diabetes mellitus and recent travel in high prevalent countries [145,146,151-153]. A recent Norwegian study interestingly identified recreational swimming in Norwegian freshwaters as a risk factor for UTI caused by ESBL-producing Enterobacteriaceae [154].

CTX-M enzymes were first discovered both in Germany and Argentina in 1989, and were present in lesser scale in some countries from the early 90s (Argentina, Japan, and Germany)[155,156]. Presently 150 variants are registered in the Lahey clinic database [141]. The CTX-M family is subdivided in five groups according to amino acid sequence homology; 1, 2, 8 9 and 25[157]. Different enzymes predominate in different parts of the world; CTX-M-15 (group 1) in most of Europe, Canada, the Middle East, and India), CTX-M-14 (group 9) in China, South-East Asia, and Spain, and CTX-M-2 (group 2) in Argentina, Israel and Japan[158]. However, CTX-M-15 is presently diffusing in most parts of the world and is dominating globally.

Mobilization of CTX-M genes from chromosomal *Klyvera spp*. to plasmid localization involve specific mobile genetic elements upstream of the $bla_{\text{CTX-M}}$; (i) IS*Ecp1*, associated with most genes within the CTX-M groups 1,-2, and 9, (ii) IS*CR1* embedded in a class 1 integron complex associated with CTX-M group 2 and -9, and (iii) phage-related sequences related to $bla_{\text{CTX-M-10}}$ in Spain [157]. Analyses of DNA sequence homology suggest that at least 9 mobilizing events have occurred in the 5 $bla_{\text{CTX-M}}$ subclasses or clusters (1, 2, 8, 9, 25, respectively) into plasmid location [67,159].

Dissemination of $bla_{\text{CTX-M}}$ genes involves both clonal spread, diffusion of plasmids and transposons and translocations of resistance genes between various mobile genetic elements

building a hierarchical structure of movable genetic modules (Figure 6)[67,160]. The frequent differences in the genetic environment observed associated with *bla*$_{CTX-M}$ nevertheless indicate that dissemination is attributed to a series of independent events[160,161]. The interaction of both efficient vertical expansion, horizontal dissemination of particular mobile genetic elements and frequent new-capture-events contribute to the overall increase in CTX-M enzymes[159,160]. Co-selection processes are promoted and also facilitate their success by prominent resistance to other antibiotics such as aminoglycosides, fluoroquinolones and trimethoprim/sulfametoxazole[67,162]. Subsequent *bla*$_{CTX-M}$ mutations (or *bla*$_{CTX-M}$ recombinations) affecting the active site of these enzymes have subsequently changed and expanded their spectrum of hydrolyses, analogous to mutations in *bla*$_{TEM}$- and *bla*$_{SHV}$-genes [67,160,163,164].

Some frequent associations have been acknowledged between these modules at different levels; e.g. (i) between *bla*$_{CTX-M}$ and certain plasmid Inc-groups (*bla*$_{CTX-M-15}$ with IncF plasmids, *bla*$_{CTX-M-3}$ and *bla*$_{CTX-M-1}$ with IncN, IncL/M and Inc A/C plasmids, *bla*$_{CTX-M-14}$ with IncI and IncK plasmids, and *bla*$_{CTX-M-9}$ with IncHI2 and IncFI plasmids) [73], and (ii) among particular successful multi-drug resistant "high-risk "clones such as ST131 and ST405 and *bla*$_{CTX-M-15}$, and ST38 and ST393 to *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-9.}$ Although these clones may also carry other *bla*-types locally prevalent as they seem to possess a flexible ability to acquire diverse resistance or virulence traits [65,67]. Co-selection processes seem important for the success of *bla*$_{CTX-M-15}$ within ST131, and most probably apply to other successful clones as well[85,162]. In ST131 fluoroquinolone resistance probably preceded the acquisition of *bla*$_{CTX-M-15}$ [165].

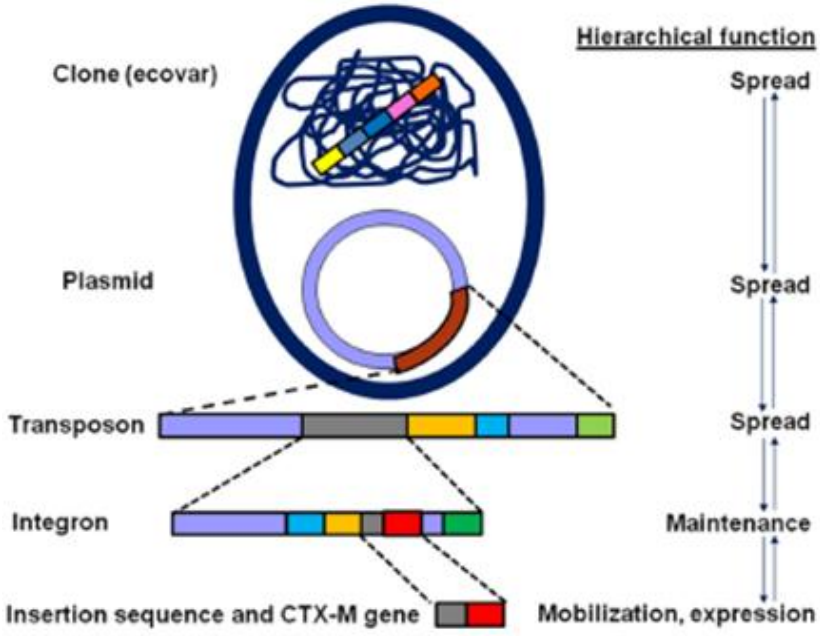



*Figure 6. Mobilization and dissemination of bla*$_{CTX-M.}$ *(Figure reprinted from Frontiers in Microbiology [67]). Specific mobile elements are associated with the mobilization and expression of the bla*$_{CTX-M}$*, and dissemination is accomplished through successful clones of various PFGE types, diffusion of plasmids and transposons and the translocation of resistance genes between mobile genetic elements [73,166,167].*

A huge global reservoir is suggested by the identification of ESBL-producing Enterobacteriaceae, mainly *E.coli* carrying *bla*$_{CTX-M}$ in drinking water, sea water, food, food animals, wild animals

(including birds), and companion animals [168-171]. Transmissions from these reservoirs to humans is fuelled by diverse factors including the frequency of travel and other population exchange to high endemic areas, high population densities promoting human-to-human transmissions, fecal-oral transmissions by water particularly in deprived areas, and by contaminated food products [168,172,173]. The study of ESBLs in this study (**paper 1, 2 and paper 3**) is concerned with human clinical Enterobacteriaceae isolates as the end point. The importance of investigating other (i) ecological niches as well as the (ii) factors related to human carriage - duration, dynamics, host risk factors and molecular epidemiology is however increasingly being realized as important in order to understand this end point.

*Bla*$_{\text{CTX-M}}$ *E.coli* has been recovered from animal populations. Presently, the most common genes associated with resistance in animals in Europe are *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CMY-2}}$, whereas *bla*$_{\text{CTX-M-15}}$ has infrequently been encountered in this context[174-176]. Regarding CTX-M-15-producing *E.coli* ST131, a recent review suggested that humans presently might be the major reservoir with occasional transfer to the animal populations[177]. The impact of food animals as a source of ESBLs impacting human health appears to play different roles in different countries (e.g. in the UK and the Netherlands)[178,179]. The national differences that likely exist underline the need for building national competence and gain epidemiological knowledge at national or regional levels in order to take appropriate actions.

The role of human-to-human transmissions in *E.coli*-producing ESBLs is supported by studies investigating transmission dynamics and fecal carrier rates in human households[168,173,180-182].

**Plasmid –encoded AmpC-β-lactamases:** *bla*$_{\text{CMY2}}$ is the most frequently isolated family of these enzymes and was first detected in Enterobacteriaceae in Greece 1996. These enzymes have commonly been associated with resistance in animals. A recent survey from our country documented the frequent presence of *bla*$_{\text{CMY2}}$ within the broiler production pyramid [183].

**Carbapenemases:** The vast majority of the presently identified acquired carbapenemases in Enterobacteriaceae belong to Ambler class A, B or D and primarily to functional groups 2f, 2d and 3[184,185]. Carbapenemases are usually linked to other resistance gene determinants, thus giving rise to multi drug resistant (MDR), extremely drug resistant (XDR) or *de facto* pan-drug resistant isolates[186,187].

Plasmid-encoded carbapenemases in clinically relevant non-fermenters (like *Pseudomonas aeruginosa* and *Acinetobacter spp*.) appeared in Japan in early the 1990s where carbapenems were more freely used in parenteral therapy after introduction than in the rest of the world[188]. Also in Japan in the early 1990, the first plasmid-encoded carbapenemase in Enterobacteriaceae was detected[189]. However, in Enterobacteriaceae, the carbapenems retained almost complete

activity for nearly 20 years after imipenem was launched in 1985[121]. In many countries carbapenem consumption in empirical and definite treatment has increased as a response to rising ESBL prevalence rates. Since the early-mid 2000s, hospital outbreaks of carbapenemase-producing Enterobacteriaceae (CPE), mainly *K.pneumoniae*, have occurred; reaching endemic levels in some areas[58,190]. The current epidemiological situation of CPE in Europe varies significantly from an endemic situation in Greece and Italy to reports of sporadic occurrences or single hospital outbreaks in all of the Nordic countries. However, presently CPE are still mostly recovered from hospital settings, and linked to travel to high prevalent areas[158]. Recently, and worryingly, carbapenemases, mainly; *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-48}}$ and less commonly *bla*$_{\text{KPC}}$, have been identified in *E. coli*[191-193]. In reality, the history of the dissemination of ESBLs may very well repeat itself with the carbapenemases; with certain carbapenemases participating in hospital outbreaks mainly involving *K. pneumoniae* (and to a certain extent *Enterobacter spp.*) like TEM and SHV-ESBLs in the 1980-90s, while others (e.g. NDM-1s and OXA-48s) may disseminate initially at a lower, but less controllable rate in community-acquired *E.coli*-isolates, mimicking the situation displayed in community-acquired urinary tract *bla*$_{\text{CTX-M-15}}$ *E.coli* isolates for the last decade [186,194]. The epidemiology of class B and D carbapenemases will not be detailed as they were outside the scope of this study. Briefly; among metallo-enzymes, VIM was first detected in Enterobacteriaceae in Greece in 2003, and clusters of VIM-producing *K.pneumoniae* are now reported worldwide [121]. NDM-1 was first detected in Sweden in 2008 in a patient transferred from a hospital in India. The Indian subcontinent and possibly the Balkans are currently considered the main reservoirs[195]. The OXA-48 was first detected in Turkey in 2001, and North-African countries represent the main reservoirs. Sporadic cases and nosocomial outbreaks have been identified in several countries in Europe[196]. Reports of OXA-48 -producing *E.coli*, in some cases harboring fewer resistant determinants, underline the potential for community spread and enhanced diagnostic problems[197]. Dissemination of *bla*$_{\text{OXA-48}}$ is strongly linked to the dissemination of a single 62 kb IncL/M plasmid (pOXA-48a). Within this plasmid a composite transposon (Tn*1999*) is inserted into the *tir* gene encoding a transfer inhibitory protein thereby promoting a higher transfer frequency of the plasmid and to its epidemiological success and dissemination[198].

***K.pneumoniae carbapenemases (KPCs).*** Another aspect of the epidemiology of the "newer β-lactamases" in Norway is highlighted by this study (**paper 4**)[1,2]. A KPC-producing *K.pneumonia*e was detected for the first time in Norway in a patient recently hospitalized in a high-prevalent country (Chania, Crete). Subsequently, this patient facilitated a prolonged, low-frequent nosocomial outbreak underlining the potential for local spread in hospitals by such isolates in our country.

KPC-producing Enterobacteriaceae has been primarily linked to *K.pneumoniae* and has been tightly associated with nosocomial outbreaks. Some reports of outbreaks in LTCF exist as well as community-onset cases most often in patients with recent and extensive health care contact[199].

Even if KPC-enzymes have been most frequently identified in *K. pneumoniae* reports have also documented their presence in *E. coli*, *Enterobacter spp*. and various other Enterobacteriaceae[200]. Diffusion into non-fermenter species, including *Acinetobacter spp.* and *Pseudomonas spp.*, has also been reported[201]. KPC-type carbapenemases are currently the predominating Ambler classA carbapenemases with $bla_{KPC-2}$ and $bla_{KPC-3}$ being the two most frequently isolated alleles. The first $bla_{KPC}$ ($bla_{KPC-1}$) was first identified in the USA through the ICARE surveillance system in 2001 in a *K. pneumoniae* isolate originating from 1996 and has subsequently been reported worldwide[200-202]. In Europe, the first imported case was reported from France in 2005 from a patient that had been hospitalized in New York[203]. High prevalence rates and extended outbreaks of KPC-producing *K.pneumoniae* have been reported from the metropolitan hospitals in New York in addition to reports of cases across the USA, China, and from Italy and Greece in Europe and from Israel[200]. The success of the $bla_{KPC}$-genes is favored by their location on a Tn*3*-like transposon structure (Tn*4401* with different isoforms) capable of mobilizing $bla_{KPC}$ genes with high transposition frequency, self-transferable plasmids of diverse sizes and replicon types, and in successful STs such as the ST258 [204] [136]. Similar $bla_{KPC}$ genetic environments have been seen in distinct *K.pneumoniae* isolates[205-207].

**SURVEILLANCE.** There exists no continuous global scale antimicrobial resistance surveillance system monitoring the prevalence of ESBL-producing clinical isolates in different regions [157]. The most valid up-to-date data are retrieved from the EARS-Net database in which reduced susceptibility to 3rd generation cephalosporins is used as a surrogate marker of ESBL production [208]. Despite the fact that that the proportion of isolates resistant to 3rd generation cephalosporins varies significantly between countries, increasing rates has been reported throughout Europe in recent years. The highest rates of resistance are reported from Southern and Eastern European countries, but even in the Scandinavian countries, rates are increasing. In EARS-Net results from 2012, the prevalence rates of 3rd generation cephalosporins resistance in blood culture isolates ranged from 4.4 % (Sweden) to 38.1 % (Bulgaria) with <5%, 5-10%, 10-25% , and >25% from 3, 11, 11, and 3 countries, respectively) in *E. coli* and from 1.7% (Finland) to 74.8% (Bulgaria) with <5%, 5-10%, 10-25% , 25-50% and >50% from 3, 2, 10, 7, 8 countries, respectively) in *K. pneumoniae* [208]. Apart from lack of phenotypic or molecular confirmatory tests, differences in methodology, selection of participating laboratories, and blood culture practices could introduce biases into these figures. International surveillance of UTI isolates does not exist. Outside Europe, the prevalence rates of ESBLs are generally lower in the USA, higher in Asia and South America [121,209]. Non-governmental surveillance systems and monitoring programs (funded by pharmaceutical companies) from different geographical regions such as SMART, SENTRY, TRUST, TEST, and MYSTIC have also reported and published prevalence rates. High prevalence rates of *E. coli* ESBLs are recorded from China (55 %) and India (79 %)[210]. Interestingly, in the data from India, the prevalence rates were equally high among *E. coli*

collected from hospital and community settings. Of note, ESBL rates may vary greatly among different population groups within a nation[121,211].

The prevalence rate of ESBL production in *E. coli* and *K. pneumoniae* isolates in Norway has been reported in NORM from 2003-2013. According to NORM 2012 and 2013 the prevalence of ESBLs in *E.coli* collected in 2012 and 2013 peaked ≥ 5 % in blood culture (5.5% in 2012 and 5.0% in 2012, respectively) and ≥ 2.0% in urinary tract isolates (2.2 % in 2012 and 2.0 % in 2013, respectively). Two $bla_{\text{CTX-M-15}}$ outbreaks have been reported from Stavanger University hospital, involving *E. coli* a minor outbreak in a medical department in 2007 and *K. pneumoniae* in an outbreak originating from the intensive care neonatal unit in 2009 [212,213]. According to the EARS-Net 2012 report the prevalence of carbapenem resistant isolates remain <0.1% in *E.coli* blood culture isolates in Europe except in Bulgaria (0.9%) and Greece (1.4%). The prevalence rates of carbapenem resistance in blood culture *K. pneumoniae* isolates ranged from zero (7 countries) to 60.5% (Greece) with <1%, 1-5%, 5-10%, 10-25%, and >25% reported from 20, 4, 2, 1, and 2 (Greece, Italy) countries, respectively [208].

CPE are still rarely encountered in Norway. During 2007 - October 2014 54 CPE isolates (19 $bla_{\text{KPC}}$, 16 $bla_{\text{NDM}}$, 10 $bla_{\text{OXA48-like}}$ (2011-2014), 7 $bla_{\text{VIM/IMI,}}$ 1 $bla_{\text{OXA48-like+NDM,}}$ 1 $bla_{\text{unknown}}$) have been detected in Norway usually related to imported cases (pers.comm. Ø. Samuelsen, Norwegian National Advisory Unit on Detection of Antimicrobial Resistance). A single long-term nosocomial outbreak of KPC-producing Enterobacteriaceae has been reported (**paper 4**). Since July 2012, all detected carbapenemases are reported to the Norwegian Surveillance System for Communicable Diseases (MSIS), Norwegian Institute of Public Health (Folkehelseinstittutet).

**REDUCED PERMEABILITY**. In this study we examined the possible contribution of reduced permeability to low-level resistance to 3rd generation cephalosporins in *E.coli* in clinical isolates in Norway, specifically in order to explore their consequences on phenotypic methods of detection (**paper 3**). Susceptibility to 2nd and/or 3rd generation cephalosporins and carbapenems may be affected by impermeability or efflux, usually interplaying with concomitant β-lactamases (Figure 7)[113,115,118,214-221].

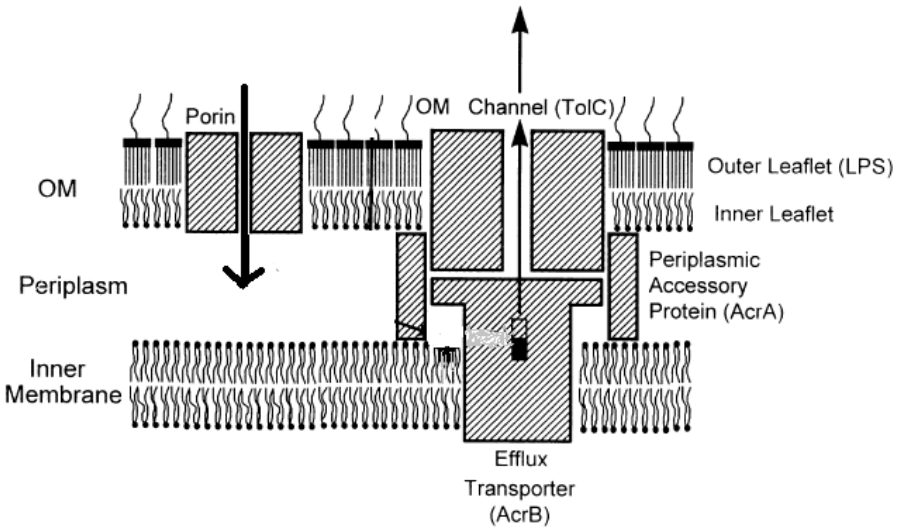



*Figure 7. Porins serve as main entrances of β-lactam antibiotics. Significant resistance may ensue from the co-operate efforts of β-lactamases (not depicted) and porin deficiencies. 2 nd generation cephalosporins may act as substrates for AcrA-TolC efflux pumps. (Modified reprint of figure with permission from Seminars in cell and developmental biology [222]).*

The outer membrane of gram negative bacteria reduces penetration of multiple toxic compounds, promoting bacterial survival in hostile environments[223,224]. Outer membrane proteins, or porins, serve as main entrances of hydrophilic nutrients and hydrophilic antimicrobials such as quinolones and β-lactams, and it also help in the extrusion of toxic waste products[222,225]. For some substrates, including some antibiotics, the permeability barrier in *E.coli* function together with active efflux systems regulated through global or local regulators, including activation of RNA antisense *micF* that down-regulates the expression of *ompF* (*micF* transcripts inhibit the translation of OmpF porin mRNA)[219,222,226-228]. Thus, transcriptional regulation of genes encoding porins and efflux-pumps are interconnected [229]. Importantly, the efflux mechanism coupled with outer membrane modifications or loss has been documented in several clinical MDR isolates[114,118].

OmpF and OmpC are the major non-specific (or hydrophilic) porins in *E.coli* with OmpF being the preferred route of β-lactam antibiotics[219,230]. In *K.pneumoniae* two proteins, OmpK35 and OmpK36, homologs of OmpF and OmpC, respectively, have been identified[219]. OmpK37, a porin that is usually quiescent, may allow the passage of carbapenems, but not to other β-lactams [219,231]. Loss of porins have been associated with reduced susceptibility to β-lactams, but the actual role and contribution of porins to decreased susceptibility is often complex[219].

Even though the molecular mechanisms involved in porin deficiency in *E.coli* have been most intensively investigated, more porin deficient isolates are observed in clinical ESBL- and

carbapenemase-isolates of *K.pneumoniae* and *Enterobacter spp*. than in *E.coli* [121,219,232]. Furthermore, in *K.pneumoniae*, OmpK35 deficient isolates are regularly observed in ESBL-positive isolates[233,234]. Additional loss of OmpK36 in such isolates as response to antibiotic pressure contributes to a significant rise in β-lactam resistance and to increased co-resistances (e.g. quinolones)[219]. More recently, studies have suggested that even OmpK36 changes may often contribute significantly to the observed β-lactam phenotype in clinical ESBL- and carbapenemase-producing *K.pneumoniae* [232,235,236 237,238]. The high fitness costs observed in *E.coli* associated with porin deficient mutants have been suggested as a major reason why these isolates are infrequently observed among clinical isolates although they are supposed to emerge frequently in response to therapy [220,239].
In summary, porin deficient mutants may evolve through selection during antimicrobial exposure, but are usually replaced by wild-type bacteria due to reduced fitness as porin changes usually impedes nutrition [121,220,240]. Thus, they may become a problem during therapy, but have thus far shown a limited potential for epidemic spread[115,186,220,241]. Additionally, lack of transferability reduces their epidemiological potential[186].

Nevertheless, nosocomial outbreaks with porin deficient isolates containing ESBL positive or plasmid encoded AmpC β-lactamases in Enterobacteriaceae conferring low-level carbapenem resistance have been documented, particularly in *K.pneumoniae* [242,243]. Ertapenem is generally most affected by this mechanism, while the zwitterionic meropenem is less affected than imipenem[244]. Considerable variations in carbapenem-MICs are observed in all prevalent carbapenemases representing a particular challenge in detection (see sections A6 and E4) [184]. Changes in porin expression partly explain such observed MIC-range and has been linked to variation in carbapenem-MIC in KPC-producing isolates[232,235].

Altered or deficient porins, OmpF and OmpK35 in *E. coli* and *K. pneumoniae* , respectively, have shown to contribute to high-level resistance to cefuroxime (and cefamycins)[113,118,214,237,245]. Most often this is observed in isolates with concomitant broad-spectrum- β-lactamase activity; however resistance has occasionally been documented in isolates without measurable β-lactamase-activity [214]. Generally, 3rd generation cephalosporins exhibit higher OMP-diffusion rates than the more hydrophobic substance cefuroxime [246]. There are, however, differences among 3rd generation cephalosporins as cefpodoxime is more affected by porin changes in *E.coli* isolates[216]. Accordingly, porin loss has to conjoin with concomitant β-lactamases in order to significantly increase resistance to 3rd generation cephalosporins[221,237]. Low-level resistance to 3rd generation cephalosporins (particularly ceftazidime) in *E.coli* or *K.pneumoniae* has been documented in porin deficient isolates displaying SHV-1 hyperproduction[247,248]. Most often low-level resistance to 3rd generation cephalosporins (particularly ceftazidime) is attributed by the combined effects of TEM-1 or SHV-1-enzymes and reduced permeability[249-252]. The combined effects TEM-1 or

OXA-1 hyperproduction and porin loss may produce reduced susceptibility to 4th generation cephalosporins[253].

**EFFLUX.** Multidrug efflux pumps have been associated with resistance to a number of antibiotic classes, including quinolons, aminoglycosides, and tetracyclins, and may contribute to a MDR phenotype[118]. β-lactams containing lipohilic side chains, including 2nd generation cephalosporins and cefoxitin, are substrates for active efflux through AcrAB-TolC pumps (Figure 7)[254,255]. In three studies by Källman on clinical strains of *E.coli* and *K.pneumoniae*, respectively, the contribution of efflux to reduced susceptibility to cefuroxime was suggested for *E.coli* through increased expressions of *acrA* of the chromosomally encoded AcrAB efflux pump , but could not be confirmed for *K.pneumoniae* [113,118]. 3rd generation cephalosporins does not possess the same 6-(or 7- ) substituent group lipophilicity, and consequently are poorer substrates for the pump[255]. The role of efflux among MDR-Enterobacteriaceae has infrequently been observed in outbreaks involving ESBL- or carbapenemase-producing in Enterobacteriaceae. [114,234,254,256].

## A.6 LABORATORY DETECTION OF ESBLS, AMPC CEPHALOSPORINASES AND CARBAPENEMASES IN ENTEROBACTERIACEAE

In 2003-04, the epidemiological situation concerning the prevalence and panorama of ESBLs in Norway were essentially unknown and unexplored. Methods for detecting ESBLs varied between laboratories, and guidelines based upon the national epidemiology were desperately needed. This study was initiated to fill this gap in knowledge while focusing on various manual agar-based phenotypic methods for confirmation of ESBL and/or AmpC-hyperproduction in isolates from infected patients. Such methods are convenient for routine use as they are flexible and easy to implement in the clinical laboratory. The clinical strains materials used were well characterized in order to evaluate test properties and diagnostic performance of the various phenotypic tests (**paper1 and 3**).

### DIAGNOSTIC TESTING OF THE "NEWER" B-LACTAMASES IN CLINICAL ISOLATES OF ENTEROBACTERIACAE

**General considerations**.

In routine laboratories different criteria must be considered when choosing a strategy for screening and confirmation of ESBL-, plasmid mediated ampC- and carbapenemase – producing Enterobacteriaceae. These criteria include the accuracy of the test applied, the convenience of and time-spending in performing the test, the cost-effectiveness and rapidity of the test itself as well as "sample taken to sample answer"- time in the individual laboratory [257-259].

The choice of an "optimal" algorithm may depend on several contributing factors; such as out- or inpatient status of the patient, laboratory skills (including knowledge of molecular methods), size and annual number of tests, instrument facilities (including automated AST systems and molecular platform supply), demand on personnel time resources, and overall budget capacities.

Aside from test-specific properties, the prevalence of the β-lactamase in question in the population (general or *a priori* selected) being tested is superimposed to these factors and will inevitably affect the performance (e.g. the PPV) of the test. Thus, the optimal solution and strategy may vary between nations, regions, laboratories and within laboratories over time as costs, platforms, methods, skills and the epidemiology of the β-lactamases themselves evolve.

**Test- and diagnostic properties.**

***Inhibitor testing.*** Inhibitor –based testing are most frequently applied in Europe for phenotypic testing to confirm or infer ESBLs, plasmid mediated AmpCs or carbapenemases in Enterobacteriaceae.

***Two-step algorithms.*** A two-step algorithm, or serial testing, is generally applied in most laboratories using manual methods to either confirm - or infer that molecular testing is needed to confirm - the presence of a given β-lactams in question (either ESBL, plasmid mediated ampC, or carbapenemase) in isolated colonies: (i) Step one - screening by one or more indicator substrates and consecutively; (ii) Step two - confirmatory testing using one or more of the indicators in combination with an inhibitor substrate looking for different kinds of synergy effects. The two-step algorithm combines a sensitive "screening" test by one or more indicator substrates and a more specific inhibitor-based "confirmatory" test. Specific issues relate to phenotypic detection of AmpC (i.e. need of molecular testing) and carbapenemase (i.e. need of several inhibitors) as will be addressed separately in this text.

***Diagnostic testing in research and routine settings.*** The test properties (sensitivity and specificity) and diagnostic properties (NPV and PPV) for a confirmatory test to a given screening substrate or a given combination of indicator substrates used in a screening procedure could be outlined in a "2x2"-table (Table 3). Gold standards are pursued and explored during research investigation. The establishment of a gold standard, in general, may be easy to accomplish in some situations, but more difficult in others.

*Table 3. 2x2 table of frequencies illustrating sensitivity, specificity, PPV and NPV. The prevalence in the given population tested affect PPV and NPV .*

| | **Gold standard characterization[1]** | |
|---|---|---|
| **Test result** | ESBL POSITIVE ENTEROBACTERIACEAE | ESBL NEGATIVE ENTEROBACTERIACEAE |
| Positive | TRUE POSITIVE (a) | FALSE NEGATIVE (b) |
| Negative | FALSE NEGATIVE (c) | TRUE NEGATIVE (d) |

*Table modified from [260-263]. [1]eg. confirmation by PCR and/or sequencing.*

- Prevalence of ESBL-Enterobacteriaceae = (a+c)/(a+b+c+d).
- Sensitivity = a/(a+c). Specificity = d/(b+d).
- Positive predictive value, PPV = a/(a+b). Negative predictive value, NPV = d/(c+d).

The diagnostic properties or performance of a given test depend on the prevalence of ESBL-producers in the population tested as defined by the *a priori* prevalence of ESBL in a certain defined population (a nation, a region, hospitalized patients, non-hospitalized patients, individuals defined by certain acknowledged risk factors, e.g. hospitalization abroad, etc, ). Low prevalence reduce the positive predictive value (PPV) [262-264].

## BASIC PRINCIPLES OF STRATEGIES USED TO DIAGNOSE; DETECT OR INFER ESBLS, PAMPCS AND CARBAPENEMASES - BY MANUAL PHENOTYPIC METHODS IN CLINICAL ISOLATES FROM INFECTED PATIENTS.

Recommendations for the detection of ESBLs, AmpCs and carbapenemases in Enterobacteriaceae have been published by several national and international organizations i.e. CLSI (USA), HPA (UK), CRG (Dutch), SFM (French), NordicAst (Nordic), NWGA (Norwegian), with diverse guidelines on preferred phenotypic tests for screening and confirmation of enzyme production. Recently, guidelines for the detection of ESBL-, acquired AmpC-, and carbapenemase-producing Enterobacteriaceae were released by EUCAST, and efforts for harmonization in Europe are expected [57].

### Phenotypic detection of ESBL.

***ESBL screening substrates:*** 3rd, and/or 4th generation cephalosporins and/or aztreonam are indicator antibiotics used to screen for ESBLs. ESBLs exhibit various hydrolytic profiles and activities towards these antibiotics, [120,129] and consequently the test properties and performance of a given test substrate will accordingly vary with the composition of encoded ESBL types and chosen screening cut-offs [265].

***Confirmatory ESBL testing:*** Phenotypic tests are based on *in vitro* inhibition of ESBLs by clavulanic acid. Visualized synergy between one or more indicator substrates (one or more 3rd generation cephalosporin or monobactam) and clavulanic acid confirms a positive test[266,267]. Several phenotypic confirmatory ESBL tests have been applied over the last 25 years, including the double disc (synergy) test (DDS), the combined disc method (test) (CDT), the gradient tests including Etest ESBL, or the MIC broth micro-dilution method [140,268-270].

Detection of ESBLs is more challenging in bacteria co-producing large amounts of AmpC-cephalosporinase than in bacteria with low or absent constitutive AmpC production. It is generally agreed that a positive confirmatory ESBL test is valid when 3rd generation cephalosporins are used in the presence of inducible chromosomally encoded class C – cephalosporinases. However, the synergy effect may be compromised when AmpC is stably expressed. Clavulanic acid may induce AmpC, which in turn would hydrolyze the indicator cephalosporin, and mask the synergy between clavulanic acid and the ESBL enzyme[271].

Presently, two strategies for phenotypic detection of ESBLs in bacteria producing significant amounts of AmpC are applied; (i) to add an AmpC inhibitor (i.e. cloxacillin) to the culture medium, or (ii) to test clavulanic acid synergy with a 4th generation cephalosporin, as they are largely immune to AmpC hydrolysis [266,272,273]. A third approach is also being suggested, which uses double-impregnated disks with cloxacillin and clavulanic acid, however this method waits

further evaluation [57]. Recent EUCAST guidelines advocate for testing with a 4th generation cephalosporin [57].

**Phenotypic detection of AmpC-cephalosporinase (activity).** Several methods for phenotypic detection of AmpC-cephalosporinase in *E.coli* non-hyperproducers and Enterobacteriaceae lacking chromosomal AmpC i. e. *Klebsiella spp*., *Proteus mirabilis, Citrobacter koserii, Shigella spp*., and *Salmonella spp*. have been proposed [274]. However, contrary to the ESBLs, no standard diagnostic phenotypic test has been recommended for detection of plasmid encoded AmpC by CLSI or HPA. On the other hand EUCAST has recently recommended a method using cefoxitin MIC > 8 mg/L coupled with cefotaxime or ceftazidime MIC > 1 mg/L for screening and cloxacillin inhibition for confirmation of AmpC production [57].

***AmpC screening substrates:*** Cefoxitin is the most commonly used screening substrate for detection of ampC-cephalosporinases; however, it does not provide optimal sensitivity, as enzymes of the AAC-1 family are susceptible[267]. Specificity may be improved when reduced susceptibility to ceftazidime or cefotaxime is complemented as screening criteria [57]. Multidrug resistance has been proposed as a criterion to enhance specificity, but this criterion seems to offer poor discrimination between inherent and acquired AmpC production [275]. Cefotetan, the other available cephamycin antibiotic, has also been suggested as an indicator associated with improved specificity but markedly reduced sensitivity[276]. A lack of specificity is anticipated for both indicators as they are influenced by metallo-β-lactamases and porin loss[267].

***Confirmatory AmpC testing:*** Proposed methods for AmpC confirmation include easy-to-use inhibitor-based tests using various derivatives of boronic acid or cloxacillin, in which the inhibitor is included in the media, disks or in the ellipsometric gradient strips[277,278]. However, inhibition by boronic acid derivatives may potentially produce false positive results in KPC-producers[279]. Secondly, the inhibitor capacity of cloxacillin is better against the CMY- compared to the DHA- family of AmpC enzymes[280]. Furthermore, data on AmpC inhibitory-based tests are both sparse and discordant[276,280].

Other confirmatory methods have also been proposed, but they have generally been abandoned for being too cumbersome and difficult to implement in the clinical laboratory. Unfortunately, in *E.coli*, none of the described phenotypic methods can reliably distinguish between chromosomally and plasmid-encoded AmpC. Final diagnosis has to rely on molecular methods, either conventional PCRs, real-time multiplex PCRs, or a microarray assay[281,282].

**Carbapenemases.**

Options, strategies and methods for the detection of carbapenemases in various epidemiological settings are presently issues of debate[58]. Concerns include (i) finding optimal screening substrates and strategies, and (ii) finding optimal inhibitors and inhibitor combinations for use in confirmatory tests[283]. Consequently, there is a lack of standardization and international consensus on phenotypic methods for carbapenemase detection[184]. However, EUCAST has recently proposed a set of guidelines [57].

***Carbapenemase screening substrates:*** The range of MICs displayed by clinical Enterobacteriaceae isolates harboring specific carbapenemases is displayed in Table 4. Accordingly, whatever carbapenem used in screening there is a narrow gap between some carbapenemase-producing Enterobactericeae and wild type isolates. The fact that MICs for carbapenemases in Enterobacteriaceae come close to or even overlap with the wild type distribution is reflected upon in recent decisions by breakpoint committees deciding on screening cutoffs close to the ECOFFs[58]. Nevertheless, meropenem (using ECOFF as screening cut-off) is favored as screening substrate by EUCAST according to recent guidelines currently providing the best separation between the wild type and clinical isolates in most situations [57] (Figure 3, Table 4).

*Table 4. MIC-ranges for carbapenems in reported clinical Enterobacteriaceae expressing the main carbapenemases (table reprinted from [186] by permission from Clin Microb Inf) as well as S/I and screening cut-offs recently recommended by EUCAST [57]. According to EUCAST the meropenem breakpoints and cut-offs offer the best balance between sensitivity and specificity. Imipenem is not recommended in screening as a stand-alone substrate in screening due to poor separation of wild type and carbapenemase-producing isolates, and ertapenem is not recommended for routine use in screening due to, in general, the low specificity of this substrate.*

| | **Imipenem (mg/L)** | **Meropenem (mg/L)** | **Ertapenem (mg/L)** |
|---|---|---|---|
| **KPC** [1] | 0.5 to > 32 | 0.5 to > 32 | 0.5 to > 32 |
| **IMP/VIM/ NMD**[1] | 0.5 to > 32 | 0.5 to > 64 | 0.38 to > 32 |
| **OXA-48/-181**[1] | 0.25 to 64 | 0.38 to 64 | 0.38 to > 32 |
| **S/I breakpoint**[2] | ≤2 | ≤2 | ≤0.5 |
| **Screening cut-off**[2] | >1 | >0.12 | >0.12 |

[1] *From [186].* [2] *From [57].*

Setting screening cut-offs is further complicated by the fact that susceptibility to carbapenems is influenced by several factors including bacterial species, β-lactamase type or variant, levels of enzymatic expression, and presence of additional resistance mechanisms [186]. Consequently, a wide range of MIC-values to all carbapenems may be displayed by these isolates [284] as was also recognized in this study (**paper 4**). ESBL or AmpC enzymes coupled with porin loss may mediate resistance to carbapenems. Breakpoints, no matter how close to the wild type distribution, will not be able to reliably discriminate between Enterobacteriaceae strains with low level- MIC carbapenemases and strains with non-carbapenemase mediated resistance due to the combined effect of porin loss and the presence of ESBL or AmpC enzymes.

***Confirmatory carbapenemase testing:*** None of the current phenotypic tests offers optimal sensitivities and specificities. Currently, the broth microdilution and disk diffusion methods are generally considered to be superior to semi-automated systems and ellipsometric gradient tests (Etest)[184,285].

*Modified Hodge Test (MHT):* MHT by cloverleaf technique has been extensively used as a method to detect un-typed carbapenemase activity and it is currently the only method recommended by the CLSI[59,286]. However, this is a time-consuming technique as it takes 24-48 hours to complete. Additionally, the MHT may produce false positive results mainly due to CTX-M-producing strains with reduced outer membrane permeability and high-level AmpC- producers[287,288]. The designing of inhibitor-based MHTs, with boronic acid and cloxacillin has been proposed to improve specificity[289]. Lack of sensitivity has been observed in detection of metallo-enzymes. MBLs are zinc dependent enzymes, and supplementing zinc sulfate (ZnSO4) into the culture media (Muller Hinton) has shown significantly to improve performance of MHT in detecting NDM-1 producers [290]

*Inhibitor-based tests:* Several inhibitor-based tests have been developed for specific detection of carbapenemases. Inhibitory tests may be performed concomitantly or separately to the MHT[289]. Recent guidelines from EUCAST consider CDTs with different carbapenemase inhibitors to be best validated, while the MHT is not recommended due to overall low sensitivities and specificities. These guidelines propose an algorithm using boronic acid derivatives and dipicolinic acid to inhibit class A and B carbapenemases, respectively, and cloxacillin to differentiate between the combined effect of AmpC hyperproduction and porin deficiency, and a true carbapenemase. In cases of no synergy, testing for high–level temocillin is recommended to infer OXA-48 mediated resistance [57].

*Inhibitor-based test for KPC:* The CDT using boronic acid (usually 3-aminophenyulboronic acid) as inhibitor for class A carbapenemases is currently the most extensively used and evaluated method for detection[287,291-293]. Boronic acids ability to inhibit AmpC β-lactamases naturally comprises its ability to selectively detect class A carbapenemases in isolates with high-level AmpC activity. To overcome this predicament, studies have been performed where cloxacillin has been added, either in the agar medium or on disks to selectively inhibit AmpC, demonstrating it as an effective strategy[294,295]. Co-production of ESBLs and carbapenemases are common in isolates, and co-production of more than one type of carbapenemases does occasionally occur. False negative boronic acid tests have been reported in *K. pneumoniae* isolates when co-production of KPC-2 and VIM-1 is recorded[296].

*Inhibitor-based test for MBLs:* The ability of EDTA (ethylene diamine tetra-acetic acid) and dipicolinic acid as metal chelating agents for the detection of MBL-producing Enterobacteriaceae has been evaluated in different formats in several studies[184]. CDT with these agents have shown excellent sensitivity in detecting IMP and VIM type MBLs, although false positives have been reported in strains conferring reduced susceptibility to carbapenems due to ESBL- or plasmid mediated ampC-production coupled with impermeability, particularly if EDTA is used for inhibition [295]. In order to control false positive synergy tests it has been suggested to test the chelating EDTA agent alone to evaluate its intrinsic effect [297].

*Inhibitor-based test for OXA:* Although the emergence of OXA-48 in Enterobacteriaceae represents a particular challenge for laboratories, no specific inhibitor-based disk, tablet or Etest is currently available. Isolates producing OXA-48 enzymes regularly do not co-produce ESBLs or AmpCs, and therefore mediate low level of carbapenem resistance and are sensitive to 3rd generation cephalosporins[196,298]. High-level temocillin resistance (MIC ≥ 128 mg/L) coupled to lack of synergy to KPC/MBL-inhibitors has been suggested as a possible indicator for OXA-48 production as such high-level temocillin resistance is infrequently observed in non-carbapenemase β-lactamases [57,293,298]. The combined results of low carbapenem-MICs and a strong inoculum effect are suspected to account for a large portion of false negative results encountered during detection[184].

Some technologies facilitating the options for alternative strategies in detection of the "newer β-lactamases" have emerged in recent years. The phenotypic techniques and methods briefly presented below were not available or in common use when our studies were performed (**paper 1 and 3**).

***Carbapenemase detection by colorometric hydrolysis of carbapenems:*** A Carba NP test has recently been developed in the Le Kremlin-Bicêtre laboratory in Paris by Nordmann, Poirel and colleges using colorimetric detection of acid produced by β-lactamase-induced cleavage of the β-lactam ring following hydrolysis[299]. The test is primarily used in screening for carbapenemases using fresh colonies (e.g. from an antibiogram agar plate or a selective agar used for screening of carriers) that following a preparation step giving cell-free extract to exclude errors resulting from impermeability or efflux are inoculated in two wells for each test, one containing no antibiotic, the other imipenem. Initial results by Nordmann and colleges showed a 100% sensitivity and specificity for detection of carbapenemases in a strain collection of 162 carbapenemase-producing (including low-level carbapenem resistant isolates) and 46 non-carbapenemase producing Enterobacteriaceae displaying reduced susceptibility to carbapenems[299]. External evaluations however, showed slightly lower sensitivities, and more studies are awaited. Tijet and colleges obtained false negative results with mucoid strains or strains linked to low level carbapenemase activity, particularly OXA-48 like enzymes [300]. Further, Østerblad and colleges question false negative results among OXA-181 Enterobacteriaceae isolates[301]. An equivalent test even exists for detection of ESBLs using cefotaxime as indicator and tazobactam as inhibitor[302]. An analogous imipenem hydrolysis-based rapid test to the Carba NP test, the Rosco Rapid CARB screen kit (Rosco Diagnostica) test, is now commercially available[303].

***Spectrophotometric assay:*** This is an inexpensive, yet technically demanding and labor-intensive technique and thus suited mainly for reference laboratory purposes[184,304]. In Norway, the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res) provides these services. Spectrophotometric assays can reliably differentiate carbapenemase-

producing isolates from non-carbapenemase-producing isolates (i.e. ESBL or AmpC hyperproducing isolates with concomitant impermeability changes)[304]. Enzyme type could be inferred by hydrolysis with and without the presence of inhibitors; i.e. clavulanic acid for KPCs, EDTA for MBLs, and NaCl for OXA-enzymes[184].

***Mass spectrometry:*** Matrix-assisted laser desorption ionization-time of flight mass spectrometry, in short MALDI-TOF, has the potential to detect degradation products from different enzymatic mechanisms of resistance. The first two studies examining MALDI-TOF for the detection of carbapenemases were published in autumn 2011[305,306]. In a second study by Hrabak et al. MALDI-TOF accurately identifying carbapenemase-activity in all carbapenemase-producers when tested for 108 carbapenemase-producing isolates (including NDM-1, KPC-2/3, VIM-1 and OXA-48) of different Enterobacteriaceae-species as well as 35 non-carbapenemase-producers, but carbapenem-resistant Enterobacteriaceae [307]. Further evaluation is warranted, however when fully evaluated, this may indeed prove to be a cost effective technique that is useful to evaluate carbapenemase activity also outside reference laboratories[186].

**Molecular detection.**

Confirmation by molecular methods is comparatively faster than phenotypic confirmation as the extra overnight incubation step is superfluous[308]. However, the need to confirm resistance mechanism in clinical isolates by molecular methods on a routine basis remains questionable basically due to the inability of these methods to detect novel beta-lactamases, their relatively high cost and their requirement of trained personnel.

***Molecular detection of ESBLs:*** Currently, molecular methods are not considered as primary tools in most routine clinical settings[186,309]. Detection and grouping of the different CTX-Ms by multiplex PCR, as well as detection of locally prevalent *bla*-genes has been accommodated by molecular tests to improve epidemiology and potentially rapidly confirm ESBL production in routine settings[310,311].

Commercial multiplex real time PCR kits such as Hyplex ESBL ID ® and Check-MDR ESBL ® are also available for the detection of frequently isolated ESBL-genes (TEM-ESBLs, SHV-ESBLs, CTX-Ms, and OXA-ESBLs)[312]. The turnaround time of the Check-MDR ESBL kit is about 4.5 hours compared to > 7 hours needed for the microarray assays that enable genotypic information of importance primarily for epidemiological purposes[313]. Also non-commercial DNA microarray has been suggested for epidemiological investigations, and in the future possibly even in a routine clinical setting[308]. The possibility of utilizing microarray techniques for detection of ESBL and carbapenemase genes directly from blood cultures is currently being explored[314].

***Molecular detection of pAmpC:*** Molecular methods are necessary to reliably differentiate plasmid- from chromosomally encoded AmpC β-lactamases in *E.coli*, and real time multiplex PCR are developed detecting the most prevalent plasmid mediated AmpC-alleles [282].

***Molecular detection of carbapenemases:***

In Norway, being a low prevalent country, confirmation by molecular methods is recommended for all suspected carbapenemase-producing Enterobacteriaceae. Presently, these services are provided by the Reference Centre for Detection of Antimicrobial Resistance [315]. Reliable and accurate detection of typed carbapenemases rely on molecular methods. Several options for molecular based detection by singleplex, multiplex, gel or real-time based PCRs by *in house* methods or commercial kits including Hyplex® and Check-Point® (TAT 4.5 hrs) exist[316,317]. Also, commercial microarray kits (Check Points MDR CT-101/2/3 (TAT >7 hrs)) are available as confirmatory methods enable genotyping of most known carbapenemases[313].

## A.7 INVESTIGATION OF TRANSMISSION OF MDR ENTEROBACTERIACEAE IN NOSOCOMIAL OUTBREAKS

In this study (**paper 4**) we investigated an outbreak of *bla*$_{KPC}$ producing Enterobacteriaceae in a Norwegian hospital following an outbreak alarm called for by the infection control unit at that hospital. A particular interest was paid to the possible involvement of the environment as a route of transmission in this prolonged, low frequent outbreak.

**INVESTIGATION OF NOSOCOMIAL OUTBREAKS.** According to CDC a nosocomial infection is defined as "an infection that is associated with a stay in a health institution, and which was not present or in the incubation period (= 48 hours) at the time of admission"[318]. An outbreak is defined as "a temporal increase in the frequency of infection (or colonization) by a certain bacterial species, caused by enhanced transmission of a specific strain … or multiple strains"[111]. Baseline endemic incidence levels of nosocomial infections exist for various species and organisms at many hospitals. A statistical significant deviation from this level would define an epidemic or nosocomial outbreak. Statistical tools may aid in monitoring and improving the awareness of an emerging nosocomial outbreak[319]. The awareness of an on-going outbreak may however be brought to attention by a number of ways, and the alertness of clinical or laboratory staff is very often crucial. In rare or unique cases, such as carbapenemase producing Enterobacteriaceae in a Norwegian hospital, a single case that is not related to import, should elicit an outbreak investigation.

Outbreak investigations aim to remove, if possible, the common source and/or unveil the modes of transmission responsible for spread in order to implement adequate infection control measurements.

**EPIDEMIOLOGY AND CLINICAL SURVEILLANCE.** In suspected outbreaks, case definitions allows for calculation of incidence- or epidemic curves. Case definitions usually specify characteristics of the suspected outbreak organisms (identification procedures, resistance pattern, day of first isolation, type of material) and often provide characteristics of affected patients (age, sex, ward (when case detected), illness, start of illness, procedures, devices, and other acknowledge risk factors)[320]. In an outbreak situation, the use of epidemic curves makes it possible to visualize the baseline situation and look beyond the junction in time where measurements of infection control have been taken. In some instances, the index case may also be identified. By collecting conventional epidemiological information more timely, accurate information is provided by "time and space"- charts unveiling time and ward overlaps between patients, and allows for the formation of cross-transmission hypothesis[321]. The mode of spread varies between different pathogens, and various approaches of investigation may therefore be applied according to the organism involved. Knowledge of the ecology of different organisms in hospitals, including MDR Enterobacteriaceae gives important clues on how to execute an

investigation. Accordingly, surveillance cultures from fecal samples of patients in order to detect asymptomatic carriers in outbreaks of MDR Enterobacteriaceae are regularly performed. The ratio of positive samples at any given time provides additional information on the course and control of the outbreak. Based on information from conventional epidemiology and patient screening results, additional environmental screenings may be performed, whereas screenings of health care personnel are only occasionally carried out.

**INFECTION CONTROL MEASUREMENTS.** Implementing control measures in hospital outbreaks of MDR Enterobacteriaceae is a dynamic process aimed at preventing further transmissions, based upon real time information of the defined outbreak. Possible infection control measurements include active surveillance culturing, contact precautions, cohorting, antibiotic restriction guidelines, training of health care workers, and potentially environmental cleaning. Often a bundle of measurements are executed[322,323 324].

**EPIDEMIOLOGICAL TYPING.** In an outbreak situation, all clinical isolates and isolates collected during screening programs should be saved to enable subsequent molecular typing. Combining "time and place"- information given by conventional epidemiology and genetic relatedness of bacteria within a given species by molecular typing methods aim at unveiling the modes of transmission responsible for local spread and detect, if present, the common source of the outbreak.

Historically, typing was performed through phenotypic methods such as serological typing in Enterobacteriaceae, which typed the antigenicity of the lipopolysaccharide, flagellae and capsules (O, H, K) [325]. Biochemical fingerprinting, such as The Phene Plate (PhP®) system developed at Karolinska Institutet, Sweden, is still used in different settings[326,327]. However, phenotypic methods are generally considered less reproducible than molecular methods, despite recent advances in mass spectrometry and proteomic approaches[111]. Selecting for the appropriate molecular typing method involves considerations of both the biology (genetic stability) of the organism and the context of which the analysis is being performed.

As previously stated (section A4), van Belkum et al.) no ideal molecular typing method is currently available and the choice of an appropriate typing method(s) is in general dependent on sets of "performance and convenience criteria" [107] related to the biology of the organism, and the epidemiological context. Speed, costs, local skills and equipment, possibilities for sharing of results, and spectrum of applicability [111] has to be considered when a typing method or system is chosen. Consequently, these criteria also would have to be evaluated in the context of investigating any local hospital outbreak with a given organism. More than one typing method may be necessary to display different aspects of an outbreak, or when the epidemiological linkage is weak. Additionally, typing of transferable genetic elements may be required in outbreak situations.

**MODES OF TRANSMISSION IN ENTEROBACTERIACEAE.** In this study (**paper 4**) we tried to explore the modes of transmissions in an outbreak of *bla*$_{KPC}$ producing Enterobacteriaceae characterized by persistence, but a low-frequency of new cases (**paper 4**). Transmission between patients, health care workers and the environment are the main modes of transmission of Enterobacteriaceae within hospitals [4]. The human gut represents the main reservoirs of Enterobacteriaceae and patient reservoirs usually constitute the dominant source of transmissions in high-frequent outbreaks of MDR Enterobacteriaceae[186]. Health care workers, by transient carriage of MDR Enterobacteriaceae mainly on their hands, may act as vehicles for dissemination between patients[13,328,329]. Thus, measurements of hand hygiene are crucial in preventing transmission to patients. In fact, such carriage in most outbreaks of Enterobacteriaceae represents a greater portion of transmissions in the ICU than the direct transmissions between patients[330], whereas patient-to-patient transmissions probably contribute significantly in LTCF and rehab units[331]. Newer techniques may enhance our understanding of the skin microbiome and dynamics [332]. Enterobacteriaceae in general is believed to survive only a short period on hands (few minutes), however inoculums, strain and host related factors may modify survival time [333]. (Skin and) hand carriage of *K.pneumoniae* may be common in hospitalized patients [8]. Results in older studies regarding potential differences in hand survival between *E.coli* and *K.pneumoniae* were contradictionary[328,334]. Transmissions may be facilitated in some health care workers exhibiting prolonged skin carriage due to specific host related factors, including dermatitis[330].

Prolonged carriage in the gastrointestinal tract of MDR Enterobacteriaceae associated outbreak strains, when looked for, is rarely encountered in health care workers except in *Salmonella spp.* [330]. Norwegian health authority guidelines currently advice against screening health care workers in outbreaks of MDR Enterobacteriaceae in health care institutions [335].

**Role of the environment.** In our study (**paper 4**) the possible role of the environment to nosocomial infections with KPC-producing *K.pneumoniae* was specifically addressed. In general, the causal role of environmental contamination to infection often remains controversial [336]. The clinical significance of the environment to transmissions in nosocomial outbreaks has generally been considered to be higher in MRSA, VRE, and *Clostridium difficile* than in Enterobacteriaceae[337,338]. Nevertheless, the environment may also play a role in transmission in nosocomial outbreaks in Enterobacteriaceae, although species and strain differences likely occur [339] (Figure 8).

A common environmental source is sometimes recognized during outbreak investigations involving Enterobacteriaceae. Diagnostic instruments and devices such as endoscopes, bronchoscopes, coupling gel used in ultrasonographic examinations, roll boards, stethoscopes and thermometers can act as sources for dissemination of MDR Enterobacteriaceae[330]. Inadequate cleaning procedures are sometimes exposed in such outbreaks [340].

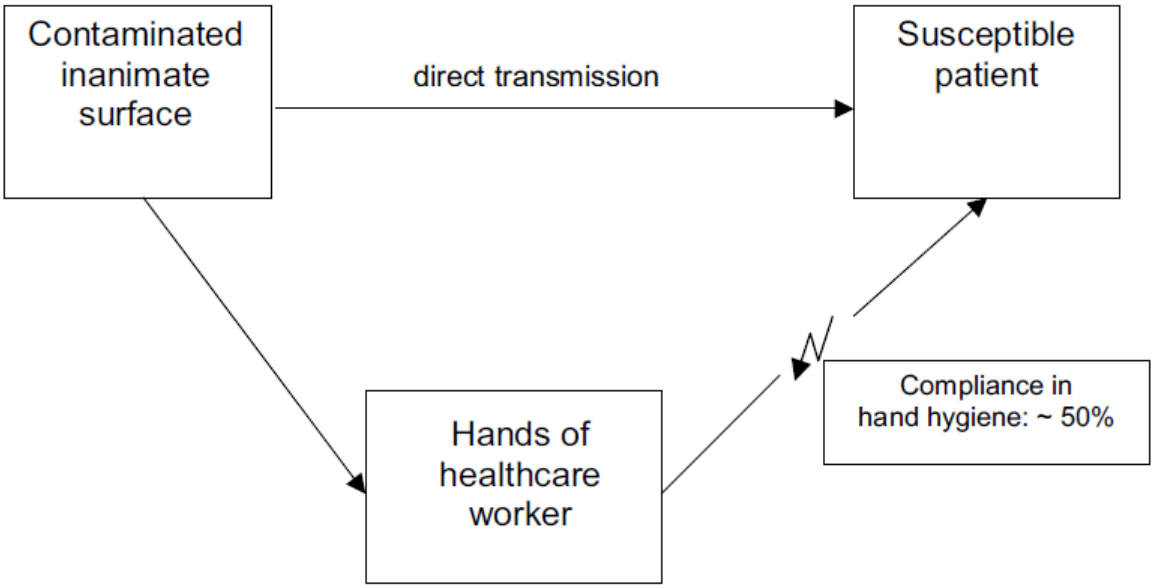



*Figure 8. Modes of transmission from inanimate surfaces to susceptible patients via direct transmission or the hands of health care workers. (From [338]).*

Survival time on inanimate surfaces and the infectious dose needed for cross-transmission vary greatly between different bacteria, even if contradictionary data are published [338] (Figure 8). Persistence on dry surfaces is believed to be better for gram-positive bacteria (being less susceptible to drying up than Enterobacteriaceae); however Enterobacteriaceae may also survive on dry inanimate surfaces for months[338,341,342]. The ability of *K.pneumoniae* to resist desiccation has been regarded and recognized for decades as important in facilitating cross-transmissions [143]. *E. coli* survival in dry environments is facilitated by protein nutrition that may be derived from body fluids [343]. Survival of Enterobacteriaceae is significantly prolonged in humid conditions [338]. Accordingly, the involvement of the environment as a source of transmission of Enterobacteriaceae has primarily been centred to moist inanimate areas, including sinks[20,22-27,344].

Examining environmental contamination by carbapenem- resistant Enterobacteriaceae Lerner and Carmelli found the near-patient-touch sites to be more frequently colonized than distant locations, and that the sampling method used significantly affected recovery rates. Particularly, contact plates were more effective on regular and flat surfaces, whereas swabs were more effective from irregular and bumpy surfaces (e.g. pillows, bed linens and mattresses)[345]. Accordingly, applying the correct harvesting and detection technique may be critical and challenging in detecting environmental Enterobacteriaceae.

The involvement of the environment stresses the significance of sufficient cleaning of the hospital environment[345,346]. Terminal cleaning regimens performed to control hospital spread of nosocomial infections (including infections by MDR organism) prevents cross-contamination via

the environment between patients entering and leaving the same room. These regimens are supposed to be more effective in preventing transmissions among MDR Enterobacteriaceae than MRSA, VRE and *C. difficile* and non-fermentative gram-negative rods (*Acinetobacter spp*. and *Pseudomonas spp*.) [347,348]

The epidemiology of classical ESBLs and KPCs in *K.pneumoniae* and the CTX-Ms in *E.coli* confirm the observation of *K.pneumoniae* as a frequent source of health-care associated infections, and *E.coli* as a precipitator of principally of community-acquired infections[187]. Clinical studies focusing on differences between *E. coli* and *K. pneumoniae* regarding transmission rates in hospitals has revealed that *K.pneumoniae* is considerably more prone to cross-transmissions between patients than *E.coli,* and consequently that this fact could justify differences in the measurements of contact precautions applied for ESBL-producing *E.coli* and *K. pneumoniae* [150,322,323,349,350]. With regards to clonal outbreaks of ESBL-producing *E.coli*, although reported[212,351], they are comparatively few in relation to *K.pneumoniae*. Better persistence in the hospital environment could account for the higher rate of transmissions in *K.pneumoniae* ESBL-producing isolates than in *E. coli* ESBL-producing isolates[352-354]. Contrary, influx of *E. coli* ESBL-producing isolates appear to contribute significantly to the number of these isolates recovered in hospitals [149,211,355,356].

# B. AIMS OF THIS STUDY

From the start, the overall aim of this study was to identify and quantify the different mechanism involved in reduced susceptibility to extended-spectrum cephalosporin, evaluate methods for the detection of ESBL producing Enterobacteriaceae and investigate the molecular epidemiology of ESBLs in Norway. Moreover, the epidemiology and modes of transmission of carbapenemase-producing Enterobacteriaceae were explored in an emerging nosocomial outbreak in a Norwegian hospital.

The following **research questions** were formulated:

- What are the ESBL genotypes in clinical isolates of *E. coli* and *K.pneumoniae* in Norway, and which are the routine diagnostic tests and substrates appropriate at the Norwegian diagnostic laboratories for the identification of ESBL producing Enterobacteriaceae? (**paper 1 and 3**)
- Is Norway part of the ongoing global spread of CTX-M producing *E.coli*, and what is the molecular epidemiology of the Norwegian isolates on the level on strain, plasmid and genotype? (**paper 2**)
- What are the mechanisms involved in resistance to 3$^{rd}$ generation cephalosporins in *E. coli* and *Klebsiella* spp., what are the prevalence and consequences of these mechanisms on the phenotypic detection of ESBLs? (**paper 3**)
- Following a nosocomial outbreak of KPC-producing *K.pneumoniae*; what are the characteristics and modes of transmission of this outbreak (**paper 4**).

Correspondingly aims of the four sub-studies (paper 1-4) were:

- **Paper 1:** to examine the distribution of different *bla*-genotypes among ESBL producing *E. coli* and *K. pneumoniae* strains at point of entry into Norway in 2003, with special attention on appropriate substrates for phenotypic detection and methods for rapid and accurate identification of ESBLs at nationwide clinical laboratories.
- **Paper 2:** to examine the molecular epidemiology of the CTX-M producing *E. coli* subpopulation of isolates from paper 1 on the strain, clone and plasmid level in order to understand the relationship between clonal and plasmid spread in the Norwegian isolates.
- **Paper 3:** to examine the panorama- and prevalence of different resistance mechanisms responsible for high- and low-level resistance to 3$^{rd}$ generation cephalosporins in *E. coli* and *Klebsiella* spp. populations prior to the dissemination of ESBLs in Norway.
- **Paper 4:** to examine a long-term low-frequency hospital outbreak of KPC-producing *K.pneumoniae* through conventional- and molecular epidemiology at strain, clone and plasmid level.

# C. MATERIAL AND METHODS

## STRAIN MATERIALS

In **paper 1** consecutive clinical isolates of *E.coli and K.pneumoniae* isolates with reduced susceptibility to one or more oxyimino-cephalosporins tested by any routine method were obtained from eighteen of 24 Norwegian diagnostic microbiology laboratories during March through October 2003. The laboratories (n=18) that agreed to participate in this prospective laboratory-based study after written invitation, and covered > 90% of the Norwegian population. At the reference laboratory (K-res) consecutive non-duplicate isolates of *E. coli* (n=**87**) and *K. pneumonia* (n=**25)** with confirmed reduced susceptibilities to oxyimino-cephalosporins by Etest (MIC > 1 mg/L according to NWGA 2003 guidelines) were included. Breakpoints for cefpodoxime had not been established by NWGA and MIC > 1 mg/L was thus defined as screening cut-off in this study.

The strain collection described in **paper 2** included the subpopulation of CTX-M-producing *E.coli* isolates (n=**45**) identified in paper 1.

In **paper 2** the subpopulation of CTX-M-producing *E.coli* isolates (n=**45**) identified in paper 1 were included into the study.

**Paper 3** was a nationwide observational, cross sectional multi-center study designed to describe the (point)-prevalence of specific mechanisms conferring resistance to 3$^{rd}$ generation in human clinical *E.coli* and *Klebsiella spp.* isolates in Norway. Consecutive *E. coli* (n=1172 urine, n=1041 blood) and *Klebsiella spp*. (n=369 blood) isolates were collected from all of the 23 Norwegian diagnostic laboratories giving a total study population of 2582 isolates. Each laboratory delivered a maximum number of *E.coli* isolates (50 isolates from blood culture and 50 from UTIs) and *K.pneumoniae* (25 isolates from blood culture) isolates within a defined time frame. The 2582 isolates were collected as part of the NORM surveillance program in 2004 after initial examination by participating laboratories for susceptibility to amoxicillin-clavulanic acid, oxyimino-cephalosporins and aztreonam in a disk diffusion panel (see laboratory methods). Additionally, blood culture isolates were also examined by Etest (bioMerieux, Marcy Etoile, France) in participating laboratories. Three laboratories with 289 isolates (including 249 *E. coli*, 33 *K. pneumoniae* and 7 *K. oxytoca*) were excluded from the study as they failed to standardize their agar disk diffusion method). Thus, 20 laboratories were approved to include strains in this study. A total of 159 isolates considered to display non-wildtype susceptibility profile were sent to K-res when they met one or more of the following criteria by disk diffusion or Etest (blood culture isolates); cefpodoxime ≤ 27 mm, cefotaxime ≤ 30 mm or ≥ 1 mg/L by Etest, ceftazidime ≤ 29 mm or ≥ 1 mg/L by Etest, cefpirome ≤ 29 mm, cefepime ≥ 1 mg/L by Etest, and aztreonam ≤ 31 mm. 150 isolates met the disk diffusion criteria, whereas 9 blood culture isolates met the Etest criteria only. In order to further correct for inter-laboratory variation with regards to the performance of disk diffusion, all isolates were examined at K-res. Isolates with reduced

susceptibility to CTX, CAZ, cefepime (FEP), ATM (MIC > 1 mg/l by Etest) and/or **CPD (MIC> 2 mg/l** by Etest) were included for further analysis. Fifty-four (*n*= **54**, 35 *E.coli*, 11 *K.pneumoniae*, and 8 *K.oxytoca*) of the 159 (34%) non-duplicate isolates met these final criteria for inclusion.

In **paper 4** six clinical KPC- producing *K.pneumoniae* isolates were recovered in clinical samples from patients at Sørlandet Sykehus/Hospital HF (SSHF) from November 2007 to May 2010. Additionally, as part of an outbreak investigation (i) one KPC-producing *K.pneumoniae* isolate and one KPC-producing *Enterobacter asburiae* isolate were recovered from one patient during a fecal screening programme and (ii) KPC-producing isolates from 4 different locations (sink drains) in the ICU-A during environmental screening. 11 strains from 7 patients and 6 strains (n=4 *K.pneumoniae* and n=2 *Enterobacter asburiae*) from two environmental location (sinks, room 5 and 6) were examined by phylogenetic and plasmid analyses. All of these environmental isolates were recovered during environmental screen that took place in June 2010. Additionally, in December 2010 a *bla*$_{KPC}$ *K.pneumoniae* isolate was again recovered from the sink in room 6 trigging a second environmental screen. In the December screen, two additional sinks that were not examined in June were identified with a *bla*$_{KPC}$-positive *K.pneumoniae* (room 9) and *bla*$_{KPC}$-positive *E.asburiae* (room 9 and the rinsing room), respectively. None of the isolates retrieved in the December 2010 screen were examined by phylogenetic and plasmid analyses.

Relevant reference strains were used throughout the study for quality control purposes. For detailed information I refer to the individual papers.

## SETTING

**Paper 1, 2 and 3**: All strain collections were in a nationwide setting.

**Paper 4:** This part of the study was conducted during and following a nosocomial outbreak at Sørlandet Sykehus HF (SSHF), a 683-bed general hospital enterprise located in three different cities in the southern part of Norway (Arendal, Kristiansand, and Flekkefjord) serving altogether 292 000 inhabitants. Due to sharing of some specialized medical functions, a certain degree of interchange of patients between the hospitals occurs. Two hospitals belonging to Sørlandet Hospital, SH-Arendal (SH-A), and SH-Kristiansand (SH-K), as well as a tertiary hospital (Oslo University Hospital – Rikshospitalet (OUH-RH)) were involved in this outbreak.

## CLINICAL AND DEMOGRAPHIC/EPIDEMIOLOGICAL DATA FROM PATIENTS

In **paper 1 and 2** a limited amount of anonymous epidemiological data was retrieved from a registration form containing information on sex, age, in- or outpatient status, hospital department, and specimen type.

In **paper 3** a limited amount of epidemiological data was retrieved from a dataset in NORM 2004: Related to patients these data were sex, age, and specimen type (blood or urine). Furthermore it was possible identify the hospital origin of each isolate.

In **paper 4** clinical data and risk factors were collected as part of the routine management of the outbreak investigation trying to establish conventional epidemiological links. Data collection included; type of specimen, investigation of time and place overlaps btw clinical patients, prior antibiotic treatment, other risk factors such as ICU admission, recent surgery and ventilator use, length of stay, hospitalization abroad an age of the patients. With regard to the **conventional epidemiology,** time and place- analysis was completed in retrospect during a thorough examination of time and place charts from the individual patients.

## ACTIVE SURVEILLANCE CULTURING (ASC)

In **paper 4** a screening programme was implemented during outbreak investigation involving a 12-bed (8 single rooms and one 4-bed room) surgical/medical intensive care unit in SH-A (ICU-A). A fecal screening programme was performed during May 2010 to April 2011 as part of routine management and included 136 patient in the ICU-A. An environmental screening was performed in June and December 2010 and involved sinks in the ICU-A (n=19), the neighboring post-operative unit, the coronary unit and taps for water to dialysis machines in the ICU-A. The laboratory methods are further detailed below.

## ETHICS STATEMENT

No human biomaterial is collected in these studies.

In **paper 1, 2, and 3** bacterial strains collected from isolates that are anonymous to the researcher. P**aper 4** focuses on the molecular characteristics of bacterial isolates collected as part of the clinical management and microbiology routine work. Fecal screening was performed according to the guidelines from the local hospital and collection of clinical data as part of outbreak investigations for implementation of appropriate infection control measurements, and no extra samples were taken or used from the patients. The age and gender of patients were not disclosed in publication. Consequently, ethical approval was not required for the study.

## LABORATORY METHODS

**Species identification:** Bacterial identification was performed using the VITEK2 IDGNB system (bioMérieux, Marcy l'Etoile, France) (**all papers**) and/or API ID32E (bioMérieux) (**paper 1, 2 and 3**). In cases of low discrimination 16S rRNA gene sequence typing (**paper 1 and 2**) and/or MALDI-TOF (Microflex LT, Bruker Daltonics) with the MALDI Biotyper 3.0 software version (**paper 4**) was performed for confirmation.

**Antimicrobial susceptibility testing (AST):**

Susceptibility testing was performed at local laboratories (i) and at the reference laboratory (ii): i) In **paper1** susceptibility testing to oxyimino-cephalosporins by any routine method was performed according to NWGA guidelines (2003). In **paper3** a specific ESBL **disk diffusion** screening protocol using amoxicillin-clavulanic acid, oxyimino-cephalosporins and aztreonam disks as substrates were employed by the laboratories **(**NORM 2004 Appendix 5) [357]. Of note, in this protocol confluent growth on PDM agar was used contrary to semi-confluent growth that was the routine method at that time. Specific screening cut-offs were set in this study. Additionally, in blood culture isolates **Etests** to oxyimino-cephalosporins and aztreonam was performed according to the manufacturer's instructions and interpreted according to EUCAST guidelines. The clinical cases in **paper 4** was detected by routine methods in the local laboratory; either by Vitek AST 029 or by disk diffusion in which panels included cefpodoxime or cefotaxime/ceftazidime and/or meropenem (Becton Dickinson) depending on the specimen tested. ii) At the reference laboratory susceptibility testing to β-lactams was performed with **Etests** according to the manufacturer's instructions (bioMérieux or AB Biodisk) (**all papers**) and non-β-lactam antibiotics (**paper 4**) and interpreted according to the EUCAST guidelines valid at the time of testing during the period 2003-2011. Susceptibility testing to non-β-lactam antibiotics was performed by Vitek2 AST N023 in **paper 1** and AST-029 and AST-041 in **paper 3.** .

**Phenotypic detection of ESBL production:**
Isolates expressing a reduced susceptibility (MIC>1 mg/L) to an oxyimino-cephalosporin (cefpodoxime and/or cefotaxime and/or ceftazidime) (**paper 1**) or reduced susceptibility (MIC>1 mg/L) to aztreonam and/or cefotaxime and/or ceftazidime and/or reduced susceptibility (MIC>2 mg/L) to cefpodoxime (**paper 3**) was examined by confirmatory tests. An "ESBL phenotype" in this study was defined by a significant increase in susceptibility to oxyimino- cephalosporins tested in combination with clavulanic acid by the Etest and/or the combined disk method (**paper 1 and paper 3**).

***ESBL Etest (bioMérieux):*** In **paper 1 and 3** ESBL production according to the manufacturer's instructions was defined as a >8-fold decrease (≥3 two-fold dilutions) in the MIC of cefotaxime, ceftazidime, or cefepime in the presence of clavulanic acid or the presence of so-called phantom or deformity zones.

***Combined disk method (CDT)*** (Oxoid, Basingstoke, United Kingdom): Disks containing cefpodoxime, ceftazidime, or cefotaxime with and without clavulanic acid were used in paper 1 and cefotaxime and ceftazidime with and without clavulanic acid were used in **paper 3**. An ESBL phenotype was defined as an increase of ≥ 5 mm in the zone around the disk containing clavulanic acid compared to the zones of corresponding disks without clavulanic acid.

***Double-disk synergy (DDS) method:*** A modified version of the DDS test a.m. Jarlier was applied in **paper 1**. A positive synergy test was defined a clearly visible extension of the edge of the inhibition zone of any indicator disk used towards the amoxicillin-clavulanic acid disk. Aztreonam (30 µg), cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (5 µg), and cefpirome (30 µg) disks (Oxoid) were used as indicators and placed around an amoxicillin (20 µg)-clavulanic acid (10 µg) disk at a distance of 25 to 30 mm center-to-center.

These tests are convenient for routine use as they instrument independent, flexible, and easy to implement in most laboratories. The DDS test is a simple and inexpensive test, but depends on the subjective reading of interactions between zones of inhibition. Furthermore, the optimal spacing between disks may vary with the individual strain. Sensitivity of the DDS-method may be improved by reducing disk spacing for bacteria hyperproducing cephalosporinases (narrowed to 20 mm)[266]. Re-examination of some strains yielding equivocal results with the disks placed closer together (15 mm) or more apart (30 mm) may be needed for strains with a very high or low resistance levels, respectively[266]. The CDT is regarded as an inexpensive and objective, method. Continuous quality controls with QC-strains are critical in order to detect the occasional occurrence of batch variation. In ellipsometer gradient tests such as Etest ESBL strips, interpretation are objective in most cases although rely on interpretation of synergy phenomena in others. Furthermore, undetermined results due to out of scale MICs do occur. Etest ESBL strips are comparatively expensive.

**Phenotypic detection of AmpC production:**

Isolates expressing a cefoxtitin - MIC≥16 mg/L (regardless of MIC to oxyimino-cephalosporins) were examined by AmpC phenotypic tests (**paper 3**). An "AmpC phenotype" in this study was defined for isolates displaying a significant increase in susceptibility to cefoxitin in combination boronic acid (*in house* prepared) in a combined disk method (a.m. Coudron) (**paper 3**).

***AmpC Etest (bioMérieux):*** In **paper 3,** AmpC production according to the manufacturer's instructions was defined as a >8-fold decrease (≥3 two-fold dilutions) in the MIC of cefotetan in one end of the strip as opposed to the MIC of cefotetan in the presence of cloxacillin (inhibitor) in the other and of the strip.

***Combined disk method - a.m. Coudron, the boronic acid inhibitory test.*** Disks containing cefoxitin (30 µg) (Becton Dickinson) with and without 20 µl boronic acid (120 mg phenylboronic acid (Sigma-Aldrich) in 3 ml DSMO and 3 ml dd$H_2O$) were used in **paper 3**. The disks containing boronic acid were *in house* prepared 30 minutes prior to use and left to dry in room temperature. An AmpC phenotype was defined as an increase of ≥ 5 mm in the zone around the disk containing boronic acid compared to the zones of corresponding disks without boronic acid (when using semi-confluent growth, *in house* validated method, K-res) [358].

***Cefoxitin-EDTA disk test a.m. Black:*** This test was used in **paper 3** and is a biological "AmpC disk"- assay that was proposed by Black in 2005. An agar plate is inoculated with a lawn of *E.coli* ATCC 25922 and a cefoxitin disk is placed upon it. Test strain colonies are inoculated on a separate Tris-EDTA containing disk. In principle, the β-lactamase is released from the test strain colonies, by the permeabilizing effect of the Tris-EDTA. Enzymatic inactivation of cefoxitin causes flattening of inhibition zone around the positive test strains[359].

**Detection of *bla*$_{KPC}$ and KPC producing- Enterobacteriaceae from human fecal carriers and environmental samples:**

***ChromID ESBL® (bio-Meriux) agar:*** In **paper 4 s**creening was performed by ChromID ESBL® (bio-Meriux) agar medium plates and/or an enrichment medium (TS-broth containing 2 mg/L cefpodoxime) and interpreted according to the manufacturer's instructions.

***Real-Time PCR of blaKPC:*** In **paper 4** Real-Time PCR of *bla*$_{KPC:}$ was performed directly from the swab (day one), enrichment medium (day two), and/or colonies from the ChromID ESBL agar (Further detailed below section)[360].

**Isoelectric focusing (IEF):**

IEF was applied on a few isolates in **paper 1** and in all isolates in **paper 3**. IEF is a conventional technique for separating different molecules by their isoelectric points (IEPs), i.e. the pH at which a particular molecular carries no net electrical charge. IEF was used to separate β-lactamase genes from crude cell extracts, performed in precast Ampholine PAGplate polyacrylamide gels with a pH range of 3.5–9.5 (GE Healthcare, Oslo, Norway), using a Multiphor II Apparatus (GE Healthcare). B-lactamase activity was detected by staining the gels with nitrocefin solution (0.5 g/L). The isoelectric points (pIs) of the studied β-lactamases were determined by comparison with reference β-lactamases *bla*TEM-1 (pI 5.4) and *bla*SHV-1 (pI 7.6), as well as with naturally coloured IEF Protein Standards pI 4.45–9.6 (Bio-Rad Laboratories, Hemel Hempstead, UK).

**DNA extraction for downstream applications:**

Bacterial DNA extraction in all bacterial strains was performed in a Qiagen BioRobot M48 (Qiagen, Hilden, Germany) using MagAttract® DNA Mini M48 kit (Qiagen).The MagAttract DNA M48 Mini Kit is designed for automated purification of total DNA from among others, cultured cells, using a silica-based DNA purification method with magnetic particles. In short; a starting material of ca. 2 x $10^6$ cells are suspended in provided lysis buffer, the DNA binds to the

silica surface of the magnetic particles, the magnets are separated, washed, separated for a second time and finally eluted to yield high purity and quality DNA.

**Molecular characterization of β-lactamases and porins:**

***Conventional PCR and DNA sequencing:*** PCR is an invaluable tool in molecular biology, daily used in laboratories around the world in a wide array of downstream applications. The method is based upon a thermal cycling approach, which includes heat-stable polymerase for the replication of target DNA in each cycle for amplification and subsequent detection.

Published conventional PCRs in single- and multiplex setups were used for the detection of the various ESBL, AmpC and carbapenemase determinants i.e. CTX-M, SHV, TEM, OXA, KPC, ACC, CIT, DHA, EBC, FOX, and MOX (**paper 1, 2 and 3**). Primers were designed for the amplification of porin genes *ompC* and *ompF* in *E. coli* **(paper 3)**. Mapping of the genetic environment for CTX-M producing *E. coli* was determined by various PCRs (**paper 2**) followed by DNA sequencing. All conventional PCRs were performed using JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA) as PCR reaction mix in a GeneAmp 9700 PCR system (Applied Biosystems, Foster City, California). Separation and visualization of PCR amplicons was performed in a conventional agarose gel electrophoresis with subsequent ethidium bromide staining. When required PCR amplicons were purified using EXO-SAP treatment and sequenced in an ABIPRISM™ 3130XL capillary sequencer (Applied Biosystems).

***Real Time PCR (qPCR):*** Real Time PCR or quantitative PCR (qPCR) follows the principles of conventional PCR, with the additional possibility of real time detection of target DNA amplification and relative or absolute DNA quantification. For the detection of $bla_{KPC}$ (**paper 4**), a TaqMan based Real-Time PCR was performed directly on suspensions from rectal swabs, enrichment medium, and colonies from a ChromID ESBL agar plate (TaqMan 7500 system, Applied Biosystems).

***Real Time Reverse Transcriptase -PCR (qRT-PCR):*** Reverse transcriptase technology complements qPCR by allowing real time quantification of gene expression, through conversion of RNA to DNA, and subsequent amplification of cDNA for real-time detection. qRT-PCR experiments were performed for the detection and quantification of chromosomal *ampC* genes using qPCR Mastermix Plus (Eurogenetec) (**paper 3**). Synthesized cDNAs were diluted 1:10 and amplified in triplicate. Amplifications of synthesized cDNA were also run without reverse transcriptase to confirm the absence of DNA in the RNA samples. The **ampC** target gene was normalized against the reference gene glyceraldehyde 3-phosphate dehydrogenase (**gapA**) using Q-Gene. The **ampC** mRNA mean normalized expression level was calibrated as fold differences using the mean normalized expression level of ATCC 25922 as 1.0. Standard deviations were calculated for the fold differences according to the standard deviations for the **ampC** mRNA mean normalized expression levels.

***PCR primers used in this study***: For detailed information, I refer to the individual papers. Specific problems were not acknowledged with these primers, and have to our knowledge neither been reported with these primers.

**Epidemiological strain typing:**

Epidemiological strain typing is very important in order to detect outbreaks, identify sources of infection and surveillance. Historically, typing was performed through phenotypic methods such as serological typing. However, phenotypic methods are generally considered less reproducible than molecular methods, despite recent advances in mass spectrometry and proteomic approaches. More than one typing method may be necessary to investigate the chromosomal relatedness of different strains.

***Serotyping:*** Serological typing of Enterobacteriaceae make use of the antigenicity of the lipopolysaccharide, flagellae and capsules (O, H, K). In **paper 2** serotyping was performed using diagnostic antisera at the International *Escherichia coli* and *Klebsiella* Centre (WHO) in Copenhagen.

***Pulsed Field gel Eletrophoresis (PFGE):*** PFGE make use of rare cutting restriction enzymes in producing 30 – 1000 kb sized DNA fragments from purified genomic DNA. Digested DNA fragments are separated in an agarose gel by electrophoresis in which the electric field across the gel is constantly changed at regular intervals, allowing fragments to be separated by size and subsequently visualized for comparison by a fluorescent dye under ultraviolet light. Dispersed parts of the genome are investigated and various genetic events may be detected. Fragment size differences less than 5 kB may not be recognized, and plasmid DNA from large plasmids may produce single bands disrupting interpretation of the chromosomal DNA restriction patterns created. This method is most commonly used in outbreak investigations[107,361]. Interpretation has been standardized in guidelines assigning indistinguishable, closely related isolates (corresponds to one single genetic event giving one to three band differences), possibly related isolates (corresponds to two independent events giving four to six band differences) and unrelated isolates (corresponds to three or more independent events giving more than six band differences). Results remain inherently challenging for inter-laboratory comparisons even after meticulous standardization of procedures, software programs (i.e. GelCompare®) and database systems for comparison such as the US PulseNet and European Harmony programs[362,363]. In general, the discriminatory power, epidemiological concordance and intra-laboratory reproducibility are high. However, the method is technically demanding and laborious (high workload), and results slow to generate (at least 3 days).

In this study (**paper 2 and paper 4**) we typed strains with PFGE following extraction of genomic DNA and digestion with *Xba*I (New England Biolabs). Plugs were run on a multidirectional gel electrophoresis using clamped homogenous electric field (CHEF), provided by Chef-DR® III System (BIO-RAD, Hemel Hemstead, UK). Electrophoresis was run at 12°C with pulse time 1-20s, at 6 V/cm on a 120° angle in 0.5xTBE buffer for 21 hours. DNA relatedness was based on criteria of Tenover et al.

***Multi Locus Sequence Typing (MLST):*** MLST is a sequence-based method assessing variation in several (usually 5-10) housekeeping genes obligate in a given bacterial species[364,365]. Standardized PCR amplifications and subsequent sequencing of amplicons (typically 450-500 bp each) are assessed by assigning arbitrary numbers to any unique allelic combination. In this manner a string of numbers corresponding to the number of loci sequenced is created. A given string of numbers is subsequently assigned to a specific sequence type. Standardization creates data that are highly reproducible and unambiguous. Furthermore, data are easily computerized and exchangeable between laboratories. Large databases for assigning STs and software for studying genetic relatedness and clustering (eBurst) are freely available on the Internet [366]. However, the method lacks sufficient discriminatory power for most bacteria in strict, localized outbreak investigation, and is primarily suitable for public health and evolutionary surveys. To circumvent this, adding more heterogeneous loci into the analysis has been attempted[367]. Besides, even if the capacity for analysis by this system is high, the method is time-consuming, laborious, and comparatively expensive. MLST schemes have been developed for a wide variety of different organisms, and different MLST schemes are available for *E. coli.*

In our study (**paper 2 and 4**) we used the most widely used and accepted scheme for *E. coli* developed by Achtman et al., targeting seven housekeeping genes; *adk, fumC, gyrB, icd, mdh, purA,* and *recA* [368].The collection of MLST data on *E. coli* is available online (http://web.mpiib-berlin.mpg.de/mlst/). Similarly, the MLST scheme developed for *K. pneumoniae* by Brisse et al. also targeting seven housekeeping genes, *rpoB, gapA, mdh, pgi, phoE, infB* and *tonB* was used for our strains[369]. The collection of MLST data on *K.pneumoniae* is available online: (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). Following PCR amplification of the target housekeeping genes, purified PCR amplicons were sent to Macrogen (Seoul, Korea) for DNA sequencing. Sequence alignment was performed in BioEdit sequence Alignment Editor and typing according to online databases.

***E.coli phylogeny – triplex PCR:*** Phylogenitical classification is used for typing purposes of *E. coli* into potentially virulent and commensal lineages. Originally, phylogenetic groups were established within the *E. coli* reference collection (ECOR) of 72 strains according to their observed multi-locus enzyme electrophoresis (MLEE) pattern on a selection of enzymes and multicopy single-stranded DNA (msDNA). Phylogenetic analysis of the ECOR strains revealed

the distribution of these strains into initially five distinct clusters, labelled A-E, later reduced to four groups A-D.

In this study (**paper 2**) we used a rapid PCR-based approached for the phylogenetic classification developed by Clermont et al. in 2000 to classify of *E. coli* into virulent (such as extraintesinal B2 and D) and commensal (such as A and B1) strains (**paper2**)[370].The approach target three DNA fragments *chuA, yjaA* and *TSPE4.C2* in a triplex PCR to distinguish between the different phylogenetic groups.

**Epidemiological plasmid typing:**

The dynamic nature of plasmids makes typing challenging. Still, plasmid replicons are comparatively stable elements within the plasmid and convenient typing targets.
In this study (**paper 2 and 4**) we combined S1-nuclease PFGE and a PCR-based replicon typing (PBRT) to characterize and compare the plasmid content of isolates.

***PCR-based Replicon Typing (PBRT):*** This method entitles to discriminate plasmids of different incompatibility families. In 1988 Couturier et al. developed a hybridization based method for the detection of 19 replicon plasmids circulating among the *Enterobacteriaceae*. The first PCR-based replicon typing of plasmids was developed in 1996 and covered four of these nineteen replicon plasmids (IncP, IncN, IncW and IncQ)[371]. In 2005 the method was furthered developed by Carattoli (2005) with 18 primer pairs in a 5 multiplex and 3 simplex PCR setup to cover 18 of the plasmid replicons[372]. This scheme of Carattoli was used for replicon typing of our resistance plasmids **(paper 2 and paper 4**).

***S1-Nuclease-PFGE and bla-hybridization:*** Plasmid profiling was performed by PFGE of S1-nuclease (Promega, Madison, WI, USA) digested total DNA. In S1-nuclease digestion of plasmid DNA the unique properties of *Aspergillus oryzae* S1-(endo)nuclease are used for (i) the selective and effective cleavage of circular plasmids and not chromosomal DNA, and (ii) the limited action on linear double-stranded DNA [373]. In this way plasmid separation from the total genomic DNA can be achieved for typing purposes. This method is frequently used to characterize plasmids by size, and may be used in outbreak situations to infer transfer of plasmids among strains and species[21,374]. PFGE was run in a Chef-DR III System (Bio Rad, Oslo, Norway) at 14°C, with pulse time 1-20 s, at 6 V/cm on a 120° angle in 0.5xTBE buffer for 15 h. The Low Range ladder (New England BioLabs, UK) was used as plasmid size marker. Each band on the gel was considered a linearized plasmid. Plasmid DNA bands were transferred to positively charged nylon membranes using a vacuum blotting system (Vacuum Blotter, Model 785, BioRad). Resistance encoding plasmids and their replicons were confirmed through **hybridization** with resistance and replicon specific labelled probes prepared by DIG High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany).

**Plasmid conjugation:**

Transferability of the resistance plasmids were investigated by broth mating to a rifampicin resistant, plasmid-free recipient strain, E. coli J53-2. Broth mating experiments were performed as follows: donor and recipient strains were cultured ON in Luria Bertani (LB) broth at 37˚C. ON cultures were diluted 1:100 and incubated at 37˚C until reaching an optical density (OD600nm) of approximately 0.5. Donor and recipient cultures were mixed 1:10 and incubated at 37˚C with careful shaking for four hours and ON. Aliquots of 100 µl mixed culture were plated on LB plates containing appropriate antimicrobial concentration dilution.

## STATISTICAL METHODS

Throughout this thesis, SPSS16.0 was used for statistical analysis. Chi-square ($\chi^2$) test was used to compare categorical data between (two) groups. Descriptive statistics functions were used to analyze and evaluate histograms, Q-Q-plots, and boxplots (median, SD and outliers) of raw data in **paper 3**. Bayesian statistics were used to analyze diagnostic testing (sensitivity, specificity, NPV, PPV).

# D. RESULTS

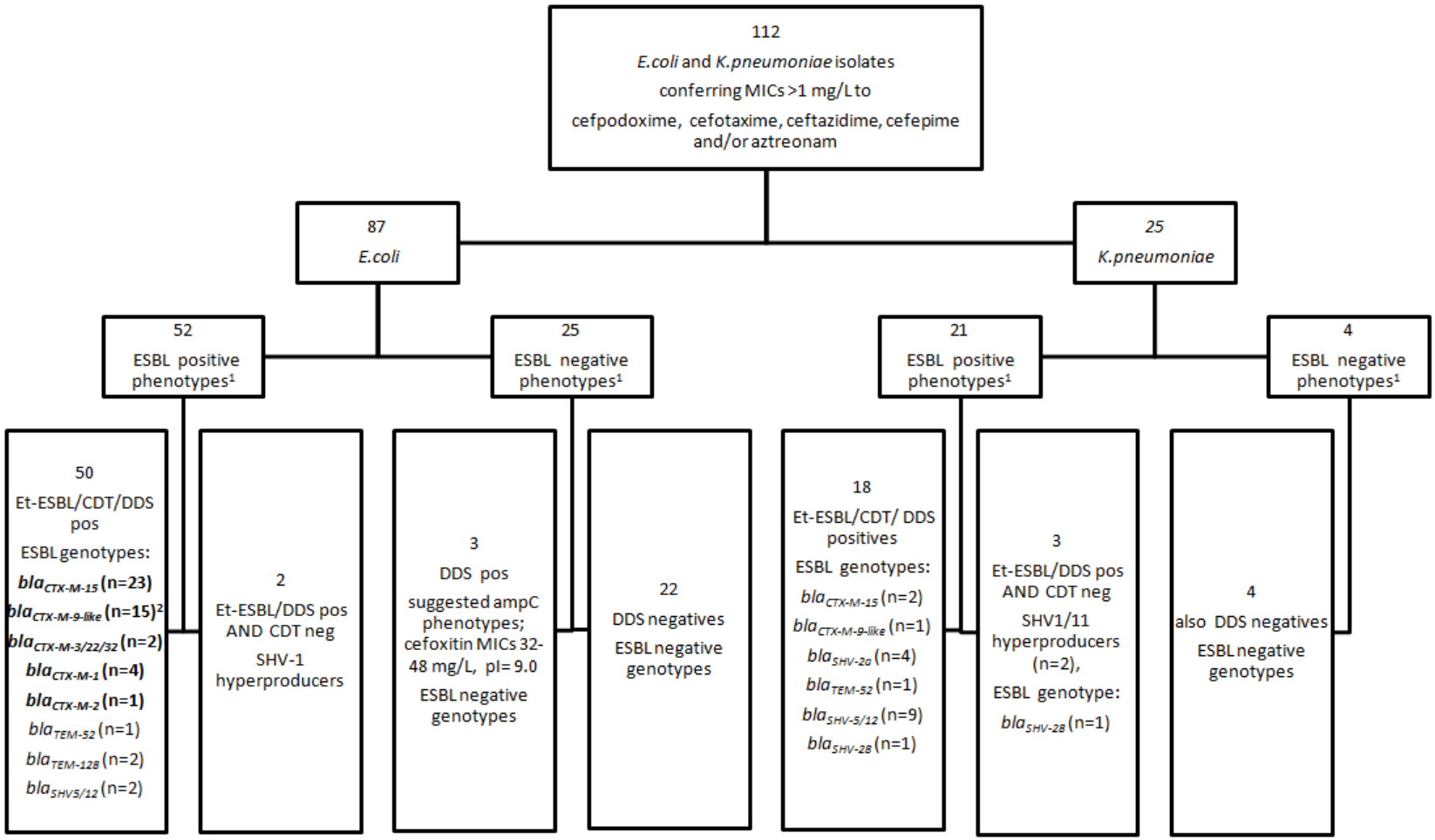
**PAPER 1.**

This part of the study aimed to examine the distribution of different *bla*-genotypes among ESBL producing *E. coli* and *K. pneumoniae* strains at point of entry into Norway in 2003 in a nation-wide study (N-ESBL). A special attention were set to determine appropriate substrates for phenotypic detection and evaluate methods for rapid and accurate identification of ESBLs at the clinical laboratories.

- **ESBL phenotypes vs. genotypes.** An ESBL phenotype as defined (see material and method section) was detected in 60% of *E. coli* (52/87) and 84% of *K. pneumoniae* (21/25) isolates, with an ESBL genotype confirmed for 50/52 *E. coli* and 19/21 *K. pneumoniae* isolates (Figu**r**e 9). The 35 *E.coli* isolates with a negative ESBL phenotype expressed moderate to high-level cefoxitin-MICs (48->256 mg/L) as well as various levels of reduced susceptibility to oxyimino-cephalosporins and aztreonam without clavulanic acid synergy (data not shown), but was not examined further in this study.

- **Distribution of ESBL-families.** In *E. coli*, 45/50 (90%) isolates belonged to CTX-M, 2/50 to SHV, and 3/50 to TEM ESBL-genogroups, respectively. In *K. pneumoniae*, 15/19 (74 %) belonged to SHV, 3/19 isolates to CTX-M, and 1/19 to TEM ESBL families, respectively (Figure 9).

- **Inferred $bla_{\text{SHV-1 /11}}$ hyperproducers (n=4)**. Two *E.coli* isolates were considered SHV-1 hyperproducers (K2-34 and K8-3) containing a $bla_{\text{SHV-1}}$ genotype by sequencing and a phenotypic profile consistent with hyperproduction with moderate increase in ceftazidime MIC (2-4 mg/L), wild-type cefotaxime MIC (0,125- 0, 25 mg/L) and a piperacillin-tazobactam MIC > 256 mg/L (Figure 9). IEF-analysis revealed a single β-lactamase pI band of approximately 7.5 consistent with SHV-like enzymes. An analogous MIC-pattern was observed in tw*o K.pneumoniae* isolates sequenced as $bla_{\text{SHV-1}}$ and $bla_{\text{SHV-11}}$, respectively. Accordingly, these isolates were also regarded as hyperproducers.




[1]*See material and method section.* [2]*In* **Paper 2** *these isolates were typed as follows; bla* $_{\text{CTX-M-14}}$ *(n=11),* $bla_{CTX\text{-}M\text{-}9}$ *(n=3),* $bla_{\text{CTX-M-27}}$ *(n=1).*

*Figure 9. ESBL phenotypes, genotypes, and classification of results from confirmatory testing (Etest ESBL, CDT and DDS) in the N-ESBL study. 112 clinical E.coli and K.pneuminiae isolates with reduced susceptibilities to oxyimino-cephalosporins (MIC >1 mg/L) were evaluated.*

- **Indicator substrate utility for ESBL-detection and properties of confirmatory tests.** The usefulness of individual oxyimino-cephalosporins and aztreonam, or combinations were evaluated using reduced susceptibility MIC > 1 mg/L according to NWGA 2003 and a screen cut-off for cefpodoxime of MIC>1 mg/L. Cefpodoxime alone and the combined use of cefotaxime and ceftazidime in screening failed to detect 1/69 isolates each (K2-79 and K5-30, respectively, both *K.pneumoniae*). Three isolates were lost when cefotaxime alone were used (K2-79, K5-30, and K4-61, all *K.pneumoniae*). More isolates would have remained unnoticed by the potential use of ceftazidime (n=20) or aztreonam (n=9) as single screening substrates. In particular, the $bla_{\text{CTX-M-9}}$ genogroup *E.coli* strains expressed low MICs of ceftazidime (mean, 0.55 mg/L) and aztreonam (mean 1.8 mg/L) except one $bla_{\text{CTX-M-16}}$ strain.

*Table 5: Test properties of individual confirmatory ESBL-tests.*

| **Test** | Sensitivity | | | Specificity | | |
|---|---|---|---|---|---|---|
| | **Overall (%)** | ***E. coli* (%)** | ***K. pneumoniae* (%)** | **Overall (%)** | ***E. coli* (%)** | ***K. pneumoniae* (%)** |
| CDT | 68/69 (99) | 50/50 (100) | 18/19 (95) | 43/43 (100) | 37/37 (100) | 6/6 (100) |
| Etest ESBL | 69/69 (100) | 50/50 (100) | 19/19 (100) | 39/43 (91) | 2/37 (95) | 4/6 (67) |
| DDS | 69/69 (100) | 50/50 (100) | 19/19 (100) | 36/43 (84) | 5/37 (86) | 4/6 (67) |

  - ***CDT:***
    - Identified an ESBL phenotype in 50 *E. coli* isolates; including all the confirmed ESBL genotypes, with no false positives or negatives.
    - Identified an ESBL phenotype in 18 *K.pneumoniae* isolates; including 18 confirmed ESBL genotypes, with one false negative. The false negative *K.pneumoniae* isolate carried $bla_{\text{SHV-28}}$ with MICs to cefotaxime and cefpodoxime < 1 mg/L, and to ceftazidime 6 mg/L.
  - ***Etest ESBL:***
    - Identified an ESBL phenotype in 52 *E. coli* isolates; including all the confirmed ESBL genotypes, with two false positives that were considered SHV-1 hyperproducers.
    - Identified an ESBL phenotype in 21 *K. pneumoniae* isolates; including 19 confirmed ESBL genotypes and two false positives. The false positive *K.pneumoniae* isolates were considered SHV-1/11 hyperproducers.
  - ***DDS:***
    - Identified an ESBL phenotype in 55 *E. coli* isolates including all the confirmed ESBL genotypes, and five false positives. Two of the false positive isolates exhibited a SHV-1 hyperproducer phenotype. Three false positive isolates were suggested AmpC hyperproducers with an AmpC phenotype and falsely identified as ESBL producers due to CLA synergy with cefpirome and aztreonam. All of

these three isolates (K2-44/K2-62/K2-66) had common features including negative PCR results for *bla*$_{CTX-M/SHV/TEM}$, moderately elevated cefoxitin MICs (32-48), and a single β-lactamase band of pI=9.0. Their oxyimino-cephalosporin and aztreonam Etest MIC-profiles were also similar (cefpodoxime 24-48, cefotaxime 2-4, ceftazidime 2-6, and aztreonam 2-4) indicating a common mechanism.

- Identified an ESBL phenotype in 21 *K. pneumoniae* isolates; including 19 confirmed ESBL genotypes and two false positives. The false positives were considered SHV-1/11 hyperproducers. (The results for *K.pneumoniae* were identical to the Etest ESBL).

- **Clinical and demographic data from patients related to ESBL-producing species (Table 6) and genotypes.**

*Table 6. Clinical and demographic data from patients with ESBL-producing E.coli and K.pneumoniae.*

| | ***E.coli* ESBL (n=50)** | ***K.pneumoniae* ESBL (n=19)** | **Total ESBL (n=69)** |
|---|---|---|---|
| **Gender** | | | |
| Male | 15 | 9 | 24 |
| Female | 32 | 7 | 39 |
| Unknown | 3 | 3 | 6 |
| **Age** | | | |
| < 16 y | 0 | 2 | 2 |
| 16-65 | 25 | 6 | 31 |
| >65 y | 22 | 8 | 30 |
| Unknown | 3 | 3 | 6 |
| **Out /Inpatient** | | | |
| Out | 22 | 3 | 25 |
| In | 25 | 14 | 39 |
| Unknown | 3 | 2 | 5 |
| **Specimen** | | | |
| Blood | 0 | 0 | 0 |
| Urine | 34 | 8 | 42 |
| Respiratory tract | 6 | 2 | 8 |
| Abdominal secretion | 4 | 3 | 7 |
| Skin-related wounds | 5 | 4 | 9 |
| Other | 0 | 1 | 1 |
| Unknown | 1 | 1 | 2 |
| **Hospitalization abroad within last 12 months** | | | |
| Yes | 2 | 2 | 2 |
| No | 1 | 2 | 3 |
| Unknown | 47 | 15 | 62 |

ESBL-producing strains were detected by most laboratories (sixteen of 18). *bla*$_{CTX-M}$ *E. coli* isolates were recovered in fourteen laboratories. 50 % (14 inpatients/1 unknown/14 outpatients) of the *bla*$_{CTX-M}$ *E. coli* isolates urinary tract isolates were identified in outpatients representing eight of 18 laboratories.

- **Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim/sulfamethoxazole (Figure 10).**

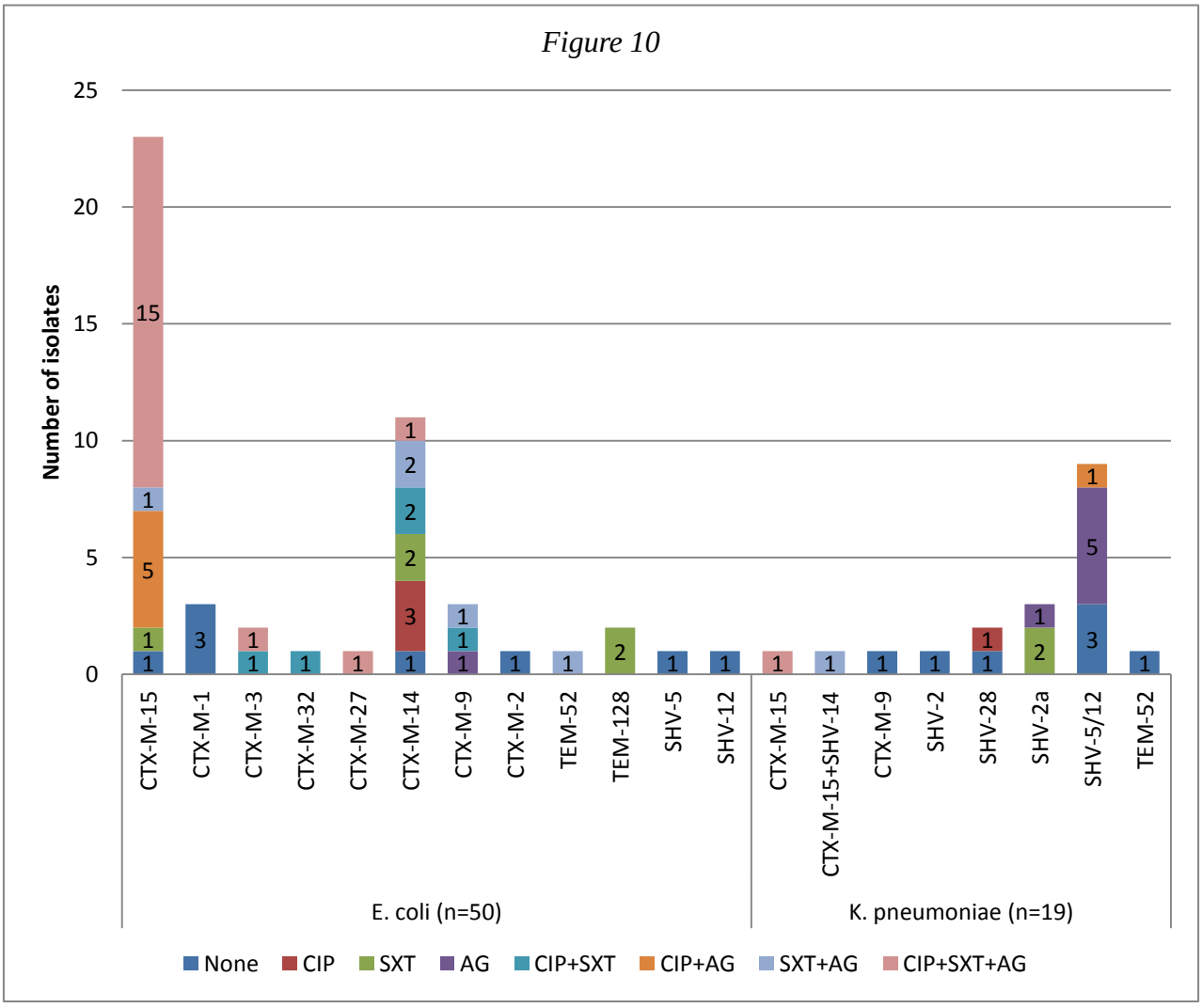



*Figure 10: Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim/sulfamethoxazole in ESBL-producing E.coli and K.pneumoniae isolates (n=69) related to genotype.*

**PAPER 2.**

This part of the study aimed to examine the molecular epidemiology of the CTX-M producing *E. coli* subpopulation from paper 1 on strain, clone and plasmid level in order to get further insight into the relationship between clonal and plasmid spread in the Norwegian isolates.

- **Epidemiology of genotypes.** In the subgroup of 45 *E.coli* CTX-M isolates from paper 1, the predominant *bla*-types were $bla_{\text{CTX-M-15}}$ (n=23/45; 51%) and $bla_{\text{CTX-M-14}}$ (n=11; 24%) (Figure 9, isolates given in bold).

- **Genetic environment of $bla_{\text{CTX-M}}$.** Eighteen of 23 $bla_{\text{CTX-M-15}}$s and five of 11 $bla_{\text{CTX-M-14}}$s were linked to *ISEcp1* upstream. One $bla_{\text{CTX-M-14}}$ was linked to *qacEΔ1sul1* upstream.

- **Epidemiology on clone and strain level.** PFGE demonstrated both genetic diversity (n=23 single-types) and regional clusters (n=22 clustered-types) included in six clusters (C1-C6) of 2 to 8 isolates. Sequence typing identified 19 different STs with the major types being ST131 (9/45 isolates, 20%), ST964 (7/45 isolates, 16 %), and ST38 (5/45 isolates, 11 %) The virulent phylogenetic groups B2 and D comprised n=32/45; 71 % of the isolates.

- **Epidemiology on plasmid level.** S1 nuclease-PFGE typing identified 22 single- and 21 multiple plasmid isolates. Plasmid replicon analyses by PBRT identified 10 different replicon types. All $bla_{\text{CTX-M-15}}$ (n=23) and $bla_{\text{CTX-M-9}}$ (n=3) were identified on replicon IncFII plasmids or on multireplicons comprising FII and additional FIA or FIB replicons. Contrary, $bla_{\text{CTX-M-14}}$ was located on plasmids with various replicons (FII, I1, FII-FIB, FII-FIA-FIB or I1-Y). Three $bla_{\text{CTX-M-1s}}$ were located on IncN-plasmids (Figure 11).

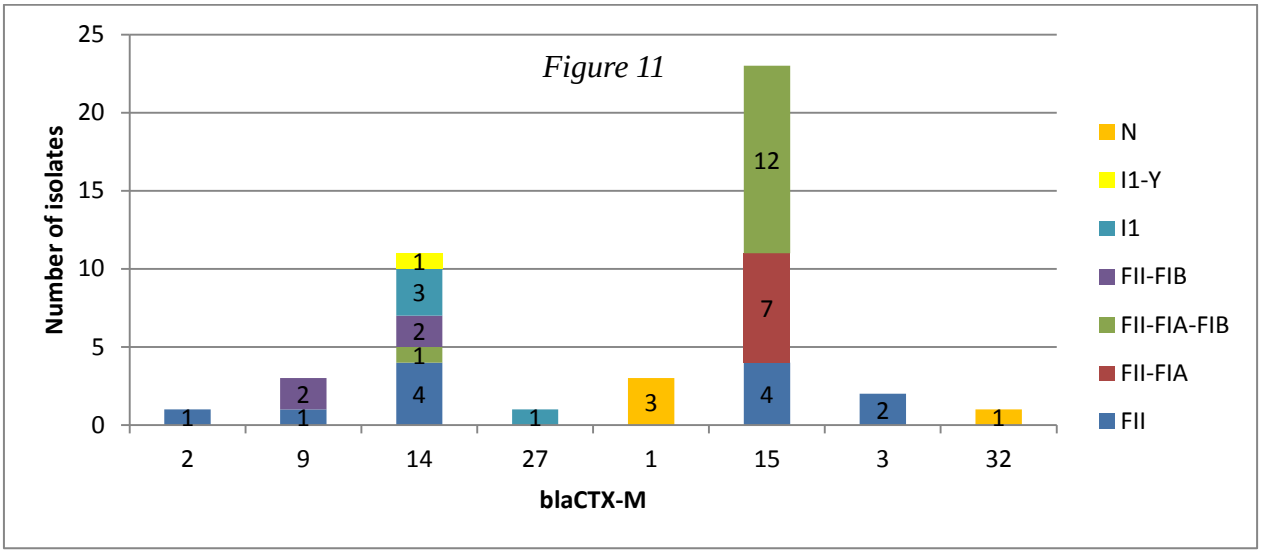



*Figure 11: Distribution of replicon types in plasmids carrying $bla_{CTX\text{-}M}$.*

- ***In vitro* dynamics of plasmid transfer by conjugation.** Plasmid transfer by conjugation was confirmed for 9/14 *bla*$_{CTX\text{-}M}$-containing plasmids. Co-transfer of other resistance phenotypes was only observed for trimethoprim/sulfamethoxazole (3/9; 33%). This observation contrast the presence of co-resistance to other important classes of antibiotics in 39/45=87 % of the isolates. Thus, the majority of co-resistance determinants were assumed to be located on other plasmids or the chromosome.

- **Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim/sulfamethoxazole related to sequence type.**

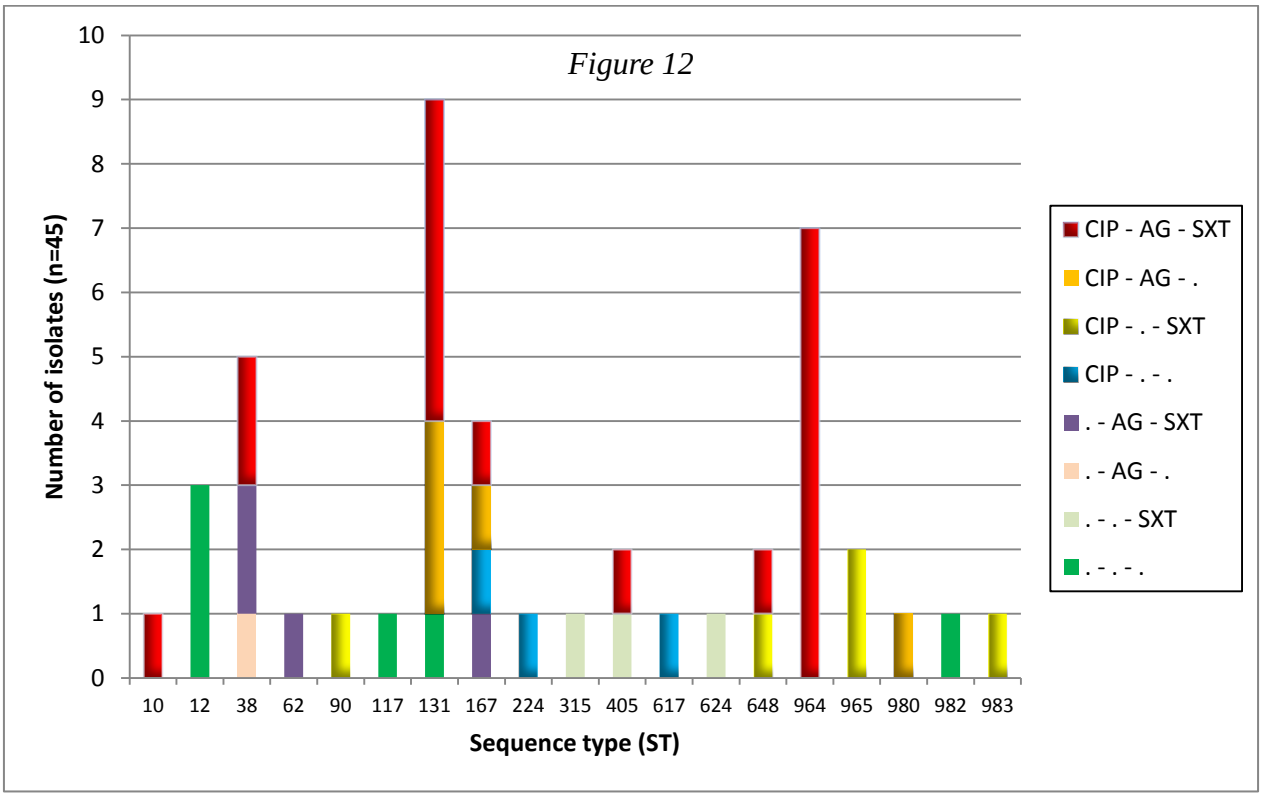



*Figure 12: Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim/-sulfamethoxazole in E.coli bla$_{CTX\text{-}M}$ isolates related to sequence type. Prominent MDR is displayed in several ST types, foremost in ST131 and ST964. (Note: All shaded bars represent ciprofloxacin resistant isolates).*

- **ST131 in emerging *bla*$_{CTX\text{-}M}$ *E.coli*.** Nine of the 45 isolates (20%) belonged to the virulent B2 O25:H4 ST131 *E.coli* clone. Genetic diversity was displayed as the ST131 divided into two distinct clusters and three single pulsotypes by PFGE. *bla*$_{CTX\text{-}M\text{-}15}$ was carried on multireplicon FII plasmid (FII replicon co-located with FIA or FIB replicons) in eight of these isolates and linked to an upstream IS*Ecp1* element in 4 of the 8 isolates.

- **Dispersal of strains:** Seven of 45 isolates (16%) clustered into an indistinguishable PFGE and MLST type (C6/ST964) with identical serotype (O102:K20, 23:H6), phylogenetic group (D) and a ~ 100kb, IncFII-FIA-FIB plasmid profile with $bla_{\text{CTX-M-15}}$ linked to an IS*Ecp1* element upstream. Five additional clusters of 2-4 isolates each were identified by indistinguishable XbaI-PFGE patterns.

**PAPER 3.**

This part of the study aimed to examine the panorama- and prevalence of different resistance mechanisms responsible for high- and low-level resistance to 3rd generation cephalosporins in *E. coli* and *Klebsiella* spp. populations in a nation-wide study (RESPECT) prior to the dissemination of ESBLs in Norway.

- **Prevalence rates of reduced susceptibility to oxyimino-cephalosporins and aztreonam (as defined in the method section) in *E.coli* and *Klebsiella spp.*** The overall prevalence of reduced susceptibility was 2.3 % (54/2292). Prevalence rates were 1.8% (35/1963) in *E. coli*, 1.1% (11/270) in *K.pneumoniae*, and 13.6% (8/59) in *K.oxytoca*. These isolates were subjected to confirmatory testing as described and classified into three phenotypic groups; (i) ESBLs, (ii) AmpCs, and (iii) neither-ESBL-nor-AmpCs (Figure 13).

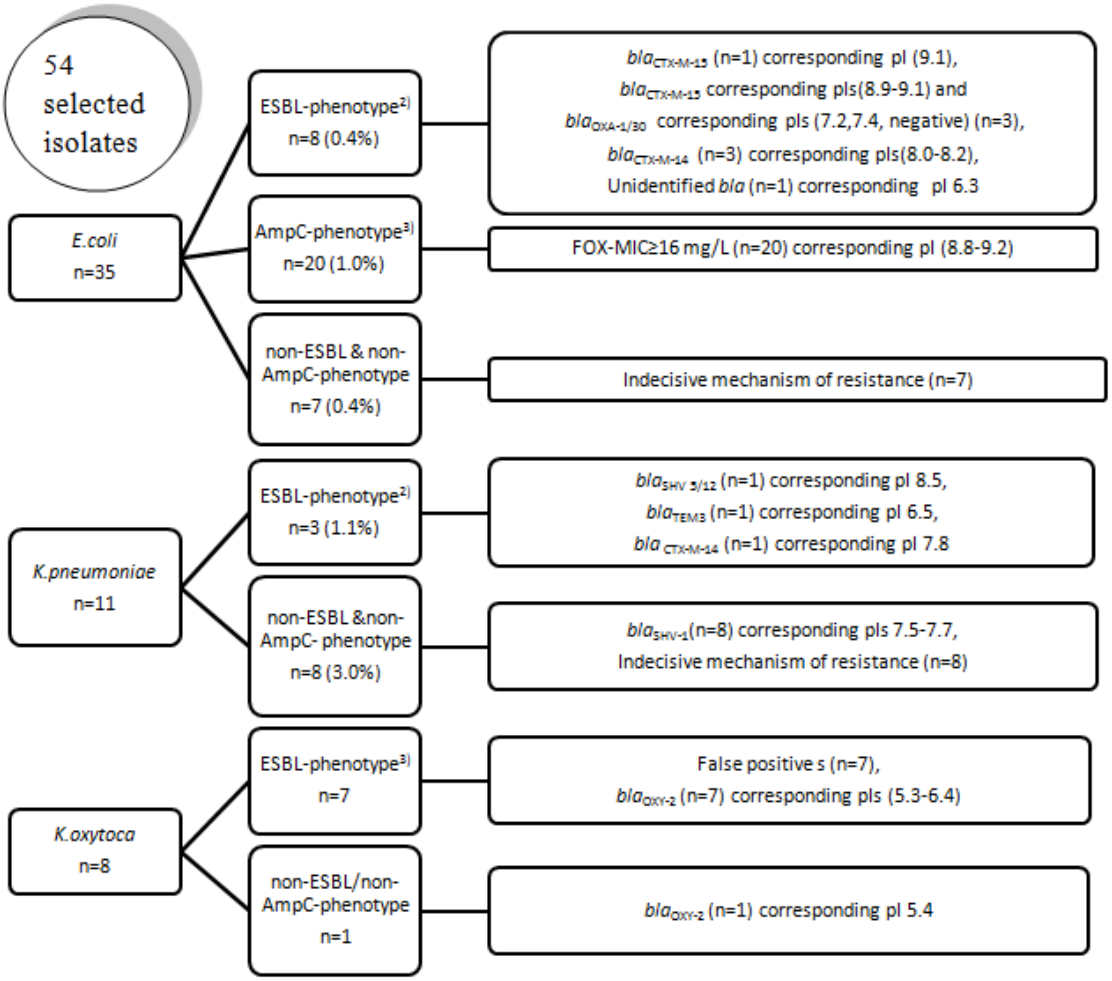


*Figure 13. Selected isolates (n=54) and inferred mechanisms of resistance to extended- spectrum cephalosporins. Prevalence rates are displayed in parantesis. The results of phenotypic confirmatory tests (ESBL or AmpC) and isoelectric focusing (performed in all isolates) were used to guide characterization and typing of β-lactamases by PCRs and sequencing.*

- **Prevalence rates of ESBL and AmpC-phenotypes and performance of confirmatory tests.**
    - ***E.coli:*** In *E.coli*, a low ESBL-phenotype prevalence rate 0.4% (8 of 1963 *E.coli*) was observed. *Bla*$_{\text{CTX-M}}$ genes (n=4 *bla*$_{\text{CTX-M-15}}$, n=3 *bla*$_{\text{CTX-M-14}}$) were confirmed by PCR and sequencing in all but one isolate. This isolate displayed an unidentified β-lactamase with an IEF band at pI 6.3. There were no discrepancy between the two phenotypic ESBL confirmatory methods (unpublished results). The prevalence rate of the AmpC phenotype was 1.0% (20 of 1963 *E.coli* isolates). Plasmid-mediated *ampC* genes were not detected. Chromosomal AmpC–hyperproduction was inferrred by a visible IEF band corresponding to AmpC (8.8-9.2) in all 20 isolates. Furthermore, increased fold expression of *ampC* normalized to *gapA* [5-85] was recorded by quantitative reverse transcriptase-PCR of *ampC* transcripts in 12/12 isolates examinded with an cefoxitin-MIC ≥ 24 mg/L. No definite resistance mechanism could be inferred in seven of 1963 isolates, resulting in a 0.4% prevalence rate. Four of these isolates (K31-57, K32-60, K32-25, and K32-31) were randomly investigated by OmpF and OmpC and subsequent porin sequencing. Analyses were performed by comparing alignments the Omp sequences of these strains to that of the *E.coli* control strain (*E.coli* K-12). No stop codons and consequent truncations were displayed. Investigation of porin expression was not performed in this study.

    - ***K.pneumoniae:*** In *K.pneumoniae*, only three isolates were recovered with an ESBL phenotype giving a prevalence rate of 1.1% (3 of 270 *K.pneumoniae*). *Bla*$_{\text{ESBLs}}$ were confirmed by PCR in all isolates (*bla*$_{\text{TEM-3}}$, *bla*$_{\text{SHV5/12}}$, and *bla*$_{\text{CTX-M-14}}$, respectively). No definite mechanism for reduced susceptibility to ESCs could be inferred in 8/270 isolates, resulting in a prevalence of 3.0%. Regardless of results in IEF and confirmatory tests, all isolates with cefoxitin-MIC ≥16 mg/L were tested and came out negative by multiplex-*ampC* PCR. The structure and function of porins were not investigated in *K.pneumoniae* isolates in this study.

    - ***K.oxytoca:*** In *K.oxytoca*, the isolates displayed MIC-profiles consistent with OXY-hyperproduction with reduced suseptibility or resistance to cefotaxime and aztreonam. All isolates displayed single IEF bands with pIs [5.3-6.4] consistent with OXY-2 group of enzymes. Discordant results were observed by the the Etest ESBL and CDT. A false positive ESBL-phenotype was recorded in seven of 8

*K.oxytoca* attributed to a positive CT/CTL Etest. A negative ESBL test was correctly assigned for all hyperproducing *K.oxytoca* isolates by CDT .

- **Comparison of AmpC-phenotypic tests.**
  - Isolates with a cefoxitin-MIC ≥ 16 mg/L (n=23) were considered putative AmpC-hyperproducers. The cefoxitin-boronic acid inhibitory test a.m. Coudron yielding 20 positive results was used to infer an AmpC phenotype in this study (see method section). However, AmpC Etest and the cefoxitin-EDTA test a.m. Black were performed for comparisons (unpublished results) (Table 7):

*Table 7: Performance of three phenotypic tests for investigation of putative AmpC production.*

| **Test** | Putative AmpC Hyperproducer | | Non-putative AmpC Hyperproducer | |
|---|---|---|---|---|
| | *E. coli* | *Klebsiella* | *E. coli* | *Klebsiella* |
| **Boronic acid** | 20/23 | 0/6 | 3/12 | 0/13 |
| **AmpC Etest** | 19/23 | 0/6 | 3/12 | 0/13 |
| **EDTA** | 15/23 | 0/6 | 2/12 | 0/13 |

Among 23 putative AmpC producers (cefoxitin-MIC ≥16 mg/L) in *E.coli*, 20, 19 and 15 produced a positive test by the cefoxitin-boronic acid test a.m. Coudron, the AmpC Etest, and the cefoxitin EDTA disk test a.m. Black, respectively. In *E.coli*, a positive test could also be displayed in some putative non-AmpC producers (n=3/12) for the cefoxitin-boronic acid test a.m. Coudron and the AmpC Etest), and in one of 12 tests by the cefoxitin EDTA disk test a.m. Black. In *K.spp* both putative AmpC producers (n=6) and non-putative AmpC producers (n=13) displayed a negative test in all three tests.

- All isolates in the RESPECT study remained fully susceptible to carbapenems.

- **Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamehoxazole in ESBL-producing *E.coli* (n=8) and *K.pneumoniae* (n=3) related to genotype.**

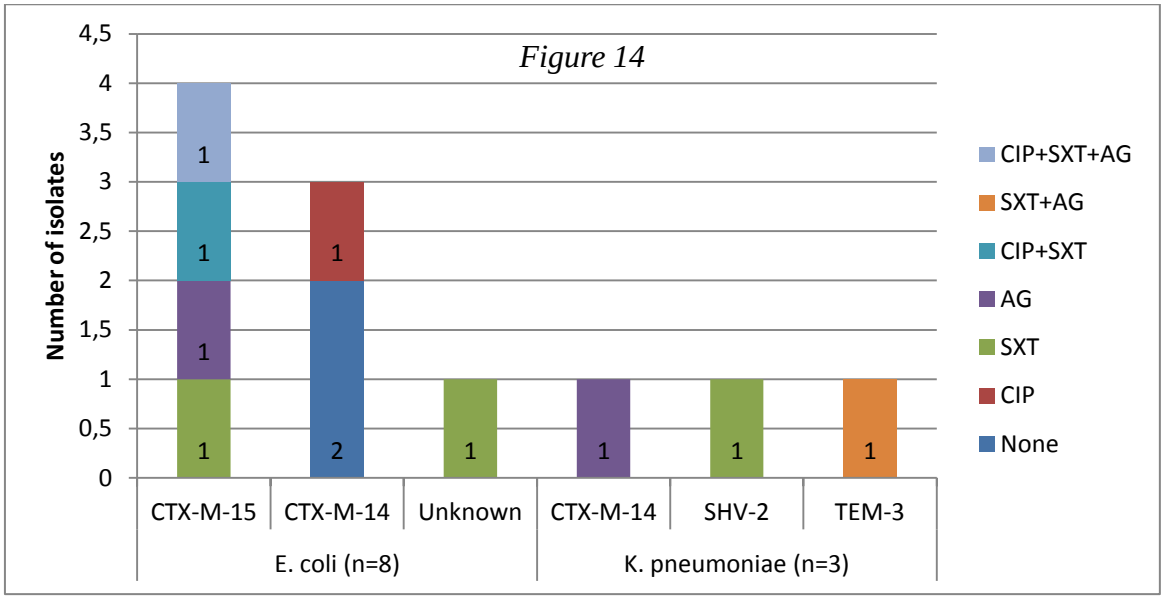



*Figure 14. Co-resistances to aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole in ESBL-producing E.coli (n=8) and K.pneumoniae (n=3) related to genotype. (Altogether co-resistance was evaluated for 80 ESBL-producing Enterobacteriaceae in paper 1 and paper 3 – see discussion).*

**PAPER 4**

This part of the study aimed to examine a long-term low-frequency hospital outbreak of KPC-producing *K.pneumoniae* through conventional- and molecular epidemiology at strain, clone and plasmid level in order to reveal modes of transmissions.

- **The index case:** Travel history revealed that the index patient had been hospitalized in Chania, Crete in September 2007 prior to hospitalization at SSHF- location Arendal (SSA) whereas none of the other patients had a history of recent foreign travel or hospital stay abroad.

- **Conventional epidemiology–time and place relations between patients:** In retrospect, conventional time and place - analysis confirmed epidemiological links between patients 1-2, 4-5, and a possible link between patients 3-5. Still, no links between clinical patients existed for long time intervals between the patients 1-3 and 5-6 representing time intervals of 10 and 11 months respectively (Figure 15).

- **Environmental detection of *bla*$_{\mathbf{KPC}}$:** KPC-producing bacteria were detected in 4 of 19 tested locations in the ICU-A: sinks in room 5, 6, 9 and the rinsing room.

- **Detection of *bla*$_{\mathbf{KPC}}$ in human carriers (feces):** KPC-producing bacteria were found in fecal screen of 1 of 136 tested patients (patient 7).

- **Molecular epidemiology of *bla*$_{\mathbf{KPC}}$ isolates at clone, strain, plasmid and gene level:** Clinical isolates (recovered during the hospital stay in all patients (1-7) and from 2 of the 4 environmental locations (sinks in room 5 and **6**) were investigated by phylogenetic typing and plasmid analysis as described:

    - A ~97 kb IncFII plasmid harboring *bla*$_{\mathrm{KPC}}$ was found in all isolates examined.

    - KPC-producing *K.pneumoniae* isolates were recovered in samples examined in patients 1-7, room 5 and 6, while KPC-producing *E.asburiae* were additionally recovered in the feces of patient 7 and the sink in room 5.

    - PFGE revealed one cluster and two single types among the *K.pneumoniae* isolates, and a single cluster of *E.asburiae*.

    - The cluster in *K.pneumoniae* was detected in patient 1, 2, 3, 4, 6, and 7 and room 5 and consisted of two related PFGE-types (A1 and A2) belonging to MLST-type ST258. Single pulsotypes were detected in patient 5 (PFGE/MLST-type B/461) and in room 6 (D/27).

    - A closely related cluster (C1 and C2) of *E.asburiae* strains was detected in the fecal sample from patient 7 and from room 5.

- **Loss of plasmids harboring *bla*$_{KPC}$**: Three urinary samples, all requested in outpatient settings, were subsequently recovered from patient 1, 4, and 3 (26, 16, and 4 months after initial diagnosis, respectively). ST258/A2 *K.pneumoniae* strains were detected in all of these samples. Compared to the primary isolates in these patients, the latter isolates were devoid of the 97kb plasmid containing *bla*$_{KPC-2,}$ and devoid of *bla*$_{TEM-1}$, however *bla*$_{SHV-12}$ and *bla*$_{SHV-11}$ were retained.

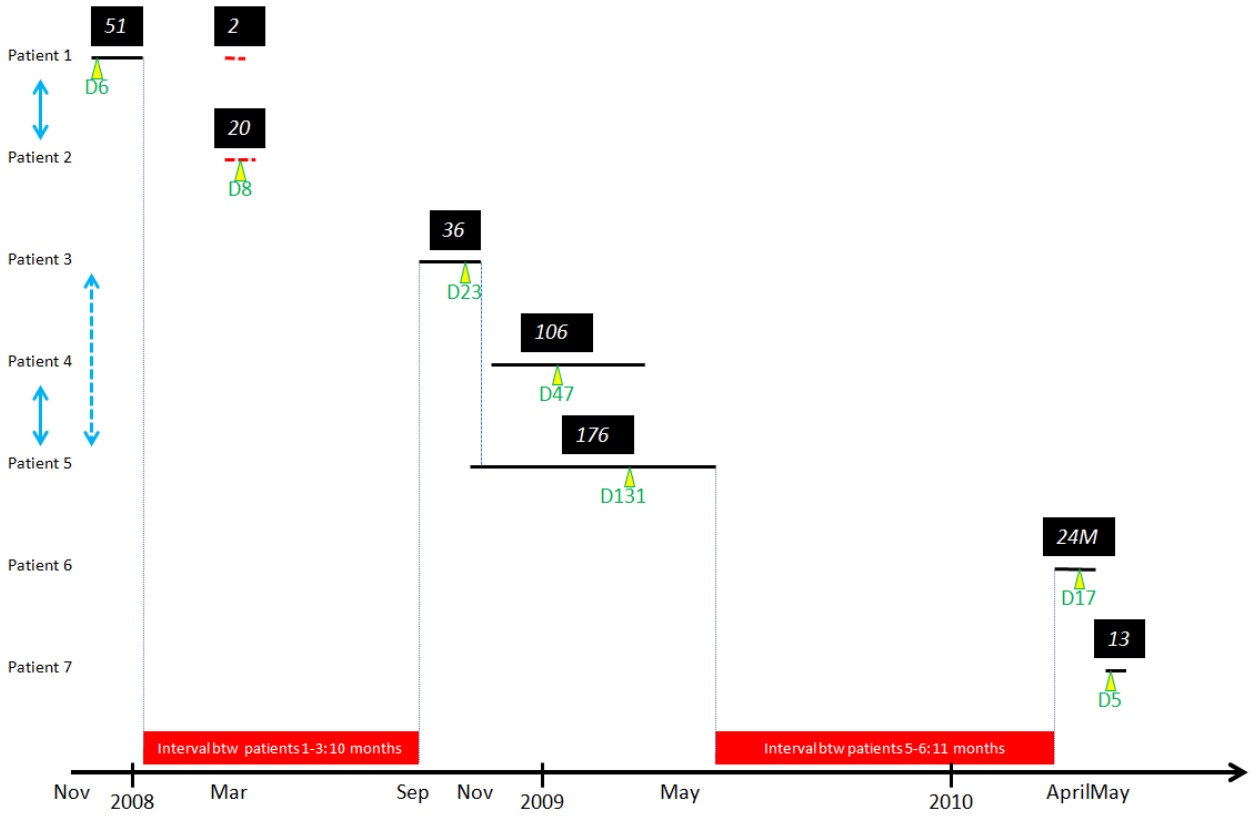



*Figure 15. Time and place ward relations between patients are displayed. Times of hospitalizations at SSA are indicated by black lines and at SSHF-location Kristiansand (SSK) by dotted red lines. The simultaneous hospitalizations of the patients 4 and 5 at Rikshospitalet, Oslo (RH) are not visualized. Epidemiological links between the patients 1 (med-2A, SSK) and 2 (med-2C, SSK) in neighboring wards at the same corridor, and the patients 4 and 5 (ICU-A and RH), are indicated by light blue vertical lines, whereas the possible link between the patients 3 and 5 (two days apart in ICU-A) is indicated by vertical dotted light blue lines. No relation could be established between the patients 1 and 3 and between the patients 5 and 6 (red bars, 10 and 11 months intervals, respectively). Length of stay is visualized by black bars and length of stay prior to diagnosis in green figures (D (number of days)). The identified KPC-producers were located at 12 different wards in three hospitals.*

- **Risk factors and outcome data for colonization or infection with KPC-producing *K.pneumoniae (Table 8)*.**

| P atient | Hospitalization abroad (country) | LOS (days) /LOS(days) prior to diagnosis | Anti G-negative antibiotics prior to diagnosis[3] | Other risk factors[2] (I/S/R/D) | Specimen | Role in infection | Location on discharge/ Discharged to: |
|---|---|---|---|---|---|---|---|
| P1 | Greece | 51/6 | MEM, PTZ | I/R | Expectorate | Uncertain | Home |
| P2 | No | 20/8' | None | None [1)] | Urine | None | Nursing home |
| P3 | No | 36/23 | MEM, TOB, CTX | I/R/D | Blood | Yes | Physical rehabilitation |
| P4 | No | 106/47 | MEM,TOB | I/R/D | Expectorate | Uncertain | Physical rehabilitation |
| P5 | No | 178/131 | MEM, IMI | I/S/R | Urine | Uncertain | Nursing home |
| P6 | No | 24†/17 | MEM | I/S/R | Expectorate | None | Diseased (in hospital) |
| P7 | No | 13/5' | CTX+MZL | I/R | Feces (screen) | None | Home |

1) *Except urinary catheter.*
2) *I= admission to ICU, S= recent surgery (laparotomi), R= artificial ventilator use, D= subjected to haemodialysis.*
3) *MEM= meropenem, IMI=imipenem, TOB= tobramycin, MLZ=metronidazole, CTX=cefotaxime*

# E. DISCUSSION

## E.1 STRAIN COLLECTIONS AND METHODS – SOME CONSIDERATIONS

The data presented in **paper 1 and 2** were recovered from isolates collected from eighteen of the 24 diagnostic laboratories in Norway covering > 90 % of the Norwegian population. Selection biases may have been introduced due to the lack of screening guidelines and practices at the time of collection. Consequently the selection of strains was heterogeneous and dependent on whatever methods and indicators the individual laboratory had in place at that time. Prior to the study, 3rd generation cephalosporins including cefotaxime and ceftazidime were regularly used for antimicrobial susceptibility testing of Enterobacteriaceae in systemic isolates. Conscious ESBL screening policies was however not implemented in routine examination of other materials such as urinary tract isolates in most laboratories. Laboratories using automated systems (mostly Vitek2), would nevertheless, receive susceptibility results on 3rd generation cephalosporins even for urinary tract isolates from outpatients.

As a laboratory-based study poor clinical data quality was recovered as illustrated by the lack of information on prior hospitalization abroad. In **paper 3** a major strength was the nationwide participation of clinical laboratories and the ability to provide denominator data and corresponding prevalence rates for different mechanisms of resistance. However, the decisions regarding inclusions and exclusions raised questions of rigorous deliberations. When examining low-level resistances, the accuracy and precision in antimicrobial susceptibility testing (AST) is pivotal. We found that three laboratories (no.5, no.9, and no.22) had to be excluded based on histogram (not displayed) and boxplott-analysis (Figure 16) of their disc test performance. All three laboratories would have entered >20% of their isolates for examination to K-res. The reason for this was multifaceted; first; we used "or-or" based criteria in inclusion (5 substrates) meaning that failing to deliver high quality data on one substrate would be enough for exclusion (Figure 16: low accuracy zone-data from hospital no.5 on CTX); second; low precision data also retrieved an inproportional high number of isolates from individual hospitals (Figure 16: low precision data from hospital no.9 on CAZ and CPD). For the same reasons, it is possible that some laboratories are being slightly underrepresented (candidates may be hospitals no.23 and no. 19).

The "or-or" criteria will however contribute to lessen the significance of the latter. A possible factor contributing to the lack of inter-laboratory calibration could be the use of confluent growth in the ESBL screening procedure in NORM 2004, whereas most laboratories used semi-confluent growth for routine examination (in 2004).

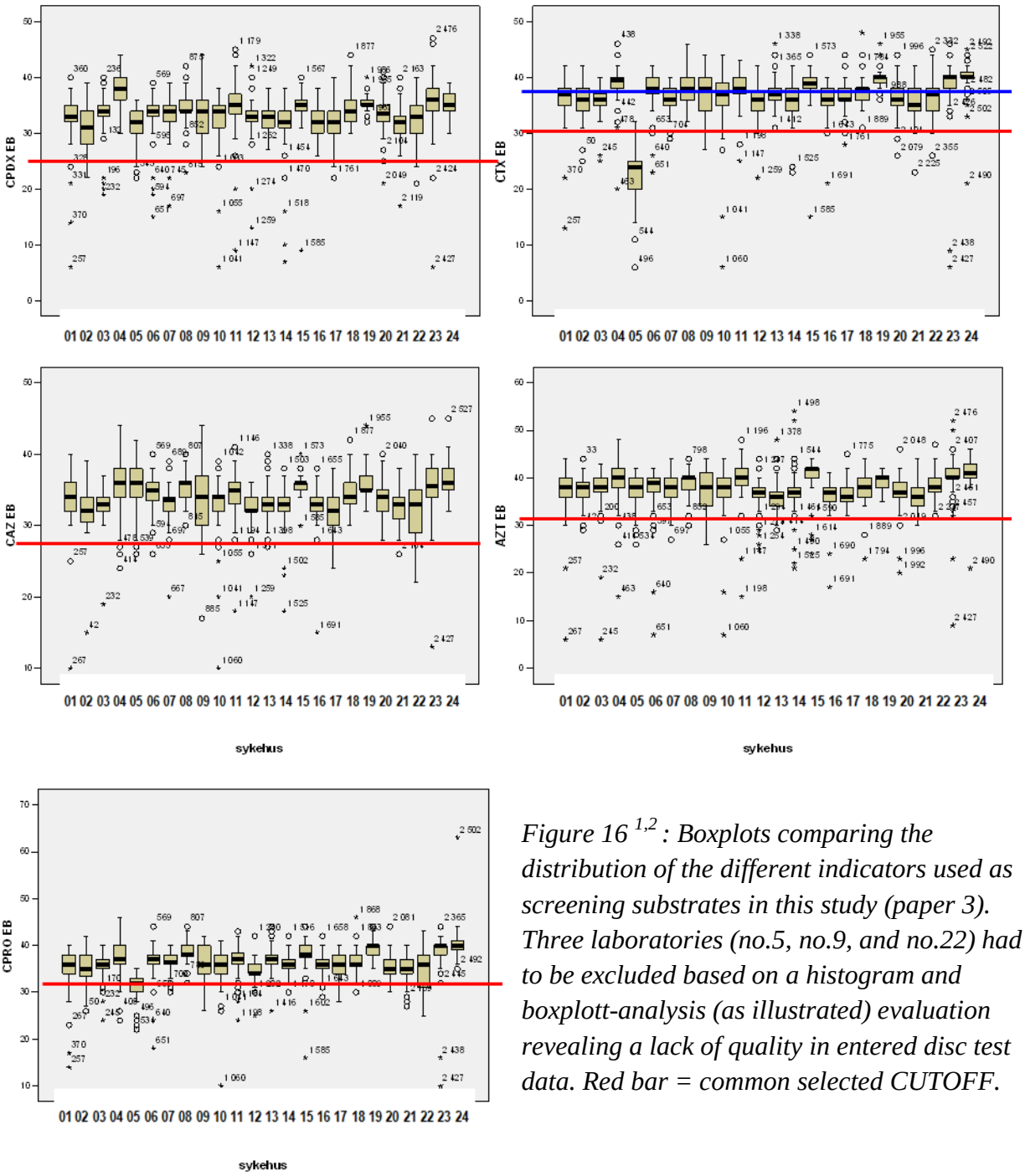



*Figure 16 [1,2] : Boxplots comparing the distribution of the different indicators used as screening substrates in this study (paper 3). Three laboratories (no.5, no.9, and no.22) had to be excluded based on a histogram and boxplott-analysis (as illustrated) evaluation revealing a lack of quality in entered disc test data. Red bar = common selected CUTOFF.*

1) *Abbreviations in this SPSS printout as follows; CTXEB=cefotaxime, CAZEB =ceftazidime, CPOXEB=cefpodoxime, AZTEB= aztreonam, CPROEB= cefpirome. Y-axis; mm sone diameter, X-axis (sykehus= hospitals). Boxes indicate the lower and upper quartiles. The bold lines indicate the medians. Whiskers indicate the m inimum and maximum valus and dots frepresent outliers.*
2) *One laboratory serving two hospitals delivered two separate strain collections no.7 and no.14)*

Several methods for setting exact cut-offs have been suggested including; the "eyeball" method (a.m. Kahlmeter), and various statistical methods such as the normalized resistance interpretation, the iterative statistical method, the 95% rule, and multimodal analysis. However, all of them explore a given strain material as a whole. If the individual laboratories had been their own reference, the accuracy problem due to inter-laboratory variation in zone disc reading would have been eliminated. This was however considered to be an unorthodox solution and was thus abandoned.

An additional bias in collection of urinary tract samples is to be mentioned; in 2004 all laboratories regardless of size sent the same number of isolates to NORM. This collection methodology, favoring the smaller laboratories compared to the bigger ones, has since changed, and collection is now in accordance with the size of each participating laboratory.

Taken together, we found the final representation in our material to be satisfactory.

## E.2 EVALUATION OF MANUAL AGAR-BASED PHENOTYPIC METHODS FOR DETECTION OF ESBL AND AMPC IN CLINICAL E.COLI AND KLEBSIELLA SPP. ISOLATES.

The N-ESBL study **(paper 1)** was the first study to examine ESBLs in Norway. Isolates were collected during 2003 at diagnostic laboratories in Norway using their routine methods of AST to detect potential ESBL-producing *E.coli* or *K.pneumoniae* with the objective to aid in development of national guidelines on appropriate screening and confirmatory testing practices based upon national ESBL epidemiology.

**Screening substrates - appropriate indicators.** In **paper 1** 68/69 ESBL-producing *E.coli* was detected by either cefpodoxime alone or by the combined use of cefotaxime and ceftazidime. Screening by cefotaxime alone also performed well (66/69), however 3 $bla_{SVH}$-ESBLs in *K.pneumoniae* were missed by this approach. Hence, following this study (**paper 1**), we could recommend usage of either cefpodoxime alone or combined usage of cefotaxime and ceftazidime as appropriate screening substrates for ESBLs in Norway.

Cefpodoxime is regarded as the single sole screening indicator cephalosporin for ESBL production, by this "saving" one spot on the agar plate, as it is significantly hydrolyzed by the vast majority of ESBLs [375]. However, it is associated with low specificity and the combined use with ceftriaxone or cefotaxime and ceftazidime offer improved specificity[216,257]. The latter substrates are frequently used in therapy, and accordingly represent an advantage in the early guidance of treatment of inpatients. The RESPECT study (**paper 3**) was designed to yield prevalence data, and only a small number of ESBLs were recovered (n=11). Even with a low number of ESBL enzymes recovered, the results confirmed the findings in the N-ESBL study with the dominance of *E. coli* CTX-M-15 and CTX-M-14 isolates, supporting the recommendations given concerning choices of screening substrate.

**ESBL confirmatory methods -test properties.** Three different commercially available phenotypic methods for confirmation of ESBL production, the CDT method, the DDS test and the ESBL Etest, were evaluated to substantiate the recommendations given in **paper 1**. These tests are convenient for routine use as they instrument independent, flexible, and easy to implement in most laboratories. Test- and diagnostic properties of the ESBL confirmatory tests vary according to the composition of species and enzymes tested.

Some non-ESBL-genotypes presented with positive results in one or more ESBL confirmatory tests:

(i) We observed that two SHV-1 hyperproducing *E. coli* and two SHV-1/11 hyperproducing *K. pneumoniae* mediated increased MIC to ceftazidime and showed CLA synergy, falsely suggesting ESBL production (**paper1**). This phenotype was more likely a source of false positive CAZ ESBL Etest, whereas the CAZ-CDT remained unaffected. A difference in CLA concentration between these tests may be the cause of this discrepancy. Even though most commonly observed in *K.pneumoniae* hyperproducing chromosomally encoded $bla_{\text{SHV-1}}$, false positive confirmatory test may also occur due to the combined effects of a normal or low level SHV-1 or TEM-1- expression and porin changes [249,376,377]. In *E.coli*, a false positive ESBL phenotype has also been associated with increased expression of TEM-1, OXA-1 and OXA-30 with concomitant loss of porins[216,253].

(ii) Three isolates displaying similar single pI of 9.0 (corresponding to ampC) and antibiograms consistent with AmpC-phenotypes (K2-44, K2-62, K2-66) were falsely identified as ESBL producers due to CLA synergy with cefpirome and aztreonam by the DDS method (**paper 1**). These findings highlight the problems of inferring results from visual inspection, as this method lack objective interpretation criteria. We did not repeat the DDS test to observe how disk spacing affected the results in these three isolates.

(iii) One *K.pneumoniae* SHV-28 producing isolate was repeatedly negative by the DDS method even though ceftazidime-MIC was 4 mg/L. Nevertheless, this method was favored due to observed fewer false positive results. It was thus advocated, in addition to the Etest ESBL method, to the clinical laboratories in Norway. In **paper 3,** as in **paper 1,** the results of the CDT and ESBL Etest were compared in *E.coli* (n=35), *K.pneumoniae* (n=11) yielding identical performances with no false positives and no false negatives (unpublished results).

(iv) The test properties of ESBL tests in *K.oxytoca* (n=8) were also evaluated (**paper 3).** The Etest ESBL test assigned false positive test results in seven of eight *K.oxytoca* isolates. On the contrary, the CDT method accurately described all of these isolates as ESBL-negative. The most likely explanation is that the β-lactamase-inhibitor is outnumbered in stoichiometric terms in the ESBL Etest, but not in the CDT. The importance of considering the MIC-profile within a certain species, rather than a single test alone is underlined by this example [378] [378,379]. The antibiogram and IEF-results displaying pIs of [5.3-6.4] consistent with OXY-2 group of enzymes in all isolates (n=8) contradicts the presence of other β-lactamases. In line with our observations, *K.oxytoca* is regarded a frequent source of false positive ESBL phenotypes. Most commonly, this phenomenon is due to hyperproduction of chromosomally encoded OXY-penicillinases in *K.oxytoca.* Hyperproduction is related to promoter mutations upstream of the $bla_{\text{OXY}}$-gene analogous to *ampC* promoter mutations in *E.coli* entailing the classical phenotype; reduced susceptibility or resistance to cefotaxime and aztreonam and most frequently high-level resistance

to piperacillin-tazobactam, but usually full susceptibility to ceftazidime[380]. Less frequently, an ESBL phenotype in *K.oxytoca* may be related to the production of a plasmid-mediated ESBL. An analogous phenomenon may be observed in *P. vulgaris* and *C. koserii* due to inherent classA β-lactamases[381].

In our study, we examined confirmation of ESBL production by phenotypic methods in *K.spp* and *E.coli* species, i.e. species without or (in most cases) only low-level of chromosomal AmpC β-lactamase expression (**paper 1** and **paper 3**) [267,379,382]. Confirmation of ESBL production by phenotypic methods in Enterobacteriaceae producing significant levels of AmpC-cephalosporinases is more challenging. Recent prospective clinical trials adopting species-specific rules and the use of 4$^{th}$ generation cephalosporins (or cloxacillin agar in which the AmpC cephalosporinase is inhibited) have accomplished high sensitivities and specificities in detecting ESBLs also in these species leaving only a small fraction of isolates to be resolved by molecular methods[257].

An AmpC- and/or reduced permeability phenotype could be inferred from the proportion of *E.coli* isolates (n=35) in **paper 1** with significantly increased cefoxitin-MIC. This phenotype was however not further examined in this part of the study. In **paper 3**, an AmpC phenotype was recognized in 20 *E.coli* isolates.

**AmpC confirmatory methods – test properties.** AmpC phenotypes in *E.coli* and *Klebsiella spp.* were defined by positive cefoxitin-boronic acid test a.m. Coudron (**paper 3)**. In *Klebsiella spp,* no AmpC phenotypes were recognized, and neither was it indicated by IEF, or multiplex *ampC*-PCR. However, in *E.coli,* 20 of 35 isolates displayed an AmpC phenotype. An AmpC phenotype in *E.coli* may be attributed to endogenous or plasmid mediated AmpC hyperproduction. No phenotypic test can reliably distinguish between the two modes of AmpC hyperproduction[134]. Chromosomally encoded AmpC hyperproduction in *E.coli* was supported by a visible IEF band corresponding to AmpC with pIs of [8.8-9.2] and a negative multiplex $bla_{ampC}$-PCR in this study (**paper 3**). Chromosomally encoded AmpC hyperproduction in *E.coli* results from mutational or, less common, insertional changes in the promoter or attenuator regions of *ampC* resulting in a 8-280-fold increase in *ampC* expression by qRT-PCR in accordance with the result obtained in this study, except for in one isolate in which there was only a 5-fold increase[132]. Nevertheless, an AmpC phenotype was strongly inferred in this isolate by the clearly visible pI band of 9.0, cefpodoxime MIC = 256 mg/l and cefotaxime- and ceftazidime-MIC = 8 mg/L (**paper 3**).

Two additional confirmatory tests, one *in house* biological assay using EDTA as permealizing agent releasing β-lactamase from test strain colonies (the cefoxitin-EDTA test a.m. Black) and one commercially available inhibitor (cloxacillin)-based test (AmpC Etest), were evaluated.

Performance among putative AmpC producers in this study (**paper 3,** unpublished results, Table 7) was quite similar in the cefoxitin-boronic acid test a.m. Coudron and the AmpC Etest. Fewer positive isolates were recovered by the cefoxitin-EDTA disk test a.m. Black. Possibly this is due to the subjective reading of flattening of inhibition zones, which may be difficult to recognize in "weak positive" isolates. The cefoxitin-EDTA disk test a.m. Black has, however, been recommended and displayed superior performance to inhibitor-based test in a proportionally large study comparing different methods of AmpC-detection[280].

Still, there is no broad consensus concerning AmpC detection in Enterobacteriaceae and this clearly represents an obstacle in defining the global epidemiology[280]. The cefoxitin-EDTA disk test a.m. Black would be considered too cumbersome for use in most laboratories. Older proposed non-inhibitory based methods such as the three dimensional enzyme extraction tests and the cefoxitin-agar-based-test (CAM-assay) would fit the same category[383,384]. Another enzyme-based test termed the cefoxitin-Hodge test, a modified three-dimensional test, has been proposed more recently [274].The guidelines recently recommended by EUCAST may subsequently aid this situation. The use of inhibitor based methods has been forwarded by this organization, and cloxacillin is preferred for inhibition in putative AmpC hyperproduction due to its improved selectivity to AmpC producing enzymes compared to boronic acid that also inhibit class A carbapenemases [57]. This situation did not apply in our study as all isolates remained fully susceptible to carbapenems, and accordingly the possibility of AmpC hyperproduction combined with impermeability causing non-susceptibility to carbapenems were less likely. Even so, evaluations of different formats and inhibitors are still generally sparse and discordant[275,276,280,385,386].

**Negative ESBL/AmpC confirmatory tests**. A neither-ESBL-nor-AmpC phenotype was seen in 0.7 % (15/2293) of the isolates (7 *E.coli* and 8 *K.pneumoniae*) in this study (**paper 3**). A low leveled MIC elevation to ESC was observed in all of these isolates. In *E.coli*, a partial explanation could be strain selection close to the cefpodoxime ECOFF allowing occasional wild type isolates to be included. Three of the seven isolates were selected solely because of their cefpodoxime MIC. In the other isolates, low-level resistances (MIC≤ 2mg/L) to one or more antibiotics (cefotaxime, ceftazidime, cefpirome or aztreonam) were encountered. Except for varying degrees of visible AmpC bands on IEF in four of seven isolates, broad spectrum β-lactamases (including TEM-1/2, SHV-1 or OXA-1) were not indicated by IEF. Structural aspects of porins in *E.coli* were explored by PCR and sequencing. No homogenous results were retrieved to explain the observed phenotypes. Porins were not specifically examined by SDS-PAGE or real time RT-PCR for quantification of mRNA encoding specific porins in order to explore porin function from a (semi)-quantitative perspective [113]. However, in order for porin deficient isolates to display significant resistance to 3rd generation cephalosporins a concomitantly produced β-lactamase generally has to be present[214,216,221,237]. Even if positive boronic acid tests were

negative, cAmpC hyperproduction cannot be ruled out in these *E.coli* isolates. In *K.pneumoniae*, 6 of the 8 isolates were included solely due to reduced susceptibility to ceftazidime (MIC 2 mg/L). This pattern is consistent with the presence of the intrinsic *K.pneumoniae* SHV-1 coupled to altered permeability[112,249,377].

## E.3 THE EPIDEMIOLOGY OF MECHANISMS MEDIATING REDUCED SUSCEPTIBILITY TO 3$^{RD}$ GENERATION CEPHALOSPORINS IN *E.COLI* AND *KLEBSIELLA SPP.* IN NORWAY.

The bacterial strains in this study (**paper 1, 2**, and **3**) were used to examine methods of detection and the epidemiology in ESBL-producing *E.coli* and *K.pneumoniae* collected at a stage (2003 and 2004) where the prevalence rates of ESBLs in *E.coli* and *Klebsiella spp*. were still at a very low level in Norway. In **paper 1** no denominator data and accordingly no prevalence data could be given. However, by using a "wide catch" strategy, we were able to get a good picture of the panorama and relative proportions of ESBL-genes present in a low prevalent setting. This may otherwise be challenging in a low prevalent situation having to address many non-ESBL isolates. In **paper 3**, taking advantage of the NORM surveillance system, we were able to build a large denominator database and wanted to explore the prevalence of different mechanisms mediating reduced susceptibility to 3$^{rd}$ generation cephalosporins. In a situation where prevalence to ESBLs was low, we assumed that other mechanisms of resistance mediating low or high-level resistance to 3$^{rd}$ generation cephalosporins could greatly affect detection strategies. In subsequent years the prevalence of ESBLs in Norway has increased significantly. The non-ESBL mechanisms conferring reduced susceptibilities to ESCs that are encountered in this study presumably remain at fairly steady-state low levels with regard to overall prevalence (**paper 3**). Consequently, the positive predictive value (PPV) of ESBL testing is improving**.**

**Prevalence of non-ESBL mechanisms in Norway:**

***E.coli hyperproducing chromosomally encoded AmpC.*** To our knowledge, our study is the first nationwide study reporting the prevalence of *E.coli* hyperproducing chromosomally encoded AmpC from a representative national collection of strains displaying an AmpC phenotype (**paper 3**). Our prevalence rate (1.0%) is in line with those observation recorded from local and hospital surveys from different parts of the world in the 1980s and 1990s [120,241,387,388]. In a recent study, a somewhat lower prevalence rate was reported from a single Danish hospital survey (0.4%)[387]. In a recent report from a nationwide laboratory-based study from the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res), the prevalence of *E.coli* AmpC phenotypes not attributed to plasmid mediated AmpC enzymes was 0.3% during the years 2010-12. Of note, only multi-drug resistant isolates (defined as resistance to 2 of 3 of the following classes of antibiotics; aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole) were selected in this study, probably underestimating the prevalence of putative ampC hyperproducers by chromosomally encoded AmpC [385,386].

The occurrence of the AmpC phenotype attributed to the emergence of chromosomally encoded hyperproducing *ampC* mutants has been related to the selection of sporadic *ampC* gene promoter mutations during antibiotic therapy *de novo* in the individual patient [240,241]. However reports on large nosocomial outbreaks of AmpC-producing *E. coli* are few in numbers, suggesting that this phenotype has a weak epidemic potential [241]. In a large Canadian study (29,323 *E.coli* isolates tested) from hospitalized patients in twelve different hospitals 232 *E.coli* isolates displaying MICs ≥32 mg/L were examined by *ampC* promoter region sequencing and subtyped by PFGE, whereas MLST was not performed. A variety of PFGE-fingerprints and promoter regions suggested spontaneous emergence of mutation in sensitive strains. Intra- and interhospital dissemination were evaluated by PFGE demonstrating a limited potential for nosocomial dissemination of this phenotype with small outbreaks of 2-4 isolates recovered some places, and a larger outbreak recorded at one site (17 isolates). Interhospital dissemination was detected on two occasions[241]. The 20 hyperproducing cAmpC *E.coli* strains detected in this study (**paper 3**) were in accordance with this observation in the sense that isolates were widely distributed and recovered from 13 of 20 included hospitals. Ongoing outbreaks could not be documented; however, conclusions that are more firm are impossible to make, as epidemiological typing was not performed. Keeping in mind that, two studies of from the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res) have indicated that clonal outbreaks by *E.coli* hyperproducing ampC indeed may occur. In these studies epidemiological typing were performed by PFGE and MLST, and suggested a regional clonal outbreak (Bergen area) of ST131 *E.coli* hyperproducing ampC due to the insertion of an IS911-element into the promoter region of the $bla_{\text{ampC}}$ gene creating a stronger promoter. Thus, even if this phenotype may in general have a weak nosocomial potential and for the greater part be attributed to *de novo* occurrence following antibiotic exposure, clonal outbreaks seem to take place (even in our country)[389,390]. Corvec et al. applied MLST to investigate the origin and dissemination of clinical isolates hyperproducing chromosomally encoded *ampC* in 12 clinical isolates collected over a long period of time (12 years) from a single university hospital in France as well as 12 isolates of animal origin. In all (12/12) human isolates and 6/12 animal isolates a particular MLST (ST23) and pattern of *ampC* promoter polymorphism in positions ( -88, -82, -42, -18, -1, +58) compared to the *E.coli* control (*E.coli* K12) were detected. Only 1 of 10 cephalosporin susceptible control isolates belonged to this ST and displayed this pattern of mutations. Fourteen different PFGE fingerprints were displayed in 14 of 14 typeable strains belonging to ST23. This study inferred a common prevalent ST type among AmpC-phenotype human and animal isolates[391]. Furthermore, it suggests that mutant ST23 is prone to be selected in the human gut under antibiotic pressure. Thus, the fitness cost of these mutations emerging during antibiotic therapy in the ST23 lineage would be of particular interest[391,392].

Reduced susceptibility to extended spectrum-cephalosporins due to hyperproduction of chromosomal AmpC β-lactamase alone in *E. coli* is mostly moderate. Additional non-enzymatic mechanisms of resistance, such as loss or change in outer membrane proteins, could be suspected

in isolates with MICs >4 mg/L to 3rd generation cephalosporins[120,217,393]. Alternatively, a non-wild type AmpC-enzyme capable of hydrolyzing 3rd generation cephalosporins (preferably ceftazidime) have uncommonly been reported[393]. In this study (**paper 3**), 25 % of the *E.coli* isolates (5/20) with an AmpC phenotype exerted relatively high MIC levels to ESC (cefpodoxime ≥128 mg/L, cefotaxime ≥4 mg/L, and ceftazidime ≥8 mg/L). These findings suggest an additional mechanism of resistance in these isolates, although this was not further explored in our study[217,393].

No plasmid mediated *ampC* isolates were recovered among 2293 isolates investigated in this study (**paper 3**), but sporadic occurrence of *bla*$_{CMY-2}$ has been described in a previous Norwegian study (in strains collected 2003-07)[134,394]. *Bla*$_{CMY-2}$ has been detected in Norwegian broilers at alarming rates[183], but current pieces of evidence suggest that resistance in clinical isolates from humans is still low in Norway [387]. In a recent report from a nationwide laboratory-based study from the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res), the prevalence of plasmid mediated ampC in *E.coli* was ≤ 0.2% during the years 2010-12 [385]. A recent publication examining strain collection recovered during point prevalence surveys in Sweden during 2007-11 stated that the prevalence of *E.coli* ESBL and plasmid-mediated AmpC (combined) was 4% in a mixed collection of urinary tract isolates and blood culture isolates in 2001. During the period the relative contribution of pAmpCs to the total burden of ESBLs and pAmpC was at a constant level of ~5%. Thus, the prevalence of pAmpC (~0.2%) is in line with observations from our country [395].

***K.oxytoca hyperproducing chromosomally encoded OXY-2 β-lactamases.*** The prevalence of OXY-hyperproducing *K.oxytoca* (13.6%) was documented for the first time in Norway in this study **(paper 3**). *K.oxytoca* hyperproducers are prone to selection during cephalosporin therapy[396]. The prevalence in our study corresponds well to results from other studies typically ranging from 10-20% varying in different studies depending on the population[131]. Nosocomial outbreaks of hyperproducing OXY-type have been reported[131]. In this study (**paper 3**) all the blood culture *K.oxytoca* isolates (n=8) were strains from patients from different hospitals, but molecular typing was not undertaken.

Six different *bla*$_{OXY}$-subtypes have presently been reported, with *bla*$_{OXY-1}$ and *bla*$_{OXY-2}$ being the most prevalent ones[380]. All of the *K.oxytoca* isolates in this study displayed band of enzymatic activity with pIs corresponding to β-lactamases belonging to the OXY-2- group of enzymes[397]. Rare cases of *bla*$_{OXY-2}$ variants have been detected which display reduced susceptibility to ceftazidime making them phenotypically indistinguishable from ESBLs[398]. A *bla*$_{OXY-1}$ -carrying plasmid has recently been recovered in *K. pneumoniae* in Spain[399].

Prevalence and magnitude of resistance to ESCs vary considerably between stably derepressed *Enterobacter spp.* mutants, *K.oxytoca* hyperproducing *bla*$_{OXY}$-genes and *E. coli* hyperproducing *ampC*-genes. According to the NORM 2008 report >30% of blood culture isolates of

*Enterobacter spp.* confer resistance to ESCs in Norway, whereas findings of this study document resistance to ESCs in 13.6% of *K.oxytoca* and 1.0% of *E.coli* due to OXY- and AmpC hyperproduction, respectively[400]. Stably derepressed *Enterobacter spp*. mutants are more easily selected during ESC therapy than *K.oxytoca* and *E.coli* mutants due to different genetic organizations in their regulation of $bla_{ampC}$ expression[401]. Further, treatment failure is documented in up to 20-30% of *Enterobacter spp.* bacteremias when treated with 3rd generation cephalosporins[402,403]. Still, large differences exist between the frequency of resistance to *E.coli* mutants and *K.oxytoca* mutants. This could be attributed to difference in acquisition of mutations (rates and patterns of mutations), the ease of selection during antibiotic therapy (favorable MLST backgrounds associated with limited fitness loss), and the propensity of *Klebsiella spp*. to cause nosocomial outbreaks[131].

**Prevalence and epidemiology of emerging ESBLs/CTX-M-producing *E.coli* in Norway:**

In retrospect, the NESBL study probably documented the emergence of *E.coli* CTX-Ms in Norway (**paper 1**)**.** To study emerging resistance offers advantages as a close-up view may be more readily accessible. However, the patterns dominating the emergence of resistance may not be maintained as the epidemiology evolve[404]. In an evolving complex epidemiological situation, while it may be easier to quantify resistance mechanisms tracing resistance determinants and their causes and effects may become more difficult to examine (perhaps requiring more sophisticated and high-resolution molecular tools such as WGS).

The epidemiology in *E.coli*-producing CTX-M is complex. From the start [405-407] the spread of mobile genetic elements (tranposons and/or plasmids, including the "epidemic" plasmids) were recognized as prominent vectors that gave rise to a panorama of fingerprints by epidemiological strain typing [160]. Superimposed with dissemination of mobile genetic elements between strains are translocations within different mobile genetic elements of $bla_{CTX\text{-}M}$[161]. Subsequently, multi-drug resistant successful clones were identified by evolutionary oriented typing (i.e. MLST) and recognized as additional important vectors for these genes by their enhanced ability (i) to expand vertical *per se*, (ii) act as donors in horizontal spread to other strains, and (iii) to acquire similar resistance genes at several occasions and also flexibility to acquire different resistance genes [65,104,407-409].

***BlaCTX-M, associated plasmids and genetic surroundings:*** This study confirmed the nationwide distribution of ESBLs and the predominance of the CTX-M group of enzymes (**paper 1, 2 and 3**). Among the ESBL–enzymes recovered in isolates collected in **paper 1** and **paper 3,** by its relative contribution, 65% (52/80) were CTX-Ms *E.coli*, 5% (4/80) were CTX-Ms in

*K.pneumoniae*, 19% (15/80) were SHV-ESBLs in *K.pneumoniae*, whereas the rest (9/80; 11%) were less frequent species/*bla*-gene combinations. As the NESBL study (**paper 1**) probably hit and documented the emergence of *E.coli* CTX-Ms in Norway, accordingly, **paper 2** outlined the situation with regards to the multilevel molecular epidemiology in CTX-M-producing *E.coli* at its arrival in Norway as these enzymes "went global" and reached our country [67]. The predominance of $bla_{\text{CTX-M}}$ is in our study was in accordance with international studies conducted at that time documenting the emergence of CTX-M producing *E. coli* worldwide, including studies from the Nordic countries on strain materials collected close in time to our study [410-415]. Throughout the years 2000-2005 continuous low prevalence rates (<1.0 %) of *E. coli* and *K. pneumoniae* isolates conferring resistance to 3rd generation cephalosporins were reported in blood culture isolates in Norwegian surveillance reports (NORM). Prevalence rates of ESBLs in *E. coli* urinary tract isolates also remained low <1 % from 2004 (first year tested) through 2007. Then it escalated…

$bla_{\text{CTX-M}}$ genogroup 1 (mainly $bla_{\text{CTX-M-15}}$) dominated among the $bla_{\text{ESBL}}$ genes recovered in *E.coli* in this study (n=33), with $bla_{\text{CTX-M}}$ genogroup-9 (mainly $bla_{\text{CTX-M-14}}$) as the second largest group (n=18)) in this study. The comparatively large contribution of $bla_{\text{CTX-M-14}}$ may reflect that this enzyme was dominating in regions that were (and are) popular travel destinations in our country such as South-East-Asia and Spain. Imported cases or subsequent dissemination in our country of this enzyme may therefore be reflected in this comparatively high number. $Bla_{\text{CTX-M-15}}$ was first detected in *E. coli* from India in 1999, and within a decade it has become the most widely distributed ESBL worldwide[67,146,416]. Of note, this enzyme was already dominating in our strain collections only a few years later (from 2003 and 2004, respectively) (**paper 1** and **paper 3**).

Specific $bla_{\text{CTX-Ms}}$ are often associated with certain plasmid replicon types, i.e. $bla_{\text{CTX-M-15}}$ is strongly associated with replicon IncFII plasmids in most parts of the world [73,157]. IncF plasmids are narrow host-range plasmids well adapted to Enterobacteriaceae, *E.coli* in particular. They are prone to rearrange, and frequently observed as multireplicon plasmids[157,417]. Furthermore, among IncFII plasmids carrying $bla_{\text{CTX-M-15}}$, addiction systems have frequently been observed [157]. It seems that IncFII plasmids were abundantly distributed within clinical isolates of Enterobacteriaceae even prior to resistance gene capture and the IncF family of plasmids are commonly found in naturally occurring fecal flora of humans both with and without resistance genes [73,418]. Other epidemic replicon type plasmids IncA/C, IncL/M, and IncI1), in contrast to IncF, are tightly linked to positive selection pressure exerted by antimicrobial use, and thus seemingly less equipped to compete outside the hospital environment[73]. In this context, it is interesting to observe the abundance of IncF plasmids in these isolates in our country (**paper 2**). The reason for this remains a matter of speculation, but one might assume that IncF plasmids is favored compared to other replicon-type-plasmids due to a comparatively modest consumption of antibiotics in our country Accordingly, in this study (**paper 2**) we observed the common

observation of IncF plasmids linked to *bla*$_{CTX-M-15}$, but also in more infrequent associations such as *bla*$_{CTX-M-14}$. *bla*$_{CTX-M-14}$ is most commonly associated with IncI and IncK plasmids, however in this study this enzyme were commonly linked to IncF plasmids (7/11).

In Europe, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-14}$ and *bla*$_{CMY-2}$ are presently the genes most commonly associated with 3$^{rd}$ generation cephalosporin resistance in *E.coli* from animals, whereas *bla*$_{CTX-M-15}$ has infrequently been encountered in this context. Further, IncN plasmid has been associated with spread of *bla*$_{CTX-M-1}$ in Europe[174,175]. *E.coli* CTX-M-1 were detected in 3/45 = 7% of the isolates in this study **(paper 2**), all of them on IncN plasmids. Two isolates were detected within the same hospital with identical PFGE profiles which allows us to suggest two probable modes of acquisition of infection (i) a common source through contaminated food (within or outside the hospital), or (ii) human-to-human transmission within the hospital.

***Co-resistance*:** Co-selection processes may attribute to the success of particular ESBL-enzymes [67,162]. A multi-drug resistant (MDR) phenotype (defined as resistance to two of 3 of the following classes of antibiotics; aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole) was observed in 49% (39/80) of the ESBL isolates in this study **(paper 1** and **paper 3**). A MDR-phenotype was significantly more common in *bla*$_{CTX-M}$ than in non-*bla*$_{CTX-M}$ ESBLs, 64% (36/56) and 13% (3/24), respectively. Ciprofloxacin resistance was displayed in 81% (22/27) of the MDR *E.coli* isolates. Furthermore, in **paper 2** co-resistances in *bla*$_{CTX-M}$ *E.coli* (n=45) were related to plasmid – and strain typing results. 21 of 23 *bla*$_{CTX-M-15}$ isolates displayed an MDR-phenotype, and ciprofloxacin resistance were displayed in all eight ST131 carrying *bla*$_{CTX-M-15}$ on IncFII plasmids. The specific mechanism of resistance to ciprofloxacin was not examined in this study (**paper 2**). Chromosomally located gyrA/parC mutations or *aac(6')-Ib-cr* mediated on plasmids represent likely options. Co-transfer of *bla*$_{CTX-M-15}$ positive plasmids did not link to ciprofloxacin resistance (not co-transferred) in representatives from ST131 (C2 and C3). None of the *E.coli* *bla*$_{CTX-M-1}$ harbored on IncN plasmids (n=3) that has been linked to animal reservoirs displayed resistance to any of the three antibiotics.
The propensity of different *E.coli* STs to carry R-plasmids seems to vary [419]. Co-resistances in *bla*$_{CTX-M}$ *E.coli* (n=45) were particularly prominent in globally successful MDR-STs such as ST131 (n=9) and CC405 (i.e. the SLV ST964) (n=7) displaying MDR in the most isolates (15/16) (Figure 12). The 15 MDR-isolates were all *bla*$_{CTX-M-15}$, whereas the single non-MDR isolate was *bla*$_{CTX-M-1}$. Of note, the exact plasmid location of plasmids of non-β-lactam-antibiotics was not explored.

***Singletons isolates:*** The epidemiology of CTX-M producing *E. coli* included both singletons and clustered isolates of various numbers (**paper 2**). A significant number (23/45) of isolates were single PFGE-types, and may indicate multiple separate import cases. International travel and migration has been suspected as a major factor contributing to the emergence and dissemination of CTX-M enzymes and travel history in patients is thus crucial in order to comprehend the importance of import due to human travel between countries and continents

relative to other factors contributing to the acquirement of ESBLs in a particular region or county [67]. Unfortunately, such information was insufficient in this study (**paper 1**) and multiple alternative routes may be involved. These include within-country/local acquisition by human-to-human transmission (in or outside health institutions), food, or another locally acquired source. All these routes and sources has to be considered and may confer the dissemination of resistance genes in *E.coli* [420] with PFGE-clustered isolates such as those within the ST964 clone perhaps representing local outbreaks and/or transmissions [153,413].

ST131 and CC/ST405 (by its SLV ST964) are two acknowledged successful MDR high-risk clones that were recognized in this study (**paper 2**).

***ST131:*** ST131 *E.coli* was observed in 20% (9/45) of the isolates investigated (**paper 2**). Accordingly, this study confirmed the presence of this globally successful clone carrying $bla_{\text{CTX-M-15}}$ at an early stage in Norway [65]. The ST131 *E.coli* isolates were mainly carrying $bla_{\text{CTX-M-15}}$ on a multireplicon FII-type plasmids (and FIA or FIB, co-located) (n=8). In addition, it was carrying $bla_{\text{CTX-M-1}}$ on an IncN plasmid in a single strain. ST131 isolates may exhibit different PFGE patterns, although in the majority of cases with more than 80% similarity (corresponding to differences of 4 to 6 bands) [421]. This is in accordance with observations in this study, in which all ST131 isolates except one displayed similarities by PFGE >80% (**paper 2)**. Furthermore, in two laboratories (Haukeland UH and Ullevål UH) ST131 isolates were detected that formed distinct PFGE clusters (XI and XIII) in two and four patients, respectively, inferring a likely local spread. However, no links by conventional epidemiological tracing was performed to substantiate this. The dominant position of ST131 *E. coli* in the dissemination of $bla_{\text{CTX-M,}}$ in human clinical isolates has been demonstrated worldwide, although it is best documented through surveys in Europe, North-America, Japan and Korea [109,394,421-423]. The epidemiology of ST131 in Europe may be heterogeneous with regards to the proportion of isolates belonging to the ST131 clone [104,421,424,425]. The prevalence of this clone in *E.coli* has not been examined in more recent nationwide materials in Norway. The ST131 *E. coli* clone appears adapted and prone to acquire various resistant traits carried by different, (yet similar) plasmids circulation locally[65,121,421]. Of particular note in this context is the fact that the IncFII plasmids devoid of resistance traits were widely distributed in Enterobacteriaceae, and thus well adapted to this family even before the use of antimicrobials[67,418]. Besides being strongly associated with CTX-M-15 encoding IncF plasmids in strains collected both from in- and outpatients as observed in this study (**paper 2**), *E. coli* ST131 has also been linked [408,409,426] to other *bla*-genes such as CMY-2, SHV-12, CTX-M-14 (as in this study (**paper 2**)), and NDM-1 although not as tightly as to $bla_{\text{CTX-M-15}}$ [157].

*ST131 – the role of linked ciprofloxacin resistance and virulence.* The success of ST131 is not entirely understood; however a combination of resistance determinants, virulence factors, enhanced transmission and colonization rates for at least a proportion of strains within this clone may all be contributors to the overall success [427,428]. Ciprofloxacin resistance was identified in all

ST131 isolates except the single isolate harboring *bla*$_{CTX-M-1}$ (i.e. all ST131 carrying *bla*$_{CTX-M-15}$) in this study (as discussed above) (**paper 2**). Accordingly, fluoroquinolone resistance seems a very important trait for the success of this clone. Johnson et al. studying US isolates found that a *fimH30+* "subclone" of the ST131 clone carrying *bla*$_{CTX-M-15}$ and specific (signature) gyrA/parC-resistance traits, constitutes the majority of *bla*$_{CTX-M-15}$ isolates, and suggests that overall clonal spread and expansion rather than plasmid transfer is "driving" the *bla*$_{CTX-M-15}$/ST131 epidemic. Besides this clonal pattern of dissemination ST131 *E.coli* isolates are prone to acquire IncFII plasmids carrying *bla*$_{CTX-M-15}$ by numerous occasions[109,165]. This was also argued by Olesen when examining ESBL-producing *E.coli* from the Copenhagen area, and in a recent Italian study of ST131 *E.coli* causing urinary tract infections or sepsis from 2012, 2009 and 2006 revealing the predominance of *fimH30+* in these isolates [429,430].

Further clues to the success of ST131 have been examined. Possible advantages in the metabolism of this clone have been suggested[427,428]. Specific combinations of virulence factors rather than the number of such factors probably are important in a proportion of ST131 isolates [431]. Results regarding virulence are however conflicting as ST131 overall does not seem to be particularly virulent in animal models[109]. Thus, the virulence potential of ST131 has been focused in several reports indicating discordant result concerning adhesion, but enhanced potential for invasion of human uroepithelium and for subverting host defense mechanisms by the production of biofilm [408,421,432,433]. This notion could possibly also contribute to endured persistence in different environments and to enhanced resistance to antimicrobials *in vivo* [408]. Nevertheless, in concordance with observations made by others the ST131 isolates in this study (**paper 2**) belonged to serotype O25b:H4, phylogenetic group B2, which identifies an uropathogenic lineage. ST131 *E.coli* is unusual in the sense that resistance in general is usually far more common in other phylogenetic lineages (A, B1, and D) than lineage B2 [421].

***ST405 (SLV964):*** ST405 has been identified as a second clone important in promoting global dissemination of *bla*$_{CTX-M}$- enzymes in *E. coli*[426]. A novel single locus variant (SLV) of this clone, the ST954, was observed in a major PFGE cluster (C6). The seven isolates displayed identical phylogroup, serotype and carried *bla*$_{CTX}$ on multireplicon FII-plasmids. Two geographical areas (Stavanger and Oslo) were involved suggesting local spread. Furthermore, the same strain, was recovered in several isolates facilitating a nosocomial outbreak at a University Hospital in Stavanger in 2004, one year post collection of the isolates in this study (2003) (**paper1** and **paper2**). Again, these observations illustrate the potential of spread of these bacteria in hospital settings in Norway[212].

Of note, all ST131 and ST964 isolates detected in our study carried *bla*$_{CTX-M-15}$ on multireplicon FII plasmids known to remain stable in the host also without the presence of antibiotics. As such, these isolates expressed an epidemic potential from "both worlds"[73].

***2007- and onwards: Increasing prevalence and further CTX-M-15/CTX-M-9 shift in E.coli:*** Currently, molecular *bla*$_{ESBL}$-typing is performed annually on all Norwegian ESBL-producing *E.coli* isolates collected within the Norwegian surveillance programme for antimicrobial resistance in human pathogens (NORM). In recent years (>2007), a slight but significant rise in prevalence rates has been recorded in both blood and urinary tract *E.coli* isolates, mainly attributed to an increase of CTX-M-15 and CTX-M-9 enzymes (Figure 17).

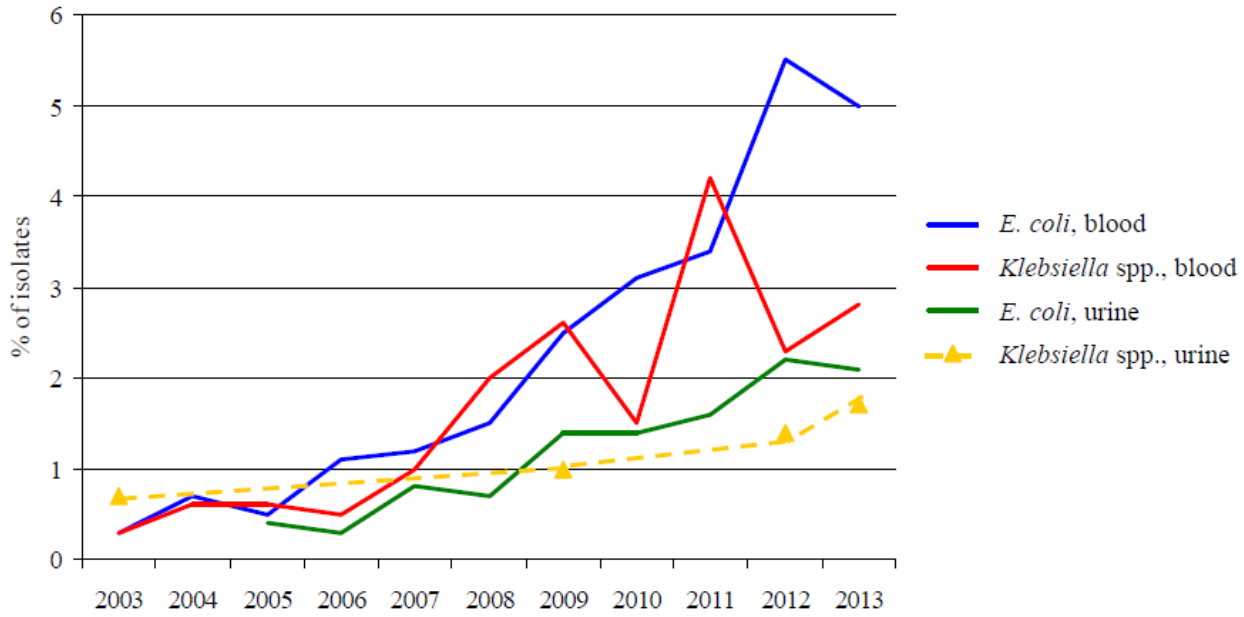



*Figure 17. Prevalence rates of ESBLs in E.coli and K.pneumoniae throughout the years 2003-12 [434]. NORM 2013. A logarithmic rise has been recognized in recent years.*

A trend, although not a significant shift, towards genogroup 1 relative to genogroup 9, is observed when comparing aggregated data of *E.coli bla*$_{CTX-M}$ genogroup1 isolates (n=33) to genogroup 9 isolates (n=18) in the N-ESBL study (2003) (**paper 1**) and the RESPECT-study (2004) (**paper 3**) to more recent data in NORM [aggregated data from 2011-13; *bla*$_{CTX-M}$ genogroup1 (n=135), *bla*$_{CTX-M}$ genogroup 9 (n=48)] (p=0.20, 33/135 vs. 18/48). As the prevalence of ST131 in CTX-M-producing *E.coli* has not been examined in later nationwide strain materials in Norway, the contribution of this clone (or other high-risk MDR clones) to the observed shift from *bla*$_{CTX}$-genogroup 1 isolates relative to *bla*$_{CTX}$-genogroup 9 isolates is thus uncertain. The observed data is in line with a recent regional study from the southwestern part of Sweden. In this investigation, the observed shift in genotypes when examining isolates from 2003-04 and 2008-09, respectively, could only partly be attributed to an increase of the ST131 clone[413]. In another recent nationwide study, evaluating point prevalence cohorts from 2007-2009-2011 in Sweden *bla*$_{CTX-M-15}$ predominated (54-58%) among *E.coli* isolates and ST131 represented 34-38% of the isolates. Distribution of CTX-M types and ST types remained stable

throughout the period in this investigation [395]. In a recently published survey from Denmark investigating isolates collected from the Copenhagen area in 2008-09, the *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M--14}}$ genotypes comprised 52% (60/115) and 19% (22/115) of the total distribution of ESBL genotypes, respectively. Further, this study revealed a positive association between *bla*$_{\text{CTX-M-15}}$ and ST131 (38%), and a negative association between ST131 and *bla*$_{\text{CTX-M-14}}$[429]. Examination of current trends in the *E.coli* CTX-M epidemic in Norway is enabled by the NORM surveillance system.

## E.4 EPIDEMIOLOGY OF EMERGING CARBAPENEMASES – IMPORTED KPC-PRODUCING *K.PNEUMONIAE* FACILIATING A NOSOCOMIAL OUTBREAK.

Nosocomial outbreaks of carbapenemases represent another aspect of the epidemiology of the newer β-lactamases in Norway that is highlighted in this study (**paper4**).

**Defining outbreaks in very low-prevalent settings –when to react?** A number of countries have reported several or occasional hospital outbreaks, or sporadic cases of KPC-producing *K. pneumonia.* In low-prevalent countries, nosocomial outbreaks are most commonly associated with import from countries with higher prevalence in hospitals and LTCFs. This underlines the potential for local spread of these bacteria in the nosocomial setting in low-prevalent countries [20,58,172,199,200,435]. This study (**paper 4**) include the first report from the Nordic countries describing a nosocomial outbreak of KPC-producing Enterobacteriaceae, and the index patient had previously been reported as the first patient in Norway to be identified with a KPC-producing Enterobacteriaceae (November 2007) [436]. The second patient to be identified in the outbreak was hospitalized at a different hospital within the same hospital enterprise. The epidemiological link that existed between patient 1 and patient 2 was overlooked at the time of hospitalization, and an outbreak investigation was not elicited. Of note, the separate investigation of patient 2 did not reveal links to foreign travel or abroad hospitalization in recent years. Patient 3-5 were identified within a 6-month interval (October 2008 and March 2009). During this time, efforts to clarify connections between the KPC-producers were ongoing, and the link of patients 1, 3-5 to the same ICU prior to diagnosis was established. PFGE was undertaken for the first time in February 2009 including patients 1-4 (isolates described as indistinguishable or closely related in this PFGE setup, unpublished result). However, declaration of an outbreak was not defined until patient 6 was detected in May 2010. Subsequently, active surveillance culturing was initiated. Thus, during the long time interval of >2.5 years, 6 *K.pneumoniae* isolates with a unique resistance genotype in the current epidemiological context were recovered, with only the index case having a recent travel history and hospitalization in a high-endemic country. In retrospect, important lessons should be learned from this development; 1) Considering the abilities of *K.pneumoniae* to disseminate within the hospital environments and the uniqueness of this resistance determinant in Norway, caution favor an outbreak investigation even after detection of a single case. 2) As the link between case 1 and 2 was missed, they were initially regarded as separate cases, and there was a lack of record of recent travel or hospitalization abroad for patient 2. Even if that was or might have been the case, it should nevertheless have favored an outbreak investigation subsequent to patient 1 identification. 3) No excuse will suffice for the delay in implementation of outbreak investigation after identifying cases 3-5 within a 6-month period.

The identified KPC-producers were located at 12 different wards in three hospitals, demonstrating the risk of dissemination associated with these patients when unnoticed.

**Risk factors and outcome:** Several risk factors common to nosocomial infections in general and to nosocomial infections with carbapenemase-producing *K.pneumoniae* in particular were observed in 5 of the 6 of the clinical patients in our outbreak (**paper 4**) [321,437-442]. Risk factors included prolonged hospital stay (>30 days) (i.e. 36-178 days in surviving patients, mean 69 days), severe illness, admission to intensive care units, antibiotic use (all our patients received meropenem prior to diagnosis) and exposure to invasive devices. All of the patients received artificial ventilation when in the ICU, and gastrointestinal surgery was undertaken in two patients. The antibiotics most favored to represent a risk factor seem to be the carbapenems, metronidazole and ciprofloxacin[443]. Ciprofloxacin probably select gut flora in two ways; by its broad effect on the gram negative facultative flora, and most probably additionally by an effect on the anaerobic flora due to high gut concentration enabled to achieve suppressive MIC-concentrations[444,445]. In this study, risk factors for nosocomial infection with KPC-producing *K.pneumoniae* were collected as an integral observation performed as part of the case definition of the outbreak. No control group was applied and our results should be interpreted accordingly.

KPC-producing *K.pneumoniae* in blood stream infections is associated with severe outcomes and resistance *per se* attributes considerable to mortality[446,447]. Only one of the patients in this study (**paper 4**) was identified with a positive blood culture, and this patient survived. The role of infection was judged as uncertain or unlikely (including the single patient in this study that diseased in-hospital) in the other patients. In general, KPC-producing *K.pneumoniae* does not seem to possess specific virulence factors [200] and low virulence has been displayed in animal models[448,449]. Nevertheless, a comparatively higher virulence has been noticed in the worldwide ST258 clone and variants (ST277, ST11) than in non-ST258 clones (ST377, ST378) associated with $bla_{\mathrm{KPC}}$ [448].

**Conventional and molecular epidemiology – exploring modes of transmission:** Transmissions between reservoirs in the patient or health care worker populations and the environment represent the principle modes of spread in nosocomial outbreaks with the patient population as the most important mode in a high-frequent outbreak[330]. KPC-producing *K.pneumoniae* has been recognized to produce explosive outbreaks in several countries and in some countries it has facilitated intra-institutional dispersion affecting hospitals and LTCF in regions or countries with rapidly increase over a short period of time (i.e. in Cyprus, Italy, Israel, Greece). LTCF may represent secondary reservoirs in these regions, as duration of carriage may remain long in a significant proportion of patient after discharge. In a study by Feldman, 50% of the patients seemed to clear carriage at 3 months, however many patients remained carriers for prolonged periods[331]. In high frequent outbreaks of Enterobacteriaceae, the contribution of the environment is supposedly outnumbered by transmissions taking place in the patient reservoir directly or indirectly from the hands of the health care workers. A high-frequent outbreak was

prevented in this study (**paper 4**) even though a successful epidemic clone was acquired in the ICU. A high-level of adherence to basic precaution measurements in the ICU may have contributed to this achievement. However, breaches to guidelines related two the index patient was identified on at least two occasions that possibly influenced the course of this outbreak. Following the detection of $bla_{KPC}$ in patient 1 contact precautions in the ICU was abandoned for several days for unknown reasons. Moreover, after the patient had been readmitted to a medical department in SSK, contact precautions were not administered as the patient record had not been electronically tagged. An infection/carrier-ratio in patients often exceeds >1:5-10 when active surveillance culturing (ASC) is undertaken [450]. Contrary, in this study only six clinical isolates were recovered in the >2.5 year duration of the outbreak, signifying the unusually low frequency of this outbreak in terms of clinical cases (**paper 4**). Conventional epidemiology and molecular typing could infer possible or likely spread transmission lines. However, long intervals for which time and place did not overlap between clinical patients existed. A significant reservoir among asymptomatic carriers bridging the gaps between clinical cases could not be identified. At least at the time when ASC was initiated, only one carrier was identified among 136 tested patients. In turn, this forced us to explore other possible reservoirs and modes of transmission.

*"…wet dirt is dangerous…." Florence Nightingale.*

*Nightingale F. (1859). Notes on Nursing, revised Edition 1952. Gerald Duckworth and co.Ltd.*

In this study, KPC-producing bacteria were recovered from as much as 21% (4/19) of the sinks investigated, confirming a significant environmental reservoir (**paper 4**). Sinks were heavily contaminated presumably, as wastewater inappropriately was washed down the sinks after cleaning of the patients. Although the environment was decontaminated and sinks replaced, KPC-producing *E. asburiae* and *K. pneumoniae* were still recovered during further environmental screening. A main problem in studies of nosocomial outbreaks focusing on the possible involvement of the environment is to establish evidence for the direction of transmissions. In cases were the same clone or plasmid are recognized in both environmental samples and patients it is often hard to decide which are the source and which are the recipient. In this study, analyzing the epidemiological link between patient 6 (clinical patient) and patient 7 (isolate recovered during screening) it was realized that patient 7 succeeded patient 6 in room 5 (**paper 4**). However, there was no time overlap between the patients in this ICU-equipped single room. Disinfection procedures with persulfate were carried out after patient 6 was discharged, such that the room was newly disinfected when patient 7 was admitted. The isolation of pulsotype indistinguishable KPC-producing *K. pneumoniae* ST258 from both patients and the sink drain in that room as well as the recovery of closely related KPC-producing *E. asburiae* from fecal screening in patient 7 and the sink drain, strongly suggested a possible environmental *source* of patient 7's colonization.

Sinks have been reported as wet reservoirs causing nosocomial transmissions of *K.pneumoniae* and *Enterobacter spp*. [23-27]. Subsequent backsplashes from sinks during hand washing have been suggested as a mode of occasional transmission of contaminated water containing MDR-bacteria including *K.pneumoniae* and *Enterobacter spp*. to the hands of patients or health care workers or to medical items in the vicinity of the sinks in some reports. Inadequately designed of sinks with the water spray directed over the drain may promote the backsplash. In cases of less remarkable susceptibility patterns such colonizations or infections are likely to remain unnoticed as no apparent epidemic peaks are created. Patients, health care workers or medical items may act as vehicles for transmission to susceptible patients completing the chain of transmission[22,26,27,451].

Long-term bacterial contamination of sinks is promoted by biofilm formation in the plumbing system of the sinks. Biofilms are complex polymer matrixes consisting of cells and matrix reducing the penetration of antibiotics [452]. Some *Enterobacter spp.* and *Klebsiella spp*. strains are able to participate effectively in biofilm formation, which allows the pathogens to persist in the environmental reservoir[453,454]. Bacteria in biofilms are less responsive to the action of to chlorine and other disinfectants, and flushing with these substances may only help temporarily [451].

Various measures were applied to get rid of KPC-producing *K.pneumoniae* colonizing the sinks in our study (**paper 4**). First, attempts were made to physically remove the biofilm by replacing sinks and sink traps. Secondly, connecting pipelines were disinfected by various measurements such as chlorine and quaternary ammonium-solutions (Persulfate ®) and hot ("boiling") water. However, these measurements were undertaken only twice; following the dismantling of sinks (June 2010), and after KPC-producing bacteria reappeared in samples from the sink in room 6 (December 2010). In order to stop re-growth of the biofilm a continuous effort is probably needed. Self-disinfecting siphon systems may offer an effective solution; however, the one-time investment costs are high [451]. Some *K.pneumoniae* strains are reported to be heat resistant creating additional pressure on the environmental cleaning procedures; this was, however, not examined in our study [15]. A "bed-bath"-system for cleaning of patients were implemented and probably effectively short-cutted the presumed sink-to-patient mode of transmission. Accordingly, subsequent cases were prevented. It is important to acknowledge in this context that *K.pneumoniae* strains originating from the environment (whether planktonic or biofilm of origin) is of clinical significance as they in general possess the same set of virulence factors as clinical isolates and thereby possess the same ability of intestinal colonization and represent a continuous challenge with possibilities to cause disease in the immunocompromised host [15,16,18].

In inanimate materials, the ability of *K.pneumoniae* to form biofilms supersedes that of many other Enterobacteriaceae-species [453]. Jones et al. examined biofilm formation in a model mimicking the environment in wet pipes, demonstrating the superiority of *K.pneumoniae* over *E. coli* and *Salmonella spp*. to form biofilms in that environment [455]. However, considerable strain-to-strain variation exists in the ability to form biofilm [456,457]. Interestingly, biofilm production is

prominent in some strains belonging to *E. coli* ST131, enhancing both virulence *in vivo* and supposedly the persistence of these strains in different environments [408].

***The ~97 kb IncFII plasmid****: bla*$_{KPC}$-genes have been identified on plasmids of different sizes and incompatibility groups, including IncN, IncL/M and IncF[73,204]. A ~ 97 kb IncFII plasmid was identified in all isolates carrying *bla*$_{KPC-2}$ in this outbreak (**paper 4**). Dispersion into four distinct PFGE types of *K.pneumoniae* and *E.asburiae* indicated *in vivo* mobility. Successful *in vitro* conjugation of *bla*$_{KPC}$- plasmids supported this observation. Superimposed, the plasmids were coupled to the highly successful *K.pneumoniae* - clone (ST258) in the majority of the isolates, including all except one of the clinical cases. Thus, both efficient clonal dissemination and plasmid diffusion was demonstrated in our outbreak[21,65].
The intestinal microflora of individual patients and the biofilm environments in inanimate surfaces such as that provided by sinks both represent suitable and possible compartments for plasmid transfer in Enterobacteriaceae [83,458-461]. Interspecies plasmid transfer (*in vivo*) in patients has been indicated in several previous reports[459,462,463]. Furthermore, biofilms are convenient locations for plasmid transfer by conjugation including plasmids carrying resistant determinants[452,458].

The exact compartment or ecological niche of plasmid diffusions remains uncertain in our outbreak (**paper 4**). ST258 *K.pneumoniae* PFGE identical strains were recognized from both clinical patients, the carrier patient (P7) and in the sink-environment, whereas *E. asburiae* isolates were recognized in the carrier patient and in the environment of this patient (sink in his room). Subsequently, *E.asburia* isolates were identified on two additional locations (room 9 and the rinsing room). Of note, the KPC-producing *E.asburiae* remained clinically silent throughout the outbreak, and as fecal screening was not performed in any of the clinical patients, carrier status, including the index case, remains unknown. Thus, the extent of involvement *bla*$_{KPC}$-producing *E. asburiae* in clinical patients remains elusive. Intra-species transfer of plasmids within the gut of patient 5 could explain the observation in patient 5 (ST461 *K.pneumoniae*).

***ST258 K.pneumoniae*****.** The widely distributed *K. pneumoniae* ST258 was detected in the index case, and involved in 4/5 of the subsequent clinical patients. The success of this clone in facilitating an outbreak of *bla*$_{KPC}$ in a nosocomial setting in our country was thus documented by our study (**paper 4**)[65]. By investigating the evolution of clones by means of MLST, an analogous situation to *bla*$_{CTX-M-15}$ carrying ST131 *E.coli,* is being disclosed for hyperepidemic *K. pneumoniae* clone ST258 and its single locus variants (ST11, ST14, ST437) in the emergence and dissemination of *bla*$_{KPC}$. Questions however remain on whether this clone is of recent emergence or a predominant strain from before the arrival of *bla*$_{KPC}$ prone to adopt and receive various plasmid DNA variants[65,464]. In our study, we observed the ability of this clone to act as donor in horizontal transmission of plasmids carrying *bla*$_{KPC.}$

***The AST phenotype – variability of co-resistances and MICs to carbapenems***: The initial presentation of the ST258 *K.pneumoniae* strains in the index case, and the subsequent observation of this strain was associated with a MDR-phenotype (**paper 4**). However, dispersion into *E. asburiae* and other *K.pneumoniae* ST-types (ST27 and ST461) were associated with susceptibility to non-β-lactams, except for low-level ciprofloxacin resistance in the *E.asburiae* related strains. The ST27 (E6) and ST461 *K.pneumoniae* (P5) strains may be local strains acquiring the ~97 kb plasmid. The local prevalence of these strains was however not explored further in our study in order to justify this presumption. In general, KPC-producing isolates are of linked or associated with a number resistance determinants, including several β-lactamases (*bla*$_{\text{OXA-9/TEM-1/SHV-11/12/CTX-M-15}}$), and non-β-lactam resistant determinants like *aac(6')-Ib*, chromosomally encoded colistin-resistance as well as porin deficiency, contribute to MDR- or extreme-drug resistant (XDR) phenotypes [110,187,232,465]. Alarming resistance profiles were associated with the ST258 clone in our study leaving few therapeutic options. Gentamicin susceptibility often remains in *bla*$_{\text{KPC}}$ producing isolates (corresponding to the *aac(6')-Ib* resistant determinant) however this antibiotic may not apply to all clinical situations. Colistin susceptibility was variable. Combination therapy seems to be associated with increased survival in these infections [447].

A wide range of meropenem-MICs [2,32] were observed for KPC-producing Enterobacteriaceae for the different strains both inside and outside species borders (*K. pneumoniae* and *E. asburiae*) in our study [232] (**paper 4**). These observations emphasize again the need for accuracy when performing susceptibility testing in detection of carbapenemases-producing Enterobacteriaceae (CPE) as well as applying appropriate screening cut-offs. Carbapenem MICs in CPE are influenced by several factors including bacterial species, β-lactamase type or variant, levels of enzymatic expression, and presence of additional resistance mechanisms as previously mentioned.

***Detection of fecal carriers during outbreaks***: Several issues relates to the screening of carriage for carbapenemases-producing Enterobacteriaceae such as optimal screening type (rectal, peri-rectal, stool), and added benefits and disadvantages of overnight enrichment broths prior to PCR- or culture-based detection. Currently, no optimal standard is set, and methods have to be adapted to local epidemiology, staff, costs and equipment. Nevertheless, a given result should be reported within 24-48 hours (whether negative, confirmed, or suspected). Singleplex-PCRs may be an attractive option in targeted detection during outbreaks. In this study (**paper 4**) an evaluation of different approaches; agar-based and PCR-based were attempted in parallel. However, as only one positive *bla*$_{\text{KPC}}$ carrier was detected among the 136 patients tested, the parallel evaluation was stopped (after the first 50 patients had been screened). The single KPC-positive patient was detected by all of the three arms of detection in over study (i.e. by *bla*$_{\text{KPC}}$-PCR directly (i) and following ON- enrichment (ii), by the ChromID ESBL® agar –directly

without ON-enrichment (iii). The latter method was familiar to the laboratory as it was validated and in routine use in the laboratory for ESBL detection).

Most studies show an added benefit of enrichment following culture based surveillance testing of CPE carriers[466-468]. MIC and density of fecal carriage impacts sensitivity in culture based testing. ChromID ESBL®, as used in this study (**paper 4**), is a favorable choice displaying good sensitivities in most surveys studying CPE detection of single non-OXA-48 isolates, and may be particularly favored in strains conferring low-level carbapenem resistance and/or specimen containing low densities of CPE (< $10^3$ CFU/ml) [469-472]. Direct simplex PCRs is generally considered more sensitive than culture based detection in detection of low loads and/or low-level carbapenem-MICs. Few data exist on multiplex PCR approaches, but comparable sensitivity to culture based methods were achieved by one report examining a commercial multiplex PCR (CPE direct, Check Point ®) [473]. Of note, PCR provide genotype, but no isolates available for susceptibility testing or typing and both methods may be combined for investigational purposes.

# F. CONCLUDING REMARKS

The following conclusions can be drawn from the results from this study:

From paper 1

i. Cefpodoxime alone or the combined use of cefotaxime and ceftazidime performed best as screening substrates for ESBL detection in Norwegian isolates of *E.coli* and *K.pneumoniae*. Consequently, the following guidelines in routine diagnostic microbiology based on national epidemiology were advocated; in screening you have to use both cefotaxime and ceftazidime OR cefpodoxime alone.
ii. In confirmation, the CDT method displayed similar sensitivity, but higher specificity than the Etest ESBL and the DDS method. The most frequent source of a false positive CAZ ESBL test was SHV1/11-hyperproduction in *E.coli* and *K.pneumoniae.*
iii. The emergence of *bla*$_{\text{CTX-M}}$ –producing *E.coli* in Norway was documented being predominated by the 1(mainly *bla*$_{\text{CTX-M-15}}$) and 9 genotypes.

From paper 2

i. The emergence of *bla*$_{\text{CTX-M}}$ (prevalent: *bla*$_{\text{CTX-M-15}}$ *and bla*$_{\text{CTX-M-14}}$)–producing polyclonal *E.coli* in Norway was documented with major high-risk clones (ST131 and CC405) being present. Locally clustered strain expansions were also documented.
ii. IncF plasmids were prevalent and a dominant position of IS*Ecp1* linked IncF plasmids were documented in *bla*$_{\text{CTX-M-15}}$ positive *E.coli* isolates. Furthermore, an unusual association between *bla*$_{\text{CTX-M-14}}$ and IncF plasmid with (IS*Ecp1* upstream) was observed.
iii. Co-transfer of non-β-lactam resistance in *bla*$_{\text{CTX-M}}$ harboring plasmids was only documented for trimethoprim-sulfamethoxazole (3/9; 33%). Co-resistance to other classes of antibiotics (39/45=87 %) was therefore assumed located on other plasmids or the chromosome. A MDR phenotype was associated with several STs, including ST131 and ST964.

From paper 3

i. The prevalence of *E.coli* AmpC- hyperproducers (1.0%) and *K.oxytoca* OXY2-hyperproducers (13.6 %) were documented for the first time in a nationwide material in our country
ii. *K.oxytoca* hyperproducing OXY2-type β-lactamase were identified as a frequent source of false positive ESBL test, whereas the same problems did not occur with the CDT method.
iii. Plasmid mediated AmpC was not observed.

From paper 4

i. The emergence and first nosocomial outbreak of KPC-producing *K.pneumoniae* and in the Scandinavian countries was documented.
ii. The involvement of the successful ST258 *K.pneumoniae* as well as the demonstration of efficient interspecies and intergenus spread of an IncF plasmid carrying $bla_{KPC2}$ was described.
iii. The establishment of local environmental reservoirs (sinks) of KPC-producing Enterobacteriaceae was documented, and the possible contribution of the environment in the maintenance and prolongation of this low frequent outbreak was inferred.

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PAPER 1

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PAPER 3

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