Extended-spectrum ß-lactamase producing *Klebsiella pneumoniae*

A neonatal intensive care unit outbreak, long-term colonization in children and plasmid characteristics

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A neonatal intensive care unit outbreak, long-term colonization in children and plasmid characteristics

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Scientific environment

This study is part of a collaboration project between the Department of Paediatrics, Department of Infection Control and the Department of Medical Microbiology, Stavanger University Hospital, Stavanger, Norway.

Supervisors and collaboration partners at the Department of Medical Biology and the Department of Pharmacy, UiT the Arctic University of Norway, Tomsø, Norway, and at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (K-res), Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway.

Collaboration partners at the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden.

In affiliation with the Department for Clinical Medicine, University of Bergen, Bergen, Norway.

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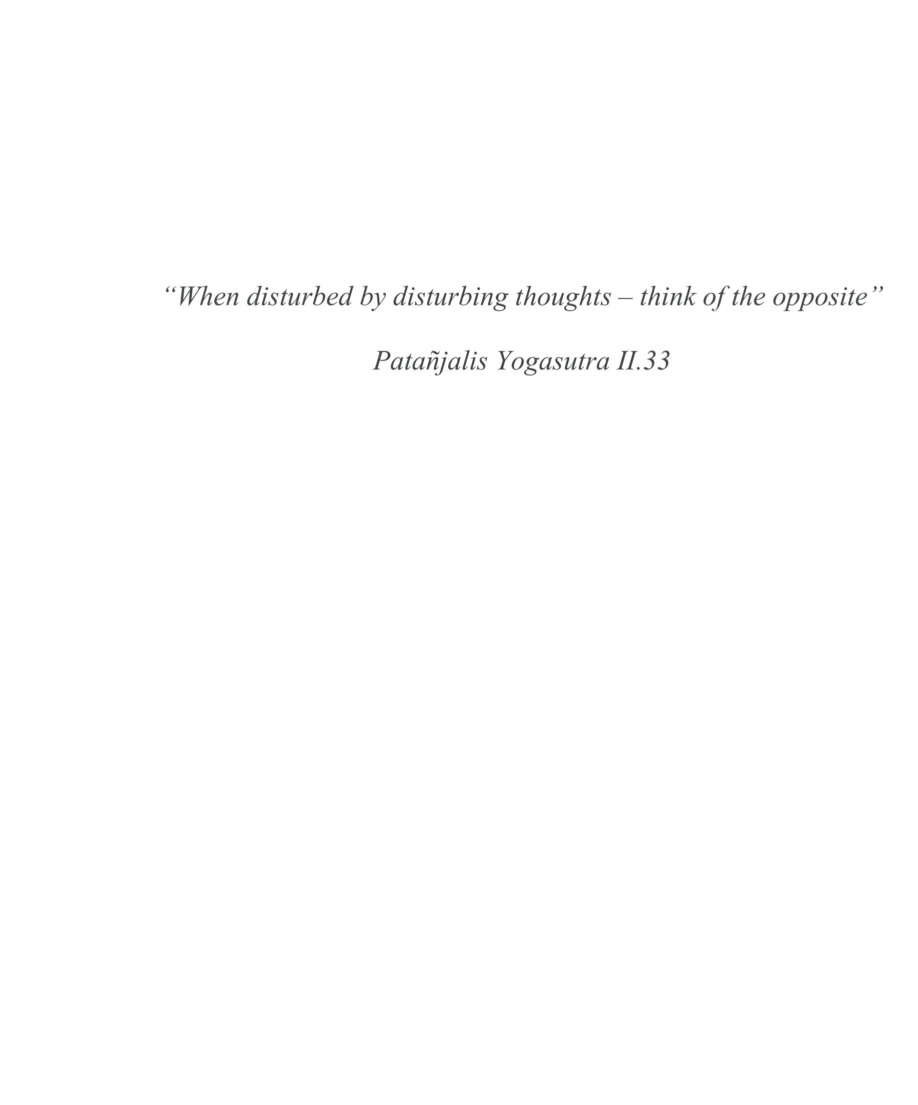

"When disturbed by disturbing thoughts – think of the opposite"

Patañjalis Yogasutra II.33

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Summary of the Thesis

Background The increasing level of antimicrobial resistance among clinical relevant bacteria, especially the increasing prevalence of extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae, is of great concern in most parts of the world including Norway. During 2008-09 an outbreak caused by ESBL-producing (type CTX-M-15) *Klebsiella pneumoniae* occurred in the neonatal intensive care unit (NICU) at Stavanger University Hospital. This was the first reported NICU outbreak caused by ESBL-producing Enterobacteriaceae in Scandinavia.

Objectives The objectives of this study were: (i) to describe the characteristics of the NICU outbreak and the *K. pneumoniae* outbreak strain(s), (ii) to investigate the duration of intestinal ESBL-carriage in the children affected by the outbreak, risk factors for prolonged carriage and intra-household transmission during colonization, and (iii) to characterize the ESBL-encoding plasmid of the outbreak strain, and examine the plasmid's dynamics in relation to its *K. pneumoniae* host strain.

Methods Several microbiological and molecular methods were employed in this study: culture-based ESBL-screening of environmental and patient samples, detection of ESBL and virulence genes by PCR, bacterial strain typing by *Xba*I pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing, plasmid profiling by *S1*-nuclease PFGE, PCR-based plasmid replicon typing, whole genome sequencing of plasmid DNA, and plasmid transfer, stability and fitness cost experiments. Kaplan-Meier survival analysis was performed to determine carriage times of the *K. pneumoniae* strain in colonized infants. Cox-regression analysis was performed to identify risk factors for prolonged carriage.

Results In total 58 children were affected by the NICU outbreak, caused by multidrug resistant CTX-M-15-producing *K. pneumoniae* (CTX-M-15-Kp). Of these, 56 were colonized by the ST17 outbreak strain (two children were colonized by an unrelated ST485 strain). No commonly encountered virulence factors were detected in the

outbreak strain. One child suffered a severe infection (sepsis) caused by CTX-M-15-Kp, but no children died of reasons related to the outbreak. A probable index case was identified as one child and its mother, from which several CTX-M-15-Kp positive breast milk samples were obtained. The outbreak was contained by enforced infection control measures and strict cohorting of patients and health care workers. In a follow-up study of colonized children and their families, CTX-M-15-Kp carriage for up to two years was confirmed in some of the children. Median carriage time was 12.5 months. Risk factors for prolonged carriage were delivery by caesarean section and treatment with antibiotics (ampicillin and gentamicin) during the NICU stay. Intra-household transmission of CTX-M-15-Kp was documented in 33% of the households. CTX-M-15 was encoded by a ~180 kb $IncFII_K$ pKPN3-like plasmid (pKp848CTX) in the ST17 outbreak strain. pKp848CTX encoded multiple antibiotic, heavy metal and thermoresistance determinants. The plasmid could not be transferred from its *K. pneumoniae* host to *Escherichia coli in vitro*, consistent with *in vivo* observations. pKp848CTX was stably maintained by its host strain during intestinal colonization for up to two years. Segregational plasmid loss ranging from 0% to 17.5% in evolved populations was detected *in vitro*, but only negligible fitness costs associated with plasmid carriage were uncovered.

Conclusion The NICU outbreak was caused by a CTX-M-15-producing ST17 *K. pneumoniae* strain, which spread efficiently among patients. The virulence properties of the outbreak strain seem to be low. Vertical mother-to-neonate transmission due to contaminated breast milk was the most likely the source of the outbreak. Children may become long-term faecal ESBL-carriers after colonization during a NICU outbreak, and represent a reservoir for intra-household ESBL-spread after hospital discharge. $Bla_{CTX\text{-}M\text{-}15}$ was encoded by a pKPN3-like $IncFII_K$ plasmid, pKp848CTX, which seems to be well adapted to CTX-M-15-Kp. The plasmid's inability of self-transfer when hosted by this strain may have limited the extent of the outbreak and the duration of ESBL-colonization.

List of papers

I. Rettedal S, Löhr IH, Natås O, Giske CG, Sundsfjord A and Øymar K**.** **First outbreak of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in a Norwegian neonatal intensive care unit; associated with contaminated breast milk and resolved by strict cohorting.** *APMIS* 2012; 120: 612-21.

II. Löhr IH, Rettedal S, Natås OB, Naseer U, Øymar K and Sundsfjord A. **Long-term faecal carriage in infants and intra-household transmission of CTX-M-15-producing *Klebsiella pneumoniae* following a nosocomial outbreak.** *J Antimicrob Chemother* 2013; 68: 1043-8.

III. Löhr IH, Hülter N, Bernhoff E, Johnsen PJ, Sundsfjord A, Naseer U. **Persistence of a pKPN3-like *bla*CTX-M-15 encoding IncFIIK plasmid in its *Klebsiella pneumoniae* ST17 host during two years of intestinal colonization.** *Submitted.*

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Abbreviations

AmpC	Ampicillinace C (β-lactamase/ $ESBL_{M-C}$-type)
AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
BSI	Blood stream infection
BHI	Brain heart infusion
CC	Clonal complex
Cfu	Colony-forming units
CI	Confidence interval
CMY	Cefamycinase ($ESBL_{M-C}$-type)
CTX-M	Cefotaximase-Munich ($ESBL_{A}$-type; Place of isolation)
CTX-M-15-Kp	CTX-M-15-producing *Klebsiella pneumoniae*
DHA	Dhahran ($ESBL_{M-C}$-type; Place of isolation)
DNA	Deoxyribonucleic acid
DIG	Digoxigenin
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off value
EDTA	Ethylene Diamine Tetra Acetic acid
ESBL	Extended-spectrum β-lactamase
$ESBL_{A}$	Ambler class A ESBL
$ESBL_{CARBA}$	ESBL with hydrolytic activity against carbapenems (may be subdivided into $ESBL_{CARBA-A}$, $ESBL_{CARBA-B}$ and $ESBL_{CARBA-D}$)
ESBL-E	ESBL-producing Enterobacteriaceae
ESBL-Kp	ESBL-producing *Klebsiella pneumoniae*
$ESBL_{M}$	Miscellaneous ESBL (may be subdivided into $ESBL_{M-C}$ and $ESBL_{M-D}$)
EUCAST	European Committee on Antimicrobial Susceptibility Testing

GES	Guiana extended-spectrum ($ESBL_{CARBA-A}$-type)
HC	Household contact
HCW	Health care worker
HGT	Horizontal gene transfer
HR	Hazard ratio
ICU	Intensive care unit
IMP	Imipenemase ($ESBL_{CARBA-M}$-type)
Inc type	Incompatibility type
IS	Insertion sequence
IS*CR*	Insertion sequence common region
IQR	Inter-quartile range
K-antigen	Capsular antigen
Kb	Kilo bases
KPC	*Klebsiella pneumoniae* carbapenemase ($ESBL_{CARBA-A}$-type)
K-res	Norwegian National Advisory Unit on Detection of Antimicrobial Resistance
LB	Luria Bertani
m	Malthusian parameter
MALDI-TOF MS	Matrix-assisted laser-desorption ionization-time-of-flight mass spectrometry
MBL	Metallo-β-lactamase ($ESBL_{CARBA-B}$-group)
MDR	Multidrug resistant (i.e. resistant to ≥ three antibiotic classes)
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable number tandem repeat analysis
MRSA	Methicillin resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase ($ESBL_{CARBA-B}$-type)
NICU	Neonatal intensive care unit

NORM/NORM-VET	Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway
OD	Optical density
ON	Overnight
OXA	Oxacillinase (β-lactamase/$ESBL_{M-D}$ and $ESBL_{CARBA-D}$-type)
PBP	Penicillin binding protein
PBRT	PCR-based replicon typing
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pMLST	Plasmid multilocus sequence typing
PNSP	Penicillin non-susceptible *Streptococcus pneumoniae*
RFLP	Restricted fragment length polymorphism
RNA	Ribonucleic acid
SE	Standard error
S/I/R	Susceptible/intermediate/resistant
ST	Sequence type
SHV	Sulfhydryl-variable (β-lactamase/$ESBL_A$-type)
TEM	Temoneira (β-lactamase/$ESBL_A$-type; named after a patient)
Tn	Transposon
UTI	Urinary tract infection
WHO	World Health Organization
VIM	Verona integron-encoded metallo-β-lactamase ($ESBL_{CARBA-B}$-type)
w	Relative fitness
WGS	Whole genome sequencing

List of genes

bla	β-lactamase gene
cps operon	Encodes the synthesis of capsular polysaccharides
rmpA	Encodes a regulator of the mucoid phenotype A in *Klebsiella pneumoniae*
wcaG	Encodes capsular fucose synthesis, which may enhance bacterial escape from phagocytosis
kfu	Encodes a siderophore involved in iron acquisition
fimH	Encodes a type 1 fimbrial adhesin
mrkD	Encodes a type 3 fimbrial adhesin
cf29A	Encodes the non-fimbrial adhesion factor CF29K
allS	Encodes the allantoin regulon (also used as a marker for *K. pneumoniae* ST23)
cepA	Encodes biocide efflux pumps
*qac*Δ*E*	Encodes biocide efflux pumps

Preface

Antimicrobial resistance (AMR) has reached the agenda of global policy makers. Its importance was recently emphasised by the World Health Organization in a comprehensive report, including data from 114 countries. It is clear that AMR is no longer a potential, but a current major threat to global public health and it is time to take action (1). In particular, the increasing prevalence of multidrug resistant (MDR) Gram-negative bacteria, expressing extended-spectrum β-lactamases (ESBLs) and associated resistance mechanisms, causing hospital outbreaks and difficult-to-treat human infections has been of concern since the turn of the millennium. The diverse use and misuse of antibiotics across sectors (in humans, animals and agriculture), is considered the primary driver of AMR. The global spread of MDR human pathogens is a multifaceted challenge, and also hugely influenced by migration and tourism, the lack of access to clean water, open rather than closed sewage systems, high population densities and inadequate healthcare in many parts of the world.

Compared to most parts of the world, we still have low, but increasing, levels of AMR in Norway (2). During 2008-09, an outbreak caused by ESBL-producing *K. pneumoniae* occurred in the neonatal intensive care unit (NICU) at Stavanger University Hospital. This was the first ESBL outbreak reported from a Norwegian NICU, and thus attracted much attention. Huge efforts were made by the NICU staff, the lab staff, the infection control team and the hospital management to control the outbreak. The gap of knowledge concerning different aspects of such an outbreak encouraged the initiation of several studies.

This thesis is based on three sub-studies focusing on: (i) the characteristics of the NICU outbreak and the *K. pneumoniae* outbreak strain, (ii) the duration of intestinal ESBL-carriage in colonized children and intra-household transmission, and (iii) the characteristics of the ESBL-encoding plasmid and its dynamics in relation to its *K. pneumoniae* host strain.

It is beyond the limit of this thesis to cover all aspects of the emergence of MDR Gram-negative bacteria. Thus, in the following introduction, I will concentrate on topics relevant for this study.

1 Introduction

1.1 Enterobacteriaceae

Enterobacteriaceae is a heterogeneous family of Gram-negative, non-sporulating, facultative anaerobic rods, belonging to the γ-proteobacteria. Most Enterobacteriaceae have type 1 pili (fimbriae), which enable bacterial adhesion to epithelial cells, and some species have flagella, which make them motile. Essential biochemical characteristics of Enterobacteriaceae include fermentation of sugars (different species ferment specific sugars), reduction of nitrate to nitrite, oxidase-negativity and catalase-positivity. Enterobacteriaceae thrive well at temperatures between 25°C and 37°C, and inhabit a wide spectrum of environmental, animal and human niches, such as the mammalian intestine, water, soil and plants (3).

The most clinically relevant Enterobacteriaceae may be divided into opportunistic pathogens, such as *Citrobacter* spp., *Enterobacter* spp. and *Klebsiella* spp., and overt pathogens, such as *Shigella* spp., *Salmonella* spp. and *Yersinia* spp. (4). *E. coli* may be divided into commensal strains and pathogenic strains. *K. pneumoniae* and *E. coli* are the most frequently observed Enterobacteriaceae in human clinical samples, and may cause common infections such as pneumonia, urinary tract infections (UTIs) and bloodstream infections (BSIs) (3, 5).

1.2 *Klebsiella pneumoniae*

K. pneumoniae belong to the genus *Klebsiella*. According to Ørskov's classification, *K. pneumoniae* may be divided into three subspecies: *K. pneumoniae* subsp. *pneumoniae, ozaenae and rhinoscleromatis* (6). In this thesis, *K. pneumoniae* subsp. *pneumoniae* is meant when *K. pneumoniae* is written.

The genus *Klebsiella* is named after the German-Swiss pathologist Edwin Klebs, who was the first to observe bacteria in the airways of patients who died of pneumonia in 1875. In 1882 the German pathologist and microbiologist Carl Friedländer detected bacteria in the fibrous exudate of patients who had died of lobar pneumonia, and it soon became apparent that two bacterial species could cause

pneumonia, *Streptococcus pneumoniae* and *K. pneumoniae*, the latter also referred to as Friedländers bacillus (7).

1.2.1 Biochemical characteristics

Biochemical characteristics used for the identification of *K. pneumoniae* include: negative indole-test, production of lysine decarboxylase (but not ornithine decarboxylase), fermentation of specific sugars (e.g. D-glucose, lactose, sucrose, L-arabinose and maltose) and sugar-alcohols (e.g. D-mannitol). Furthermore, *K. penumoniae* is non-motile and usually produce a prominent acidic polysaccharide-based capsule (8). Biochemical characteristics are still being used for species identification of bacteria isolated from clinical samples. However, biochemical identification is increasingly performed by automated systems, such as Vitek2 (bioMèrieux, Marcy l'Etoile, France) or Phoenix (BD Diagnostics, Sparks, USA). Moreover, new identification approaches, such as MALDI-TOF mass spectrometry, has been taken into use in many laboratories with great success.

1.2.2 Pathogenicity and virulence factors

The pathogenicity of *Klebsiella* spp. may be associated with virulence factors, such as capsular antigens (O- and K-antigens), adhesins, siderophores and lipopolysaccharides (endotoxins). The capsule is considered essential to the virulence of *Klebsiella*, as it protects the bacterium from phagocytosis and prevents killing of the bacteria by bactericidal serum factors (6).

Some serotypes or capsular types (K-types) of *K. pneumoniae*, e.g. K1, K2, K5, K54 and K57, have been associated with invasive human infectious diseases. K1 was observed among isolates causing Friedländer's pneumonia, and has more recently been associated with pyogenic liver abscesses (9, 10). Brisse *et al*. studied the association between K-type, sequence type (ST) and virulence gene content. The authors concluded that K-types are not associated with specific *K. pneumoniae* clones, and that K-types are distributed among unrelated clones by horizontal transfer of the *cps* operon, which encodes the synthesis of capsular polysaccharides. Furthermore, the

virulence gene content was found to be associated with specific clones, rather than with K-types (11).

During recent years, several genes encoding virulence factors in *K. pneumoniae* have been described: the plasmid-borne *rmpA* regulates the mucoid phenotype (12), *wcaG* is associated with enhanced bacterial escape from phagocytosis (13), *kfu* is involved in iron acquisition, *fimH* encodes type 1 fimbriae, *mrkD* encodes type 3 fimbriae and *cf29A* encodes the non-fimbrial adhesion factor CF29K (11). *K. penumoniae* ST23, which is frequently of serotype K1, is considered to be a particular virulent clone. Presence of *allS* is a marker for ST23 (14). Calhau *et al*. recently detected several virulence genes and pathogenicity islands (PAIs) in a collection of clinical ESBL-producing *K. pneumoniae* isolates from renal transplant patients (15). In a recent Danish study, the virulence factors aerobactin, *kfu* and *rmpA* were detected in a hypermucoviscous *K. pneumoniae* ST23 blood isolate from a patient with a liver abscess (9).

Klebsiella spp. are known to be inherent or intrinsically resistant to ampicillin, ticarcillin and piperacillin due to chromosomal SHV-1-production. Furthermore, all Enterobacteriaceae are intrinsically resistant to penicillin G, glycopeptides, fusidic acid, macrolides (with some exceptions), lincosamides, streptogramins, daptomycin and linezolid (16). Acquired resistance to other relevant antibiotic groups is increasingly reported in clinical *K. pneumoniae* isolates, and will be described more in detail further below.

Biocides, including pesticides, preservatives for food, desinfectants and antiseptics, have been extensively used in agriculture, in food industry and in hospitals for decades. Bacterial tolerance to biocides has been observed, and concern has been raised on their impact on the selection of antimicrobial resistance (AMR) in human pathogens (17). Efflux pumps extruding both multiple biocides and antibiotics have been described in Enterobacteriaceae and other Gram-negative bacteria (18). Reduced susceptibility to the antiseptics chlorhexidine, trigene and benzalkonium chloride was recently documented in clinical *K. pneumoniae* isolates. The biocide resistance genes *cepA* and *qac∆E* (encoding efflux pumps) were detected in most of the isolates. There was, however, no genetic linkage between determinants encoding reduced biocide

susceptibility and antibiotic resistance (19). Furthermore, bacterial resistance to heavy metals, such as copper and silver, also frequently used as antiseptics, has been reported in clinical MDR *K. pneumoniae* strains (20, 21).

Altogether, the combination of antibiotic, biocide and heavy metal resistance in nosocomial pathogens, such as *K. pneumoniae*, may favour their selection and dissemination in the hospital environment.

1.2.3 Colonization in human and dissemination in hospitals

In humans, *K. pneumoniae* may colonize the skin, the naso- and oropharynx and the intestinal tract (22-24). Carriage rates of *K. pneumoniae* are generally low in healthy humans, but have been found to increase dramatically in hospitalized patients, especially in patients treated with broad-spectrum antibiotics (25-27). The healthy human skin is not a common reservoir for Gram-negative bacteria, but *K. pneumoniae* has been found to colonize the hands of hospitalized patients and health care workers (HCWs) for several hours (28, 29). *K. pneumoniae* may spread remarkably well in the hospital environment, and frequently cause nosocomial infections and outbreaks, especially in intensive care units (ICUs). Medical equipment, the gastrointestinal tract of patients and the hands of HCWs are considered the most important reservoirs for the spread of *K. pneumoniae* in the hospital environment (28, 30).

1.2.4 *K. pneumoniae* infections

Historically, *K. pneumoniae* was known as the cause of community-acquired pneumonia or Friedländer’s pneumonia, which particularly occurred in immunocompromised persons, such as chronic alcoholics. Friedländer’s pneumonia was a severe infection with high mortality if untreated (6). This disease has become rare in most parts of the world. Today, *K. pneumoniae* is primarily known as a nosocomial pathogen, but may also be associated with community-acquired pneumonia or UTI, especially in elderly or immunocompromised persons. More lately, community-acquired liver abscesses with metastatic spread caused by highly virulent

K. pneumoniae strains (often of serotype K1 and ST23) are increasingly observed in healthy subjects, especially in South East Asia (31).

Immunocompromised hospitalized patients have an increased risk of opportunistic *K. pneumoniae* associated infections (6). Furthermore, the rate of nosocomial *Klebsiella* infections has been found to be four times higher in patients who carry the bacteria in their intestine than in non-carriers (32).

K. pneumoniae belong to the top three or four pathogens causing NICU infections (33). A combination of factors may explain why *K. pneumoniae* infection is so common in the NICU setting. Neonates, in particular premature neonates, have an immature immune system and lack a protective normal flora (33, 34). Invasive procedures and ventilator support allow bacteria from the NICU environment to access the bloodstream and lungs (33). Antibiotic treatment interferes with the development of the neonatal intestinal flora, and especially ampicillin has been associated with increased *Klebsiella* carriage rates in neonates hospitalized in NICUs (35).

Lower UTIs caused by *K. pneumoniae* may be successfully treated with per oral agents such as nitrofurantoin, trimethoprim and pivmecillinam. Agents available for treatment of upper UTIs and systemic *K. pneumoniae* infections include broad-spectrum penicillins in combination with β-lactamase inhibitors (e.g. piperacillin-tazobactam), fluoroquinolones, trimethoprim-sulfamethoxazole, aminoglycocides, broad-spectrum cephalosporins and carbapenems. However, resistance is emerging to most of these antibiotics, and the treatment options of *K. pneumoniae* associated infections are becoming more and more limited.

1.3 Antibacterial agents

The antibacterial effect of penicillin was discovered by Alexander Fleming in 1928. Since the first antibacterial agents were taken into clinical use during the late 1930s and 40s, antibacterial chemotherapy has played a crucial role in the treatment of infectious diseases. Today's specialised modern medicine, like intensive care, cancer therapy and advanced surgery, rely on potent antibacterial agents.

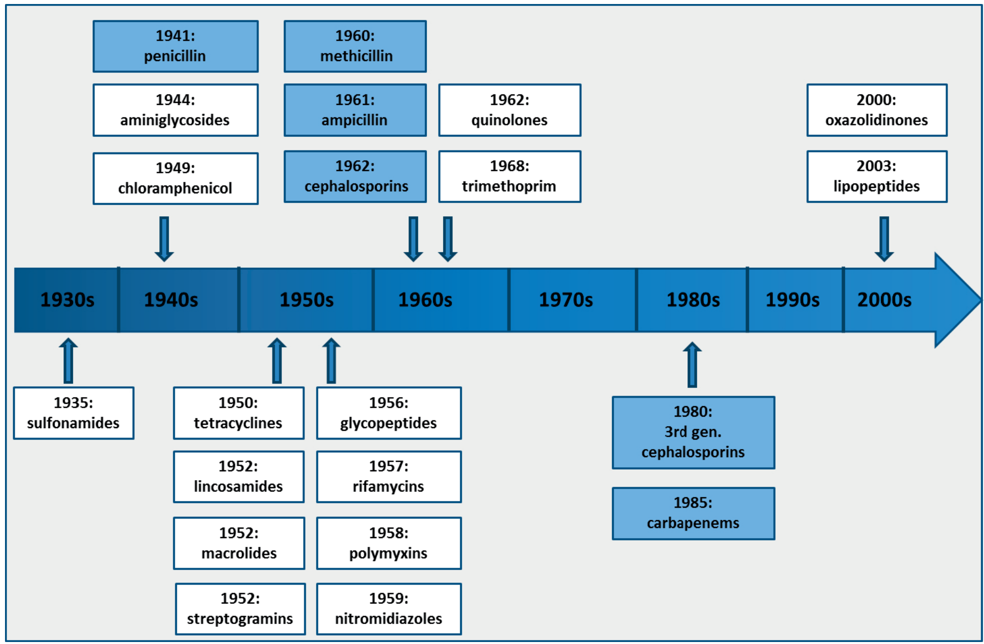

Figure 1. Historical timeline for the introduction of new antbiotic classes (white boxes) and new groups of β-lactams (blue boxes).

In the 1940s to 1960s, several antibiotic classes with different modes of action were detected and developed for clinical use (Figure 1). However, after the introduction of trimethoprim in 1968, no new classes of antibiotics effective in the treatment of Gram-negative bacterial infections have been discovered. The 3rd generation cephalosporins and a unique class of β-lactams, the carbapenems, were introduced in the 1980s. Furthermore, a large number of broad-spectrum cephalosporins and quinolones was introduced in the years to come, but these were all chemical modifications of agents already in clinical use (36). The lack of new drugs effective in the treatment of Gram-negative bacterial infections along with the emergence of antibacterial resistance Gram-negative pathogens has become a serious threat for modern medicine.

Antibacterial agents differ in their mode of action, antimicrobial spectra, pharmacologic parameters (i.e. pharmacokinetics and -dynamics) and toxicity. Antibacterial agents are frequently classified into five groups according to their mode

of action and antibacterial target: (i) inhibitors of the cell wall synthesis (e.g. β-lactams, glycopeptides), (ii) inhibitors of the DNA/RNA synthesis (e.g. quinolones, nitroimidazoles, rifampicin), (iii) inhibitors of the folic acid synthesis (e.g. sulphonamides, trimethoprim), (iv) inhibitors of the protein synthesis (e.g. aminoglycocides, macrolides, tetracyclines) and (v) inhibitors of the cytoplasmic membrane (e.g. polymyxins) (37).

1.3.1 β-lactams

Due to their diversity, broad spectrum of activity and low toxicity, β-lactams are the most prescribed antibiotics worldwide (38). All β-lactams have the β-lactam ring in common. Due to differences in their side chains, β-lactams may be classified into the following main groups: penicillins, cephalosporins, monobactams and carbapenems (Table 1) (37). β-lactams target the bacterial cell wall synthesis and act by binding covalently to penicillin binding proteins (PBPs). PBPs are bacterial enzymes involved in the synthesis and crosslinking of peptidoglycan, which is a major component of the bacterial cell wall. PBPs are located in the inner cytoplasmic membrane or in the periplasmatic space of Gram-negative bacteria. When PBPs are inactivated by β-lactams, the peptidoglycan synthesis is inhibited and the bacterial growth is affected. Irregularities in the cell wall synthesis lead to loss of integrity and finally cell lysis (39).

Table 1. Main goups and sub-classes of β-lactam antibiotics in clinical use in Norway (2014).

Group	Sub-classes	Agents
Penicillins	Narrow-spectrum penicillins	Benzylpenicillin, benzathinepenicillin, phenoxymethylpenicillin
	β-lactamase-stable penicillins	Cloxacillin, dicloxacillin
Benzylpenicillin	Broad-spectrum penicillins	Amoxicillin, ampicillin, mecillinam, pivmecillinam
	Broad-spectrum penicillin + β-lactamase-inhibitor	Piperacillin-tazobactam
Cephalosporins	1st generation	Cephalexin, cephalothin
	2nd generation	Cefuroxime
	3rd generation	Cefotaxime, ceftazidime, ceftriaxone
	4th generation	Cefixim (treatment of gonorrhoeae)
Cefotaxime	5th generation	Ceftaroline (treatment of MRSA or PNSP associated infections)
Monobactams		Aztreonam
Carbapenems		Imipenem, doripenem, ertapenem, meropenem

1.4 Resistance to antibacterial agents

Since antibacterial agents were taken into clinical use, resistance to most classes of relevant antibiotics, including the β-lactams, has emerged among human pathogens. Sir Alexander Fleming warned in his Nobel lecture in 1945 that resistance may evolve if antibacterial drugs are misused or under-dosed: “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing occasionally happened in the body” (Alexander Fleming, Nobel lecture “Penicillin”, 1945).

1.4.1 Definitions

- **Minimum inhibitory concentration:** The minimum inhibitory concentration (MIC) is the lowest antimicrobial concentration that inhibits visible growth of microorganism after overnight (ON) incubation *in vitro* (40). MICs are used in microbiological laboratories to measure the activity of an antimicrobial agent against a specific microorganism.
- **Clinical susceptibility and resistance:** Since the 1970s, clinical breakpoints (based on MIC values) have been determined and used in microbiology laboratories to categorize microorganisms as susceptible (S), intermediate (I), or resistant (R). The S/I/R categorization provides guidance to clinicians with respect to the potential success of a given agent in the treatment of an infection, caused by a specific organism. S, I and R has been defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (41).
- **Microbiological resistance:** A microorganism is defined as wild type for a species by the absence of acquired resistance mechanisms to the drug in question. The epidemiological cut-off value (ECOFF) is a MIC value, which identifies the upper limit of measured MIC values for a given wild type population. ECOFFs are considered when setting clinical breakpoints, and they are useful indicators of developing resistance in surveillance studies. There is no systematic relationship between clinical breakpoints and ECOFFs (41).
- **Setting clinical breakpoints:** From a clinical point of view, AMR is a relative phenomenon influenced by the following factors, which must be considered when setting clinical breakpoints: available formulations of the agent (e.g. oral or intravenous), standard and maximum dosing, clinical indications, target organism, MIC distributions and ECOFFs for individual species (wild-type), pharmacokinetic (PK) and pharmacodynamic (PD) data, clinical data relating outcome to MIC values, information on resistance mechanisms and the clinical significance of the resistance mechanisms. Of notice, clinical breakpoints for susceptibility testing should not divide wild-type distributions of the target species (42).

- **Antimicrobial susceptibility testing:** Antimicrobial susceptibility testing (AST) may be performed in several ways. The most common method used for AST in routine laboratories is conventional disk diffusion, which categorize microorganisms as S, I or R. The use of plastic strips, containing an antimicrobial concentration gradient (gradient test), is a convenient way to generate MIC data on agar plates. Broth dilution is considered the gold standard of MIC determination, but is not commonly used in routine laboratories (43). Recently, a standardized disk diffusion method (The EUCAST method) was validated and implemented in several European countries, including Norway (44, 45). Furthermore, automated AST systems (e.g. Vitek2 and Phoenix) are commonly used for AST in routine laboratories, and offer the convenience of combining species identification and MIC determination for relevant agents.

1.4.2 Mechanisms of resistance

Antibacterial resistance may be intrinsic (natural) or acquired. Intrinsic bacterial resistance to antibacterials, produced by other bacteria or fungi, existed in the environment before antibacterial compounds were taken into clinical use. Bacteria, furthermore, have the remarkable ability of environmental adaptation by changing their genome through mutations or by horizontal gene transfer (HGT), or by differential gene expression (46, 47). The extensive use of antibiotics in humans, but also in food animals and fish farming, has led to a selective pressure in several environmental niches promoting acquisition of resistance determinants.

Resistance may be acquired in three ways: (i) mutations in chromosomal genes, causing altered antibacterial targets or transcriptional changes, e.g. mutation in chromosomal topoisomerase/gyrase genes leading to fluoroquinolone resistance (48), (ii) acquisition of new genes by horizontal gene transfer (HGT) , e.g. plasmid-mediated acquisition of β-lactamase encoding genes (49), or (iii) mutations in previously acquired genes, e.g. mutation in the β-lactamase genes $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$, resulting in production of enzymes with a broader spectrum (50).

Four biochemical mechanisms of antibacterial resistance have been described (51): (i) inactivation or modification of antibiotics by antibiotic-modifying enzymes, e.g. β-lactamases (50) and aminoglycoside modifying enzymes (52), (ii) modification of the target molecule, e.g. modifications of PBPs in *S. pneumoniae* (53) and *Enterococcus faecium* (54), (iii) restricted access to the target of an antibiotic due to reduction of porins in the outer membrane of Gram-negative bacteria, and (iv) efflux of one or more antibiotic groups from the bacterial cell due to efflux pumps in the cytoplasmic membrane. Bacteria may combine two or more of these mechanisms. Furthermore, in *E. faecium* a bypass mechanism has been described to cause glycopeptide resistance (55).

1.4.3 Mechanisms of β-lactam resistance

Resistance to β-lactams may be PBP-mediated, or caused by production of β-lactamases. Porin loss or down-regulation may cause β-lactam resistance alone, or in combination with β-lactamase production. In *Pseudomonas aeruginosa* down-regulation of the porin OMPD2 in combination with production of chromosomal AmpC β-lactamase may result in an imipenem resistant phenotype (56). PBP-mediated resistance may be caused by acquisition of foreign PBPs, e.g. acquisition of the gene encoding PBP2a in methicillin resistant *S. aureus* (MRSA) (57), or by modifications of PBPs, e.g. penicillin non-susceptible *S. pneumoniae* (PNSP) due to mosaic PBPs (58).

β-lactamase production is the most common mechanism of β-lactam resistance in Gram-negative bacteria. β-lactamases are enzymes, which may inactivate β-lactam antibiotics by hydrolysing the amide bond of the β-lactam ring (59). β-lactamases may be classified based on their primary structure according to Ambler (60), or due to their functional characteristics (i.e. the enzymes abilities to hydrolyse different β-lactam classes) according to Bush-Jacoby-Medeiros (61-64). The serine β-lactamases (Ambler class A) share several highly conserved amino acid sequences with PBPs, from which they probably evolved (65, 66). As for the β-lactam antibiotics, also the β-lactamases vary in their spectrum of activity depending on the structure of their side chains, and

they may be subdivided into: (i) narrow-spectrum β-lactamases (penicillinases), (ii) broad-spectrum β-lactamases (ampicillinases), (iii) extended-spectrum β-lactamases (ESBLs), which may hydrolyse 3rd and 4th generation cephalosporins and monobactams, and (iv) carbapenemases, which may hydrolyse all β-lactams, including the carbapenems.

1.5 β-lactams and β-lactamases in a historical perspective

As penicillin was taken into clinical use during the early 1940s, it was considered the "magic bullet" against all Gram-positive bacteria (65). Infections caused by *S. aureus*, *Streptococcus pyogenes* and *S. pneumoniae* could effectively be cured. However, the understanding of antibiotic prescription and infection control was rudimentary, and little awareness of AMR existed.

The first β-lactamase (i.e. penicillinase) in clinical isolates of *S. aureus* was observed only a few years after the introduction of penicillin (67). Soon, a pandemic of hospital-associated staphylococcal infections due to penicillinase producing clones emerged worldwide (65). In the early 1960s, the penicillinase stabile methicillin was introduced to combat staphylococcal infections, and more attention was paid to infection control and prudent use of antibiotic. During the next two decades, several new β-lactams with a broader antimicrobial spectrum were released and widely used. Ampicillin was the first penicillin with activity against Gram-negative bacteria. In 1963, the first clinical *E. coli* isolate producing an R-factor or plasmid-mediated broad-spectrum β-lactamase (i.e. ampicillinase) was isolated in Athens from a patient called Temoneira, and thus named TEM-1 after this patient (68). Soon after, a second plasmid-mediated ampicillinase, SHV-1, was detected among *K. pneumoniae* and other Enterobacteriaceae. Both TEM-1 and SHV-1 encoding genes, carried by conjugative transposons and plasmids, spread rapidly and became ubiquitous in Gram-negative bacteria (69) and TEM-1-producing *K. pneumoniae* became endemic in many hospitals (65).

During the 1980-90s, several β-lactams with extended-spectrum were introduced. A new era of antibacterial therapy had begun. However, as environmental,

commensal and pathogenic bacteria were exposed to the new agents, novel β-lactamases with extended-spectrum rapidly emerged among clinical isolates. Already in 1979, induction of the chromosomal AmpC β-lactamase of *Enterobacter cloacae* was recognized (70, 71). In 1983, the first *K. pneumoniae* isolate resistant to 3rd generation cephalosporins was discovered in Germany (72). The new β-lactamase, which conferred resistance to cefotaxime, was a mutation-driven alteration of the existing SHV-1 enzyme, and thus designated SHV-2 (73). The first TEM-derived β-lactamase conferring resistance to cefotaxime, TEM-3 (initially named CTX-1), was reported in *K. pneumoniae* from France a few years later (74, 75). In 1988, the term extended-spectrum β-lactamase (ESBL) was introduced (76). Due to widespread use of broad-spectrum cephalosporins, such as ceftazidime and cefotaxime, numerous TEM- and SHV-mutants with extended spectrum evolved. During the early 1990s, ESBL-producing *K. pneumoniae* emerged as a nosocomial pathogen, causing hospital-acquired infections and hospital outbreaks (77, 78). In 1989, a clinical cefotaxime resistant *E. coli* isolate, producing a non-TEM/SHV ESBL, was recognized in Munich. The new enzyme was designated CTX-M due to its predominant activity against cefotaxime rather than ceftazidime, and the geographical reference (79). A shift from the predominance of TEM- and SHV-enzymes among *K. pneumoniae* in the hospital setting to the dissemination of CTX-M-enzymes among *E. coli* and *K. pneumoniae*, also in the community, was observed (80-82).

The first plasmid-mediated AmpC β-lactamases were reported in *K. pneumoniae* in 1990 (83). In 1991, the first oxacillinases (OXA-10-group) conferring resistance to 3rd generation cephalosporins were detected in Turkey (84). Since 1990, several new ESBL-groups conferring resistance to the carbapenems have been detected in clinical isolates all over the world. Imipenem resistance in *P. aeruginosa* due to a plasmid-mediated metallo-β-lactamase (MBL) was reported from Japan in 1991 (85). The first carbapenem hydrolysing OXA-enzyme (OXA-23) was described in *Acinetobacter baumanii* in 1993 (86). OXA-48 was identified in a Turkish carbapenem resistant *K. pneumoniae* isolate in 2001 (87). The first *K. pneumoniae* carbapenemase (KPC-1) was reported from USA in 2001 (88). In 2009, a carbapenem resistant *K. pneumoniae* isolate, producing a novel MBL, designated New-Delhi

metallo-β-lactamase-1 (NDM-1), was isolated from a Swedish patient (89). NDM-1 has the potential to spread rapidly among clinically relevant Enterobacteriacea (90). Thus, the detection of this enzyme attracted much attention and publicity. The current spread of the carbapenamases KPC, VIM, IMP and NDM among Enterobacteriaceae cause great concern (91).

1.6 Extended-spectrum β-lactamases (ESBLs)

1.6.1 ESBL-classifications and definitions

ESBLs may, like other β-lactamases, be classified based on their primary structure according to Ambler (60), and their functional characteristics according to Bush-Jacoby (64). Due to these classification systems, a "classical ESBL" is a molecular class A and a functional class 2be enzyme, which hydrolyses extended-spectrum cephalosporins and monobactams, and is inhibited by clavulanic acid. These classification systems were convenient as long as the TEM- and SHV-derived ESBLs were dominating. However, due to the emergence of several new enzyme groups or families, including about 400 TEM- and SHV-ESBLs, 150 CTX-M- variants (92), the plasmid-borne AmpCs and the diverse spectrum of carbapenamases, the scientific nomenclature has become too complex for daily clinical use. Also the ESBL-definition has been debated (93). In 2009, Giske *et al.* proposed to include plasmid-mediated AmpC, OXA-enzymes and carbapenamases in the ESBL-definition, and a simplified ESBL-classification was introduced (94). ESBLs were proposed classified in to three main categories: $ESBL_{A}$, $ESBL_{M}$, and $ESBL_{CARBA}$. The "classical ESBLs" were designated $ESBL_{A}$ in the novel classification. $ESBL_{M}$ was subdivided into $MSBL_{M-C}$ and $ESBL_{M-D}$, whereas $ESBL_{CARBA}$ was subdivided into $ESBL_{CARBA-A}$, $ESBL_{CARBA-B}$ and $ESBL_{CARBA-D}$ (Table 2). In contrast to former definitions, the novel ESBL-definition includes all acquired β-lactamases with hydrolytic activity against extended-spectrum cephalosporins and/or carbapenems. The ESBL-definition and -classification, proposed by Giske *et al*., will mainly be applied in this thesis.

Table 2. The Extended-Spectrum β-Lactamase (ESBL) classification scheme of Giske *et al*. (modified from reference 94).

ESBL-classes		Examples	Definitions	Ambler[a]	Bush-Jacoby[b]
$ESBL_A$	"Class A ESBLs"	CTX-M, TEM- and SHV-ESBLs	Non-susceptibility to extended-spectrum cephalosporins and monobactams Inhibited by clavulanic acid	Class A	Group 2be
$ESBL_M$	"Miscellaneous ESBLs"		Non-susceptibility to extended-spectrum cephalosporins		
$ESBL_{M-C}$	Plasmid-mediated AmpC	CMY and DHA	Inhibited by cloxacillin and boronic acid	Class C	Group 1
$ESBL_{M-D}$	OXA-ESBLs	OXA-10-group	Inhibitor not known	Class A	Group 2de
$ESBL_{CARBA}$	"ESBLs with hydrolytic activity against carbapenems"		Non-susceptibility to extended-spectrum cephalosporins and at least one carbapenem		
$ESBL_{CARBA-A}$	KPC and other class A carbapenemases	KPC and GES	Inhibited by boronic and dipicolinic acid	Class A	Group 2f
$ESBL_{CARBA-B}$	MBLs	IMP, VIM and NDM	Inhibited by EDTA and dipicolinic acid	Class B	Group 3a
$ESBL_{CARBA-D}$	OXA-carbapenamases	OXA-23 and OXA-48	Inhibitor not known	Class D	Group 2df

[a]From reference 60. [b]From reference 64.

1.6.2 ESBL-detection

$ESBL_A$-producing strains may exhibit reduced susceptibility to all cephalosporins and monobactams. However, the substrate profile differs among enzyme groups (e.g. in contrast to the SHV-ESBLs, CTX-M group 9 enzymes confer resistance to cefotaxime, but not to ceftazidime). Phenotypical tests, including the inhibitor clavulanic acid, are mainly used for the detection of $ESBL_A$ (95-97). During recent years also more rapid methods, such as the ESBL NDP test, have become available for ESBL-detection (98). For epidemiological purposes, molecular detection

may be added to determine the $ESBL_A$-type (99-101). CTX-M, TEM and SHV are the $ESBL_A$-enzymes most frequently detected, but other $ESBL_A$-types have also been described (102).

$ESBL_{M-C}$-producing strains exhibit reduced susceptibility to 1st and 2nd generation cephalosporins and cephamycins (cefoxitin and cefotetan), and intermediate susceptibility or resistance to 3rd generation cephalosporins and monobactams. In contrast to $ESBL_A$, $ESBL_{M-C}$-enzymes do not confer resistance to 4th generation cephalosporins (e.g. cefepime and cefpirome). $ESBL_{M-C}$ may be detected by phenotypic tests including cloxacillin or boronic acid (97, 103). Molecular methods are needed for epidemiological purposes, but also to differentiate plasmid-mediated $ESBL_{M-C}$-production from chromosomal AmpC-hyperproduction in *E.coli* (104, 105). Furthermore, derepressed chromosomal AmpC-production in some Enterobacteriaceae, such as *Citrobacter freundii* and *Enterobacter spp.*, may cause reduced susceptibility to 3rd generation cephalosporins and should also be discriminated from $ESBL_{M-C}$ (97). $ESBL_{M-D}$ can only be detected by molecular methods.

$ESBL_{CARBA-A}$-enzymes may confer reduced susceptibility to all β-lactam antibiotics. $ESBL_{CARBA-B}$-producing bacteria may be non-susceptible to all β-lactam antibiotics, except monobactams. OXA-48, the most common $ESBL_{CARBA-D}$-enzyme in Enterobacteriaceae (106), causes reduced susceptibility to carbapenems, but does not confer resistance to 3rd and 4th generation cephalosporins, and may thus be difficult to detect. $ESBL_{CARBA-A}$ and $ESBL_{CARBA-B}$-enzymes may be detected phenotypically by using the appropriate β-lactam/β-lactam-inhibitor combination. So far, there is no known inhibitor against $ESBL_{CARBA-D}$ enzymes, and their presence need to be confirmed by molecular tests. Of notice, OXA-48-producing strains are normally highly resistant to temocillin, which may be used as a diagnostic marker for this enzyme (107). Detection and discrimination of $ESBL_{CARBA}$-variants may be challenging. Thus, several phenotypic and molecular tests are available (97, 100, 108, 109). More recently, detection of carbapenamase production using MALDI-TOF MS has been proposed (110, 111).

$ESBL_A$ and $ESBL_M$-producing isolates are normally susceptible to carbapenems. However, the combination of $ESBL_A$ or $ESBL_M$-production and porin loss, or increased efflux, may result in a carbapenem non-susceptible phenotype (112), which should be discriminated from true $ESBL_{CARBA}$.

ESBLs, as defined by Giske *et al*., are encoded by acquired genetic elements, which frequently carry several resistance determinants, causing co-resistance to other important antibiotic groups in most ESBL-producing isolates. Thus, rapid ESBL-detection and communication of results both to clinicians and infection control staff is of great importance. Further information concerning both phenotypic and molecular detection of ESBLs and other relevant resistance mechanisms is available at the EUCAST website (113).

1.7 Spread of ESBLs

Antibiotic resistance genes may be located within the chromosome of a bacterium, or on mobile genetic elements (MGEs). Chromosomally located antibiotic resistance genes are mainly dispersed vertically to daughter cells through regular cell division (i.e. clonal spread). Resistance genes carried by MGEs may also be passed on horizontally (i.e. horizontal gene transfer) to more or less related bacteria (114). ESBL-encoding genes are mostly carried by conjugative plasmids or transposons, and thus efficiently disseminated both by vertical and horizontal modes (115).

1.7.1 Clonal spread

Some bacterial clones are referred to as “successful clones” due to favourable phenotypic traits (e.g. virulence or antibiotic resistance), which enable them to disseminate and persist in different environments, and cause human infections and outbreaks. Successful clones carrying antibiotic resistance determinants play a major role in the spread of resistance (116). Two examples are *E. coli* ST131 and *K. pneumoniae* ST258, which have contributed significantly to the worldwide spread of CTX-M-15 and KPC, respectively (117).

1.7.2 Horizontal gene transfer (HGT)

The transfer of foreign genes between organisms is referred to as horizontal gene transfer (HGT) (114). HGT most commonly take place between closely related organisms, but may also take place between different species, genera and kingdoms (for example between bacteria and plants). The association of HGT to the spread of antibiotic resistance among bacteria was recognized by Japanese investigators already in the late 1950s (118). There are three mechanisms of HGT: (i) transformation is the active uptake of free DNA from the environment by competent bacterial cells, (ii) transduction is an infection of bacteria by bacteriophages or bacterial viruses, who inject foreign DNA into a bacterial cell, and (iii) conjugation is the mechanism where a donor and a recipient cell physically connect to each other and share DNA carried by conjugative elements, such as plasmids and transposons (114). Foreign DNA captured by transformation, transduction or conjugation may be integrated into the bacterial chromosome, whereas plasmids normally exist as independent genetic entities in a bacterial cell (114).

1.7.3 Mobile genetic elements (MGEs)

The mobilization, transfer and persistence of MGEs between and within bacterial genomes, play an essential role in adaptive bacterial processes and bacterial evolution (46, 114). Processes facilitated by MGEs contribute to the intra-species variability and the distribution of genetic modules, encoding virulence or antibiotic resistance determinants, within bacterial communities. During transduction and conjugation, a multitude of different MGEs, such as bacteriophages, transposons, insertion sequence (IS) elements, integrons and plasmids, may be transferred between bacteria.

The most simplistic transposon, an IS-element, consists of a transposase-encoding gene surrounded by inverted repeated elements. A complex or composite transposon contains additional DNA, for example multiple antibiotic resistance genes flanked by two IS-elements. Transposases enable mobilization. Transposons may move within a genome, or between genomes, and cause high genome plasticity by

homologous recombination events (119). Integrons are genetic elements capable of capturing gene cassettes. Integrons are not mobile themselves, but may be incorporated into conjugative transposons. Super-integrons consisting of several gene cassettes and more than 60 different antibiotic resistance genes have been described (47). Insertion sequence common regions (IS*CR*) move genes through rolling-circle transposition, and are considered to be highly mobile genetic vehicles (120). ESBL-encoding genes are frequently integrated in transposons, integrons or IS*CR* elements, carried and disseminated by plasmids (121).

1.7.4 Plasmids

Plasmids are extra-chromosomal, self-replicating, double-stranded DNA elements, harboured by most bacterial cells. They are diverse in size, mode of replication, mode of transmission and host range. Whereas broad-host range plasmids may spread among and be maintained by a wide range of bacterial hosts, narrow-host range plasmids are only maintained by closely related bacterial hosts. The plasmid backbone harbours conserved core genes essential for replication, transfer and maintenance (Figure 2). The replicon is a highly conserved region, which encodes genes needed for plasmid replication initiation and control (i.e. copy number). A plasmid may have one single or several compatible replicons (122). Plasmids may be conjugative, mobilizable or non-mobilizable. Conjugative, or self-transferable, plasmids carry a set of mobility genes and a membrane-associated mating pair formation, providing the mating channel. Some plasmids carry mobility genes, but use the mating channel of another plasmid, and are called mobilizable (123). The plasmid core genome may also encode plasmid-partitioning and post-segregational killing systems, ensuring the maintenance of a plasmid in a bacterial population after cell division (124). In their variable regions, plasmids frequently carry virulence and antibiotic resistance determinants (Figure 2). Thus, a plasmid may supply its host with favourable phenotypic traits, which may increase the survival of the host in a given environment (125, 126).

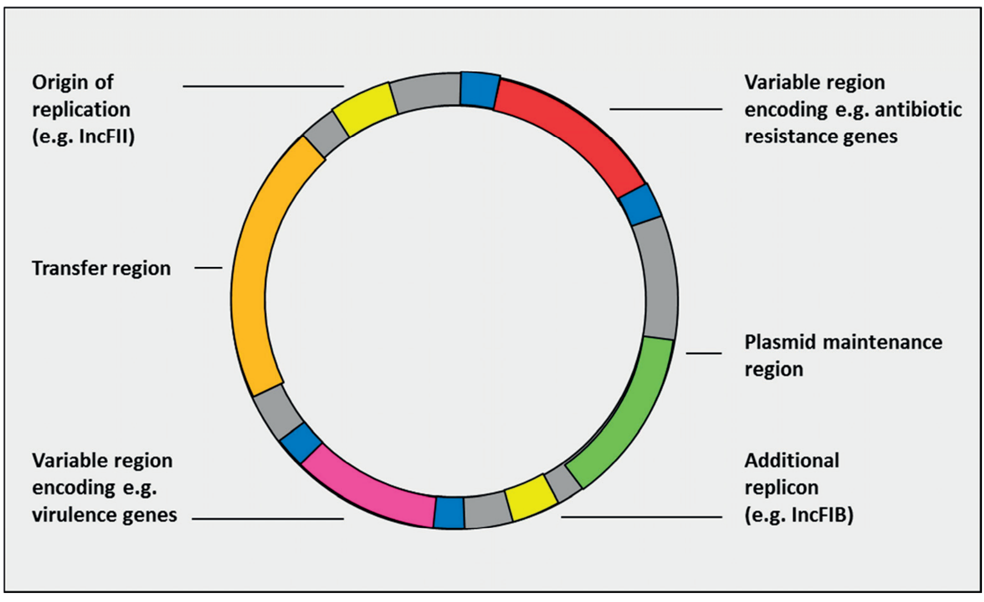

Figure 2. A simplified illustration of a typical IncF plasmid. The backbone/core regions include: replicons (yellow), transfer region (orange) and maintenance region (green). Variable regions (red and pink) are flanked by IS elements (blue). Undefined regions are grey.

Evolutionary, plasmids are dynamic entities capable of adaptation to new environments and hosts by shearing and rearranging genetic elements. Thus, the variable regions of most plasmids are genomic mosaics of genes and MGEs that may continue to jump and rearrange within a plasmid, between plasmids and between plasmids and chromosomes due to changes in their environment. Plasmids were initially thought to cause a metabolic burden or fitness cost to their bacterial host (127). However, amelioration of initial costs or even increased fitness for bacterial hosts through compensatory evolution between host and plasmid, also in the absence of selective pressure, has been demonstrated in several studies (128-130).

Historically, plasmids are classified based on plasmid incompatibility (131); i.e. the principle that plasmids with the same replicon type cannot be stably maintained by one host cell (132). Plasmid typing was traditionally performed by competition assays, where a new plasmid was introduced to an isolate carrying a plasmid of known incompatibility type (Inc type) (133). Today, a more convenient PCR-based replicon typing (PBRT) method has replaced the former competition assays (134). However,

due to the genetic plasticity of plasmids, two plasmids of the same Inc or replicon type may be very diverse in their variable regions.

1.7.5 ESBL-plasmids

Plasmids are considered effective vehicles for the spread of antibiotic resistance determinants. In Enterobacteriaceae, IncFII, IncA/C, IncL/M, IncN and IncI are among the most common plasmid types carrying ESBL-encoding genes. *Bla*$_{CTX\text{-}M\text{-}15}$ is predominantly associated with the IncF family, but also IncR, IncI1, IncA/C, IncL/M and IncN type plasmids have been reported to carry *bla*$_{CTX\text{-}M\text{-}15}$ (135-139). The IncF family consists of a heterologous group of narrow-host range low copy number plasmids, which vary in size from 50 to 200 kb. The host range of IncF plasmids is limited to the Enterobacteriaceae family. IncF replicons may be classified into IncFII, IncFIA, IncFIB and IncFIC. The IncFII replicon has been further subdivided into IncFII$_S$, IncFII$_Y$ and IncFII$_K$ due to sequence variations and preferred host, *Salmonella* spp., *Yersinia* spp. and *Klebsiella* spp., respectively (140). In *K. pneumoniae*, IncFII$_K$ plasmids have been associated with several clinical important β-lactamases, including CTX-M-15, KPC-2, KPC-3 and NDM-1. In contrast to the rather clonal spread of *bla*$_{KPC}$ among *K. pneumoniae* ST258, *bla*$_{CTX\text{-}M\text{-}15}$ seems to disseminate among unrelated *K. pneumoniae* clones (137, 141). IncFII$_K$ plasmids carrying *bla*$_{CTX\text{-}M\text{-}15}$ in *K. pneumoniae* have been associated with nosocomial outbreaks, indicating that this plasmid family may encode further phenotypic traits, which may increase the fitness and survival of their host in the hospital environment (20, 21, 142, 143).

1.8 Epidemiology of ESBL-producing Enterobacteriaceae (ESBL-E)

The overall prevalence of ESBL-producing Enterobacteriaceae (ESBL-E) in clinical isolates from Scandinavian countries is low compared to countries in the Southern and Eastern Europe (80, 81). Of notice, the ESBL-rates differ among Scandinavian countries, and the rates of invasive *K. pneumoniae* isolates resistant to 3rd generation cephalosporins were considerably higher in Denmark (10-25%) compared to Norway and Sweden (1-5%) as reported by the European Antimirobial

Resistance Surveillance Network (EARS-Net) in 2012 (144). In Scandinavian countries, $ESBL_A$-producing *E. coli* and *K. pneumoniae* are the most common ESBL-producers detected in clinical samples, CTX-M-15 being the most prevalent enzyme (145-150). CTX-M-15-producing strains have also caused hospital outbreaks in Scandinavian countries (142, 151, 152).

In Norway, the prevalence of ESBL-production among *E. coli* and *K. pneumoniae* blood culture isolates in 2012 was 5.5% and 2.9%, respectively (2). Although the ESBL-rates are still low, a 10-fold increase has been observed in Norwegian invasive *E. coli* and *K. pneumoniae* isolates from 2003 to 2012 (Figure 3).

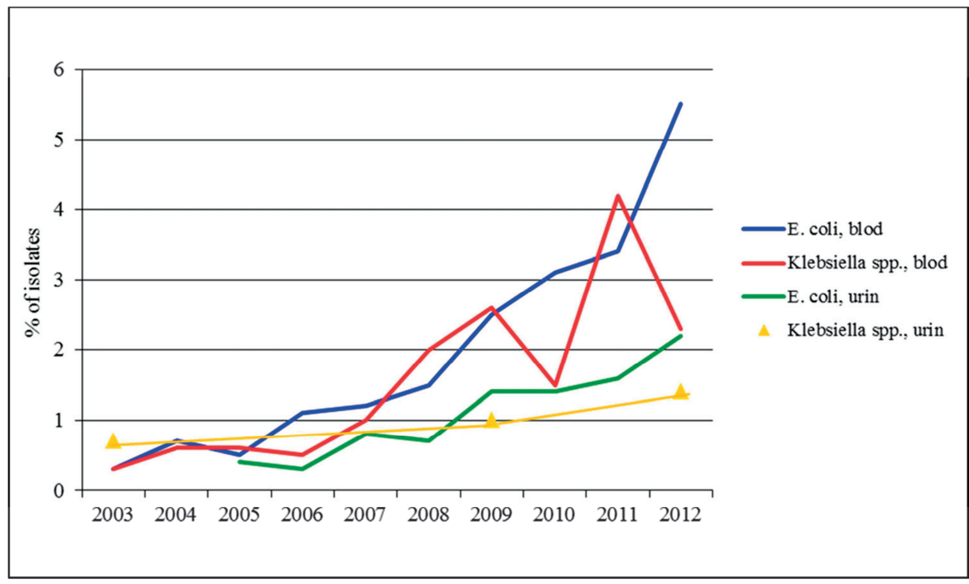

Figure 3. Prevalence of ESBL-production among Norwegian *E. coli* and *Klebsiella* spp. isolates from blood and urine 2003-2012 (Source: NORM/NORM-VET. 2012). Figure is reprinted with permission from NORM/NORM-VET.

The CTX-M-enzymes are the most common ESBLs also in Norwegian isolates, and constituted 60-80% of $ESBL_A$ detected in invasive *Klebsiella* spp. isolates from 2009 to 2012 (Figure 4). $ESBL_{M-C}$-enzymes are sporadically detected among human clinical isolates in Norway (153). However, high colonization rates (up to 43%) of $ESBL_{M-C}$-producing *E. coli* have recently been detected among Norwegian broilers (154). Sporadic occurring $ESBL_{CARBA}$-producing isolates are mostly associated with travel or previous hospitalization in countries outside Europe (155-157).

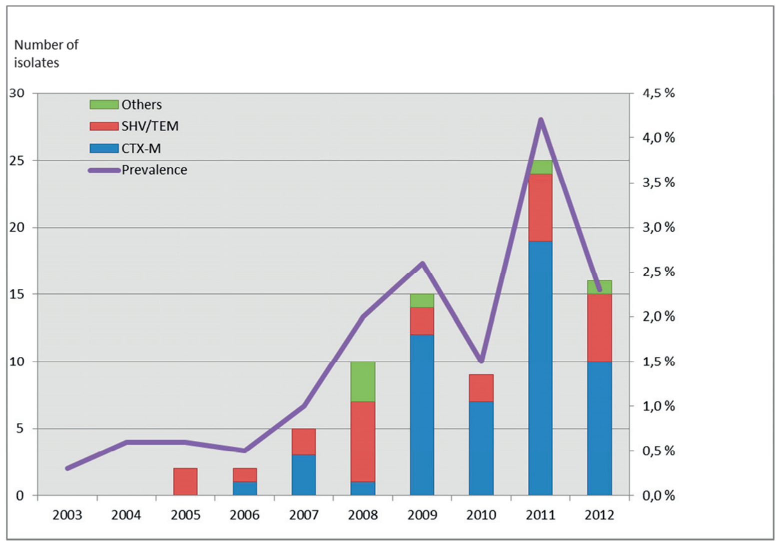

Figure 4. ESBL-enzyme distribution in Norwegian *Klebsiella* spp. blood culture isolates from 2003 to 2012 (Sources: NORM/NORM-VET. 2003-2012 and K-res). Figure is printed with permission from K-res.

EARS-Net annually reports the proportion of resistance to 3rd generation cephalosporins, carbapenems, fluoroquinolones, aminoglycocides and other relevant antibacterial agents in invasive isolates of *E. coli*, *K. pneumoniae, A. baumanii, P. aeruginosa, E. faecalis/faecium, S. aureus and S. pneumoniae* based on data from 30 European countries. Figure 5 illustrates the increasing prevalence of resistance to 3rd generation cephalosporins in European *K. pneumoniae* isolates from 2005 to 2012. In 2012, the proportion of resistance to 3rd generation cephalosporins in invasive *K. pneumoniae* isolates varied from 1-5% in Norway, Sweden and Finland to >50% in

Greece and Eastern European countries. A similar geographical distribution of resistance to carbapenems in *K. pneumoniae,* and to 3rd generation cephalosporins and carbapenems in *E. coli* was reported (144). The geographical differences in the prevalence of MDR-strains within Europe may reflect more prudent use of antibacterial agents and more comprehensive infection control practices in the North and West of Europe, compared to the South and East (144).

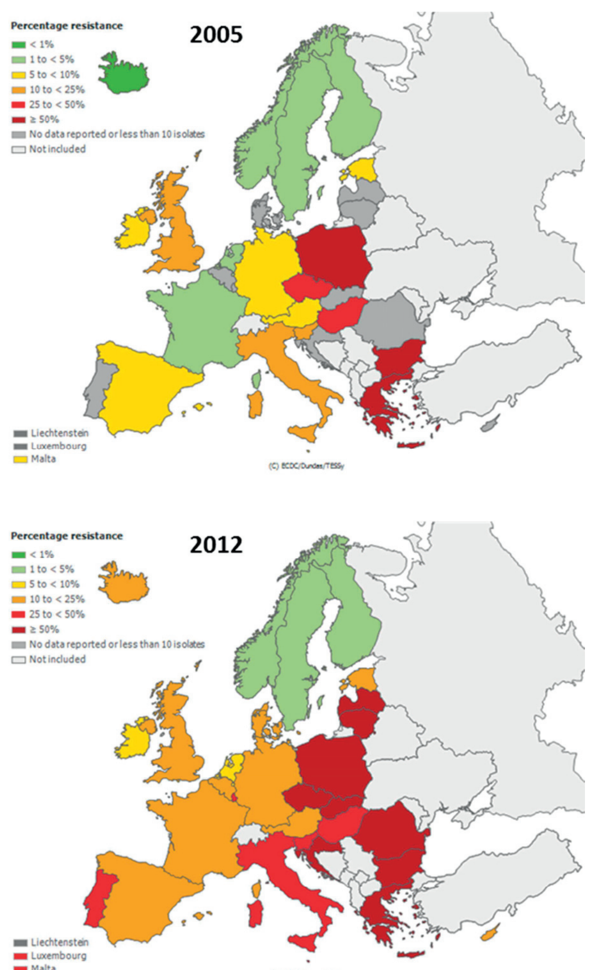

Figure 5. Proportion of 3rd generation cephalosporins resistant invasive *Klebsiella pneumoniae* isolates in European countries in 2005 and 2012 (Source: ECDC/EARS-Net Maps, http://www.ecdc.europa.eu).

Also outside Europe, the prevalence of ESBL-producing bacteria varies a lot between countries and continents, and from some areas data are still scarce. ESBLs are assumed to have emerged in Asia (158), and reports from Asia have documented alarming high prevalence of ESBL-producing MDR strains. In a recent study from India, ESBL-production was reported in 69% and 41% of clinical *E. coli* and *K. pneumoniae* isolates, respectively (159).

Due to international tourism and migration, an influx of ESBL-producing MDR bacteria from high prevalence areas, like India, South East Asia and the Middle East, to low-prevalence areas, like Scandinavia, has been documented by several studies. A recent Swedish prospective study, revealed that 68/226 (30%) travellers with an ESBL-negative pre-travel sample acquired ESBL-E during travel outside Scandinavia. The most common species acquired was *E. coli* (90%) and the most common ESBL-type was CTX-M (73%). The most important risk factors for ESBL-acquisition were travel to the Indian subcontinent, Asia and Africa North, as well as gastrointestinal symptoms (160). Similar results are reported from other travel studies performed in Sweden, Canada, Spain and the Netherlands, emphasizing the contribution of travel to the global distribution of ESBL-E (161-166).

1.8.1 The CTX-M pandemic

A number of review articles have addressed the rapidly emerging CTX-M-type ESBLs, which are now endemic in many European countries, in Asia and in South America (115, 167-173). Some CTX-M-enzymes have been associated with specific geographical areas; CTX-M-9 and CTX-M-14 are prevalent in Spain, whereas CTX-M-2 is prevalent in South America (167, 174, 175). CTX-M-15, which was first detected in India in 2001 (176, 177), has spread to all continents (178). Both the dissemination of successful bacterial clones, such as *E. coli* ST131, and plasmid-mediated spread of CTX-M-encoding genes, has contributed to the current situation (179). For the dissemination of some CTX-M-types, specific plasmid types seem to have played a major role. The spread of CTX-M-15 has been associated with IncFII plasmids (115). The early spread of CTX-M-3 in Poland and Eastern Europe was

facilitated by IncN plasmids (180). The more recent dissemination of CTX-M-14 in Spain and the UK seem to be favoured by IncK plasmids (181-184).

The CTX-M family may be subdivided into five phylogenetic clusters: CTX-M group 1, CTX-M group 2, CTX-M group 8, CTX-M group 9 and CTX-M group 25, which differ from each other by $\geq$10% amino acid residues. The most common enzymes, CTX-M-15 and CTX-M-14, are found within CTX-M group 1 and 9, respectively (173). CTX-M-encoding genes have been detected in several Enterobacteriaceae species, such as *Salmonella enterica*, *Shigella* spp., *Serratia marcescens* and *Proteus mirabilis* (172, 173), but are most commonly found in *E. coli* and *K. pneumoniae*. More rarely, non-enterobacterial species, like *P. aeruginosa*, *Acinetobacter* spp., *Vibrio* spp., *Aeromonas* spp. and *Stenotrophomonas maltophilia* have been found to produce CTX-M-enzymes (173).

Unlike the TEM- and SHV-type ESBLs, which evolved by selection of plasmid-mediated TEM- and SHV-type β-lactamase mutants, the CTX-M-encoding genes are most likely captured from the environmental metagenome (173). Mobilization of $bla_{\text{CTX-M}}$ from the chromosome of *Kluyvera ascorbata*, *K. cryocrecens* and *K. georgiana* to plasmids has been described (185-187). *Kluyvera* belong to the Enterobacteriaceae family, but are rarely involved in human infection (188). Multiple gene capture events from different *Klyuvera* spp. likely explain the heterogeneity of the CTX-M family (173). CTX-M-encoding genes are mainly carried by conjugative plasmids in Enterobacteriaceae (172). More rarely, $bla_{\text{CTX-M}}$ has also been found integrated into the chromosome, as described in *P. mirabilis* (189). IS elements, such as IS*Ecp1* and IS*CR1*, are involved in the capture of $bla_{\text{CTX-M}}$ from the *Kluyvera* chromosome and its mobilization to plasmids (120, 190, 191). The ISs have, furthermore, been described to be strong promotors for high-level expression of $bla_{\text{CTX-M}}$, conferring clinical resistance to the bacterial host (192, 193). IS*Ecp1* has been associated with all CTX-M-groups, and is likely the most relevant IS element in the capture and mobilization of CTX-M genes (172). The IS*Ecp1*-$bla_{\text{CTX-M}}$ module has also been associated with IS*26* (194) and Tn*3*-like transposons, frequently integrated in IncF plasmids (195).

Plasmids were also widely distributed among Enterobacteriaceae in the pre-antibiotic era (196, 197). IncF plasmids are known to be well adapted to *E. coli* (198). The uptake of resistance genes into plasmids already well adapted within a bacterial population may be a key factor for the successful dissemination of resistance genes (117). It has been proposed that plasmid-mediated dissemination is the cornerstone of the current CTX-M pandemic (171). To better understand the dynamics of the pandemic, $bla_{CTX\text{-}M}$-bearing plasmids must be carefully characterized and studied. New methods, such as full DNA sequencing, may shed new light on the dynamics of plasmid-mediated spread and persistence of resistance (199).

1.9 Human ESBL-E carriage

In humans, the intestine is the main reservoir for Enterobacteriaceae, and thus for ESBL-E. Intestinal colonization, moreover, normally precede Enterobacteriaceae infection (e.g. UTI), and is therefore of clinical interest (200-202). Grundmann *et al*. reported that only 15% of ICU infections were due to patient-to-patient transmission, (203), which underlines the importance of patients carrier states also when it comes to ESBL-E infection.

The first reports on human faecal ESBL-carriage were from Spain. Mirelis *et al*. reported ESBL-carriage rates of 2.1% in stools from patients at hospital admission in 2001, and a significant increase in carriage rates to 7.5% in 2002 (204). Valverde *et al*. reported an increase in faecal ESBL-carriage rates from 0.3% to 11.8% in hospitalized patients and from 0.7% to 5.5% in outpatients during the period 1991-2003. They also reported a faecal ESBL-carriage rate of 3.7% among healthy volunteers in 2003 (205). Since then, several studies have reported rates of intestinal ESBL-carriage in hospitalized patients, outpatients and among healthy volunteers in the community (206-215).

Risk factors for being colonized by ESBL-E have been identified by several studies in different settings (160, 216-219). Tumbarello *et al*. identified recent hospitalization (≤ 12 months), transfer from another health care facility, comorbidity, recent β-lactam or fluoroquinolone treatment (≤ 3 months), recent urinary

catheterization and age ≥ 70 years as risk factors for ESBL-E colonization in adult patients at hospital admission (220).

1.9.1 ESBL-E carriage in the community

Recently, Woerther *et al*. comprehensively reviewed the existing literature on carriage rates of ESBL-E among healthy subjects in the community. Based on data published between 2001 and 2011, they created a figure illustrating the ESBL-E carriage rates in six different geographic regions (according to the World Health Organization (WHO) region grouping) during this period (Figure 6 a). As illustrated by the figure, the Eastern Mediterranean and South East Asia showed the highest carriage rates and the most ascending trends of carriage (199). In some reports from Thailand and Egypt, ESBL-E carriage rates of 50-60% were documented (211, 221, 222). In contrast, carriage rates reported from Europe did not exceed 12%, as reported in a study from Belgium (223). In all areas, ESBL-E carriage rates were increasing from 2001 to 2011, and CTX-Ms were the most prevalent ESBL-enzymes worldwide (199). Furthermore, the authors estimated the number of ESBL-E carriers in the six WHO areas for 2010. In South East Asia, over 1.1 billion ESBL-E carriers were estimated, whereas 35 million carriers were estimated in Europe (Figure 6 b).

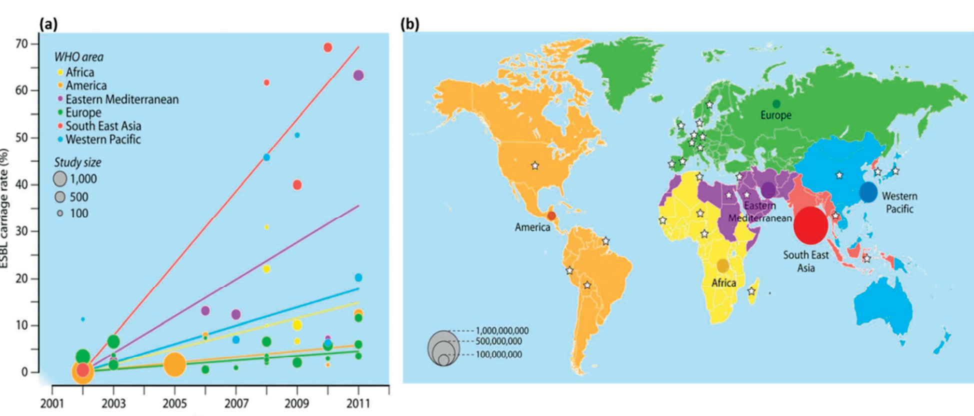

Figure 6. (a) ESBL-carriage rates in the community of the six WHO areas; based on data published between 2001 and 2011. **(b)** Estimated number of ESBL-carriers in the community of the six WHO areas in 2010; based on the data analysed in the review and the WHO 2010 population census (Source: Woerther *et al.*, Clin Microbiol Rev 2013). Figures are reprinted with permission from Clinical Microbiology Reviews.

Due to the differences in the number of expected carriers between regions, the authors suggest poor access to drinking water, poverty and a high population density to be important driving forces for ESBL-E carriage and dissemination in the community (199). In low-prevalence countries, factors such as recent use of broad-spectrum antibiotics (e.g. β-lactams and flouroquinolones) and travel to high endemic areas like Asia, the Middle East and North Africa, have been associated with increased risk of being colonized by ESBL-E (160, 217). More recently, ESBL-colonization in food animals and the possibility of ESBL-spread from animal to human via the food chain has been suggested to be an important factor for the dissemination of ESBL in the community (224-227).

ESBL-E colonization among healthy children has not yet been well studied. However, in a recent Swedish study, the ESBL-E carriage rate in healthy preschool children was found to be 2.9% (9/313) (228). Similar ESBL-E carriage rates were reported among healthy children in Boliva, Peru and Portugal (229, 230). In a recent French study, 6.7% (28/419) of children in day-care centres were found to be ESBL-E carriers (231). Both the Swedish and the French study reported transmission of ESBL-E among children attending the same preschool or day-care centre. The authors of the Swedish study suggest that crowding and insufficient hygiene standards in a preschool may have greater impact on transmission than a short course of penicillin or underlying medical conditions (228).

1.9.2 Duration of ESBL-E carriage

The duration of faecal ESBL-carriage has more recently been investigated in different settings.Various methods and study designs are used in these studies. Thus, the results may not easily be compared. Faecal carriage of ESBL-producing *E. coli* for up to 59 months was observed in 13% (5/39) of adult patients after a nosocomial outbreak in Sweden (232). In adult patients with traveller's diarrhoea, who were also carriers of ESBL-producing *E. coli*, 24% (10/41) were ESBL-carriers 3-8 months after the first sample, and 7% (3/41) were still carriers after three years (233). A prospective follow-up study of 100 healthy adult Swedish travellers revealed that 24 had acquired ESBL-producing *E. coli* during travel outside Northern Europe, of which 24% (5/24)

were still colonized after six months (166). A French study reported mean duration of faecal ESBL-E carriage of nine months in 22 children adopted from Mali (234). A recent Swedish study reported faecal carriage of ESBL-producing *E. coli* or *K. pneumoniae* in 43% (26/61) of adult patients for 12 months after ESBL-related infections (235). Birgand *et al*. demonstrated that 39% (180/446) of patients were persistent ESBL-E carriers at readmission after previous ESBL-E infection or positive screening. The median time to ESBL-E clearance in these patients was 6.6 months (236). In a study from the Netherlands, the median time until ESBL-E clearance in adults after ICU discharge was 1.4 months (237).

As described above, risk factors for being or becoming colonized by ESBL-E have been identified. Risk factors for prolonged ESBL-E carriage have until present only been studied in a few settings. Titelman *et al*. recently found that prolonged faecal ESBL-E carriage after ESBL-associated infection is associated with carriage of *E. coli* phylogroup B2 and CTX-M-group-9 (235). Birgand *et al*. concluded that prolonged ESBL-E carriage was associated with having the first positive ESBL-culture from a clinical sample, compared to having the first positive ESBL-culture recovered from a screening sample (236). Also risk factors for becoming colonized by ESBL-E during a NICU outbreak have been identified, as described in section 1.10. However, the duration of ESBL-E carriage and risk factors for prolonged carriage in children colonized during NICU hospitalization, have to our knowledge not yet been investigated.

In contrast to healthy adults and older children, newborn children have not yet an established gut microbiota. The colonization, development and maturation of a child's gastrointestinal tract begin at birth, and has been found to continue at least until five years of age (238). In particular, the early intestinal colonization process may be influenced by numerous factors, including mode of delivery, feeding regime, maternal diet and weight, probiotic use and antibiotic exposure. Infants hospitalized in NICUs are often exposed to several factors, which may alter the development and composition of their normal intestinal microbiota, and thus make them vulnerable to colonization by bacteria from the NICU environment (239-244).

1.9.3 Intra-household ESBL-E transmission

Transmission of ESBL-E within households, however, has only been investigated in a limited number of studies. ESBL-E was detected in 27.4% (20/73) of household contacts (HCs) in a study from Spain, where adults with community-acquired UTIs caused by ESBL-producing *E. coli* were index cases (218). A second Spanish study reported ESBL-E colonization in 16.7% (9/54) of HCs of persons with community-acquired ESBL-E infections (202). In a French study, intra-household transmission was suspected in 23% (5/22) of HCs of adoptive children from Mali colonized with ESBL-E (234). In a study from Hong Kong, a CTX-M carriage rate of 43.5% was found in children (0-5 years of age) upon hospital admission. In 83% (44/53) of their respective households, at least one HC was also colonized by a CTX-M-producing strain. The number of HCs colonized within a household was inversely correlated with the living space per person (215).

1.10 Nosocomial outbreaks

An outbreak or an epidemic may be defined in different ways. The World Health Organisation definition of an outbreak is: "A disease outbreak is the occurrence of cases of disease in excess of what would normally be expected in a defined community, geographical area or season. An outbreak may occur in a restricted geographical area, or may extend over several countries. It may last for a few days or weeks or for several years" (245). Tacconelli *et al*. recently defined an epidemic or outbreak of MDR Gram-negative bacteria as following: "Settings where there is an unusual or unexpected increase of cases of infections due to MDR-GNB (Gram-negative bacteria) already isolated in the hospital or an emergence of cases of infection due to a new MDR-GNB, with or without molecular analysis of strains" (246).

S. aureus, including MRSA, and MDR Gram-negative bacteria are frequently associated with nosocomial (i.e. hospital associated) outbreaks. *Klebsiella* spp., *Staphylococcus* spp., *Serratia* spp. and *Enterobacter* spp. are the pathogens most frequently involved in NICU outbreaks, whereas *Staphylococcus* spp., *Pseudomonas* spp. and *Acinetobacter* spp. more often cause outbreaks in adult ICUs (247).

Outbreaks caused by MDR bacteria are generally more common in ICUs than in other hospital wards. ICU patients are often immunocompromised and susceptible to colonization by bacteria from the hospital environment. Furthermore, broad-spectrum antibiotics and invasive procedures are frequently used in ICUs. Invasive equipment, such as bronchoscopes, has been reported to be the source of patient-to-patient transmission of outbreak strains (248-250).

ESBL-producing *K. pneumoniae* has become a common cause of nosocomial outbreaks, especially in NICUs (251-256). Factors, such as overcrowding, understaffing, insufficient hygienic barriers and frequent use of broad-spectrum antibiotics, often facilitate the dissemination of MDR strains in NICUs and between NICU patients (257). Risk factors for ESBL-colonization during a NICU outbreak, e.g. low gestational age, low birth weight, use of invasive devices, antibiotic treatment and prolonged hospital stay, have been identified by several studies (253, 258-260). The gastrointestinal tract of patients is an important reservoir for ESBL-E (261), and transient hand carriage of ESBL-producing *K. pneumoniae* among HCWs has been reported to be one of the most common mechanisms of ESBL-transmission between infants during NICU outbreaks (251, 262-264).

Gastmeier *et al*. reviewed NICU outbreaks reported in the worldwide database of health-care associated outbreaks, and found that *Klebsiella* spp. caused 20.3% of NICU outbreaks (25% of these were caused by ESBL-producing *Klebsiella* spp.), that BSI was the most frequent type of infection during NICU outbreaks (67.7%), and that the overall mortality in NICU outbreaks was 6.4%. In outbreaks associated with *Klebsiella* spp., the mortality was 11.8% (247).

1.10.1 Outbreak prevention and control

Efficient and targeted interventions are crucial to reduce the spread of ESBL-E and to prevent and control outbreaks. Recently, Tacconelli *et al*. proposed guidelines for the management of infection control measures to reduce the transmission of MDR Gram-negative bacteria among adult hospitalized patients (246). In an outbreak caused by ESBL-E, the authors strongly recommend the implementation of enhanced hand hygiene, educational programmes, active screening cultures, contact precautions and

isolation of colonized patients in single rooms, the use of alert codes to identify previously colonized patients at readmission, environmental cleaning and antimicrobial stewardship. Cohorting of patients and staff, environmental screening and screening of HCWs are considered conditional interventions. In an outbreak associated with MDR *K. pneumoniae*, also cohorting of staff was strongly recommended in these guidelines (246). The combination of screening cultures, contact precautions for colonized and infected patients, and antimicrobial stewardship have been reported to be of significant importance to reduce the ESBL incidence, also in non-outbreak settings (265). In a recent Danish multidisciplinary intervention study, including isolation precautions, communication, education, antimicrobial stewardship and restrictions, the incidence of infections caused by $ESBL_A$- and $ESBL_M$- producing *K. pneumoniae* successfully decreased in the study period (266).

Educational programs to enhance hand hygiene among HCWs, is strongly recommended to prevent and control the spread of MDR Gram-negative bacteria in hospitals, both in the endemic and epidemic setting (246). The need for strict contact precautions and isolation of colonized patients in single rooms depends on the characteristics of the colonizing microbe and the risk for spread, and of course differs between wards and patients characteristics. In ICUs or other wards with immunocompromised patients, isolation of colonized patients should always be considered (246, 267).

In most reported NICU outbreaks, multiple infection control measures, such as patient screening, enforced hand hygiene, cohorting of patients and protective clothing, were implemented. In 16.3%, the NICU had to be closed to control the outbreak (247).

1.10.2 ESBL-screening

Tacconelli *et al*. strongly recommend the implementation of active screening cultures in the epidemic setting to reduce the spread of MDR Gram-negative bacteria (246). ESBL-screening of patients upon hospital admission and during hospitalization in non-outbreak situations has been debated. Retrospective studies indicate that screening at hospital admission might reduce the rate of hospital-acquired ESBL-

infections (265, 268-270). To further address this issue, prospective studies and cost-benefit analysis are needed (270).

Although PCR-based methods are available, culture-based methods are the most reliable and most used methods for ESBL-screening. Several in-house and commercial selective media are available for ESBL-screening (e.g. Chrom ID ESBL and Brilliance ESBL) (271). Rectal surveillance swabs, as well as clinical urine, respiratory and wound samples are considered appropriate for ESBL-E screening (246). In a study assessing the sensitivity of six different anatomic sites (groin, perirectal area, finger webs, forehead, axillae, toe webs), the groin and the perirectal area were the most sensitive sites for detecting MDR Gram-negative bacteria, including ESBL-producing *K. pneumoniae* (272). Whereas one screening site, e.g. the perirectal area, is considered sufficient for ESBL-E detection, multiple sites should be cultured in order to detect MDR *A. baumannii* colonization (246). In a recent study, Snyder *et al*. detected the same MDR Gram-negative strain by perianal/rectal swabbing as also detected in clinical samples in 29/37 patients (273), thus concluding with a sensitivity of 78% of perianal/rectal surveillance swabs for the detection of colonization with MDR Gram-negative bacteria.

1.10.3 Molecular outbreak investigations

Molecular examination of epidemiological links between patients and wards, and identification of the outbreak strain(s) and the source, are important for successful outbreak prevention and control. Outbreaks caused by MDR Gram-negative bacteria may be clonal or multiclonal. *K. pneumoniae* has been found to spread efficiently by a clonal mode in the nosocomial setting, e.g. via HCWs hands (274). However, multiclonal *K. pneumoniae* outbreaks due to interclonal or intergenus dissemination of promiscuous MDR plasmids have also been described (275, 276). “Plasmid-borne” outbreaks may be difficult to identify, and thus remain undetected. Therefore, molecular typing both on strain and plasmid level is needed in order to detect and confirm epidemiological links between bacterial isolates and patients during an outbreak.

Currently, some of the most common methods used for typing of Enterobacteriaceae strains during an outbreak situation are pulsed-field gel electrophoresis (PFGE) and multiple-locus variable number tandem repeat analysis (MLVA) (277). Also in-house and commercial PCR-based typing methods, such as the DiversiLab system (bioMèrieux), are increasingly used for strain typing during outbreaks (278). Brolund *et al*. compared the discriminatory power of the DiversiLab system to PFGE on a collection of ESBL-producing *E. coli* and *K. pneumoniae* isolates, and concluded that the DiversiLab system may be useful as a primary screening method in an outbreak situation, but the results should be confirmed by PFGE (279). Multilocus sequence typing (MLST) does not have as high discriminatory power as the above mentioned methods, and is therefore more convenient for epidemiological surveillance than in local outbreak situations. More recently, whole genome sequencing (WGS) has been successfully applied for tracking both food-borne outbreaks in the community and hospital outbreaks (280, 281).

For plasmid profiling and typing, *S1*-PFGE, restricted fragment length polymorphism (RFLP) and PBRT are commonly used. However, WGS may become an important tool also for plasmid detection and typing in the near future. Brolund *et al*. described how they used WGS to analyse the plasmidome (i.e. the plasmid-content) of ESBL-producing *E. coli* strains, and concluded that their approach of analysing WGS data could become a valuable tool for tracing plasmids in an outbreak situation (282). Recently, Carattoli *et al*. suggested an approach for *in silico* detection and typing of plasmids in Enterobacteriaceae using WGS data (283).

1.11 ESBL-E: clinical impact

Enterobacteriaceae are common causes of both community-acquired and nosocomial infections (80). Whereas CTX-M-producing *E. coli* has spread efficiently in the community and frequently causes community-acquired infections (82), MDR *K. pneumoniae* has become one of the most dreaded organisms in the hospital setting (284). ESBL-E strains often express co-resistance to several commonly used antibiotic classes, such as the fluoroquinolones, aminoglycosides and trimethoprim-

sulfamethoxazole. Thus, the treatment options are often very limited in infections caused by these bacteria. In infections caused by MDR $ESBL_{CARBA}$-producing strains, there may be no effective treatment options at all. High resistance rates against drugs commonly used in the empirical treatment of critically ill patients may result in initial treatment failure, delay of adequate therapy and increased morbidity and mortality (285, 286). Furthermore, ESBL-E associated infections has been found to be a burden on the health care system, conferring prolonged hospital stay and increased costs (287).

Also in uncomplicated ESBL-E infections, such as community-acquired lower UTIs, empirical treatment often fails. In some cases, the causative agent confers resistance to all per oral drugs. Failure of empirical therapy of an otherwise uncomplicated UTI may result in a complicated UTI or BSI, which in turn requires hospitalization and intravenous drug administration. In Norway, however, the sensitivity rates to per oral drugs, such as nitrofurantoin and pivmecillinam, are still high among *E. coli* urine isolates (99.0% and 95.8%, respectively) (2). Especially pivmecillinam play an important role in the treatment of lower UTIs in Scandinavian countries, and is about to be "rediscovered" also in other countries, for example in the UK (288). The clinical outcome of pivmecillinam treatment in uncomplicated UTI, caused by ESBL-E, has been debated. A recent Norwegian study reported treatment failure of pivmecillinam in 44% of patients with UTI caused by ESBL-producing *E. coli* (289), whereas the authors of a recent Danish study concluded that pivmecillinam was bacteriologically and clinically effective for the treatment of lower UTIs caused by ESBL-E (290). Of notice, different dosing regimens are used in Norway and Denmark, 200 mg three times a day and 400 mg three times a day, respectively. Moreover, in a recent British study, concern about increasing resistance to pivmecillinam in ESBL-E was raised (291).

In order to make successful recommendations for empirical treatment, knowledge of the local epidemiology and levels of resistance are crucial. International and national surveillance of resistance, as performed by EARS-Net and NORM/NORM-VET (Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway), is of great importance. Hence, even if the

empirical treatment guidelines are based on the local epidemiology and surveillance data, it may fail. We do not know when the “superbugs” attack, even in areas with low levels of resistance. Traditional species identification and subsequent AST usually takes 48-72 hours, and we therefore have to rely on empirical guidelines before final AST-results are available. As resistance is emerging, the development of more rapid methods for detection of an antibiotic resistant geno- and/or phenotype in clinical isolates is of significant importance (98, 100). Also WGS was recently proposed to be a future approach for rapid species identification and detection of resistance (292, 293).

1.12 Summary of the introduction

AMR is emerging rapidly among Gram-negative bacteria. In particular, the emergence of plasmid-mediated ESBL-production in *E. coli* and *K. pneumoniae* causes great concern. ESBL-producing *K. pneumoniae* may cause community and hospital infections, and is increasingly reported to cause nosocomial outbreaks, especially in the NICU setting.

NICU outbreaks may have fatal consequences for affected patients. Only a small proportion of NICU outbreaks have been reported, although insight into the nature of NICU outbreaks is of importance for the improvement of outbreak prevention and control (247). Thus, we decided to investigate and describe the most important aspects of the ESBL-outbreak affecting the NICU at Stavanger University Hospital during 2008-09, including the extent and the source of the outbreak, infection control measures needed to control the outbreak and the characteristics of the *K. pneumoniae* outbreak strain.

Children colonized during the outbreak were putative faecal carriers of ESBL-producing *K. pneumoniae* when discharged from the hospital to their homes. As limited knowledge existed concerning the duration of faecal ESBL-E carriage and the risk of ESBL-E transmission within households, a follow-up study of colonized children and their families was initiated to address these to date unanswered questions.

The dynamics between an antibiotic resistance plasmid and its host bacteria have to our knowledge not yet been studied during human carriage. The collection of bacterial isolates obtained during the outbreak and the follow-up study, gave us the opportunity to study the dynamics between an ESBL-encoding plasmid and its *K. pneumoniae* host strain during long-term intestinal colonization in young children.

2 Aims of the Thesis

The overall aim of this thesis was to gain new insights into ESBL-E outbreak dynamics and control in the NICU setting, the colonization dynamics between an ESBL-producing *K. pneumoniae* strain and its newborn human host, and the dynamics between a *K. pneumoniae* outbreak strain and its ESBL-encoding plasmid.

The specific objectives were:

I. To describe the characteristics of the NICU outbreak at Stavanger University Hospital caused by ESBL-producing *K. pneumoniae* (ESBL-Kp)
   a. To determine the extent and the source of the outbreak
   b. To describe implemented infection control measures
   c. To examine the microbiological and molecular properties of the outbreak strain(s)

II. To investigate the ESBL-Kp dynamics during intestinal carriage in children colonized during the NICU outbreak, after discharge from the hospital
   a. To determine the duration of ESBL-Kp carriage in colonized children
   b. To identify risk factors associated with prolonged ESBL-Kp carriage
   c. To determine the rate of intra-household transmission of ESBL-Kp

III. To identify and characterize the ESBL-encoding plasmid of the *K. pneumoniae* outbreak strain(s)
   a. To obtain the complete DNA sequence of the ESBL-encoding plasmid
   b. To identify plasmid-encoded traits relevant for the outbreak dynamics
   c. To investigate the dynamics between the plasmid and its *K. pneumoniae* host with respect to self-transfer, maintenance and fitness cost during intestinal colonization in a human host (i.e. colonized children)

3 Material and methods

3.1 Setting

Stavanger University Hospital serves a population of 330.000 inhabitants (2010) and has about 5000 deliveries annually. The 21-bed NICU treats neonates from gestational age 23 weeks, and has approximately 550 admissions and 7000 patient-days per year. The unit has four adjacent rooms, including one isolation room. The NICU serves as a secondary referral center for a local hospital (with 1500 deliveries annually). Patients in need of surgery are transferred to Oslo University Hospital.

3.2 Study design

This thesis is based on three sub-studies: The first sub-study, "The NICU outbreak", is a retrospective descriptive study of the ESBL-Kp associated NICU outbreak at Stavanger University Hospital during 2008-09 (Paper I). The second sub-study, "The follow-up study", is a prospective cohort study including children colonized during the outbreak and their families (Paper II). The third sub-study, "The plasmid study", is based on *in vivo* and *in vitro* studies of the ESBL-encoding plasmid in *K. pneumoniae* isolates collected during the outbreak and the follow-up study (Paper III).

3.2.1 Subjects and bacterial isolates

An outbreak was suspected in late January 2009, as ESBL-Kp was detected in clinical samples from three NICU patients during one week. All children hospitalized in the NICU from 30 January to 15 April were screened for ESBL-Kp colonization (n=117). Furthermore, all children discharged from the NICU during November 2008 until the end of January 2009 were offered screening, and 127/135 children were screened. In addition, all children admitted to or discharged from the maternity wards during the outbreak period were offered screening if exposed to cross-contamination, and 247/872 children were screened. Altogether, 501 children underwent screening for ESBL-Kp during the outbreak investigations (Figure 7).

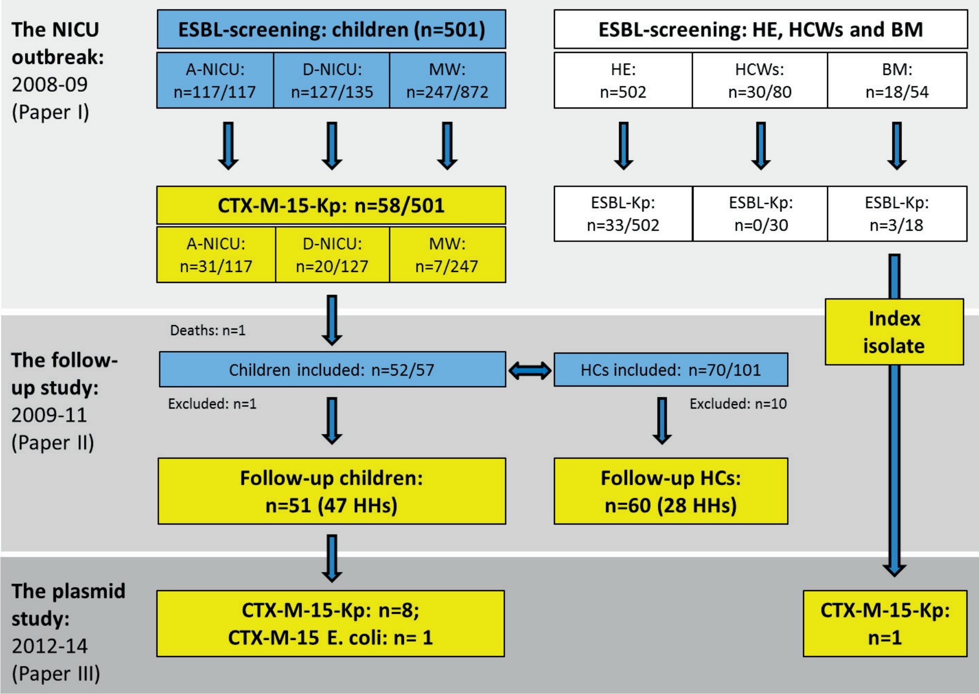

Figure 7. Simplified study design.
A-NICU=screened when admitted to the NICU; D-NICU=screened after dicharged from the NICU; MW=screened when admitted or discharged from the maternity wards; HE=screening samples from the hospital environment; HCWs=rectal samples from health care workers; BM=breast milk samples from n mothers of colonized children; HCs= household contacts; HHs=households

Included in the outbreak investigations were also environmental screening, screening of HCWs and screening of breast milk samples from mothers of colonized children. In total 502 screening samples were taken from the NICU environment and medical equipment. All HCWs in the NICU were offered anonymous screening, and 30/80 HCWs participated. Finally, breast milk samples from 18/54 mothers of colonized children were screened (Figure 7). Sampling for ESBL-screening was performed as described in 3.2.2.

During the NICU outbreak, 58 children were found colonized by ESBL-Kp, i.e. CTX-M-15-producing *K. pneumoniae* (CTX-M-15-Kp). One of the colonized children died. Thus, 57 children from 53 households (twins in four families) and their HCs (i.e. parents and siblings) were invited to participate in a follow-up study. The intended study period was one to three years after hospital discharge (or until CTX-M-15-Kp

elimination in the invividual colonized child). A total of 52/57 (92%) children from 48 households and 70/101 (69%) HCs from 33 households were included in the follow-up study. One infant and ten HCs were excluded (see exclusion criteria below) during the study period. Thus, follow-up data from 51 children (25 boys and 26 girls) from 47 households and 60 HCs (28 mothers, 25 fathers and 7 siblings) from 28 households were considered in this study (Figure 7). Seven of the 51 children had not been admitted to the NICU, but were likely colonized by cross-transmission in the maternity wards. Two children had most of their NICU stay in another hospital. Demographic data of the 42 children hospitalized and colonized in the NICU in our hospital are listed in Table 3.

Table 3. Demographic data of children colonized in the NICU (n=42).

Variables	
Male gender	22 (52)[a]
Gestational age (weeks)	34 (30-37)[b]
Birth weight (g)	2055 (1226-2969)[b]
Length of hospital stay (days)	19 (10-61)[b]
Treated with antibiotics*	33 (79)[a]
Length of antibiotic treatment* (days)	5 (3-7)[b]
Caesarean section	24 (57)[a]
Total parenteral nutrition	16 (38)[a]

[a]Number (%); [b]Median (IQR); *Ampicillin and gentamicin intravenously

All putative ESBL-Kp isolates recovered during the NICU outbreak and the follow-up study were phenotypically characterized (as described in 3.4). A selection of 152 ESBL-Kp isolates was subjected to molecular characterization (as described in 3.5). Of these, 60 isolates were recovered during the outbreak: the first screening isolate obtained from all colonized children (n=58), one blood culture isolate recovered from one infected child (n=1), and the first breast milk isolate from the mother of the index case (n=1). And 92 isolates were recovered during the follow-up study: the last follow-up isolate from all children (1/51 children had no positive follow-up sample) (n=50), an additional follow-up isolate from children colonized > 12 months (n=23), the first screening isolate from 12 HCs colonized during the follow-

up study (n=12) and the last follow-up isolate from seven HCs (5 colonized HCs had no positive follow-up sample) (n=7).

Finally, a selection of nine CTX-M-15-Kp isolates and one CTX-M-15-producing *E. coli* isolate, recovered during the outbreak and the follow-up study, was included in the plasmid study: the first and the last faecal CTX-M-15-Kp isolate from four children colonized for 18-24 months (n=8), the first CTX-M-15-Kp breast milk isolate from the mother of the index case (n=1) and one CTX-M-15-producing *E. coli* isolate recovered from one child during follow-up (n=1) (Figure 7).

3.2.2 ESBL-screening

During the outbreak investigations, all children hospitalized in the NICU were repeatedly screened for ESBL-Kp colonization by faecal or rectal swabbing. From the children hospitalized in the NICU as the outbreak was detected (n=24), additional swab samples were collected from nasopharynx and conjunctiva. Only one faecal screening sample was obtained from discharged children. HCWs were screened once by rectal swabbing (four months into the outbreak). One or more breast milk samples from mothers of colonized children were screened if available (if mothers were donating their breast milk to other infants, screening was required). Environmental samples were repeatedly collected from, e.g. sinks, bath tubes, breast milk pumps, the milk kitchen, respiratory therapy equipment and incubators.

During the follow-up study, faecal and rectal swab samples were obtained from colonized children and their HCs, respectively (for details, see Appendix 1). The intended sample schedule included a monthly sample from the children and their HCs during the first year, and thereafter every three months (for up to three years after hospital discharge). Home sampling was performed by the parents, and the samples were sent by post to the lab. All screening samples (except breast milk samples) were collected using sterile cotton-tipped swabs (COPAN®) and transported in Stuart's medium. All samples were subjected to culture-based $ESBL_A$-screening as described in 3.4.

The following were excluded from the follow-up study:

- Children providing less than three follow-up samples.
- HCs that did not provide follow-up samples regularly until elimination of CTX-M-15-Kp from their respective colonized child.
- Children colonized in the maternity wards, or hospitalized in another NICU before transferred to our NICU, were excluded from the risk analysis.

3.2.3 Definitions

- **CTX-M-15-Kp colonization:** Identification of CTX-M-15-Kp in faecal or rectal samples from children without clinical symptoms or signs of systemic infection (The NICU outbreak).
- **Systemic CTX-M-15-Kp infection:** Isolation of CTX-M-15-Kp from clinical samples (blood or cerebrospinal fluid) from children with symptoms or signs of systemic infection (The NICU outbreak).
- **CTX-M-15-Kp elimination:** Three or more consecutive negative faecal samples (screening interval ≥ month) in previously colonized children (The follow-up study)
- **Intra-household transmission:** Identification of CTX-M-15-Kp in rectal samples of HCs (The follow-up study).

3.2.4 Clinical data and questionnaires

Date of birth, admission and discharge were recorded for all children admitted to the NICU or maternity wards during the period from November 2008 to April 2009 (The NICU outbreak). For the risk analysis, data concerning gender, gestational age, birth weight, mode of delivery, length of hospital stay, mode of nutrition and antibiotic treatment during hospitalization (Table 3) were obtained from medical records of children hospitalized in the NICU during the outbreak (The follow-up study).

Questionnaires were answered by the parents and returned together with screening samples during the follow-up study. Information concerning nutrition, infections, re-hospitalization and use of antibiotics in the children after hospital

discharge was obtained. Furthermore, information about travel outside Scandinavia was recorded for all household members during the study period (Appendix 2).

3.3 Ethical considerations

The study was approved by the Norwegian Data Inspectorate and the Regional Committee on Medical Research Ethics (096.09). The Regional Committee on Medical Research Ethics also approved the extension of the follow-up period from one to three years. Signed statements of informed consent were obtained from one or both parents on behalf of participating children and from all participating parents. In this study no interventions or procedures were performed, which could have posed any risk to the participants.

3.4 Phenotypical detection of $ESBL_A$-producing isolates

Culture-based $ESBL_A$-screening

All screening samples were inoculated on modified MacConkey agar with a 30 µg cefotaxime tablet (Rosco Diagnostics AS, Taastrup, Denmark) and on Chrom ID ESBL agar (bioMèrieux, Marcy l'Etoile, France) for $ESBL_A$-screening. To potentially increase the sensitivity during the follow-up study, swabs were also cultured in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Maryland, USA) supplemented with cefotaxime (1mg/L) directly after plating. Agar plates and broth samples were incubated for 24-48 h at 35°C. Any putative ESBL-producing strain (one colony of each phenotype) growing on the Chrom ID ESBL agar was sub-cultured on modified MacConkey agar for further identification. Colonies growing within the cefotaxime inhibition zone on the primary modified MacConkey agar were sub-cultured if no growth was detected on the Chrom ID ESBL agar after 48 h of incubation. Bacterial growth outside the cefotaxime inhibition zone was used as a control for a valid sample. If no growth of ESBL-producing bacteria was suspected on either of the two plates after 48 h of incubation, the corresponding BHI broth sample was re-inoculated on a

Chrom ID ESBL plate (The follow-up study). *K. pneumoniae* ATCC 700603 (ESBL-positive) and *E. coli* ATCC 25922 (ESBL-negative) were used as control strains.

Antimicrobial susceptibility testing (AST) and species identification

AST was performed by agar disk diffusion (Becton Dickinson) on all putative $ESBL_A$-producing isolates. MICs to relevant antibiotics (e.g. mecillinam, nitrofurantoin, piperacillin-tazobactam, ciprofloxacin, gentamicin, tobramycin, trimethoprim-sulfamethoxazole, cefotaxime, ceftazidime, cefepime, aztreonam and meropenem) were determined by Etest (bioMérieux) on selected isolates. Interpretations were according to clinical breakpoints recommended by the Norwegian Working Group on Antibiotics 2009-2011 (294), which were in line with the EUCAST recommendations for the same period (295). An $ESBL_A$-phenotype was confirmed by the double disk approximation test and by the combined disk method using cefotaxime/cefotaxime-clavulanate and ceftazidime/ceftazidime-clavulanate discs (96). $ESBL_A$-producing isolates were identified to the species level using Vitek2 ID-GN (bioMèrieux). All $ESBL_A$-producing isolates were stored at -70°C for subsequent molecular analysis.

3.5 Molecular characterization of $ESBL_A$-producing isolates

Detection of $ESBL_A$-encoding genes

Screening for the $ESBL_A$-encoding genes bla_{SHV}, bla_{TEM} and bla_{CTX-M} was performed by PCR as previously described (99). $Bla_{CTX-M-15}$ was detected by using $bla_{CTX-M-group1}$-specific primers (296, 297) and subsequent DNA sequencing of the PCR products using the Genetic analyser 3130 (Applied Biosystems, Foster City, CA, USA). Editing and alignments of DNA sequences were performed using SeqScape version 2.5 (Applied Biosystems).

Bacterial strain typing

Clonal relatedness was examined by PFGE of *Xba*I-digested (New England BioLabs, Ipswich, UK) genomic DNA using the Chef-DR® III System (BioRad, Oslo, Norway). Briefly, PFGE was run at 12°C, with pulse time 1-20 s, at 6 V/cm on a 120° angle in 0.5xTBE buffer for 21 h. PFGE-patterns were analysed and compared in BioNumerics version 6.6 (Applied Maths NV, St-Martens-Latem, Belgium), and results were interpreted according to the criteria of Tenover *et al*. (298).

One representative isolate per PFGE-type was subjected to MLST. MLST of seven *K. penumoniae* housekeeping genes was performed as described by Diancourt *et al*. (299).

Detection of virulence determinants in *K. pneumoniae*

One representative isolate per PFGE-type was subjected to screening for K-types commonly associated with invasive disease (K1, K2, K5, K20, K54, K57) and virulence genes common in invasive *K. pneumoniae* isolates (*rmpA*, *wcaG* and *allS*). Screening was conducted by PCR as described (11, 14).

3.6 Plasmid analyses

Plasmid typing and profiling

Plasmid replicons of CTX-M-15-producing isolates were detected by PCR using a commercial kit (DIATHEVA, Fano PU, Italy) developed by Carattoli *et al*. (134, 140). Plasmid profiling was performed by PFGE of *S1*-nuclease (Promega, Madison, WI, USA) digested total DNA (296, 300). Briefly, PFGE was run in the Chef-DR® III System (BioRad) at 14°C, with pulse time 1-20 s, at 6 V/cm on a 120° angle in 0.5xTBE buffer for 15 h. Each band on the gel was considered a linearized plasmid. The Low range ladder (New England BioLabs) was used as a plasmid size marker. Plasmid DNA bands were transferred to positively charged nylon membranes using a vacuum blotting system (Vacuum Blotter, Model 785, BioRad). CTX-M-15-encoding plasmids were identified through Southern blot-hybridization with $bla_{\text{CTX-M}}$-specific probes. The replicon types of both CTX-M-15-encoding plasmids and

additional plasmids were determined through hybridization with probes specific for detected replicons. The DIG High Prime DNA Labelling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany) was used for labelling of PCR-generated probes and detection.

Plasmid sequencing

Plasmid DNA from one CTX-M-15-Kp isolate (Kp848) was purified using the Qiagen large construct kit (Qiagen). A shotgun library was constructed and single-end pyrosequencing reads were obtained applying the 454 Genome Sequencer FLX-system (Roche Diagnostics, Indianapolis, IN, USA). Reads were assembled *de novo* using the Newbler software (Roche Diagnostics). Gap closure was performed by sequence-based bridging. Artemis version 15 (Welcome Trust Sanger Institute, Hinxton, UK) was used for alignment and annotation. Plasmid sequence comparison with previously published plasmids was performed using WebAct and ACT version 12 (Welcome Trust Sanger Institute). Plasmid MLST (pMLST) was performed as proposed by Villa *et al*. (140) using the pMLST version 1.2 of the Center for Genomic Epidemiology (Lyngby, Denmark) (301).

Plasmid transfer

***In vivo*:** To detect possible self-transfer events of the CTX-M-15-encoding plasmid from CTX-M-15-Kp to other Enterobacteriaceae strains during intestinal colonization, all CTX-M-15-producing isolates, other than CTX-M-15-Kp, recovered during follow-up of 49 children (n=1) were characterized by PBRT, *S1*-nuclease PFGE and Southern blot-hybridization as described above.

***In vitro*:** Transferability of the CTX-M-15-encoding plasmid from CTX-M-15-Kp to a rifampicin resistant plasmid-free recipient strain, *E. coli* J53-2, was investigated by broth mating as follows: strains were cultured ON in Luria Bertani (LB) broth (Sigma Aldrich, Oslo, Norway) at 35°C, ON cultures were diluted 1:100 and incubated until reaching the logarithmic growth phase (measured by OD_{600nm} of ~0.5). Donor and recipient cultures were mixed 1:9 and incubated at 35°C with careful

shaking. After 4 h and 24 h of incubation, aliquots of 100 µL mixed culture were plated on LB plates (Sigma Aldrich) containing cefotaxime (2 mg/L) and rifampicin (100 mg/L). The clinical *K. pneumoniae* strain, Kp2200, containing a self-transferable IncI1 CTX-M-15-encoding plasmid was used as control. Furthermore, re-transferability of the CTX-M-15-encoding plasmid was tested by filter mating between CTX-M-15-Kp and corresponding rifampicin resistant plasmid-free segregant isolates (i.e. CTX-M-15-Kp isolates that lost the CTX-M-15-encoding plasmid during serial transfer; see below) under the following conditions: ON cultures were diluted 1:1000, mixed 1:1 and spotted in 40 µL droplets on LB plates. After 8 h of incubation at 37°C, cell lawns were scraped off, re-suspended in phosphate buffered saline (PBS) and plated on LB plates supplemented with cefotaxime (8 mg/L) and rifampicin (100 mg/L).

Plasmid stability

In vivo**:** To detect CTX-M-15-Kp isolates that possibly had lost the CTX-M-15-encoding plasmid during intestinal colonization, the two first ESBL-negative faecal follow-up samples from 35/49 children were screened for ESBL-negative isogenic *K. pneumoniae* strains (i.e. *in vivo* segregants devoid of the CTX-M-15 phenotype). Frozen ESBL-negative samples were inoculated on modified McConcey agar and incubated ON at 35°C. On the next day, all Gram-negative strains detected were identified to the species level by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany), and subsequently all *K. pneumoniae* isolates (n=24) were subjected to typing by *Xba*I-PFGE as described above.

In vitro**:** The *in vitro* stability of the CTX-M-15-encoding plasmid in CTX-M-15-Kp was investigated by serial passage of two independent populations of eight ancestor isolates (the first and the last isolate from the four children colonized the longest) for ~two months at 37˚C without antibiotic selection. Twice a day, aliquots of 10 µl were serially transferred into 1 ml of fresh LB broth, allowing ~6.64 generations per transfer. At the endpoint of the experiment, 150 µL aliquots from 10^{-5}-dilutions of the 16 evolved populations were plated on plain LB plates and incubated at room

temperature ON. To determine the rate of plasmid-free segregants (i.e. CTX-M-15-Kp colonies devoid of the CTX-M-15-encoding plasmid and thus the CTX-M-15 phenotype) in each sample, 120 colonies were randomly picked from each plate and streaked on plain LB plates and on LB plates supplemented with cefotaxime (8 mg/L) in parallel. A selection of putative segregants (at least one representative per ancestor isolate) were further analysed by relevant gradient tests (Liofilchem, Roseto degli Abruzzi, Italy), *bla*$_{CTX-M}$ PCR, *Xba*I-PFGE, *S1*-nuclease PFGE and PBRT as described above.

Fitness cost of plasmid carriage

The biological cost associated with carriage of the CTX-M-15-encoding plasmid in CTX-M-15-Kp *in vitro* was estimated in two ways: (i) by pairwise head-to-head competitions between plasmid-free segregant isolates and their corresponding plasmid-carrying ancestor isolates in mixed culture competitions, and (ii) by comparing the growth rates of plasmid-free segregants and their corresponding plasmid-carrying populations in monocultures.

For preconditioning, evolved CTX-M-15-Kp plamid-carrying acestor isolates and their corresponding plasmid-free segregant isolates (all recovered at the endpoint of the serial transfer experiment and frozen at -70˚C) were inoculated on LB plates, with cefotaxime 8 mg/L for plasmid-carrying isolates and without cefotaxime for segregant isolates, and cultured at 37˚C ON. Then, single colonies were picked and cultured in LB broth ON. The ON cultures were serially diluted 1:1000 and incubated for another 14 hours before fitness experiments were conducted.

Pairwise head-to-head competition experiments were performed in triplicates and repeated three times as previously described (302, 303). Approximately 2.5×10^6 cells of each competitor were transferred into 1 ml LB broth. The mixed cultures were incubated shaking at 37°C for 12 hours, allowing eight to nine generations per competition. Initial (N_0) and final (N_{12}) densities (cfu/mL) of each competitor were determined by selective and non-selective plating. Relative fitness (*w*) measurements were estimated as described (302, 303). Briefly, from the initial (N_0) and final densities (N_{12}) the population growth of each competitor, known as its Malthusian

parameter (m), was determined using the equation $m=\ln(N_{12}/N_0)$. The value w for each segregant isolate was estimated as a ratio of the Malthusian parameter of the plasmid-free segregant (m_1) to that of the corresponding plasmid-carrying ancestor isolate (m_2) (302).

Growth rates of plasmid-carrying ancestor isolates and their corresponding plasmid-free segregant isolates were determined during logarithmic growth using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, USA). Approximately $2x10^5$ cells of each population were inoculated into 200 µl LB broth and incubated shaking at 37°C in monocultures. Each isolate was assayed in three independent cultures, with three technical replicates per culture, in at least two independent experiments. Absorbance was recorded at 650 nm at regular intervals of five minutes. Growth rate calculations were based on OD_{650} values between 0.1 and 0.6, where growth was observed to be exponential using the program GrowthRates (304). Every growth rate calculation was also manually inspected. Relative growth rates were calculated by dividing the growth rate of plasmid-free segregant isolates by the growth rate of the corresponding plasmid-carrying ancestor isolate.

3.7 Statistics

Kaplan-Meier survival analysis was performed to calculate the median time of faecal CTX-M-15-Kp carriage in children and colonized HCs. For each child, the duration of CTX-M-15-Kp carriage was calculated as the average of the time from hospital discharge until the last CTX-M-15-Kp positive sample, and the time from discharge until the first CTX-M-15-Kp negative sample. For colonized HCs, the duration of carriage was calculated as the average of the time from the first CTX-M-15-Kp positive sample until the last positive sample, and the time from the first positive sample until the first negative sample. In subjects without complete follow-up, the first missing sample was considered the first negative or last positive sample in children and HCs, respectively (The follow-up study).The logrank test was used to compare the carriage times between children and parents. For testing the effect of various variables (risk factors) on carriage times in children, univariate and

multivariate Cox regression analysis was performed. Variables with *P*<0.25 in the univariate analysis were included in the stepwise selection. Relations between different variables were evaluated in a multivariate Cox regression model. The assumption of proportional hazards between groups over time was investigated by log minus log plots. Hazard ratios (HR) are reported with 95% confidence intervals (CI). Comparison of proportions between groups was analysed by Mc Nemar's test for related samples. *P* values< 0.05 were considered statistically significant. PASW version 18 (SPSS Inc, Chicago, IL, USA) was used for analysis (The follow-up study).

Students *t*-tests were used for the pairwise comparison of the relative fitness and the relative growth rates between isolates. Relative growth rates and *w* values are reported with 95% CI. Generation times are reported with mean ± standard error (SE). *P* values <0.05 were considered statistically significant. *t*-tests were performed in Excel (The plasmid study).

4 Summary of results

The main results of the three sub-studies included in this thesis are summarized in this section. Further details are given in the respective papers.

4.1 The NICU outbreak

As the outbreak was recognized, screening revealed that 22/24 (92%) infants hospitalized in the NICU were colonized by ESBL-Kp. Furthermore, screening of 501 children during the outbreak investigations revealed that altogether 58 children were colonized during the NICU outbreak (Figure 7). Colonized children had their stay in the NICU (n=51) or in the maternity wards (n=7) between 27 November 2008 and 17 April 2009. A probable index case was identified as a newborn child transferred to our NICU from another Norwegian NICU on 27 November. ESBL-Kp was recovered from several frozen breast milk samples from this child's mother, collected both before and after the transfer (for details see Table 1 and Figure 1, Paper I). ESBL-Kp was detected in 33/502 environmental samples, mainly taken from sink drains. Thirty HCWs accepted rectal screening, but none were found colonized. The implementation of strict infection control precautions (e.g. enhanced hand hygiene) and cohorting of patients and nurses in two separate units were considered to be of significant importance for the rapid outbreak control. One child suffered a systemic infection caused by the outbreak strain (ESBL-Kp positive blood culture), but recovered after treatment with meropenem. No patients died of reasons associated to the outbreak.

All ESBL-Kp isolates expressed resistance to extended-spectrum cephalosporins and aztreonam, as well as clavulanic acid synergy. Most isolates displayed co-resistance to trimethoprim-sulfamethoxazole and gentamicin. All isolates were susceptible to carbapenems and ciprofloxacin. Molecular analyses were performed on the first faecal isolate obtained from each of the 58 colonized children, the blood culture isolate from one child and the first breast milk isolate from the mother of the index case (n=60). The presence of $bla_{\text{CTX-M-15}}$ was confirmed in all isolates. Fifty-eight isolates displayed indistinguishable PFGE-patterns, designated PFGE-type I. Faecal isolates from two infants showed unrelated PFGE-type II

patterns. Isolates of pulsotype II were, in contrast to isolates of pulsotype I, susceptible to gentamicin. Pulsotype I isolates (including the index breast milk isolate and the invasive isolate) were of ST17 and pulsotype II isolates were of ST485. Both strains were PCR-negative for all tested virulence determinants.

4.2 The follow-up study

All $ESBL_A$-producing *K. pneumoniae* isolates recovered during follow-up expressed the typical CTX-M-15 phenotype and co-resistances. Molecular analysis was performed on a selection of faecal ESBL-Kp follow-up isolates (n=92) from 51 children and 12 colonized HCs. $Bla_{\text{CTX-M-15}}$ was confirmed in all isolates. Pulsotype I (ST17) was confirmed in follow-up samples from 49 children and 11 HCs, and pulsotype II (ST485) was confirmed in follow-up samples from two children and one corresponding HC.

The median carriage time of CTX-M-15-Kp in 51 children, after discharge from hospital, was estimated to 12.5 months (inter-quartile range (IQR) 9.5-17.5) by Kaplan-Meier survival analysis (Figure 1, Paper II). The longest period of carriage observed was ~24 months. Stable antimicrobial susceptibility patterns and PFGE-profiles from the first to the last CTX-M-15-Kp isolate in all children supported stable persistence of CTX-M-15-Kp during long-term intestinal carriage.

Clinical data from 42 children hospitalized in the NICU during the outbreak were available for risk analysis. Risk factors for prolonged faecal carriage, assessed by multivariate Cox regression analysis, were delivery by caesarean section (HR=4.5, 95%, CI 1.6-12.6, *P*=0.004) and treatment with antibiotics during hospitalization (HR=2.4, 95% CI 1.1-5.5, *P*=0.029) (for further details see Table 1, Paper II).

Transmission of CTX-M-15-Kp was observed in 12/60 (20%) HCs (i.e. 8/28 mothers, 3/25 fathers and 1/7 siblings) from 9/28 (32%) households. Median carriage time in 11 parents was 2.5 months (IQR 1.0-5.0) (*P*<0.001 compared to infants).

4.3 The plasmid study

Plasmid typing and profiling of selected CTX-M-15-Kp ST17 isolates (the first and the last faecal isolate from four children colonized for 18-24 months, and the index breast milk isolate) revealed the presence of a ~180 kb $IncFII_K$ CTX-M-15-encoding plasmid in all nine isolates. In 8/9 isolates the presence of an additional ~70 kb IncR plasmid was confirmed. The last isolate from one child (Kp848), was devoid of the ~70 kb IncR plasmid (Figure 1 and Table 1, Paper III).

Sequencing of the CTX-M-15-encoding plasmid of the CTX-M-15-Kp ST17 outbreak strain uncovered a 182 204 kb $IncFII_K$ plasmid, named pKp848CTX (GenBank accession number LM994717). A conserved transfer region and several plasmid maintenance systems were encoded by pKp848CTX. Plasmid pKp848CTX carried a Tn*3*-like transposon, encoding the antibiotic resistance genes $bla_{CTX\text{-}M\text{-}15}$(linked to IS*Ecp1*) and $bla_{TEM\text{-}208}$. Furthermore, the aminoglycoside resistance gene *aac(3)-IIa*, multiple heavy metal (i.e. mercury, arsenic, copper and silver) and thermoresistance determinants were identified on pKp848CTX. The core regions of pKp848CTX, as well as the conserved heavy metal and thermoresistance encoding region, were highly similar to corresponding regions in pKPN3 (Gene bank accession number CP000648) and pUUH239.2 (CP002474) (Figure 2, Paper III).

We have no evidence of pKp848CTX self-transfer from CTX-M-15-Kp ST17 to other Enterobacteriacea during two years of intestinal colonization. In line with this finding, pKp848CTX could also not be transferred from its *K. pneumoniae* host strain (CTX-M-15-Kp ST17) to *E. coli* J53-2 or back to plasmid-free CTX-M-15-Kp ST17 segregants by mating experiments *in vitro*.

Stable *S1*-nuclease PFGE profiles from the first to the last CTX-M-15-Kp ST17 isolates from four children (Figure 1, Paper III), indicated that pKp848CTX was maintained by its *K. pneumoniae* host strain during the entire intestinal colonization period of up to two years. By screening ESBL-negative follow-up samples from 35 children, previously colonized by CTX-M-15-Kp ST17, we could not detect any isogenic ESBL-negative *K. pneumoniae* isolates (i.e. CTX-M-15-Kp ST17 isolates devoid of pKp848CTX, and thus the CTX-M-phenotype). Hence, we have no evidence of plasmid loss *in vivo*. In the serial passage experiment, however, we uncovered an

endpoint segregant frequency after ~430 generations without antibiotic selection ranging between 0.0% and 17.5% in 16 evolved CTX-M-15-Kp ST17 populations (Table 2, Paper III).

The biological cost of pKp848CTX carriage in CTX-M-15-Kp ST17 was studied *in vitro*. Plasmid-free segregant isolates were competed against their evolved corresponding plasmid-carrying ancestor isolates in mixed culture competitions, but no significant fitness cost associated with pKp848CTX carriage could be identified by this approach. However, a statistical significant reduction of fitness was seen for one segregant isolate (Kp2180) suggesting a subtle benefit of plasmid carriage (Table 2, Paper III). Growth rates of plasmid-free segregant isolates and their corresponding evolved plasmid-carrying ancestor isolates were measured in monocultures and compared. Generation times of the CTX-M-15-Kp ST17 isolates, with and without plasmid pKp848CTX, varied between 30.24 and 33.14 minutes (mean 31.17 ±0.658). Two plasmid-free segregant isolates (Kp2158 and Kp848) displayed increased growth rates indicating a significant biological cost of plasmid carriage (Table 2, Paper III). Moreover, one segregant (Kp734) displayed a statistically significant reduced growth rate suggesting a small benefit of plasmid carriage after two years of colonization. The results from the mixed culture competition experiments and the growth rate comparisons were not consistent and should be interpreted with caution. Also no difference in fitness was found between the first and the last isolate of individual children (all $P>0.05$, t-tests).

5 Discussion

5.1 Methodological considerations

In this section the choice of methods, as well as their strengths and limitations, will be discussed.

Study design and study population

The first sub-study, describing the NICU outbreak, is a retrospective descriptive study. Retrospective studies will always have limitations as one cannot go back in time and implement additional measures or collect missing samples etc. In our setting, the study population was given in advance. Thorough investigations were made to disclose the extent and the source of this outbreak, which strengthens this study compared to other outbreak reports, where the source often remained unknown (247).

The follow-up study is a prospective cohort study. The design of this study was also limited by some factors, which could not be influenced. The study population (i.e. the number of colonized children during the outbreak and their respective family members) was already set as we planned the study. The first follow-up samples were intended to be obtained within a month after hospital discharge. Some infants were already discharged before we were aware of the outbreak. Hence, their first follow-up samples were obtained 3-5 months after discharge. As all these infants, except one, were still colonized when they delivered their first follow-up sample, this did not influence our overall results (e.g. median CTX-M-15-Kp-carriage time). Due to the delayed follow-up in some families, we might have missed transient colonization in HCs. Furthermore, we missed information otherwise obtained by the questionnaires in some of the children (e.g. breast feeding, infections or antibiotic treatment during the first months after discharge). Altogether, the information obtained by the questionnaires was incomplete and of poor quality, and was therefore not included in the risk analysis. Initially, follow-up was planned for one year after hospital discharge. However, the study period was extended from one to three years, which allowed more infants to reach the endpoint of the study (i.e. clearance of CTX-M-15-Kp). The

longest carriage time observed in this study was ~24 months. Thus, all children, who completed follow-up, were followed until CTX-M-15-Kp clearance.

The unique collection of isolates recovered during the NICU outbreak and the follow-up study, gave us the opportunity to perform the plasmid study. The strength of the plasmid study is the design combining modern sequencing technology with both *in vivo* and *in vitro* studies of the CTX-M-15-encoding plasmid and its *K. pneumoniae* host strain.

ESBL-screening

During the early outbreak investigations, children were screened by swabbing the conjunctiva, nasopharynx and the rectal area (or faeces). However, only 8% (2/25) and 12% (3/25) of children with ESBL-positive rectal/faecal samples had additional ESBL-positive conjunctival or nasopharyngeal samples, respectively. Bagattini *et al*. reported 47.4% positivity by rectal swabbing, followed by 35.5% and 26.4% positivity in throat and nose cultures, respectively, during a NICU outbreak caused by ESBL-producing *K. pneumoniae* (252). We did not obtain oropharyngeal swabs, which might have been of higher value than nasopharyngeal swabs. However, we concluded that rectal/faecal samples were superior to conjunctival and nasopharyngeal samples for ESBL-E screening in our setting, and continued to screen only rectal/faecal samples throughout the outbreak and during the follow-up study. Perirectal swabs are commonly used and considered adequate for ESBL-E screening both in hospitalized and healthy persons (246, 273), supporting our choice.

Children admitted to the NICU were repeatedly screened during the outbreak (every 2-3 days). Screening samples from newborn children were ESBL-negative during their first days of life, but turned ESBL-positive on the second or third day in most children before strict cohorting was established (unpublished data). This observation emphasizes the importance of repeated screening in a NICU outbreak situation, where the risk of cross-transmission between patients is considered to be high (247). Children admitted to the maternity wards, or already discharged from the NICU or maternity wards, were only screened once. Also HCWs provided only one

screening sample. Hence, transient or low levels of colonization in these groups may have remained undetected.

In the follow-up study, we defined elimination of CTX-M-15-Kp in colonized children as three or more consecutive negative faecal samples, as also suggested by Alsterlund *et al.* (232) and Kola *et al*. (267). In contrast, a recent Swedish follow-up study reported duration of carriage until the first negative sample (235). In our study, 29/41 children, who completed follow-up had four or more negative samples. None of the 41 children had a CTX-M-15-Kp positive sample after three or more consecutive negative samples. Two children, however, had one positive sample after two negative samples, and four children had one positive sample after one single negative sample. Hence, the overall results of the follow-up study support the chosen definition of CTX-M-15-Kp elimination. Both children and HCs were screened every 1-3 months during the follow-up study. Due to the chosen screening schedule, is possible that we missed transient or short-term colonization (< 3 months) in some HCs.

Statistical methods (The follow-up study)

Kaplan-Meier survival analysis was performed to determine the duration of CTX-M-15-Kp carriage (i.e. the time until CTX-M-15-Kp clearance) in infants and colonized HCs. The power of a survival analysis is not related to the total sample size, but to the number of subjects who reach the event of interest or the defined endpoint (305). In our study, CTX-M-15-Kp clearance (≥ 3 consecutive negative samples) was defined as the endpoint of the survival analysis. The majority of the infants (41/51) reached the endpoint as we extended the study period from one to three years. The remaining 10 infants stopped providing screening samples before reaching the endpoint of the study (< 3 consecutive negative samples). However, their follow-up data were included in the survival analysis as censored survival times. In most studies, where survival analysis is applied, the endpoint (e.g. death or recurrence of a disease) is well defined, whereas the start (e.g. onset of a disease) may be less well defined. In our study all infants were colonized by CTX-M-15-Kp during a four month period and we know the exact date of hospital discharge, which was defined as the start point in this study. We consider Kaplan-Meier survival analysis to be the most appropriate

statistical method for the estimation of ESBL-carriage times in a group of subjects like in our setting. This approach was also chosen by Birgand *et al*. (236). In contrast, most other carriage studies report proportions of subjects still being carriers at different time points e.g. three, six, nine and 12 months after ESBL-infection or colonization (232, 233, 235).

The logrank test is the most common used method for comparing independent groups of survival times. Thus, we chose this test for comparing carriage times between children and colonized parents. The logrank test should, however, not be used when exploring the effects of multiple variables on survival (305). Thus, we chose to perform Cox regression analysis for exploring risk factors for prolonged ESBL-carriage in children colonized during a NICU outbreak. As carriers were compared to carriers, we did not have the possibility to include controls for the risk analysis in this study. It is generally complicated to perform power analysis in advance of regression analysis. In our setting, where knowledge lacked concerning the variables of interest (i.e. expected duration of carriage and possible risk factor for long-term carriage), we did not have the possibility to perform any meaningful power analysis. Furthermore, the number of available subjects was set, and we would not have had the option to include more subjects in order to increase the power of the risk analysis. Forty-two children and six potential risk factors were included in the Cox regression analysis (Table 1, Paper II). As listed in the table, the number of children in the groups to be compared varied between the variables. Due to low numbers of subjects in some of the groups, type 2 errors may have occurred. Consequently, all negative results may not be true negative, and should be interpreted with care.

Bacterial strain typing

*Xba*I-PFGE was chosen for bacterial strain typing in this study. MLST was only performed on selected isolates in order to detect possible epidemiological links between the outbreak strain from our NICU and other outbreak strains. PFGE has high discriminatory power and is generally considered to be the gold standard for typing of Enterobacteriacea isolates in a local outbreak situation. PFGE, however, is time consuming and a more rapid typing method would have been useful, especially during

the outbreak. Currently, commercial PCR-based typing methods are increasingly used for more rapid strain typing (278). Their discriminatory power compared to PFGE is debated (279). During recent years, WGS has become cheaper and more available. WGS has high discriminatory power and may soon become an important tool for strain typing, also in outbreak situations (280, 281). However, during the outbreak in 2008-09, WGS was not available in our hospital.

Plasmid typing

Plasmid typing is challenging due to the diversity and genetic plasticity of plasmids. In this study, we chose to combine PBRT, *S1*-PFGE and subsequent Southern blot-hybridization to characterize and compare the plasmid content of the selected isolates. PBRT is easy and fast to perform and has the advantage that results (i.e. Inc types) may be compared between laboratories, and thus provides valuable epidemiological information. *S1*-PFGE is more time-consuming to perform, but gives information about the number and sizes of plasmids carried by bacterial isolates. Through Southern blot-hybridization with relevant probes, ESBL-plasmids and their Inc types may be identified. As for bacterial strain typing, WGS may have the potential to replace the above mentioned methods for plasmid identification and typing in the near future (282, 283).

Plasmid sequencing

We obtained the complete circular DNA sequence of pKp848CTX using the 454-technology of Roche on a shotgun library, and by subsequent sequence-based gap bridging. By this approach some hours of wet lab work are required to gain a circular plasmid sequence. However, sequencing technology is evolving fast and it is becoming easier and cheaper to obtain a *de novo* plasmid sequence or even the sequence of a complete bacterial genome, as the length of reads is becoming longer and consequently the number of contigs and gaps fewer. The GS FLX+ system of Roche now provides read lengths up to 1 kb and the PacBio RSII system provides reads up to 10 kb. A number of full sequenced plasmids have been published during

recent years. However, even if modern sequencing technology may open new doors into biology, there will always be a gap between geno- and phenotype. Thus, the importance of combining WGS with studies of phenotypes must be emphasized, and is one of the strengths of this study.

Plasmid transfer, stability and fitness cost experiments

Although we could not detect transfer of pKp848CTX from CTX-M-15-Kp ST17 to *E. coli* J53-2 or to clonally identical plasmid-free CTX-M-15-Kp segregants *in vitro*, we cannot principally exclude that pKp848CTX may have been transferred below our limits of detection ($1x10^{-9}$/donor and $3.3x10^{-9}$/donor, respectively). Also *in vivo*, it is possible that transconjugants remained undetected. Furthermore, the possibility of *in vitro* self-transfer of pKp848CTX after transformation into other hosts cannot be excluded, as this was not in the scope of this study.

Our *in vivo* results suggested that pKp848CTX was stably maintained by CTX-M-15-Kp ST17 during intestinal colonization. To test the stability of pKp848CTX in its *K. pneumoniae* host *in vitro*, we performed a serial passage experiment for ~ two months (achieving ~ 430 generations). We assumed the plasmid to be stably maintained by its host also *in vitro*, therefore we only screened for segregants at the endpoint of the experiment. Considering the high variability in the endpoint segregant frequencies between the evolved populations, it would have been interesting to have data on plasmid loss rates at several points during the serial transfer experiment. Although, such data would probably not have influenced our conclusion that segregants emerge stochastically and escape bottlenecks at different time points resulting in variable endpoint segregant frequencies as discussed in paper III.

To assess the biological cost of pKp848CTX carriage in CTX-M-15-Kp ST17, we first performed 24-hour mixed culture competition experiments. Due to the rapid growth of both the plasmid-carrying and the plasmid-free segregant isolates, we chose to modify the experimental setup and performed 12-hour competitions, which were found to be more convenient for this strain. As we could not uncover any significant fitness cost associated with plasmid carriage by this approach, we also compared the growth rates of the plasmid-free segregant isolates and their corresponding plasmid-

carrying mother isolates to test whether we could capture fitness costs below the resolution of mixed culture competitions. However, the growth rate results should be interpreted with caution since they merely reflect a short time-span of logarithmic growth, whereas the mixed culture competition experiments include the complete growth cycle of the two competitors.

5.2 Discussion of the results

The main results of the three sub-studies are discussed in the respective papers. In this section, some results will be discussed more in detail and linked together when relevant.

5.2.1 The NICU outbreak

The extent of the outbreak

By screening hospitalized children and children already discharged from the NICU or maternity wards when the outbreak was recognized, we identified 58 children colonized by CTX-M-15-Kp. However, the number of colonized children may be underestimated. Some children already discharged from the NICU before we became aware of the outbreak, were missed for screening. Furthermore, discharged children only delivered one single screening sample and transient carriage may have remained undetected in some of these children. As discussed in section 5.1, the results of the follow-up study indicate that three consecutive negative screening samples would be appropriate in order to rule out ESBL-colonization.

Infection control measures

The implementation of strict infection control measurements, e.g. enhanced hand hygiene, intensified cleaning, strict barriers between patients and cohort nursing within the NICU, was not sufficient to interrupt the outbreak and prevent spread of ESBL-Kp to more children. Consequently, the original NICU was closed for new admittances, and a second preliminary NICU was established to care for new admittances. In our setting, cohorting of colonized and non-colonized patients and two groups of staff into two separate units was considered to be of great importance for the rapid control of the outbreak. The infection control measurements implemented to contain the outbreak in our NICU are in line with those implemented in most other reported NICU outbreaks (247). Laurent *et al*. reported that cohorting was the most important measure in controlling an outbreak caused by ESBL-producing *K.*

pneumoniae in an adult ICU, where routine infection control measures failed to interrupt the outbreak (306). In line with this finding, Tacconelli *et al*. recently recommended cohorting of both patients and staff to control MDR *K. pneumoniae* outbreaks among adult patients (246). In our opinion, this recommendation is also relevant in the NICU setting.

Transient carriage of ESBL-producing *K. pneumoniae* on HCWs hands has been found to be a common cause of transmission, especially between infants during NICU outbreaks (254, 263, 264). Although we did not screen HCWs hands, we suggest that patient-to-patient transmission via HCWs hands may have been an important mechanism for the efficient spread of ESBL-Kp between infants during the outbreak in our NICU. In this study, HCWs (37.5%) were only screened by rectal swabbing. In contrast to other studies, none were found colonized (247, 307). The significance of faecal ESBL-colonization in HCWs, if detected, may be debated; colonization in HCWs may as well be a result of intensive patient care as HCWs being the source of the outbreak, and as long as HCWs practice good hand hygiene their ESBL-carrier state should not cause transmission to patients.

Extensive screening of patients and hospital environment was performed during the outbreak to identify the source and to gain control. ESBL-Kp was detected in 6.5% of the environmental screening samples, but most of these were taken from sink drains and were considered to be contaminated by patients, or by the hands of HCWs or parents, rather than being the source of the outbreak. After the outbreak, the NICU was decontaminated using a hydrogen peroxide dry aerosol decontamination system (308) including silver ions (O. B. Natås, personal communication). Patients admitted to the NICU after the decontamination procedure were screened weekly for three months (18 April to 3 July), and no new cases were identified. Also environmental samples taken after the decontamination procedure, were all ESBL-negative indicating successful eradication of the outbreak strain from the hospital environment.

The source of the outbreak

The patient population is considered the most important reservoir in nosocomial ESBL-E outbreaks (95). In this study, a probable index case was identified as a child

transferred to our NICU from another hospital on 27 November 2008. CTX-M-15-Kp ST17 was isolated from frozen breast milk samples from this child's mother, collected before and after transfer. None of 32 children discharged from the NICU between 1 November and 27 November were found colonized, which strengthens the hypothesis that this child was the index case. In contrast, the source remained undetected in 48.6% of reported NICU outbreaks (247).

In paper I, we suggest that mother-to-neonate transmission due to contaminated breast milk was the most probable mechanism of ESBL-colonization in the index case. Transmission of group B streptococci and Enterobacteriaceae from mothers to neonates via breast milk has been reported previously (309, 310). However, other transmission routes have to be considered. The faecal and vaginal carrier state of the mother at the time she gave birth is unknown. Vertical transmission during delivery has been reported in cases where ESBL-producing *E. coli* was isolated from urinary, genital or faecal samples of mothers (311-314). The index case in our study was born by caesarean section, which reduces the risk for transmission during delivery. Children born by caesarean section are rather colonized by bacteria present in the hospital environment than by the maternal vaginal or faecal flora (239). Hence, a further possibility is that the index case was colonized by a strain present in the NICU environment in the hospital where it was born. In this hospital ESBL-screening was performed on all clinical Enterobacteriaceae isolates from NICU patients as they became aware of the outbreak in our hospital, but CTX-M-15-Kp was not detected (P. E. Akselsen, personal communication). As screening was implemented three months after transfer of the index case, we still cannot rule out that CTX-M-15-Kp was silently present in the other NICU before the index case was transferred.

The mother of the index case had no typical risk factors for ESBL-colonization, e.g. origin from or recent travel to endemic areas or history of broad-spectrum antibiotic use. And it is still unknown if the mother was in fact colonized before her child, and if yes, we don't know if she brought the CTX-M-15-Kp strain from the community to the hospital, or if she was colonized in the hospital where she gave birth. CTX-M-15-Kp was detected in several breast milk samples from the mother of the index case, and asymptomatic excretion and subsequent colonization of her child

via breast milk is possible. On the other hand, neonate-to-mother transmission can also not be excluded. The mother's breast, and subsequently the expressed breast milk, may have been contaminated by her infant, if the infant was colonized first. The infant received expressed breast milk from its mother through a nasogastric tube, but was also laid to its mother's breast as often as possible.

In areas with high ESBL-E carriage rates in the community, the influx of ESBL-E from the community to hospitals is of concern, as emphasized by Ben-Ami *et al*. (315). Denkel *et al*. recently concluded that the mother is the most important risk factor (OR 7.4, 95% CI 2.1-26.7, $P = 0.002$) for ESBL-E colonization of very low birth weight infants (314). In a Spanish study, 9.6% (39/406) of mothers giving birth were colonized by ESBL-E. Transmission of ESBL-E was found in seven mother-neonate pairs (18%) (313). In a recent study in the South West Region of Norway, we found that 2.9% (26/896) of pregnant women were colonized by ESBL-E before giving birth, and mother-to-neonate ESBL-E transmission was confirmed by PFGE in five mother-neonate pairs (19%) (S. Rettedal *et al*., unpublished data). The results of the above listed studies indicate that screening of mothers might be of importance in order to reduce the risk of ESBL-E import to NICUs, at least in settings with high carriage rates in the community.

Properties of the outbreak strain

Molecular investigations revealed that two CTX-M-15-Kp strains were coexisting in the NICU during the beginning of the outbreak: 56 children were found colonized by the CTX-M-15-Kp ST17 strain and two children by an unrelated ST485 strain. In paper I, we suggest plasmid-mediated transfer of $bla_{\text{CTX-M-15}}$ between the two *K. pneumoniae* strains involved in the outbreak. However, subsequent plasmid profiling and typing did not confirm this hypothesis. $Bla_{\text{CTX-M-15}}$ was encoded by a ~180 kb IncFII_{K} plasmid in the ST17 outbreak strain (Paper III), and by a ~75 kb IncI1 plasmid in the ST485 strain (supplementary unpublished data). Thus, two unrelated *K. pneumoniae* strains, carrying unrelated CTX-M-15-encoding plasmids, seem to have coexisted in the NICU during the first weeks of the outbreak.

The CTX-M-15-Kp ST17 outbreak strain is closely related to the CTX-M-15-producing *K. pneumoniae* ST16 strain, which caused a large hospital outbreak in Uppsala University Hospital in 2005 (21, 142). ST16 and ST17 are single locus variants belonging to the clonal complex (CC) 16. CTX-M-15-producing *K. pneumoniae* ST16 has also been detected in the Copenhagen area (278), indicating that strains belonging to CC16 are common in Scandinavia.

No patients died of reasons associated with the NICU outbreak in our hospital, and only one patient suffered a systemic infection caused by CTX-M-15-Kp. Due to the low infection-colonization rate (1:57) during the outbreak, it seems plausible to assume that CTX-M-15-Kp ST17 had low virulence properties. In line with this assumption, both the ST17 and the ST485 strain lacked all the virulence determinants we screened for. The zero mortality during the NICU outbreak in our hospital contrasts to mortality rates reported from other NICU outbreaks. Gastmeier *et al*. reported an average mortality during NICU outbreaks associated with *Klebsiella* spp. of 11.8% (247).

The ST17 outbreak strain seems to have outdone the ST485 strain during the first weeks of the outbreak, which indicates higher fitness of the ST17 strain under the given conditions. Empirical antibiotic treatment of systemic infection in the NICU, before and during the first months of the outbreak (until we became aware of the outbreak), was ampicillin and gentamicin in combination. The ST17 strain, which spread efficiently in the NICU environment, was co-resistant to gentamicin, whereas the ST485 strain lacked this resistance trait. Thus, the gentamicin resistant phenotype, in addition to the ESBL-phenotype, may have favored the clonal dissemination of the ST17 strain.

Characteristics of the outbreak plasmid

The DNA sequence of the CTX-M-15-encoding IncFII$_K$ plasmid of CTX-M-15-Kp ST17 (pKp848CTX), uncovered a pKPN3-like backbone similar to that in pUUH239.2, the CTX-M-15-encoding plasmid in the Uppsala *K. pneumoniae* ST16 outbreak strain (21). Interestingly, these two plasmids from related *K. pneumoniae* strains seem to have a common ancestor and several additional common traits, such as

heavy metal and thermoresistance (Figure 2, Paper III). A plasmid-encoded thermoresistance gene cluster similar to that in pKp848CTX and pUUH239.2 was recently described in Danish *K. pneumoniae* isolates (316). A thermotolerant phenotype, along with heavy metal resistance, has been proposed to increase bacterial survival and persistence in the hospital environment and on medical devices (143, 317). Copper, silver and thermoresistance have been described to have impact on the effectiveness of sterilization and decontamination methods used to decontaminate the hospital environment and invasive equipment (143, 316, 318). Thus, it is possible that a plasmid-mediated heavy metal and thermoresistant phenotype, if significantly expressed, may have contributed to the survival and spread of CTX-M-15-Kp ST17 in the NICU environment during the outbreak. The CTX-M-15-encoding IncI1 plasmid of the ST485 strain has not been sequenced. Hence, it is unknown if this plasmid encodes similar traits.

5.2.2 The follow-up study

Duration of ESBL-carriage

This is the first study to report the duration of faecal ESBL-carriage in children colonized during a NICU outbreak. The median carriage length of CTX-M-15-Kp was 12.5 months after discharge from hospital, and some children were carriers for up to two years, which is longer than most of the carriage times observed in adults after recent ICU hospitalization or ESBL-infection (236, 237). However, Titelman *et al*. found that 43% of adult patients were still colonized by ESBL-E 12 months after infection (235), and Alsterlund *et al*. reported that 13% carried ESBL-producing *E. coli* for up to 59 months after a nosocomial outbreak (232). These reports indicate that long-term ESBL-E carriage may also be expected in adults, especially after ESBL-E infection or nosocomial outbreaks. Knowledge about expected carriage times is of importance for infection control purposes during hospitalization and in the case of re-admittance (246). Due to our results, screening of children colonized by ESBL-Kp during a NICU outbreak may be considered for 1-2 years after discharge, if re-admitted to hospital. Of notice, we only studied the carriage times of one *K.*

pneumoniae strain carrying an ESBL-plasmid, which was not self-transferable. Thus, the results might have been different if the carriage times of diverse strains were studied. Altogether, further studies are needed for the estimation of expected carriage times of diverse ESBL-E strains in different populations.

Risk factors for prolonged ESBL-carriage

Factors, such as low gestational age, low birth weight, the use of invasive devices, antibiotic treatment and duration of hospital stay, have been associated increased risk of ESBL-E colonization in infants hospitalized in NICUs (253, 258-260). We investigated risk factors for becoming colonized by CTX-M-15-Kp during the outbreak in our NICU. In line with previous studies, we found that prematurity (gestational age < 37 weeks) and treatment with antibiotics (i.e. ampicillin and gentamicin in combination) were independent risk factors for becoming colonized (319).

Risk factors for prolonged duration of colonization in children colonized during a NICU outbreak have until present not been investigated, but it seems plausible that some of the factors mentioned above could also influence the duration of carriage. Thus, we examined the impact of gender, gestational age, treatment with antibiotics (ampicillin and gentamicin in combination) during NICU hospitalization, mode of delivery and feeding regime on the duration of CTX-M-15-Kp colonization. Treatment with antibiotics and delivery by caesarean section were found to be significant risk factors for prolonged CTX-M-15-Kp carriage.

Mode of delivery is the first factor which may affect the early intestinal colonization (239). Several studies report lower counts of *Bacteroides* spp. and bifidobacteria in infants delivered by caesarean section compared to vaginally delivered infants from three days up to six months of age (320-322). Hence, it is biological plausible that children born by caesarean section and subsequently hospitalized in a NICU may be at risk for long-term faecal carriage of a nosocomial *K. pneumoniae* strain if colonized.

Early antibiotic treatment in infants has also been associated with delayed intestinal colonization of beneficial bacteria (241, 243, 323, 324). In a recent study, the

gut microbiota of nine infants, who received antibiotic treatment with ampicillin and gentamicin within 48 hours after birth was compared to that of nine controls, using high-throughput sequencing and quantitative PCR. The authors showed that the gut microbiota of infants that underwent antibiotic treatment had significantly higher proportions of Proteobacteria, and significantly lower proportions of Actinobacteria, Bifidobacterium and Lactobacillus, compared to controls at four weeks. By eight weeks, the levels of Proteobacteria were still high in antibiotic treated infants, whereas the levels of Actinobacteria, Bifidobacterium and Lactobacillus had recovered (325). These findings emphasize, that antibiotic treatment during the first days of life may provide a favourable niche for intestinal colonization by Enterobacteriaceae, and furthermore, promote long-term carriage of Enterobacteriaceae, including those producing ESBL (325).

Intra-household ESBL-transmission

We documented transmission of ESBL-Kp to 20% of HCs, which is in line with transmission rates reported by others (202, 215, 218, 234). Our data suggest that children colonized by ESBL-E during hospitalization may constitute a source for ESBL-E spread within their households. In contrast to the studies referred to above, the index cases were young children (< three years of age) in this study, and the parents were aware of the carrier state of their child and were continuously informed about the screening results. Furthermore, the parents were educated to practice good hand hygiene when changing diapers etc. to prevent spread. These factors may have reduced the transmission rate, and it is possible that young children represent a higher risk for intra-household transmission of ESBL-E than demonstrated by this study.

The median carriage length of CTX-M-15-Kp in colonized parents was considerably shorter than in the children in this study. This observation may indicate that the risk of long-term carriage of an ESBL-Kp strain is higher in children colonized in the neonatal period than in healthy adults, which would be biological plausible. However, our carriage data in adults are limited, and further studies are needed to investigate the duration of faecal carriage in healthy children and adults.

5.2.3 The plasmid study

We determined the complete DNA sequence of the CTX-M-15-encoding $IncFII_K$ plasmid (pKp848CTX) from the CTX-M-15-Kp ST17 NICU outbreak strain, and investigated the plasmids dynamics and persistence in its *K. pneumoniae* host during two years of intestinal colonization in four children.

Plasmid pKp848CTX

The backbone of pKp848CTX revealed high sequence similarities to pKPN3 and other pKPN3-like plasmids (Figure 2, Paper III), including the Swedish pUUH239.2 (21), the Czech pKPN_CZ (143), the American pBK32179 (326), the Italian pKPN-IT (327), and the Korean pKP09085, pKP02022 and pKP007 (328). The worldwide dissemination of pKPN3-like plasmids into diverse *K. pneumoniae* strains indicates high stability and robustness of this backbone. Of notice, plasmid pKp848CTX seem to have evolved by the transposition of a Tn*3*-like transposon into a pKPN3-like backbone, whereas the related pUUH239.2 evolved by IS*26*-mediated integration of a completely different antibiotic resistance cassette into a similar backbone (21).

The Tn*3*-like transposon of pKp848CTX, encoding $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}208}$, is similar to transposon structures described in pEK204 and pEK516, isolated from *E. coli* ST131 strains in the UK (329). Furthermore, pKp848CTX carries the aminoglycoside resistance determinant *aac(3)-II* and a mercury resistance gene cluster. Similar mercury resistance gene clusters are present in pKPN4 and in the KPC-3 encoding pKPN4-like plasmids pKpQIL and pKpQIL-IT (141, 327). Conserved heavy metal and thermoresistance regions, similar to that in pKp848CTX, are present in several other pKPN3-like plasmids, such as pUUH239.2 and pKPN_CZ, (21, 143, 327, 328). Both pUUH239.2 and pKPN_CZ were isolated from *K. pneumoniae* strains associated with nosocomial outbreaks, suggesting that plasmid-mediated heavy metal and thermoresistance traits may favor the dissemination of the host in the hospital environment (as discussed in 5.1).

Plasmid transfer

Although pKp848CTX encodes a conserved transfer region, we have no data confirming *in vivo* or *in vitro* transfer of pKp848CTX from CTX-M-15-Kp ST17 to *E. coli* or other Enterobacteriaceae strains. Also the attempt to conjugate pKp848CTX back to plasmid-free CTX-M-15-Kp ST17 segregant isolates failed. Plasmids similar to pKp848CTX could not be detected in any other intestinal strains during up to two years of follow-up of 49 children. Also in colonized HCs, we found no evidence of plasmid transfer *in vivo* (unpublished data). Thus, in contrast to pUHH239.2 and pKPN_CZ (20, 21, 143), pKp848CTX does not seem to be self-transferable, at least not when hosted by CTX-M-15-Kp ST17.

In line with the findings from Rogers *et al*. (330), our results suggest that CTX-M-15-Kp ST17 carriage was dependent on the persistence of a strain-plasmid combination rather than the persistence of the plasmid alone after HGT to other bacteria, which may have limited the persistence of pKp848CTX in the intestine of children during long-term carriage. The plasmid not being capable of self-transfer when hosted by CTX-M-15-Kp ST17 may also have limited the outbreak to clonal spread. Hence, the extent and outcome of the outbreak could have been of another dimension if the plasmid was transferrable by HGT.

Plasmid stability and fitness cost

The dynamics between an ESBL-producing host strain and its ESBL-encoding plasmid have to the best of our knowledge not yet been studied during human colonization. We studied the CTX-M-15-encoding plasmid of CTX-M-15-Kp ST17 with regard to persistence, stability and fitness cost in relation to its host both *in vivo* and *in vitro*.

Plasmid pKp848CTX was found to be maintained by its *K. pneumoniae* host strain throughout the intestinal colonization period of up to two years in four children (who did not receive antibiotics during follow-up). Furthermore, we found no evidence of plasmid loss during carriage by screening ESBL-negative follow-up samples of these children for plasmid-free isogenic *K. pneumoniae* isolates. Also ESBL-positive samples were screened for ESBL-negative isogenic *K. pneumoniae* isolates, but again

we could not detect any segregants *in vivo* (unpublished data). These results lead to the assumption that plasmid pKp848CTX is well adapted to CTX-M-15-Kp ST17, confers negligible fitness cost to its host *in vivo*, and that the elimination of CTX-M-15-Kp in colonized children was due to outnumbering of the strain per se rather than plasmid loss.

To assess the stability of pKp848CTX carriage in CTX-M-15-Kp ST17 *in vitro*, we performed a serial transfer experiment. Surprisingly, and in contrast to the results from Sandegren *et al*., who reported no segregational loss of the similar $IncFII_K$ plasmid pUUH239.2 over 1250 generations (21), plasmid-free segregants were easily recovered with frequencies ranging between 0.0% and 17.5% after ~430 generations in serial transfer cultures without antibiotic selection. This highly variable pattern may be accountable to our 10-fold decreased bottleneck in the serial transfer regime, compared to the study of Sandegren *et al*., which reduced the probability for stochastic loss of initially rare segregants from populations.

Interestingly, pKp848CTX encodes several post-segregational killing systems (*parB*, *sopAB* and *relE*) (331-333), which are likely to prevent segregant formation through improper plasmid partitioning upon cell division. Considering the detected segregant rates of up to 17.5% in one of the evolved populations, one may question how well-tuned or functional these systems really are. However, also in the presence of active partitioning and post-segreagational killing systems, segregants may arise at low frequencies (334). In line with the assumption of de Gelder *et al*. (334), the variability in the segregant frequencies observed at the endpoint of the serial transfer in this study is likely to be representative for an evolving population, in which segregants emerge stochastically and escape bottlenecks at different time points, and thus rise in frequency at different time points resulting in different endpoint frequencies. Therefore, we hypothesize that relatively frequent segregational loss affects plasmid stability in CTX-M-15-Kp ST17 in an experimental non-selective environment to a greater extent than plasmid-conferred fitness costs. This hypothesis is supported by the results of our fitness cost experiments, where only small fitness costs, if any, could be uncovered for pKp848CTX carriage in CTX-M-15-Kp ST17.

Finally, we found no differences when comparing fitness cost of plasmid carriage in the first and the last isolates of the four children, supporting the hypothesis that plasmid pKp848CTX was readily well adapted to CTX-M-15-Kp ST17, even before it entered the NICU, and before the children were colonized.

6 Conclusions

Considering the aims of this study, the following conclusions could be drawn based on the results of the three sub-studies included in this thesis:

- The multidrug resistant CTX-M-15-producing *K. pneumoniae* (CTX-M-15-Kp) ST17 strain spread efficiently among infants during the NICU outbreak.
- Despite the high colonization rate among infants, the rate of clinical infection was low, and no death was associated with the outbreak, indicating low virulence properties of the outbreak strain.
- In total 56/58 children were colonized by CTX-M-15-Kp ST17, whereas two were colonized by an unrelated ST485 strain. The ST17 outbreak strain was in contrast to the ST485 strain co-resistant to gentamicin, which may have given this strain a selective advantage in the NICU environment.
- One child was identified as the probable index case, and vertical mother-to-neonate transmission of CTX-M-15-Kp may have been the mechanism for colonization of this child.
- Patient screening and implementation of rigorous infection control measures, such as enforced hand hygiene and strict cohorting of patients and staff, were of great importance for the rapid control of the outbreak.

- Children may become faecal carriers of ESBL-producing *K. pneumoniae* for up to two years after colonization during a NICU outbreak.
- Delivery by caesarean section and antibiotic treatment during hospitalization may be risk factors for prolonged ESBL-carriage.
- Faecal carriage in young children may represent a reservoir for intra-household spread of ESBL-producing *K. pneumoniae*.

- The CTX-M-15-encoding gene was carried by a ~180 kb pKPN3-like $IncFII_K$ plasmid, pKp848CTX, in the *K. pneumoniae* ST17 outbreak strain.

- Plasmid pKp848CTX encodes multiple antibiotic, heavy metal and thermoresistance determinants, which may have contributed to increased fitness and survival of the ST17 outbreak stain in the hospital environment.
- Plasmid pKp848CTX seems to be well adapted to its *K. pneumoniae* host conferring negligible fitness cost and being maintained throughout intestinal colonization for up to two years.
- Our results suggest that pKp848CTX is not capable of self-transfer when hosted by CTX-M-15-Kp ST17, which may have limited the extent of the NICU outbreak and the duration of ESBL-colonization in children and HCs.

7 Impact and future perspectives

It is important to investigate, characterize and report outbreaks. Such information may be useful in the prevention and containment of future outbreaks. In our hospital, procedures have been changed according to our findings. Infection control measures, such as enhanced hand hygiene and more efficient hygienic barriers between patients, were implemented in the NICU during the outbreak, and have been maintained since. The empirical treatment for systemic infections in neonates was changed from the combination of ampicillin and gentamicin to penicillin and gentamicin, to prevent future selection of *K. pneumoniae* in the NICU environment. Furthermore, Enterobacteriaceae isolated from breast milk samples are now always screened for ESBL-production to prevent ESBL-transmission from mothers to infants via contaminated breast milk. Regular ESBL-screening of children hospitalized in the NICU or at admission has been discussed, but has until present not been implemented.

Due to the increasing prevalence of ESBL-carriage in the community in most parts of the world, mothers may become an important source for NICU outbreaks. As limited data exist concerning ESBL-carriage rates in the Norwegian healthy population, we recently performed a study where the ESBL-carriage rate among pregnant women in our region, and the risk of mother-to-neonate ESBL-transmission has been studied (Rettedal *et al*., manuscript in preparation).

In the present study, we documented that children colonized during a NICU outbreak are at risk for prolonged ESBL-carriage, which has implications for infection control measures during NICU stays and at re-admissions. Due to our results, the use of alert codes to identify previously colonized patients and screening at re-admission might be considered for 1-2 years after ESBL-colonization in the perinatal period to prevent ESBL-spread within NICUs or other pediatric wards.

Plasmid stability, plasmid-host adaptation and the magnitude of plasmid-conferred fitness costs are of importance for the persistence and spread of antibiotic resistance determinants. Biological costs of an antibiotic resistance plasmid and plasmid maintenance by the host strain during long-term colonization of a human host has until present not been investigated. By combining *in vivo* and *in vitro* studies, we

gained new insight into the dynamics between a plasmid encoding antibiotic resistance determinants and its host strain. Our results suggest that multidrug resistance plasmids may be well adapted to successful host strains, and that antibiotic resistance determinants may be maintained both on the plasmid and the bacterial strain level, without antibiotic selection, for long periods of time, also *in vivo*.

Many interesting questions were raised during the plasmid study, and some of them remained unanswered. Therefore, we have initiated new projects, including WGS of the ST17 *K. pneumoniae* outbreak strain. By including the first and the last isolates from individual children, we also aim to study the microevolution of this strain and its plasmids on the DNA sequence level during long-term human carriage.

The combat against AMR is a multifaceted global task. To limit further spread of ESBL-producing and other MDR bacteria, resistance surveillance programs, adequate empirical treatment recommendations, antibiotic stewardship, increased focus on infection control and rapid detection of antibiotic resistance are of great importance (335). Increased understanding of the dynamics of spread and maintenance of resistance determinants in hospitals, in the environment, in animals, in food and in the human community is needed. The interactions between plasmids encoding multidrug resistance and their host strains need to be further studied to better understand the mechanisms for the spread and maintenance of MDR bacteria also as a potential target for interventions.

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Literature research was concluded in August 2014.

9 Errata

Page 14: The title of Paper III should be: “Persistence of a pKPN3-like **CTX-M-15-encoding IncFII$_K$** plasmid in its *Klebsiella pneumoniae* ST17 host during two years of intestinal colonization”

Page 63: The definition of CTX-M-15-Kp elimination should be: “Three or more consecutive negative faecal samples (screening interval **≥ one month**) in previously colonized children (The follow-up study)”

Page 91: In the last line, the reference to a previous chapter of the discussion should be **5.2.1.**

10 Appendix

Appendix 1

How to take a faecal sample

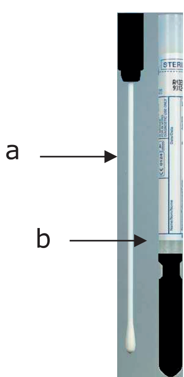
A faecal sample is easily taken in one of the following ways.

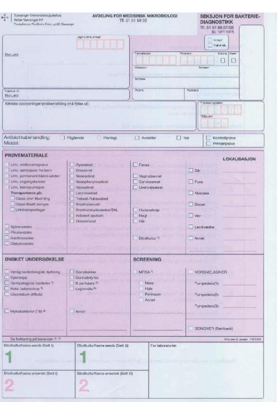
1. You can brush the rectal swab (a) around the anus immediately after defecation and before wiping.

2. Brush the rectal swab into faeces in diapers or toilet paper.

It is important that the rectal swab is visibly covered with faeces.

After the test is taken, put the swab into the tube (b) which contains charcoal. Write your names and date of birth on the tubes. Send the rectal samples together with laboratory requisitions and the questionnaire to the laboratory.




AVDELING FOR MEDISINSK MIKROBIOLOGI

SEKSJON FOR BAKTERIE-DIAGNOSTIKK

PRØVEMATERIALE

LOKALISASJON

ØNSKET UNDERSØKELSE

SCREENING

How to fill out the laboratory requisitions

Write your names and date of birth to the right on the top. Write the date the sample is taken. It is important that both samples and requisitions are well marked.

If you need something or if you have questions, you can call Kirsti Gummedal or Beate Fet, tlf. 51518807/8800.

Appendix 2

Follow-up study on newborn colonized with Klebsiella pneumoniae ESBL and transmission to family members

Date of faecal sample:

Project number:

1. What is your child's nutrition?
   Breast feeding alone
   Partial breastfeeding
   Mothers milk substitution
   Porridge or normal food is introduced

2. Did your child become any milk products containing living bacteria (like Biola) for the last month (last three months)?
   Yes
   No

3. Has your child been ill for the last month (last three months)?
   Yes
   No

If yes, what kind of symptoms did your child have?
   Fever
   Respiratory disease or pneumonia
   Diarrhoea or other gastrointestinal symptoms
Other symptoms/diseases: ______________________________

4. Has your child been treated with antibiotics for the last month (last three months)?
   Yes
   No
If yes, please write the name of the antibiotic/s:
____

5. Has your child been admitted to a hospital for the last month (last three months)?
   Yes
   No
If yes, which hospital: ______________________________

6. Has anyone in your family been abroad for the last month (last three months)?
   Yes
   No
If yes, who of you? ______________________________
Which country did you visit? ______________________________

7. How many family members are living in your house?
Number of adults:
Number and age of children:

Thank you for your participation.

Please put the questionnaire together with the faecal samples and the laboratory requisitions in the envelope, and send it to the laboratory.
If you have questions, you can call Kirsti Gummedal or Beate Fet, tlf. 51518807/8800.