Enterobacteriaceae Producing Extended-Spectrum Beta-Lactamases

Aspects of Detection, Epidemiology and Control

BIRGITTA LYTSY
Dissertation presented at Uppsala University to be publicly examined in Hörsalen, Klinisk mikrobiologi, Dag Hammarskölds väg 17, Uppsala, Friday, December 3, 2010 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Enterobacteriaceae belong to the normal enteric flora in humans and may cause infections. Escherichia coli is the leading urinary tract pathogen with septicacemic potential, whereas Klebsiella pneumoniae causes opportunistic infections and often outbreaks in hospital settings. Beta-lactams are the first choice for treatment of infections caused by Enterobacteriaceae, and might be destroyed by extended-spectrum beta-lactamases, ESBLs. ESBLs hydrolyse all beta-lactams except cephapcyn and carbapenems, and constitute a large heterogeneous group of enzymes with different origins. The phenotypic and molecular characteristics of a K. pneumoniae strain causing a major outbreak at Uppsala University Hospital between 2005 and 2008 were described. The strain was multiresistant and produced CTM-M-15, a common ESBL type in Europe. Due to the lack of obvious epidemiological links between patients, a case-control study was performed, which identified risk factors for the acquisition of the outbreak strain in urine cultures. The complex chain of transmission facilitated by patient overcrowding and the interventions applied to curb the outbreak, was revealed in the subsequent study. In the final study, the genetic background of the observed increase in ESBL-producing E. coli isolates during the K. pneumoniae outbreak was explored. The utility of six typing methods in epidemiological investigations of a local outbreak with ESBL-producing E. coli was compared. The increase of ESBL-producing E. coli isolates was not secondary to the K. pneumoniae outbreak. Twentytwo per cent belonged to the epidemic O25b-ST131 clone and only a limited number of infections were caused by nosocomial transmission. ESBL-producing Enterobacteriaceae are a challenge to clinical microbiology laboratories and infection control teams. To investigate their dissemination, typing methods need to be continuously adapted to the current situation. Proper hand disinfection and structural key problems such as over-crowding, under-staffing, lack of single rooms and bathrooms must be addressed to limit transmission.

Keywords: Extended-spectrum beta-lactamase, ESBL, pulse-field gel electrophoresis, typing methods, infection control, typningsmetoder, vårdhygien

Birgitta Lytsy, Department of Medical Sciences, Clinical Bacteriology, Box 552, Uppsala University, SE-75122 Uppsala, Sweden.

© Birgitta Lytsy 2010

ISSN 1651-6206
ISBN 978-91-554-7922-0
urn:nbn:se:uu:diva-131901 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-131901)
“I will prescribe regimens for the good of my patients according to my ability and my judgment and never do harm to anyone.”

Hippokrates (ca. 460 BC – ca. 370 BC)
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Reprints were made with kind permission from the respective publishers.
Results...........................................................................................................32
Paper I ........................................................................................................32
Paper II ......................................................................................................32
Paper III ....................................................................................................34
Paper IV ...................................................................................................35
Discussion .....................................................................................................38
Conclusions .................................................................................................46
Sammanfattning på svenska.................................................................47
Acknowledgements ..................................................................................48
References .................................................................................................50
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-PCR</td>
<td>Arbitrarily primed PCR</td>
</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum beta-lactamase</td>
</tr>
<tr>
<td>HCW</td>
<td>Health care workers</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>Rep-PCR</td>
<td>Repetitive sequence-based PCR</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>SRGA</td>
<td>Swedish Reference Group for Antibiotics</td>
</tr>
<tr>
<td>UUH</td>
<td>Uppsala University Hospital</td>
</tr>
</tbody>
</table>
Preface

The European Centre for Disease Prevention and Control (ECDC), the European Medicines Agency (EMEA) and the international network “Action on Antibiotic Resistance” (ReAct), estimated in a recent report that over 25,000 European citizens died from infections caused by resistant bacteria in 2007. These infections resulted in 2.5 million extra hospital days and extra in-hospital costs of more than €900 million. The report concluded that there is an acute need of new antibacterial agents, particularly for Enterobacteriaceae and other Gram-negative bacteria, which have developed resistance at an alarming rate in a short time.

The major forces behind the development of resistance in bacteria are exposure to antibiotics followed by selection and dissemination of resistant strains. The health care setting provides a favourable environment in this context. Resistant strains evolve and disseminate by direct and indirect contacts, where large numbers of people are congregated under one roof and where the usage of antibiotics is high. Previous infection control research has focussed on the control of resistant Staphylococcus aureus. Less research has been done on the control of resistant Enterobacteriaceae. With this thesis, I hope to contribute to the existing knowledge of the epidemiological investigation and the effective interventions for prevention and control of Enterobacteriaceae producing extended-spectrum beta-lactamases.

Introduction

**Enterobacteriaceae**

*Enterobacteriaceae* is a heterogeneous family consisting of more than 30 genera and 150 species and subspecies (1). Members of *Enterobacteriaceae* are rod-shaped, fermenting and Gram-negative. They belong to the normal intestinal flora of most animals, including humans, and may cause intestinal and extra-intestinal infections. The difference between commensalism and pathogenicity is the result of a complex balance between the status of the host and the presence and expression of virulence factors in the bacteria. Medically important species of *Enterobacteriaceae* are *Escherichia coli* and *Klebsiella pneumoniae*.

*E. coli* is the most abundant facultative anaerobe of the human intestinal microflora. Commensal *E. coli* strains rarely cause disease in humans, except in immunocompromised patients or when the normal gastrointestinal barriers are breached. A limited number of pathogenic *E. coli* clones have gained specific virulence attributes (adherence factors and other pathogenic surface structures) which enable them to cause urinary tract infections, septicemia, meningitis and diarrheal disease not only in immunocompromised patients but also in healthy individuals (2). Pathogenic *E. coli* are characterised by shared O (lipopolysaccharide) and H (flagellar) antigens that define serotypes or serogroups. *E. coli* can be sub-divided further into four main phylogenetic groups; A, B1, D and B2. Commensal strains of *E. coli* belong mainly to the A and B1 phylogenetic groups (3). Pathogenic phylogenetic lineages involved in extra-intestinal infections are mainly derived from the B2 group and its sub-groups.

*K. pneumoniae* typically causes opportunistic infections of the urinary tract, in wounds and soft tissue and in the blood stream (4-6). Infections caused by *K. pneumoniae* are often observed in hospital settings and are associated with high mortality rates in infants and the elderly (7, 8). A number of bacterial factors contribute to the pathogenesis of *K. pneumoniae*, of which the prominent capsule and lipopolysaccharides are essential for the ability of this species to form biofilm and to colonise (5, 9, 10).
Beta-lactams

Few medical discoveries have had a greater impact on human disease and mortality than the introduction of antibiotic drugs. The disease pattern in developed countries has changed from infectious diseases to degenerative diseases (11), and there has been a dramatic increase in life expectancy, especially in the young population.

The era of antimicrobial chemotherapy began in 1929, with Fleming's discovery of a powerful bactericidal substance, penicillin. In the early 1940s, spurred by the need for antibacterial agents in World War II, penicillin was found not only to cure infections but also to possess low toxicity. This promoted an intense search for similar antimicrobial agents.

Antibiotics are grouped in classes or families based on their chemical structure. The beta-lactam class of antibiotics, including penicillins, cephalosporins, monobactams and carbapenems, share the beta-lactam ring (Figure 1).

![Chemical structure of penicillin G](image)

**Figure 1.** The structure of penicillin G. The beta-lactam ring is represented by dashed lines.

The mechanism of action of beta-lactams is to disrupt bacterial cell wall synthesis by linking covalently to enzymes, i.e. penicillin-binding proteins (PBPs), located in the cell-wall (12). Beta-lactams irreversibly inactivate these peptidoglycan transpeptidases, resulting in cell death. This bactericidal effect, combined with low toxicity, has made the beta-lactams currently the most prescribed antibiotic class, constituting over 60% of all clinically used antimicrobial drugs (13).
Beta-lactamases

In 1940, almost immediately after the penicillins became available for therapy, enzymes able to destroy penicillin named “penicillinases” were detected in *E. coli* (12, 14, 15). To overcome the beta-lactamase mediated resistance against penicillins produced by Gram-negative bacteria, a broad-spectrum penicillin, ampicillin, was introduced into clinical practice in the 1950s. In the 1960s, the first generation cephalosporins reached the market. The broad-spectrum penicillins and the first generation cephalosporins have remained the first line treatment options for over twenty years.

The production of penicillinases and other beta-lactamases is the principal mechanism of resistance to beta-lactam antibiotics among members of the *Enterobacteriaceae* family (14, 16). Beta-lactamases are enzymes located in the periplasm on the outer surface of the inner membrane of the cell envelope (17). They hydrolyse the beta-lactam ring structure by binding covalently to the amide bond of the beta-lactam ring structure (18).

Beta-lactamases were present in bacteria long before the introduction of penicillins (15), and genes encoding these ancient enzymes were originally located on the bacterial chromosome (15, 19). Furthermore, these enzymes are inducible and constitutively expressed in low quantities. In 1965, the first report of a plasmid-encoded beta-lactamase in a Gram-negative bacterium appeared from Greece (20). This TEM-1 producing *E. coli* hydrolysed ampicillin, and within a few years after its first isolation, its plasmid-mediated resistance had spread over the world and into many different members of the *Enterobacteriaceae* family, including *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Additional reports soon followed, describing plasmid-encoded beta-lactamases, which were also able to hydrolyse first generation cephalosporins. At this time, Gram-negative bacteria became more prevalent in health-care-associated infections, HCAIs, and *S. aureus* declined as the most prevalent bacteria in nosocomial infections.

The spread of plasmid-encoded TEM and SHV enzymes from the mid-1970s and onwards resulted in therapeutic failures. To counter this, the pharmaceutical agencies introduced several novel classes of beta-lactams with expanded spectra in the late 1970s and the early 1980s. These drugs included the cephemycins, oxyamino-cephalosporins, carbapenems, monobactams, clavulanic acid and penicillanic acid sulfone inhibitors. For reasons of convenience, spectrum, cost and safety, the oxyimino-cephalosporins (principally cefuroxime, cefotaxime, ceftriaxone, ceftazidime and cefepime) became the most-used of these analogues. The cephalosporins are now standard therapies for pneumonias, intra-abdominal infections and urinary tract infections worldwide. By the extensive use of these compounds, the selective pressure has increased. Consequently, there has been a dramatic expansion of beta-lactamases (14, 21, 22), and new enzymes with wider substrate
ranges emerged (22). Today, acquired resistance to beta-lactams is mainly mediated by the ESBLs.

**Evolution and epidemiology of extended-spectrum beta-lactamases**

In 1983, the first report came from Germany by Knothe et al. of a mutant of SHV-1 named SHV-2 in a *K. pneumoniae* strain, which was capable of hydrolysing oxyamino-cephalosporins (23). Two years later, SHV-2 was transferable between bacteria (24). Just a few years later came reports from French hospitals of problems attributed to *Enterobacteriaceae* carrying mutated TEM-derivates which acted like SHV-2 (25-27). The term “extended-broad-spectrum beta-lactamases” was coined (28).

The first described ESBLs evolved through random point mutations in isolates with broad-spectrum beta-lactamases, i.e. TEM-1, TEM-2, TEM-13, SHV-1 and SVH-11, which were already widespread in clinical settings when expanded-spectrum beta-lactams were introduced (22, 29, 30). The ESBLs derived from TEM and SHV could differ from their progenitors by only one amino acid. This change was critical and had a profound effect on enzymatic activity, leading to hydrolysis of third-generation cephalosporins and aztreonam. The beta-lactamases had thereby an “extended-broad-spectrum” activity as compared with the “broad-spectrum” classic TEM and SHV enzymes. The term “broad” was lost and the term became “extended-spectrum beta-lactamase”.

The continuous pressure exerted by the use of newer expanded-spectrum beta-lactams promoted the development of new TEM and SHV derivates. Today, the number of TEM and SHV variants has grown to over 170 and 130, respectively (http://www.lahey.org/Studies, see the web site for updated information).

ESBLs of the CTX-M type were rare until the end of the 1980s, but Japan, Argentina and Germany reported almost concomitantly findings of this ESBL type (22, 29-32). The CTX-M family consists of over 80 different CTX-M types. (http://www.lahey.org/Studies). The CTX-Ms can be further subclassified, based on amino acid similarities and sequence homology, into five groups; groups 1, 2, 8, 9 and 25. CTX-M groups 1 and 2 evolved by the escape of chromosomal genes from *Kluyvera ascorbata* (33), whereas group 8 and 9 enzymes evolved via similar escapes from *Kluyvera georgiana* (34). The genes encoding CTX-Ms have been mobilised from *Klyuviera* spp. by several genetic events and mechanisms (29).

With the emergence of the CTX-Ms, there has been a marked shift in the epidemiology of ESBLs (29, 31, 32, 35-37). Before the mid 1990s, the ESBLs were mainly present in *Klebsiella* spp. in nosocomial settings. The
dominating ESBL enzymes were TEM and SHV derivates. Today, the CTX-Ms are the most prevalent ESBL enzymes, and *E. coli* is the main ESBL producer. The predominance of CTX-Ms has not only been observed in hospitals but also in the community, from nursing homes and long-term facilities. CTX-M-15 is the most commonly reported ESBL-enzyme in Europe (32, 37), first observed in the UK (38, 39). CTX-M-15 is derived from CTX-M-3 by a single amino acid substitution at position 240 (Asp → Gly). This substitution confers an increased catalytic activity against ceftazidime (40).

The success of the CTX-Ms over the classical ESBL-enzymes SHVs and TEMs is linked to the way by which CTX-M enzymes are spread. Through mobile genetic elements, resistance genes disseminate within the same species and also between bacteria of different species (29, 41, 42). Mobile elements involved in the dissemination of *bla*CTX.M genes have been described in recent reviews (22, 29, 37). Horizontal dissemination of genes encoding ESBLs occurs by conjugative plasmids and transposons (Figure 2).

![Figure 2. Anatomy of a resistance plasmid. The plasmid contains mobile transposons and integrons carrying resistance gene cassettes.](image)

The motility and multidrug-resistance of the CTX-Ms is sometimes associated with integrons (31, 43). Integrons are highly efficient recombination and expression systems, which are able of capturing DNA sequences known as gene cassettes by site-specific recombination (42). Integrons can move in and out of the genome and in this way remodel it, but integrons are not able to move between bacteria. The gene cassettes, which harbour genes encoding CTX-Ms, usually carry one or several other genes encoding antibiotic
resistance. Several different integron classes have been reported according to the homology of their integrase genes (43). Class 1 integron, followed by class 2 integrons is most commonly found in nosocomial and community settings. Typing of integrons may be a way to surveil the spread of ESBL-producing bacteria.

As a consequence of horizontal gene transfer by transposon-plasmid vectors, most ESBL-producing *E. coli* were clonally unrelated until a few years ago (44). Recently, CTX-M-15 was identified in an international clone of *E. coli*, which has been detected in both in-patients and out-patients (45-48). This clone belongs to the phylogenetic group B2, MLST-type 131 and exhibits a specific lipopolysaccharide type (O25b). This O25b-ST131 clone has not only a considerable ability to disseminate, it is also equipped with a high virulence potential, causing significant morbidity and mortality. This is partly explained by its capacity to produce biofilm, which might contribute to their long-term persistence in various environments and to their exhibited resistance to antimicrobial agents and disinfectants (47). It is also possible that the production of biofilm leads to an increased resistance to host immune defences.

The rapid emergence of the ESBL-production among *Enterobacteriaceae* has already had serious clinical implications. Several studies have described an association between ESBL-producing *Enterobacteriaceae* in bloodstream infections and treatment failure, excess mortality, increased treatment costs and prolonged hospital stay (7, 49-53). According to EARSS, resistance to antibiotics among Gram-negative bacteria involved in serious infections in humans has now reached 25% or more in many European countries (http://www.rivm.nl/earss/). According to data from the European surveillance system, (EARSS), the incidence of invasive *E. coli* and *K. pneumoniae* with resistance to third-generation cephalosporins increased in Sweden between 2005 and 2008 from 1.3% to 2.3% and 1.4% to 2.3%, respectively. So far, only two minor Swedish outbreaks have been reported: one from the Stockholm region caused by *E. coli* carrying CTX-M group 1 (54), and one from the southern parts of Sweden (55). The latter involved 27 patients and was caused by an *E. coli* strain carrying CTX-M-15.

Many research groups have investigated the risk factors associated with the acquisition of infections with ESBL-producing *Enterobacteriaceae* (52, 56-62). One important risk factor is prior exposure to antibiotics, predominantly third generation cephalosporins and fluoroquinolones. Other risk factors are the presence of severe underlying disease, invasive medical equipment (nasogastric feeding tubes, urinary catheters, endotracheal tubes, and central venous lines), prior surgery, a prolonged hospital stay and recent hospitalisation. Intensive care units are typically “risk units” due to their high selective pressure in combination with susceptible patients.
Definition and classification of extended-spectrum beta-lactamases

Two widely used taxonomic classification schemes for beta-lactamases are the Ambler structural classification (63) and Bush–Jacoby-Medeiros functional classification (64, 65). In strict sense, ESBLs fall into Ambler’s class A based on structural properties. Based on functional characteristics, ESBLs fall into subgroup 2be (hydrolysing broad-spectrum clavulanate-inhibited beta-lactamases, able to hydrolyse oxyimino-cephalosporin at rates of at least 10% that for benzylpenicillin). The 2be designation means that these enzymes are derived from group 2b beta-lactamases (TEM-1, TEM-2, and SHV-1); the “e” of 2be denotes that the beta-lactamases have an extended spectrum.

These classification schemes exclude several other acquired beta-lactamases with a broader spectrum, including for example plasmid-borne AmpC and OXA-type cephalosporinases, the transferable carbapenemases, the Klebsiella pneumoniae class A carbapenemases (KPC) and certain GES-variant beta-lactamases (28, 66). These beta-lactamases belong to different functional and/or structural classes but share an extended spectrum of beta-lactam hydrolysis.

Some or all of these groups are included as ESBLs depending on the author. It is still under debate whether ”ESBL” serves as a descriptor of hydrolytic activity, or not or if the term has evolved to become phylogenetic. There was recently a proposal to expand the definition of ESBLs to include all acquired beta-lactamases, with activity to broad-spectrum cephalosporins and/or carbapenems, since they are equally relevant for the clinical and infection control perspective (66). However, there is not yet a generally accepted definition of ESBL. In this work, ESBLs are defined as acquired beta-lactamases, which inhibit narrow and broad-spectrum penicillins, cephalosporins and monobactams by hydrolysis, which do not inhibit cephamycin and carbapenems and which are inhibited by site-directed beta-lactamase inhibitors such as clavulanic acid, sulfabactam and tazobactam.

Laboratory detection of Enterobacteriaceae producing extended-spectrum beta-lactamases

ESBL-producing members of the Enterobacteriaceae family are not easy to identify phenotypically. Broad-spectrum cephalosporin resistance may result from several mechanisms and not only by the production of ESBLs (15). The co-expression of AmpC enzymes may with high efficiency mask the presence of an ESBL in the same strain.
The SRGA recommends susceptibility testing with cefpodoxime (a substrate for ESBLs, AmpC and OXA-enzymes) or cefotaxime (a substrate for most CTX-Ms) and ceftazidime (a substrate for most TEMs and SHVs) as the first step for identifying ESBLs (www.SRGA.org). With either screening method, the SRGA recommends further testing with either a disc diffusion method or an Etest based method, using cephalosporins with and without clavulanic acid. None of these methods can accurately detect all strains producing ESBLs. The merits and shortcomings of the different methods for detection of ESBLs have recently been reviewed by Bradford et al. (15).

These phenotypic tests are sometimes followed by further characterisation of the enzymes by molecular methods. These methods have a higher sensitivity and specificity. On the other hand, they are more complicated to perform. Determination of isoelectric point was previously used when the number of known ESBLs was low. Today, this is not possible, since several of the SHV, TEM, CTX-M and OXA types have the same isoelectric point. DNA probes specific for TEM and SHV derivatives and RFLP analysis have also been used. The most broadly used molecular methods are PCR-based with subsequent DNA sequencing.

Outbreaks

HCAIs are infections acquired by patients or by members of the health care staff as a direct or an indirect result of health care (67). The majority of HCAIs are endemic. Endemic HCAIs are defined as sporadic infections that constitute the background incidence within a health care facility (67). The rate of endemic HCAIs may fluctuate over time, but these fluctuations are usually not statistically significant. The most common endemic HCAIs are UTI, bloodstream infections, surgical site infections and ventilator-associated infections. A substantial proportion of bacteria causing HCAIs are resistant to antibiotics (68). The bacteria causing HCAIs are often multiresistant due to the selection for and transmission of resistant bacteria in the hospital setting.

Epidemic HCAIs or outbreaks, are defined as HCAIs that occur at a significant higher rate than the usual, expected incidence rate (67, 69). Epidemic HCAIs or outbreaks, are clustered over a defined time-period and a defined geographic area. This suggests that outbreaks stem from a common source. Outbreaks are easily recognised if they involve unusual pathogens or pathogens of unusual resistance patterns, but can sometimes be hard to discover since they merge with endemic infections. Since surveillance is the cornerstone for the rapid recognition of outbreaks, hospitals must have a reliable surveillance system that allows for the identification of increased infection rates. Outbreaks account for enormous morbidity and costs and should be identified and investigated immediately on suspicion.
Outbreak investigations involve several steps (67, 69, 70). The purpose of the investigation is to identify the responsible pathogen(s), its mode of transmission, to find and remove the source(s) and to formulate recommendations to interrupt transmission. The main goal is to prevent further cases.

Initial steps in the outbreak investigation include establishing a case definition. The case definition is based on population characteristics (e.g. age, sex), clinical data (e.g. onset and duration of signs and symptoms, treatments, devices) and laboratory results. A line-list should be constructed to display all patients’ characteristics important for the investigation. After identifying all the suspected case patients and the precise time period (time of exposure) and place (affected ward or hospital) of infection, an epidemic curve should be constructed. An epidemic curve is helpful to compare the incidence rate in the epidemic phase compared to the pre-epidemic phase. Based on the assembled information a hypothesis of the source and the route or mode of transmission can be developed. In some investigations, the environment is surveyed by obtaining cultures from HCW and suspected items.

There are no standardised forms for an outbreak investigation, so the approach must be adjusted and individualised depending on the source, the host, the pathogen involved and its mode of transmission. The natural ecological niche and other characteristics of the pathogen provide useful information in the investigation. For example, *Legionella* spp and *Pseudomonas* spp are often traced to water sources, and resistant bacteria emerge from environments with high antibiotic pressure.

At first suspicion of an outbreak, isolates cultured from individuals or from the environment should be saved and stored for future strain typing at the clinical microbiological laboratory (71-73). Typing the pathogen involved in the outbreak to the sub-species level provides invaluable information to the investigation. Isolates that are epidemiologically related, i.e. cultured from patients, fomites or the environment during a discrete time frame in a well-defined area and are indistinguishable after typing, are likely to stem from a common source. By identifying and removing the source, the outbreak can be terminated.

The definition of epidemic strains or clones is that the isolates are related both epidemiologically (by time and place) and by typing methods (73, 74). Endemic strains, on the other hand, are isolates that are recovered frequently from patients in a particular health care setting of the community that do not have an obvious epidemiological linkage but are still related by typing methods. Such organisms are presumed to be clonally related but their common origin may be temporally more distant from those of outbreak strains. For example, most strains of methicillin-resistant *Staphylococcus aureus*, MRSA, are derived from a small number of ancestral clones (74). If epidemiologically related isolates do not have identical DNA-fingerprints, they could still belong to a single strain or clone. Evolutionary changes might be
responsible for rapidly evolving differences between isolates of the same original strain.

Typing systems are divided into phenotyping and genotyping methods. The first techniques are based on characteristics such as antibiotic resistance patterns and soluble protein profiles (Table 1). An objection to phenotyping methods is that bacterial genes may not always be expressed, making phenotyping methods less stable than genotyping methods. Phenotyping methods have been replaced by genotyping methods over the last two decades, even though genotyping methods are more resource and time consuming. PFGE is the traditional gold standard for molecular typing of bacteria, including ESBL-producing Enterobacteriaceae (50). Other commonly used molecular techniques for local outbreak investigations are shown in Table 1.

Once the epidemiologic and laboratory investigations are completed, infection control measures should be recommended in order to terminate the outbreak and to prevent further transmission or similar events. The selection of control measures depends on the results of the initial analysis and the results of further comparative studies. Interventions aim at interrupting transmission and/or at removing the source of the pathogen. HCW should repeatedly be educated on the recommended measures and the measures should be assessed regularly. Sometimes, revisions of current infection control measures are necessary.

Investigations of epidemic HCAIs are common and the results from these investigations are often published. Three different types of studies are generally used. They include descriptive studies, analytic studies and experimental studies. Descriptive studies are often the first investigation performed and form the hypothesis and a base for additional, more sophisticated studies such as analytic or experimental studies. Analytic studies, including cohort studies and case-control studies, explore risk factors and potential associations of a disease with the acquisition of a pathogen. Experimental studies, such as double-blinded randomised controlled trials, prove or disprove the hypothesis about the cause or the efficiency of the intervention.

Table 1. Examples of phenotyping and genotyping systems used for typing nosocomial organisms.

<table>
<thead>
<tr>
<th>Phenotyping methods</th>
<th>Genotyping methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic susceptibility pattern</td>
<td>PFGE</td>
</tr>
<tr>
<td>Soluble protein profiles</td>
<td>AP-PCR</td>
</tr>
<tr>
<td>Phage typing</td>
<td>Rep-PCR</td>
</tr>
<tr>
<td>Biotyping</td>
<td>MLST</td>
</tr>
<tr>
<td>Serotyping</td>
<td>RFLP</td>
</tr>
<tr>
<td>Colicinotyping</td>
<td>DNA sequencing</td>
</tr>
<tr>
<td></td>
<td>Plasmid analysis</td>
</tr>
</tbody>
</table>
Extended-Spectrum Beta-Lactamase Producing Enterobacteriaceae at Uppsala University Hospital

Until 2005, about 10-15 ESBL-producing strains were isolated annually at UUH and the majority of these strains were identified as *E. coli*. Starting in May 2005 and throughout the summer months, the number of *K. pneumoniae* isolates with a multidrug-resistant phenotype suddenly increased. The predominant source was urinary samples, and all isolates expressed a similar resistance pattern, including resistance to third generation cephalosporins and fluoroquinolones.

Samples positive with the multi-resistant *K. pneumoniae* strain originated from patients admitted to different wards throughout the hospital, with the exception of paediatric and psychiatric wards. At the time of the outbreak, there was no obvious relation in time and space between culture-positive patients, but all patients had a history of previous hospitalisation at UUH shortly before their first positive culture. Since an outbreak situation could not be ruled out, the chief epidemiologist at the Department of Communicable Disease Control and Prevention declared that the situation was serious and required extra resources. An outbreak investigation was initiated.
Aims of thesis

The aims of this thesis were to:

- Perform an epidemiological investigation including characterising the molecular epidemiology and the genetic background of a number of ESBL-producing *K. pneumoniae* and *E. coli* isolates obtained at UUH during 2005-2007.


- Describe the complex chain of transmission between patients and the interventions undertaken to curb the outbreak.

- Compare six different typing methods and their utility in an epidemiological investigation of a suspected local outbreak of an ESBL-producing *E. coli*. 
Materials and Methods

Summary of study designs

Paper I is a retrospective descriptive study.
Paper II is a retrospective case-control study (analytic study).
Paper III is an ambidirectional descriptive study.
Paper IV is a retrospective descriptive study.

Setting

All four studies were conducted at UUH, a 1,100 bed hospital with 52,000 admissions and 650,000 visits per year. UUH has about 80 wards and five intensive care units. The hospital serves a population of approximately 300,000 inhabitants with primary health care and 1,300,000 inhabitants with tertiary health care.

During the study period, about 10% of the patient beds were in single rooms with en-suite bathrooms. The infection control team consisted of one, and sometimes two, full time physicians, three infection control nurses for the hospital and one for the community. The clinical microbiology laboratory at UUH reported daily to the infection control team any isolation of multidrug-resistant bacteria such as MRSA, vancomycin-resistant enterococci or ESBL-producing enterobacteria.

The infection control committee, chaired by the chief physician of the hospital, included representatives from the Departments of Infection Control, Infectious Diseases, Occupational Health, Clinical Microbiology and Public Health; hospital administration, hospital information and hospital service suppliers.

One nurse and one auxiliary nurse from each of the hospital wards were appointed as link nurses, and joined together to form hygiene groups in each clinical department. These groups met monthly, supervised by their designated infection control nurse.

Infection control guidelines stipulated short-sleeved working clothes for all categories of staff, with no rings or no wristwatches permitted. An alcohol-based hand disinfectant was to be used before and after tending to a patient, before donning and after removing gloves, and when entering or leaving a patient room. Hand washing with ordinary soap was used before disin-

23
fection only when hands were visibly dirty. Gloves were used when touching secretions, excretions and contaminated or dirty equipment. Gowns or aprons were used only for close contact with a patient or a patient’s bed. Doctors’ white coats were removed before close patient contact. The precautions were the same for patients nursed in single or isolation rooms. Masks, caps and overshoes were not used in wards.

The hospital antibiotic policy was communicated to doctors through policy documents, lectures and direct contact from specialists from the Department of Infectious Diseases. In 2004, the year before the outbreak, an average of 45.9 defined daily doses (DDD) of antibacterial drugs were used in the hospital per 100 patient-days. Cephalosporins accounted for 20% of the total antimicrobial use and quinolones 12%.

Inclusion criteria of isolates, cases, controls and contacts

In paper I, a total of 64 *K. pneumoniae* isolates resistant to a third generation cephalosporin and to at least three other classes of non-beta-lactam antibiotics were included. In addition, 22 randomly chosen isolates of *K. pneumoniae* fully susceptible to cephalosporins were included to serve as controls for the PFGE. For details, see Table 2.

In paper II, cases and controls were identified retrospectively through the laboratory records. All patients included were adults (≥ 15 years old), and no symptoms had to be present for inclusion. A case was defined as a patient, hospitalised or receiving medical care at an outpatient department at the hospital, from whom the ESBL-producing *K. pneumoniae* outbreak strain was isolated in a urine sample. A control was defined as a patient with significant growth of *E. coli* in a urine sample, exhibiting no phenotypic resistance mechanism to cephalosporins and susceptible to at least three other classes of antibiotics. Control patients were matched in a 2:1 ratio to case patients according to ward and month of isolation of the bacterium.

In paper III, all patients with confirmed ESBL-producing *K. pneumoniae* isolated from any site were included. A patient was defined as a case when the isolate was identified by PFGE as belonging to the outbreak strain. Cases identified through samples taken at the discretion of the clinician were called “clinical cases”. Patients sharing a room with a known case during the week preceding the detection of the case were called “contacts”.

In paper IV, the included *E. coli* isolates had to either exhibit resistance to cefotaxim and/or ceftazidime (MIC >1 mg/L or < 24 mm) or belong to CTX-M group 1. Of the isolates, 166 were obtained from stool through active surveillance and the remaining 87 isolates were obtained from clinically infected patients. Five isolates from the *K. pneumoniae* outbreak were added for comparison of RFLP types.
Table 2. Summary of the included isolates obtained from patients in the four studies.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source of isolates (no.)</th>
<th>Patients (no.)</th>
<th>Time period</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Urine (57) Wound (5) Blood (2)</td>
<td>Case (64)</td>
<td>May – December 2005</td>
<td>I</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Urine (22)</td>
<td>Control (22)</td>
<td>December 2005</td>
<td>I</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Urine (54)</td>
<td>Case (54)</td>
<td>May – December 2005</td>
<td>II</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Urinary (108)</td>
<td>Control (108)</td>
<td>May – December 2005</td>
<td>II</td>
</tr>
</tbody>
</table>

Bacteria and media

The ESBL-producing *K. pneumoniae* and *E. coli* isolates in papers I - IV were collected at the Department of Clinical Microbiology at UUH. The isolates were stored at –70°C. Strains of ESBL-producing *K. pneumoniae* previously stored from January 2004 to May 2005 were thawed and re-examined.

The bacteria were cultured on blood and cysteine lactose-electrolyte-deficient agar (Becton, Dickinson and Company Sparks, MD, USA). The agar plates were incubated overnight at 35°C in room atmosphere. Conventional laboratory methods combined with a VITEK 2 instrument (BioMérieux, Lyon, France) were used to identify *K. pneumoniae* and *E. coli* to the species level.

In paper III, until March 2007, surveillance specimens, obtained from contact tracing and screening samples, were plated onto McConkey agar (Acumedia, Lansing, MI, USA) with 5 mg cefotaxime and 10 mg ceftazidime discs (Oxoid Ltd, Basingstoke, UK). Colonies growing within the expected zones of cefotaxime and/or ceftazidime were identified to the species level. The sensitivity of stool screening was found to be 95% compared to 47% for urine samples, hence screening was limited to faecal samples only. From March 2007 and throughout the rest of the study period, a real-
time PCR was used to detect faecal organisms with the blaCTX-M phyloge-netic group 1 gene, using a method described below.

In paper III, about 400 environmental cultures were taken in the months of February and November 2006 from frequently touched items or surfaces in wards with suspected transmission, using Rodac contact plates (Biotrace, Runcorn, UK) and incubated at 30º C for 5 days. Isolates obtained by environmental cultures were typed with standard methods described above.

Antimicrobial susceptibility testing and phenotypic detection of extended-spectrum beta-lactamases

In all four studies, antibiotic susceptibility testing was performed by the VITEK 2 instrument (BioMérieux) or by the disc diffusion method (Oxoid Ltd., Basingstoke, UK) recommended by SRGA. The species-related zone breakpoints recommended by the SRGA characterised the isolates as susceptible, indeterminate, or resistant.

In paper I, a total of 25 randomly chosen isolates from the early and the late part of the observation period were tested with Etest (AB Biodisk, Solna, Sweden) in accordance with the manufacturer’s instructions and the recommendations of SRGA.

In all four studies, first step ESBL detection criteria was resistance to ce-fadroxil (≤13 mm zone diameter) or resistance/reduced susceptibility to ce-fotaxime (<24 mm zone diameter/MIC > 1 mg/L) and/or ceftazidime (<24 mm zone diameter/MIC>1 mg/L). Phenotypic confirmation of ESBL-production was performed by a modified double disc diffusion synergy test (75) or according to the method recommended by the SRGA. All plates were incubated for 18-24 h at 35ºC in room atmosphere. The quality control strain included was E. coli ATCC 25922.

Multi-resistance was defined as resistance to a third generation cepha-losporin and to at least three other classes of non-beta-lactam antibiotics.

Molecular detection of genes encoding extended-spectrum beta-lactamases and plasmid-encoded fluoroquinolone resistance

In paper I and paper II, the K. pneumoniae isolates were screened for alleles encoding the blaCTX-M phylogenetic lineage groups 1, 2, 8, 9 and 25/26, the blaSHVs and the blaTEMs. Screening for the blaTEM was performed using primers designed for the study based on blaTEM-sequences reported in the GenBank database. For the other PCR assays, earlier described methods were used after slight modifications (76).
To identify carriers of the CTX-M-15 producing *K. pneumoniae* outbreak strain, a modified PCR assay for CTX-M group I enzymes described by Pi-tout *et al.* was applied (77). The assay was used in paper III from March 2007 onwards and in paper IV during the whole study period. ESBLs of the CTX-M group I were thereby detected in all members of the *Enterobacteriaceae* family, including *K. pneumoniae* and *E. coli*. In paper IV, the *E. coli* isolates were in addition screened for alleles encoding *bla*CTX-M phylogenetic lineage groups II-IV (corresponding to 2, 8 and 9 above), *bla*SHVs and *bla*TEMs with PCR (77).

Six ESBL-producing strains used as positive controls for the PCR assays were kindly provided by David Livermore and Neil Woodford, London, UK, and Barbro Olsson-Liljeqvist, Swedish Institute for Infectious Disease Control, Stockholm, Sweden.

Before running the faecal samples in the thermal cycler, the bacteria were enriched in Luria-Bertani broth (Becton Dickinson and Co.) under selective pressure (cefotaxime, Oxoid Ltd.) and pooled. All samples included in a PCR-positive reaction were inoculated onto McConkey agar (Acumedia, Lansing, MI, USA) with 5 µg cefotaxime and 10 µg ceftazidime discs. Colonies growing within the expected inhibition zones of cefotaxime or cefotaxime and ceftazidime were identified to the species level as described above.

In papers I and IV, PCR products from a subset of 70 isolates were sequenced at MWG Biotech, Martinsried, Germany, or locally. The BLAST tool at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) was used to identify the beta-lactamases. Multiple sequence alignments were generated with the BioEdit 7.0 sequence alignment editor (Ibis Therapeutics, Carlsbad, CA USA).

In paper I, twenty randomly chosen *K. pneumoniae* isolates belonging to the outbreak strain were tested for *qnr*-production using the method described previously by Jacoby and co-workers (78, 79). Positive control strains producing plasmid-encoded fluoroquinolone resistance (*qnr* A and *qnr*B) were kindly provided by Patrice Nordmann, Le-Kremlin-Bicetre, France.

Detection and characterization of class 1 and 2 integrons

Integrons belonging to classes 1 and 2 were detected by PCR and typed by RFLP analysis as described by Machado *et al.* (43). In brief, for each integron class two sets of primers were used, one set for integrase detection and one for amplifying the variable gene cassette region. PCR products from the latter reaction were digested with restriction enzymes. The fragments were electrophoresed, and each RFLP type was thereafter sequenced. To be able to compare the characteristics of integrons from *E. coli* isolates with those carried by the *K. pneumoniae* outbreak strain, five isolates from this outbreak were included in the integron analysis.
Biochemical fingerprinting

In paper IV, all *E. coli* isolates were phenotypically typed by the Phene-Plate™ system, PhP-24, (Bactus AB, Huddinge, Sweden), according to the manufacturer’s instructions. A single colony was used, and the microplates were incubated at 37°C. Absorbance at 620 nm was recorded at 7 h, 24 h and 48 h, using the VERSA max Microplate Reader (Sunnyvale, CA, USA) and the SOFT max PRO VERSA max (Sunnyvale, CA, USA).

*E. coli* ATCC 8739 was used as a control. The similarities between isolates were calculated as a correlation coefficient and clustered according to the unweighted pair group method using arithmetic means (UPGMA). Isolates showing ≥ 0.95 % similarity was defined as belonging to the same PhP type. PhP types consisting of only one isolate were termed singles. Two PhP clusters (n=27+5) were selected for further analysis together with 18 randomly chosen single types.

Pulsed-field gel electrophoresis

In all studies, isolates were genotyped by PFGE according to the methods described earlier by Schoonmacher *et al.* (80) and Schlichtring *et al.* (81). After preparing solid DNA-agarose plugs, the DNA was incubated and digested by the restriction enzyme. It was subjected to electrophoresis, using the Chef Mapper XI Chiller System or Gene Path Electrophoresis System (Bio-RAD laboratories, Inc., Hercules, CA, USA) in which the orientation of the electrical field changed periodically, allowing separation of the digested DNA-fragments (10 to 10,000 kb). By continuously reorienting the positive charge and by emission of electrical pulses of different durations, fragments of DNA of different sizes migrated through the gel. The electrophoretically resolved DNA-fragments yielded a pattern and a number of bands amenable for systematic analysis of relatedness. Banding patterns were compared with each other visually, and BioNumerics Software, version 4.0 (Applied Maths Bvba, St.-Martens-Latem, Belgium), created dendrograms by clustering of a similarity matrix based on band-matching Dice coefficients. Isolates showing indistinguishable pulsed-field patterns or closely related band patterns were regarded as belonging to the same clone, as suggested by Tenover *et al.* (74) or van Belkum *et al.* (73). To standardise the PFGE bands, *S. aureus* strain NCTC8325 was used.

Repetitive sequence-based PCR

In paper IV, the genetic profiles of the subset of 50 *E. coli* isolates were determined by rep-PCR (Diversilab ®), as described by Healy *et al.* (82). Bac-
terial DNA was extracted from overnight cultures using the UltraClean Mi-
crobial DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, California,
USA). Rep-PCR was carried out using DiversiLab Escherichia kit (BioMé-
rieux, Marcy l’Étoile, France) following the manufacturer’s instructions. The
PCR products were separated with the Agilent 2100 Bioanalyser. Analysis
was performed with the DiversiLab software (version 3.3) using the Pearson
correlation coefficient and UPGMA. The automatically generated report
included dendrogram, virtual gel image, scatter plot and similarity matrix.
Samples with > 95% similarity were classified into the same rep-PCR type.

Arbitrarily primed PCR
In paper IV, an AP-PCR method was also performed to genotype the subset
of 50 E. coli isolates. DNA was extracted using the MagAttract DNA Mini
M48 Kit (Qiagen, Solna, Sweden) and the BioRobot M48 (GenoVision,
West Chester, PA, USA). HotStarTaq DNA polymerase (Qiagen) and two
arbitrary primers were used. The PCR reaction was processed in a Perkin
Elmer Cetus DNA thermal cycler. The amplified products were visualised on
a 1% agarose gel stained with ethidium bromide. The gel was photographed
and the DNA bands were analysed visually. Isolates with patterns differing
by one or more bands were considered distinct types (73).

PCR-detection of the O25b-ST131 clone
In paper IV, all 253 E. coli isolates were screened for the pabB gene using an
O25b-ST131 clone allele-specific PCR described by Clermont et al. (83). It
was slightly modified before use. DNA templates were generated by boiling
bacteria in dH2O. The HotStarTaq master mix kit (Qiagen) was used, and the
amplification was performed on a Perkin-Elmer Cetus DNA thermal cycler.
PCR products were analysed by electrophoresis, and the specificity of the
PCR products was verified by sequencing. The positive control strain was
kindly provided by M. Drobní, Department of Infectious Diseases, Uppsala
University Hospital. To control the performance of the assay, the genetic
relatedness of 50 PCR-positive isolates was determined with PFGE. Twenty-
eight of them belonged to the subset, whereas the remaining 22 isolates were
randomly chosen.
Epidemiological investigation of nosocomial transmission

In paper III, epidemiological links between cases were explored, using the hospital patient registration system. The system allowed daily tracing of patients through their admissions, discharges and movements between wards. The information was collected for each case and manually transferred to a data sheet for analysis.

To investigate whether nosocomial transmission had occurred or not, a locally developed software for daily tracing of patients in hospital wards and out-patient clinics were used in paper IV. A total of 33 patients were investigated, and nosocomial transmission was suspected if patients with indistinguishable or closely related PFGE types had shared wards during any time period between January 2005 to the first positive culture.

Risk factors

In paper II, medical records of cases and controls were reviewed and relevant demographic and clinical data were registered. These data included age, gender, place of residence, duration of hospitalisation, underlying medical conditions and antimicrobial treatment lasting two days or more. Invasive procedures or insertion of invasive devices were registered. In each patient, exposure to risk factors was investigated during the previous six months before the positive urinary culture. This time period corresponded to the time interval between the admission of the index patient and the arrival of the first positive cultures for the outbreak strain obtained from other patients at the hospital.

Statistical analysis

In paper II, the prevalence of risk factors among cases and controls were summarised as percentages and counts except for age, which was summarised by the median and the inter-quartile range (IQR). Duration of hospital stay was dichotomised at the median value (nine days). Since this was a matched case-control study, conditional logistic regression analyses were used to assess the relative odds of exposure to a risk factor for cases compared with controls. As many of the risk factors were correlated, multivariable models were fitted to reduce bias from confounding. Several models were evaluated of which the most relevant are presented in Tables 3 and 4). Results are presented as odds ratios (OR) with the corresponding 95% confi-
Interventions

A steering group was formed, with the power to make financial decisions and chaired by the chief physician of the hospital. An infection control team visited wards the same day as a new case was found. Contacts were sampled within two working days if still in the hospital at the time. Cases who shared rooms with other patients were moved to single rooms if they had diarrhoea, urinary incontinence or a discharging wound.

The infection control team provided education about ESBL-producing organisms and hygiene precautions, and delivered training sessions in hand-disinfection to doctors, nurses and auxiliary staff in all departments with cases, as well as to all cleaners and janitors and to temporary staff covering holiday periods.

Patients were encouraged and helped to disinfect their hands before meals and after toilet visits. Breakfast buffets were abandoned in favour of individual patient meals distributed on trays.

A point prevalence survey in February 2006 had demonstrated bed occupancies in geriatric wards and the transplant ward between 100% and 113%. Patient rooms in the geriatric and surgical wards that had been used as offices were re-allocated. More toilets were installed in the geriatric wards to make it possible to allocate separate toilets to patients in single rooms. A new admission ward was opened. Transplant patients were moved to a newly reopened ward. In 2006, extra staff members were employed in the summer to avoid the usual reduction in numbers of beds during holiday periods.

The frequency of cleaning was increased to include weekends and cleaning of bathrooms and toilets twice daily. Cleaning procedures were not changed.
Results

Paper I

All 64 *K. pneumoniae* isolates exhibited resistance to cephalosporins and were phenotypically positive for ESBL-production. The isolates were co-resistant to fluoroquinolones, nitrofurantion, co-trimoxazole and tobramycin. All isolates had one major PFGE band pattern, suggesting the involvement of a single *K. pneumoniae* strain (Figure 3.a). In contrast, none of the 22 susceptible *K. pneumoniae* had band pattern similarities (Figure 3.b). All isolates carried the ESBL enzyme CTX-M-15. None of the 20 isolates screened were positive for *qnrA* or *qnrB* genes.

![Figure 3a. Representative PFGE profiles of the multiresistant K. pneumoniae isolates.](image)

![Figure 3b. Representative PFGE profiles of the susceptible K. pneumoniae isolates.](image)

Paper II

Fifty-four cases and 108 controls fulfilled the inclusion criteria. Two cases with their four matched controls and two additional controls, each belonging to separate cases, were excluded due to insufficient data in patient charts. Altogether, 52 cases and 102 controls had complete medical records available for review and data collection. Patient characteristics and results from different statistical models are presented in Tables 3 and 4.
Table 3. Patient characteristics and variables associated with acquisition of the outbreak strain. Odds ratios (OR) and 95% confidence intervals (CI) were derived from unadjusted conditional logistic regression models. The matched OR represents a change of 10 years in age and \( n \) indicates the number of matched strata with non-zero margins on which the analyses are based.

<table>
<thead>
<tr>
<th></th>
<th>Cases ((n=52))</th>
<th>Controls ((n=102))</th>
<th>Matched analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variables</strong></td>
<td>% (N)</td>
<td>% (N)</td>
<td>OR CI n</td>
</tr>
<tr>
<td><strong>Median age, years (IQR)</strong></td>
<td>75 (61, 84)</td>
<td>72 (53, 83)</td>
<td>1.12 (0.93, 1.33)</td>
</tr>
<tr>
<td><strong>Gender (male)</strong></td>
<td>42 (22)</td>
<td>26 (27)</td>
<td>2.25 (1.06, 4.76)</td>
</tr>
<tr>
<td><strong>≥ 9 hospital days</strong></td>
<td>90 (47)</td>
<td>29 (30)</td>
<td>18.8 (5.74, 61.2)</td>
</tr>
<tr>
<td><strong>Residency in a nursing home</strong></td>
<td>25 (13)</td>
<td>28 (29)</td>
<td>0.84 (0.36, 1.92)</td>
</tr>
<tr>
<td><strong>Diarrhoea</strong></td>
<td>44 (23)</td>
<td>8 (8)</td>
<td>9.62 (3.30, 28.1)</td>
</tr>
<tr>
<td><strong>Skin lesions requiring wound care</strong></td>
<td>67 (35)</td>
<td>19 (19)</td>
<td>7.65 (3.34, 17.5)</td>
</tr>
<tr>
<td><strong>Prior surgery</strong></td>
<td>52 (27)</td>
<td>21 (21)</td>
<td>5.00 (2.12, 11.8)</td>
</tr>
<tr>
<td><strong>Malignancy</strong></td>
<td>37 (19)</td>
<td>13 (13)</td>
<td>3.33 (1.48, 7.53)</td>
</tr>
<tr>
<td><strong>Diabetes mellitus</strong></td>
<td>23 (12)</td>
<td>25 (25)</td>
<td>0.78 (0.34, 1.93)</td>
</tr>
<tr>
<td><strong>Urinary catheters</strong></td>
<td>79 (41)</td>
<td>37 (38)</td>
<td>5.41 (2.45, 12.0)</td>
</tr>
<tr>
<td><strong>Endoscopy</strong></td>
<td>44 (23)</td>
<td>11 (11)</td>
<td>6.18 (2.48, 15.4)</td>
</tr>
<tr>
<td><strong>Nasogastric feeding tube</strong></td>
<td>19 (10)</td>
<td>3 (3)</td>
<td>18.00 (2.28, 142)</td>
</tr>
<tr>
<td><strong>Drainage</strong></td>
<td>25 (13)</td>
<td>9 (9)</td>
<td>3.09 (1.27, 7.49)</td>
</tr>
<tr>
<td><strong>Central venous catheter</strong></td>
<td>29 (15)</td>
<td>10 (10)</td>
<td>4.26 (1.50, 12.1)</td>
</tr>
<tr>
<td><strong>Mechanical ventilation</strong></td>
<td>35 (18)</td>
<td>10 (10)</td>
<td>4.66 (1.93, 11.3)</td>
</tr>
<tr>
<td><strong>Any antibiotics</strong></td>
<td>98 (51)</td>
<td>47 (48)</td>
<td>- - -</td>
</tr>
<tr>
<td><strong>Penicillins</strong></td>
<td>46 (24)</td>
<td>16 (16)</td>
<td>4.07 (1.85, 8.94)</td>
</tr>
<tr>
<td><strong>Penicillins with inhibitors</strong></td>
<td>10 (5)</td>
<td>0 (0)</td>
<td>- - -</td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
<td>4 (2)</td>
<td>1 (1)</td>
<td>- - -</td>
</tr>
<tr>
<td><strong>Cephalosporins</strong></td>
<td>67 (35)</td>
<td>22 (22)</td>
<td>7.58 (3.13, 18.4)</td>
</tr>
<tr>
<td><strong>Monobactams</strong></td>
<td>23 (12)</td>
<td>3 (3)</td>
<td>20.0 (2.56, 156)</td>
</tr>
<tr>
<td><strong>Co-trimoxazole</strong></td>
<td>25 (13)</td>
<td>12 (12)</td>
<td>2.47 (1.04, 5.88)</td>
</tr>
<tr>
<td><strong>Macrolides</strong></td>
<td>12 (6)</td>
<td>3 (3)</td>
<td>5.31 (1.05, 26.8)</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td>8 (4)</td>
<td>0 (0)</td>
<td>- - -</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td>71 (37)</td>
<td>17 (17)</td>
<td>13.5 (4.76, 38.4)</td>
</tr>
<tr>
<td><strong>Imidazoles</strong></td>
<td>15 (8)</td>
<td>2 (2)</td>
<td>8.00 (1.70, 37.7)</td>
</tr>
<tr>
<td><strong>Other antibiotics</strong></td>
<td>13 (7)</td>
<td>4 (4)</td>
<td>3.00 (0.85, 10.6)</td>
</tr>
</tbody>
</table>

33
Table 4. Variables associated with acquisition of the outbreak strain. Odds ratios (OR) and 95% confidence intervals (CI) were derived from multivariable models.

<table>
<thead>
<tr>
<th></th>
<th>Multivariable analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td></td>
<td>OR (CI)</td>
</tr>
<tr>
<td>≥ 9 hospital days</td>
<td>12.6 (3.15, 50.2)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>9.34 (1.76, 49.6)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>7.16 (1.33, 38.6)</td>
</tr>
<tr>
<td>Penicillins</td>
<td>4.72 (0.83, 26.7)</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>4.77 (1.41, 16.1)</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>3.12 (0.59, 16.5)</td>
</tr>
</tbody>
</table>

Paper III

The outbreak strain was isolated from 247 patients during the study period. Of the 247 cases, 113 were female and 134 male, with a median age of 78 years (range 4–100). A line-list covering 93 consecutive clinical and screening cases revealed that 65 (70%) had received antimicrobial therapy before the first positive culture with the outbreak strain, 56 (62%) needed some help with their personal hygiene and/or with feeding, 37 (40%) had incontinence pads, and 34 (37%) had a permanent urinary catheter.

The source patient was colonised with the outbreak strain and with MRSA and was treated in the Departments of Oncology, Dermatology and Internal Medicine during week 51 in 2004. Slightly more than four months later, in May 2005, the first outbreak case was identified. Numbers of new cases peaked during 2006. A steady decline in cases was seen from mid-January 2007, although 30–40 known cases were present in the hospital each week.

Six screening samples were needed to find one new case in contact tracing, and more than 400 in the other screening exercises. ESBL-producing *K. pneumoniae* were found only twice from environmental samples, one colony on a private telephone and four colonies on a toilet seat in a bathroom. No transmission could be demonstrated in the intensive care units.

Chains of transmission were followed for 19 consecutive cases diagnosed when the active surveillance program had been in operation for a month. These patients had visited or been admitted to the hospital on a median of 19 (range 2–37) occasions after the admission of the source patient. They had visited a median of seven different wards (range 1–22). The shortest possible incubation time was one week.
Infection control audits revealed no reasons to change the existing infection control guidelines used in the hospital, but identified several obstacles to compliance, including patient beds in corridors and treatment rooms, and a lack of hand disinfectant dispensers. Staff shortages frequently caused patients to help each other with personal hygiene.

With education, information and training, the use of alcohol hand disinfectant increased from 31 ml per patient day in 2005 to 82 ml in 2007. Overall compliance with the dress code was 94–96%, but physicians’ compliance improved from 74% to 90%.

In 2007, the hospital budgeted €3 million for the curbing of the outbreak. The funding was used for screening cultures, fewer ward closures during the summer months, employment and education of vacancy staff, more alcohol hand disinfectant and more clean laundry, renovation of fixtures in surgical and geriatric wards, more single rooms, reclaiming of patient rooms that had been converted into offices and increased cleaning frequency.

Paper IV

The majority of the 253 ESBL-producing *E. coli* isolates was obtained from active surveillance in 2007 and was isolated from stool. Among the clinical isolates, resistance to nalidixic acid was most common followed by resistance to trimethoprim-sulfamethoxazole and any of the two tested aminoglycosides. One third of the isolates were multi-resistant, and this figure increased during the study period. All tested isolates were susceptible to meropenem.

Of the isolates, 70% harboured CTX-M group 1 enzymes and 19% CTX-M group 9 enzymes. Sequencing of amplified products from a subset of isolates revealed that all isolates belonging to CTX-M group I were of the CTX-M-15 type, whereas the CTX-M group IV positive isolates were all of the CTX-M-14 type.

Integron carriage was more common among faecal samples from the active surveillance than among clinical samples. Integrons with integrated gene cassettes were detected in 47% of the isolates and 77% were of class 1. The cassettes of the integrons contained resistance genes to trimethoprim, streptomycin/spektofilmycin and/or streptothricin, a growth-promoting antibiotic drug associated with food-producing animals. Type I was the leading RFLP type among isolates belonging to the O25b-ST131 clone, whereas type III was indistinguishable from the integron found in the five investigated isolates from the clonal outbreak of *K. pneumoniae*. Eight patients (40%) were infected or colonized with both *E. coli* and the *K. pneumoniae* outbreak strain harbouring the type III integron.

With the PhP-system 46 clusters and 106 singles were identified. The largest cluster (type 3, *n*=27) and one containing two septicemic cases (type
11, \( n=5 \) were selected together with 18 singles for further analyses with other epidemiological methods. Only the rep-PCR identified correctly the true two clusters \( (n=28 + 4) \) in the selection above, and the largest cluster consisted of isolates belonging to the O25b-ST131 clone. The attention was thereafter turned to this clone.

The ability of the PhP-system to identify the epidemic O25b-ST131 clone among the 253 isolates was controlled, and the identification rate was only 49%.

Most methods had problem with this clone. The genetic diversity within the O25b-ST131 clone was high, and the minimal similarity between the 50 PCR-positive isolates, which were genetically typed with PFGE, was 62%. The identification rate for the PhP-system was only 49%. For the other methods, which were only applied on the selected subset mentioned above, the corresponding figures were as follows: PFGE 86%, AP-PCR 68%, PCR-RFLP of integrons 39%, and antibiogram-based typing 32%. The majority of the isolates belonging to the O25b-ST131-clone carried CTX-M-15 followed by CTX-M-14. Intergron class 1 and RFLP type I with the gene cassette array \( dfrA17-aadA5 \) was most prevalent.

Exploration of patients’ movements at UUH showed two transmission chains among the investigated patients. Both epidemiological and genetic links could be established in nine out of 33 patients.
Table 5. Relationship between phenotypic and genetic types after biochemical fingerprinting with the PhP system, PFGE, rep-PCR, and AP-PCR among isolates positive for the O25b-ST131 clone with PCR.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Types</th>
<th>PhP</th>
<th>PFGE</th>
<th>Rep-PCR</th>
<th>AP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>U05-07916</td>
<td>3</td>
<td>A4</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM06-00382</td>
<td>3</td>
<td>E</td>
<td>A</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>U06-07336</td>
<td>3</td>
<td>A1</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM06-011066</td>
<td>3</td>
<td>A2</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL06-01556</td>
<td>3</td>
<td>B</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL06-03762</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>ESBL06-03638</td>
<td>3</td>
<td>A3</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL06-08104</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL06-11988</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>UM07-00165</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL07-02314</td>
<td>3</td>
<td>A10</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL07-02113</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL07-02374</td>
<td>3</td>
<td>A6</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM07-04846</td>
<td>3</td>
<td>A9</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>U07-04347</td>
<td>3</td>
<td>A10</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>U07-04658</td>
<td>3</td>
<td>A9</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM06-05010</td>
<td>3</td>
<td>A1</td>
<td>A</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>U07-09030</td>
<td>3</td>
<td>A5</td>
<td>A</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>ESBL07-20031</td>
<td>3</td>
<td>A6</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM07-11158</td>
<td>3</td>
<td>C1</td>
<td>A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>ESBL07-20355</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL07-20682</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>ESBL07-21235</td>
<td>3</td>
<td>A8</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>ESBL07-21419</td>
<td>3</td>
<td>D</td>
<td>A</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ESBL07-22561</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>UM07-13964</td>
<td>3</td>
<td>A11</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>U07-12214</td>
<td>3</td>
<td>A7</td>
<td>A</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>U07-01135</td>
<td>Single</td>
<td>A12</td>
<td>A</td>
<td>V</td>
<td></td>
</tr>
</tbody>
</table>

* Isolates marked ESBL are obtained from faecal samples in the active surveillance program, B from blood, S from wounds and U/UM from urine.
Discussion

In the summer of 2005, the Clinical Microbiology Laboratory at UUH reported to the Infection Control team of an increased isolation frequency of *K. pneumoniae* with a multi-resistant profile. This finding prompted the Infection Control Team and the chief epidemiologist of Uppsala County to initiate an outbreak investigation in the autumn of the same year. The investigation clearly showed that UUH was dealing with an outbreak, and it was caused by a *K. pneumoniae* strain producing CTX-M-15. The outbreak lasted between the years 2005-2008, affected over 300 patients, and it resulted in the largest outbreak documented in Northern Europe so far. The estimated cost to curb the outbreak was at least €3 million.

Until 2005, *Enterobacteriaceae* producing ESBLs were not a clinical problem in Scandinavia. With the CTX-M family, the situation changed in the same manner as in the rest of the world (29, 31, 32). The CTX-Ms brought multi-resistance, and the focus transferred from secondary pathogens to primary pathogens. The most prevalent ESBL-producer today is *E. coli*, the leading cause of urinary tract infections and septicaemia, and it has become a significant problem not only in hospitals but also in the community (35, 84).

The *K. pneumoniae* outbreak strain described in this thesis carried CTX-M-15. Since the source patient had been hospitalised in the Middle East just before the admittance to UUH, it was presumed that the outbreak strain was imported. CTX-M-15 is prevalent on all continents. Its successful dissemination is often referred to as the “CTX-M-15 pandemia”, and it is assumed to be linked to the rapid global dissemination of the highly virulent clone *E. coli* O25:H4/O25b-ST131 (85). Dissemination of CTX-M-15 not only occurs by clonal spread, but also by conjugative plasmids and transposons (42).

Over almost half a year, the outbreak strain spread among patients without being detected. Even when the first clinical cases appeared, it was difficult to recognise the ongoing outbreak because there were no obvious epidemiological links between the patients in the different wards and buildings of UUH. A prompt genetic typing would have shown that there must have been some connection, because the isolates were all part of the same clone. A search for possible links in a silent transmission chain would thereby have been initiated much earlier.
It is usually claimed that typing of isolates should help an epidemiological investigation rather than lead it, and that typing data should be considered within the time-frame and the current epidemiological context from which bacterial isolates have been obtained (71, 73, 74). Typing data can never be a substitute for epidemiological data, but the reverse is also true. Rather, the two sets of data should be generated independently but be analysed together to determine whether a transmission has taken place or not. Much would probably be gained if clinical microbiology laboratories conducted active surveillance of ESBL-producing *Enterobacteriaceae* as a part of an infection control programme, or at least typed the clinical isolates on a regular basis, in order to discover clusters much faster. Watchful waiting is not a wise strategy when important data are missing and where ESBL-producers are concerned. It can become very costly for patients and hospitals, as the results from this thesis have shown.

For all clinical microbiology laboratories, it is essential that they have appropriate epidemiological typing methods in order to confirm or dismiss suspicions of an outbreak (73). Typing is also essential for epidemiological surveillance, which aims at recording the local epidemiology in order to adapt treatment regimens. Typing methods differ in performance and costs. They need to be adapted to both local and national (sometimes even international) epidemiological situations and different bacterial species. They should therefore be chosen with care and updated on a regular basis. Epidemiological tools in clinical laboratories are becoming in many respects so advanced, that the infection control professionals need help with interpreting the results and how to use them. Teamwork is necessary. If the infection control team formulates the epidemiological question, the clinical microbiology laboratory selects the appropriate fingerprinting methods in order to answer that question. Every typing method needs to be evaluated and validated with respect to a number of criteria. Performance criteria include stability, typability, discriminatory power, epidemiological concordance and reproducibility (73, 86). Convenience criteria include flexibility, complexity, rapidity and costs. All these things may change over time. Re-evaluations are therefore necessary.

In paper I and in paper III, the isolates were genotyped by PFGE. PFGE is the “gold standard” for epidemiological typing since it exhibits features close to an ideal typing system (86), and is a very common method where ESBL-producing *Enterobacteriaceae* are concerned (87). However, PFGE is resource consuming, demands well-trained staff, and can be problematic for certain species (88). Other common genotyping methods for ESBL-producers are rep-PCR (44) and MLST (87). When a large group of isolates must be typed rapidly, easier and cheaper methods might be more practical and convenient.

Antibiogram-based typing is still used in the clinical laboratory as an epidemiological marker (86). It is often the first step in an outbreak investiga-
tion, but, as suspected, this was the least reliable method when dealing with ESBL-producing *E. coli* isolates in paper IV. Differences in antibiotic pressure, spontaneous point mutations and acquisition or loss of mobile genetic elements may alter the antibiotic profile rapidly or over time. In contrast, the O25b-ST131 PCR assay was relatively cheap and reliable throughout the study period in paper IV, but a major drawback with assays such as this is that they only detect a specified gene sequence. Thus, when identifying new epidemic clones or outbreaks with not yet characterized strains other molecular techniques have to be used, and they differ in both capacity and performance.

All the included typing methods in paper IV identified the epidemic potential of the O25b-ST131 clone, but the number of patients who would have gone undetected varied depending on the typing method. If the size of the holes is too large in an infection control net, it will be impossible to control an outbreak. PFGE had obvious problems with the O25b-ST131 clone. The number of subtypes was high, and the minimal similarity for the clonal isolates was as low as 62%. This finding is in accordance with other studies (46, 48, 87, 89) and constitutes a problem when criteria for bacterial strain typing are considered (73, 74). One possible explanation for the relatively poor performance of PFGE is that the O25b-ST131 *E. coli* clone has been spread over large areas over long periods of time. Mutations and other evolutionary events such as DNA-transfers and rearrangements over prolonged time-periods and large areas may result in a PFGE pattern with several band differences even though they belong to the same original phylogenetic lineage.

Maybe it was more noteworthy that the commercial and expensive repPCR from DiversiLab® identified correctly the O25b-ST131 positive isolates in a selected subset. The discriminatory ability and reproducibility of this method is frequently characterised as relatively low (90), but this might be an advantage when the comparatively clonal and geographically widespread *E. coli* population is taken into account (44).

Since it was not possible to analyse 253 isolates with all the methods described in paper IV, the phenotype-based PhP-system was used in the primary survey of the epidemiological situation, whereas the O25b-ST131 PCR assay detected the clone of most concern. The PhP-system is often applied in Scandinavia. It is quicker and less labour-intensive than the other methods, and no special skill is required. Like other phenotyping methods, it is performed on living bacteria. Standardisation is critical, since biochemical properties may vary due to changes in the environmental circumstances. In paper IV, the performance of the PhP-system appeared to be comparable with the genetic methods, but a flaw in the system was revealed by the PCR assay: it only recognised 49% of the isolates, which belonged to the O25b-ST131 clone. This would pose a problem to any infection control program, and the utility of this typing system in outbreak investigations must therefore be questioned.
The epidemiology of ESBL-producing *Enterobacteriaceae* is a challenge to clinical microbiology laboratories in more than one way. In contrast to MRSA, the epidemiology of ESBL-producers can be characterised by a wide diversity of clones and mobile genetic elements. Traditional genotyping methods based on analysis of the chromosomal bacterial DNA are not able to rule out epidemics caused by mobile elements, since horizontal transmission of ESBL encoding genes cannot be detected with these methods. In paper IV, only a limited number of patients with epidemiological links exhibited indistinguishable or closely related PFGE types. However, a striking number of the *E. coli* isolates carried CTX-M-15 or CTX-M group I genes. Further analysis of the plasmids was therefore necessary. The chosen method was integron typing. The results of this analysis, in combination with the double-infected patients, indicated that an overspill of genetic elements from the two leading clones at the hospital had occurred: the *E. coli* clone O25b-ST131 had probably taken part in the dissemination of RFLP type I integrons and the *K. pneumoniae* outbreak clone in the dissemination of RFLP type III integrons. A third expansion of integrons of RFLP type XIV was found, but the origin of this expansion was never established. The most prevalent gene cassette array was *dfrA17-aadA5*, an array which has earlier been found in outbreak strains and is also widely distributed among food animals and in the environment.

Epidemiological links between cases were surveyed throughout the study period of the thesis. In paper III, the investigation was carried out manually by reviewing patients’ records one by one and by mapping patients’ movements throughout the hospital with the purpose to reconstruct chains of transmission. When hundreds of patients were included, reconstructing epidemiological links between patients was still possible but cumbersome and very time-consuming. The experience led to the conclusion that this type of work should be performed by a search engine linked to different hospital registers. There is otherwise a risk that valuable information about the transmission chain will be missed or delayed. This strategy was used in paper IV, where a locally developed semi-automatic search tool was used. There were, however, very few epidemiological connections between the patients investigated in paper IV, which could be explained by an endemic situation and missing data.

When an epidemiological investigation has confirmed the suspicion that there is an outbreak, the next step is to intervene. For an intervention to be efficient, case definitions and risk factors have to be established, and an epidemic curve has to be constructed. In paper III, the number of colonised patients was at least twice the number of clinical cases. Previous studies have shown that the undetected ratio (the proportion of patients undetected by clinical cultures among all patients colonised) for ESBL-producing *K. pneumoniae* can be as high as 69% (95). Thus, in outbreaks with ESBL-producing *K. pneumoniae*, transmission can go unnoticed if cases are found
only based on clinical symptoms. This was the situation in the beginning of the outbreak, when the case-finding strategy consisted of samples taken at the discretion of the physician. Resources for contact tracing, screening cultures and typing were indispensable to get a proper case definition and for the construction of a reliable epidemic curve. The purpose for the active surveillance was, however, not only to find all cases but also to identify wards and procedures carrying a risk for transmission, and to use the obtained information to prevent future cases (96, 97). The efficiency of different screening strategies is often difficult to evaluate, as the number of cases found per sample will always be high initially. Active surveillance of the outbreak strain could probably have been limited sooner to contact sampling and to wards at risk when these had been identified.

The case-control study described in paper II concluded that the majority of the patients had been exposed to antibiotics shortly before the first positive culture. Patients at risk of acquiring the outbreak strain were those with underlying conditions and poor functional status. This category of patients demanded longer hospital stays and a higher level of health care attendance to manage their activities of daily life, including feeding and toilet visits. The results of the case–control study in paper II and of the line list in paper III, supports the proposal that two major forces drive the emergence of resistant bacteria in a hospital population. The first force is the selection of resistant bacteria in response to the clinical use of antimicrobials, and the second force is the transmission of resistant bacteria between susceptible patients (22, 42). The risk factors of significance in paper II and paper III did not differ from those described before in other studies (52, 56, 61, 98). However, the information obtained from both studies was useful when describing the local problem to the staff, and to make them realise that if the right situation is provided for a multi-resistant bacterium, the nationality of the hospital is irrelevant. The causal factors behind patient-to-patient transmission of antibiotic-resistant bacteria are intertwined and interact with each other, and most hospitals can provide this situation by usage of antibiotics and locating patients at close proximity.

Among the antibiotics, exposure to fluoroquinolones had high relative odds. The association between exposure to fluoroquinolones and the risk of acquiring ESBLs has been described before (50). The proportion of ESBL-producing isolates resistant to fluoroquinolones has increased over time and several studies have shown that plasmid-encoded fluoroquinolone resistance is common in organisms carrying ESBLs (99). Particularly, CTX-M-15 has been associated with plasmid-encoded fluoroquinolone resistance (79, 100, 101). Plasmid-encoded resistance mechanisms include Qnr proteins (qnrA, qnrB, qnrS) and acetylases that can affect the action of certain fluoroquinolones (aac(6')-Ib-cr) or systems for pumping fluoroquinolones out of the bacteria (qepA). Plasmid-encoded fluoroquinolone-resistance encoded by qnrA and qnrS was just recently detected in Scandinavia (102). In paper I,
no qnrA or qnrB genes could be detected in the ciprofloxacin-resistant outbreak strain, leaving the not yet investigated aac (6)-Ib-cr and qnrS genes as possible plasmid-encoded candidates (103). Considering the high level of resistance observed, it is more likely that chromosomal mutations conferring resistance are involved, and the CTX-M-15 activity was probably acquired by a K. pneumoniae strain already resistant due to mutations on the chromosome (104).

The risk factor with the highest odds ratio in paper II was a prolonged period of hospitalisation. A prolonged hospital stay leads to a high exposure of direct and indirect contacts by staff and other patients, and the longer the stay, the higher the risk. It is therefore not surprising that this risk factor is well documented (52, 58, 61). The same risks exist for patients with a poor functional status, since more attendance leads to more exposure. In this context, it is very easy to accuse the staff for not following the recommended infection control measures. However, patients are not as passive as it is often assumed. In paper III, information indicating patient-to-patient transmission was obtained through interviews and audits. In geriatric wards, patients with dementia had difficulties to comply with proper hand hygiene, especially after toilet visits. It was revealed that patients helped each other without assistance by hospital staff in lavatories and washrooms. Direct routes of transmission, such as contact via the hands of patients, seem to have been important. This is supported by other studies (105). Furthermore, it was revealed that patients were moved, discharged and readmitted frequently due to shortage of beds, causing multiple patient-to-patient contacts. Understaffing and a shortage of beds are known factors that may effectively counteract proper infection control measures and increase the risk of nosocomial transmission (106-108).

It has been shown that bacteria may persist for long periods in health care settings (109). Due to a lack of evidence, there is an ongoing debate within the infection control community about the appropriate treatment of inanimate surfaces and the impact of cleaning in hospitals in order to prevent transmission of nosocomial pathogens. Transmission of ESBL-producing K. pneumoniae by contaminated surfaces has been reported in the Netherlands (110). Routine environmental cleaning has been shown to reduce transmission of resistant micro-organisms (111). The increased frequency of toilet cleaning may have had some impact on the outbreak described in this thesis. The increased frequency of cleaning of wards included only the floors, which have not been proven to be of importance in transmission of infection (112, 113). However, there was probably a psychological effect.

The K. pneumoniae outbreak strain described in this thesis appears to have been highly transmissible, in contrast to other Klebsiella strains isolated during the study period. The strain may have had a high capacity to survive on the skin, which could explain why improved hand disinfection of hospital staff and patients was probably the most important intervention (114).
In contrast to other studies (115), no transmission was observed in the intensive care units. No overcrowding was permitted in these units, and patients remained passive in their beds. Moreover, the source patient was also a carrier of MRSA, but no secondary MRSA cases were recorded during the study period. This implies that the standard of basic infection control in the hospital was relatively high. Consequently, the outbreak was terminated without extra infection control procedures (115), such as environmental disinfection or contact precautions ad modum the CDC (116). There was, however, room for improvement of the compliance to existing guidelines among the hospital staff. Interventions described in paper III were tailor-made by the infection control team, who prioritized isolation of risk patients, renewed the focus on hand hygiene (117, 118) and improved the adherence to the existing dress code. The initial efforts did not involve large extra costs. Rather, they were directed towards making existing policies known and complied to.

Infection control personnel can and do provide the tools to prevent the transmission of micro-organisms, but it is always the patient units who own the problem and have to implement the recommended infection control precautions. Ward directors should be reminded that they are accountable for cross-transmission of resistant bacteria that occur in their units. Efforts should be directed towards attitudes and a culture of zero-tolerance in order to achieve sustained adherence to existing infection control guidelines. (119).

The policy should be that everyone, no matter what their position, must comply with the recommendations, since the health care chain is only as strong as its weakest link.

In September 2006, almost 1.5 years after the onset of the outbreak, a steering group was formed. The steering group had the financial power and the mandate from the hospital management to implement expensive structural interventions. Costly interventions necessary in some wards included hiring kitchen maids, increasing the availability of patients’ beds and toilets and moving the transplant ward. In geriatric and transplant wards overcrowding was prohibited and patient transfers within the wards were prevented. Efforts to minimise overcrowding and understaffing throughout the whole hospital during the summer of 2007 probably secured sustained results.

When adequate resources were allocated, the outbreak was under control only a few months after the formation of the steering group and the infection control committee. Based on this experience, it seems reasonable to suggest that the hospital management should be actively engaged at an early stage of an outbreak with ESBL-producing Enterobacteriaceae and ensure that all types of necessary resources are available. Moreover, joint efforts of all key hospital units involved with infection control aspects are required, and these units should collaborate closely in order to control larger outbreaks efficiently. Future planning of hospitals or hospital building needs to consider
current outbreak-promoting structures. Structural adjustments are probably necessary and a wise investment. Important actions for the control of ESBL-producing *Enterobacteriaceae* are expensive. To make these decisions are therefore, in the end, not a medical but a political responsibility.
Conclusions

- The ESBL-producing *K. pneumoniae* strain obtained at UUH during the study period produced CTX-M-15 and caused the first major outbreak of this kind in Northern Europe. In contrast, the increased isolation frequency of ESBL-producing *E. coli* during the same period was caused by several strains carrying mainly CTX-M-15. Twenty-two percent of them belonged to the O25b-ST131 clone and 3% was probably secondary to the clonal *K. pneumoniae* outbreak. A limited number of infections were caused by nosocomial transmission.
- The patient-related risk factors of acquiring the *K. pneumoniae* strain were exposure to antibiotics, underlying conditions, poor functional status, a long period of hospital stay and a high level of health care attendance.
- Transmission of the *K. pneumoniae* strain occurred by direct and indirect patient-to-patient contacts through the faecal-oral route. Overcrowding facilitated transmission.
- Existing infection control procedures were applied to curb the *K. pneumoniae* outbreak. Resources were allocated to educational efforts for improving compliance to these infection control policies. Costly interventions included structural adjustments such as limiting overcrowding, allocating more staff, more beds and more toilets.
- The genetic complexity of ESBL-producing *Enterobacteriaceae* suggests that more than one epidemiological typing method should be used and that these methods should probably not all focus on the chromosome.
- Rapid, efficient tools for investigating possible epidemiological links between cases can provide important information about transmission when a large number of ESBL-producing *Enterobacteriaceae* are involved.
- The hospital management should be active in the intervention process, and a close collaboration between all key hospital units involved with infection control aspects is required.
Sammanfattning på svenska


Betalaktam-antibiotika är förstahandsval vid behandling av infektioner orsakade av *Enterobacteriaceae*, och inaktiveras av extended-spectrum betalaktamaser, ESBL. ESBL är en heterogen grupp av enzym med olika urprung som hydrolyserar alla betalaktamantibiotika utom cefamycin och karbapenemer.


I would like to express my sincere gratitude to all the people who most generously contributed with their assistance, knowledge and support to this thesis. In particular, I would like to thank

Åsa Melhus, supervisor, for sharing your outstanding knowledge of clinical bacteriology, for research supervision and inspiration, for genuine commitment and indispensable support in proofreading and writing scientific papers.

Dan I Andersson, co-supervisor, for providing access to your laboratory, for scientific guidance and support and for generously letting me go to the ESBL-conference in Venice 2006.

Linus Sandegren co-writer, for sharing your knowledge in molecular microbiology, for systematically guiding me through the basics of PCR and for your contribution in writing paper I.

Johan Lindbäck, co-writer, for interpreting and presenting the statistics in paper II and for patiently answering the same questions over and over again.

Ulrika Ransjö, senior infection control doctor and co-writer, for sharing your knowledge of hospital epidemiology and infection control, for your contribution to paper I-III, for being accessible at all times and yet teaching me to always look for answers in the literature and for consistently believing in me.

Camilla Artinger, infection control nurse and Anna Hambraeus, senior infection control doctor and co-writers, for the endless days, nights and weekends spent, tracking the movements of over 300 patients through the health-care system. Your contribution to paper III was huge.
All other co-writers and collaborators in this work: Eva Tano, Erik Torell, Staffan Sylvan, Inga Velicko, Olle Aspevall, Inger Tverin, Mette Palm, Göran Günther, Britt-Marie Eriksson, Markus Klint, Anna Heydecke, Hong Yin, Petra Edqvist and Kristofer Severinsson: Without you, this work would never have been accomplished. I sincerely would like to thank each and every one of you.

Eva Hjelm, my boss and Hilpi Rautelin, professor in clinical bacteriology, for providing the practical, timely and financial conditions to fulfil this work.

Anna Schwan, professional supervisor, for giving me the sincere feedback that is often needed and seldom given.

Margareta Edvall, infection control nurse, for loyally supporting me from day one.

All co-workers at department of infection control, Inger E Andersson, Fredrik Idving, Birgitta Perälä and Barbro Liss, for your patience when I was absent both in mind and in person.

Birgitta Sembrant, for answering my mails within the hour and for fixing all the important practical things.

Eva Haxton, for invaluable advice and practical support.

Torbjörn Söderström, former chief physician and president of the ESBL-steering and working group, for listening to infection control advise so carefully and for mandating the necessary but costly interventions.

My husband, Per, for still loving me.
References

plasmid-borne CTX-M-1-derived cefotaximases. Antimicrobial Agents &
34. Olson AB, Silverman M, Boyd DA, McGeer A, Willey BM, Pong-Porter V, et
al. Identification of a progenitor of the CTX-M-9 group of extended-spectrum
beta-lactamases from Kluyvera georgiana isolated in Guyana. Antimicrobial
35. Pitout JD, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacte-
riaceae producing extended-spectrum beta-lactamases (ESBLs) in the commu-
nitsky I, et al. Influx of extended-spectrum beta-lactamase-producing entero-
bacteriaceae into the hospital.[see comment]. Clinical Infectious Diseases
2006;42(7):925-34.
lence and spread of extended-spectrum beta-lactamase-producing Enterobacte-
riaeae in Europe.[erratum appears in Clin Microbiol Infect. 2008 May;14
Community and hospital spread of Escherichia coli producing CTX-M ex-
tended-spectrum beta-lactamases in the UK. J Antimicrob Chemother 2004;54
(4):735-43.
39. Livermore DM, Hawkey PM. CTX-M: changing the face of ESBLs in the UK.
40. Poirel L, Gniadkowski M, Nordmann P. Biochemical analysis of the cef-
tazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its
structurally related beta-lactamase CTX-M-3. Journal of Antimicrobial Chemo-
cation of an extensive outbreak in a neonatal unit caused by two sequential Kleb-
siella pneumoniae clones harbouring related plasmids encoding an ex-
42. Courvalin P. Predictable and unpredictable evolution of antibiotic resistance.
Journal of Internal Medicine 2008;264(1):4-16.
content of extended-spectrum-beta-lactamase-producing Escherichia coli
strains over 12 years in a single hospital in Madrid, Spain. Antimicrobial
44. Diaz M, Hernández-Bello, JR., Rodriguez-Bano, J., Martínez-Martínez, L.,
Calvo, J., Blanco, J., Pascual, A.: A Spanish group for hospital infections
(GEIH). Diversity of Escherichia coli strains producing extended-spectrum
tion of clonally related Escherichia coli strains expressing extended-spectrum
46. Nicolas-Chanoine MH, Blanco J, Leflon-Guibout V, Demarty R, Alonso MP,
Canica MM, et al. Intercontinental emergence of Escherichia coli clone O25:
al. The CTX-M-15-producing Escherichia coli diffusing clone belongs to a


60. Kanafani ZA, Mehio-Sibai A, Araj GF, Kanaan M, Kanj SS. Epidemiology and risk factors for extended-spectrum beta-lactamase-producing organisms: a case


95. Harris AD, McGregor JC, Furuno JP. What infection control interventions should be undertaken to control multidrug-resistant gram-negative bacteria? Clinical Infectious Diseases 2006;43 Suppl 2:S57-61.


Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 610

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)