Detection and epidemiology of extended-spectrum beta-lactamases

Tamara Platteel

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Utrecht University, Faculty of Medicine, the Netherlands

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Detection and epidemiology of extended-spectrum beta-lactamases

Detectie en epidemiologie van extended-spectrum beta-lactamases

(met een samenvatting in het Nederlands)

Proefschrift

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“Παντα ρει και ουδεν μενει”
“Alles stroomt en niets blijft”

- Herakleitos van Efeze

Voor mijn ouders

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Chapter 1

General introduction

Introduction

Since ancient times humans have been searching for methods to treat infections. Greeks and Indians used moulds and plants and Babylonian doctors gave patients beer soup mixed with turtle shells and snake skins. Since the 1870s the first steps towards the use of antibiotics were taken. At that time Sir John Scott Burdon-Sanderson observed that bacteria did not grow on culture fluid covered with moulds. Sir Alexander Fleming, a Scottish biologist, opened the door for antibiotics, with his discovery of the antibiotic penicillin in 1928. He observed that a common fungus, *Penicillium notatum*, had destroyed bacteria on a *Staphylococcus* culture plate. Upon subsequent investigation, he discovered that mould-juice had developed a bacteria-free zone which inhibited the growth of Staphylococci. He named the mould-juice penicillin. It took 11 years for an Oxford University research team, under the guidance of biochemists Ernst Chain and Howard Florey, to isolate and purify penicillin. In 1940 they reported that mice injected with a lethal dose of *Streptococcus* could be cured with penicillin. Testing on patients began immediately, and in 1943 Florey described great success using penicillin to treat wounded soldiers in North Africa. European production of penicillin was still limited during World War II, but by D-Day, June 6. 1944, US pharmaceutical companies were able to produce enough penicillin to treat all wounded British and American troops. Penicillin quickly became the primary treatment for pneumonia, diphtheria, syphilis, gonorrhoea, scarlet fever, and many other infections.

Hereafter, other antibiotics were discovered and became available for the treatment of patients. Unfortunately, bacteria strike back with their resistance mechanisms, which enable them to overcome the effect of antibiotics. This thesis is dedicated to a family of bacteria with a specific resistance mechanism, extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae.

ESBLs

Bacteria within the family of Enterobacteriaceae (among others *Enterobacter* spp., *Klebsiella* spp., *Escherichia coli*, *Proteus* spp., *Serratia marcescens* and *Citrobacter* spp.) are habitual inhabitants of the intestinal flora. Besides being commensals, they can be also pathogenic and are among the most important causes of community-acquired, as well as nosocomial infections. Species of the family of Enterobacteriaceae are important causes of urinary tract infections, bloodstream infections, pneumonias and intra-abdominal infections. *E. coli* is by far the most frequent cause of urinary tract infections, whereas *Klebsiella* spp. and *Enterobacter* spp. can cause pneumonias. Virtually all members of the Enterobacteriaceae family have been implicated in bloodstream infections and intra-abdominal infections[1].

For years, beta-lactam antibiotics have been the cornerstone for treatment of these kinds of infections. The beta-lactam antibiotics are a broad class of antibiotics, characterised by the presence of a beta-lactam ring in the molecular structure. This group includes penicillin derivates, cephalosporins, monobactams, and carbapenems[2]. Beta-lactam antibiotics act on the bacterial cell wall by binding to, and thus inactivating, enzymes which are involved in cell wall construction. Inhibition of cell wall construction ultimately leads to cell lysis and death.
Until the late 20th century beta-lactam antibiotics were reliable therapeutic options for treatment of infections due to Enterobacteriaceae. Though, this reliability has been challenged since the early 21st century, as resistance to these antibiotic classes rose. The most important way to escape from the effect of beta-lactams is production of beta-lactamases, which are enzymes that hydrolyse the beta-lactam ring, rendering the antibiotic unable to bind the enzymes involved in cell wall construction. Other mechanisms to confer resistance to beta-lactam antibiotics are the presence of efflux pumps, an altered permeability of the cell membrane and mutation of the penicillin binding enzymes.
The clinical most relevant beta-lactamases are the ESBLs, the AmpC cephalosporinases, and carbapenemases, posing resistance to frequently used beta-lactam antibiotics.

ESBLs are able to hydrolyse penicillins, cephalosporins and monobactams (aztreonam), but not the cephamycins (cefoxitin, cefotetan) and carbapenems (imipenem, meropenem, doripenem, and ertapenem). These enzymes can be inhibited by beta-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam. Under normal circumstances, infections caused by Enterobacteriaceae can be treated by trimethoprim/sulfamethoxazole, fluoroquinolones, aminoglycosides, and beta-lactam-antibiotics. In case of an ESBL-producing isolate co-resistance to other antibiotics than beta-lactam antibiotics is often present, narrowing the possibilities of antibiotic treatment even further. Genes encoding for ESBL are nearly always present on plasmids. A. plasmid is a circular piece of DNA that replicates autonomously from the chromosome. Plasmids can encode different functions including antibiotic resistance. Often multiple resistance genes are present on these plasmids rendering the isolate multidrug resistant. Furthermore, plasmids can be easily transmitted between bacteria (horizontal gene transfer). This will be further explained in “The spread of ESBLs”.

AmpC beta-lactamases hydrolyse penicillins and cephalosporins (especially cephamycins) but are not inhibited by beta-lactamase-inhibitors like clavulanic acid. They are typically encoded on the chromosome of multiple species of Gram-

negative bacteria, including *Enterobacter* spp., *Serratia* spp. and *Citrobacter* spp. In these species the expression of AmpC is inducible and can be expressed at high levels by mutation, potentially changing initial susceptibility into resistance during therapy. Since plasmids have acquired AmpC-genes, these genes also appear in bacteria lacking or possessing poorly expressed chromosomal AmpC, such as *E. coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*[3].

As carbapenems are the only widely marketed beta-lactam antibiotics, which cannot be hydrolysed by ESBLs and AmpC beta-lactamases, their use is increasing.
For years carbapenems were almost universally active against Enterobacteriaceae, but nowadays carbapenemases threaten the activity of these antibiotics as well. In carbapenemase-producing Enterobacteriaceae the co-resistance to non-beta-lactams is variable, but many carbapenemase producers are only susceptible to tigecyclin, colistin and/or fosfomycin, which are not "ideal" antibiotics. An increased mortality has been observed in patients treated with tigecyclin as compared to other antibiotics[4], colistin use is associated with nephrotoxicity, and fosfomycin is not recommended to be used as monotherapy in serious infections because of borderline susceptibility of many Enterobacteriaceae and the potential for emergence of resistance by mutation[5,6].
Like ESBLs carbapenemases in Enterobacteriaceae are almost exclusively plasmid-mediated.

Bacteria possessed the ability to hydrolyse beta-lactam antibiotics already before penicillin was developed. In 1940 the first beta-lactamase was identified in an isolate of *E. coli*[7]. The first plasmid-mediated beta-lactamase was described in 1965[8]. As this first beta-lactamase-encoding plasmid was first found in an *E. coli* from a blood culture from a Greek patient called Temoniera, the name TEM was born. This beta-lactamase soon spread to other members of the Enterobacteriaceae. Around the same time SHV-1 (sulfhydryl variable), another plasmid-borne beta-lactamase, was found in *E. coli* and *K. pneumoniae*[9].
New antibiotics, like the oxyimino-cephalosporins, were developed by the pharmaceutical industry, to overcome the effects of these beta-lactamases. The use of these new antibiotics, belonging to the group of extended-spectrum beta-lactams, quickly increased, as did the resistance to these new antibiotics[10].
The first ESBL, as the enzyme conferring resistance to the new broad-spectrum beta-lactam antibiotics was later named, was found in a *K. pneumoniae* isolate in Germany in 1983[11-13]. From that time on more and more ESBLs belonging to the TEM-, as well as the SHV-family were identified.

At the same time as ESBLs got their name, a new family of ESBLs, CTX-M (active on cefotaxime, first detected in Munich), was detected in isolates from humans in Germany[14]. Chromosomal beta-lactamase genes from *Kluyvera* species have been identified as a possible source for the CTX-M beta-lactamases now found in Enterobacteriaceae[15,16].
Beside ESBLs belonging to the TEM, SHV and CTX-M family, other ESBL families like PER, VEB and GES have been identified, but these are less prevalent.

The spread of ESBLs

Increasing resistance rates are mostly due to spread of resistance genes between bacteria (horizontal gene transfer) and not to the evolution of resistance by mutation. In addition, the survival and spread of already resistant bacteria, when they replicate themselves and their plasmids, is observed. Transmission of already resistant bacteria between patients, is referred to as clonal spread.
Mutation is a change in the nucleotide sequence in the genome of a bacterium, most often due to unrepaired damage to the DNA or errors in replication[17]. This random process can bring advantage to the bacterium as well as disadvantage.
Horizontal gene transfer enables bacteria to adapt rapidly to their environment, as in this way they acquire complete DNA sequences from another bacterium in a single transfer.
Genes that are responsible for antibiotic resistance in one species of bacteria can be transferred to another species of bacteria through various mechanisms like transformation, transduction and conjugation. Transformation is genetic alteration, resulting from induction, uptake and expression of foreign genetic material. This is relatively common in bacteria. Transduction is the process in which bacterial DNA is moved from one bacterium to another by a virus (bacteriophage). Bacterial conjugation refers to a process that involves the transfer of DNA to a recipient during cell-to-cell contact (Figure 1). Bacterial conjugation and clonal spread are the primary mechanisms for the increased prevalence of ESBL-producing bacteria.

Epidemiology of EBSL-producing bacteria

Reports on the prevalence of ESBL-producing bacteria tend to show differences in the different settings like the community, long-term care facilities and hospitals, but in general they do agree that the prevalence of ESBL-producing bacteria is rising. As there are large differences in the populations under study as well as microbiological procedures, comparisons between studies are difficult. Besides, the distinction between carriage and infection is not always as straightforward as would be expected.

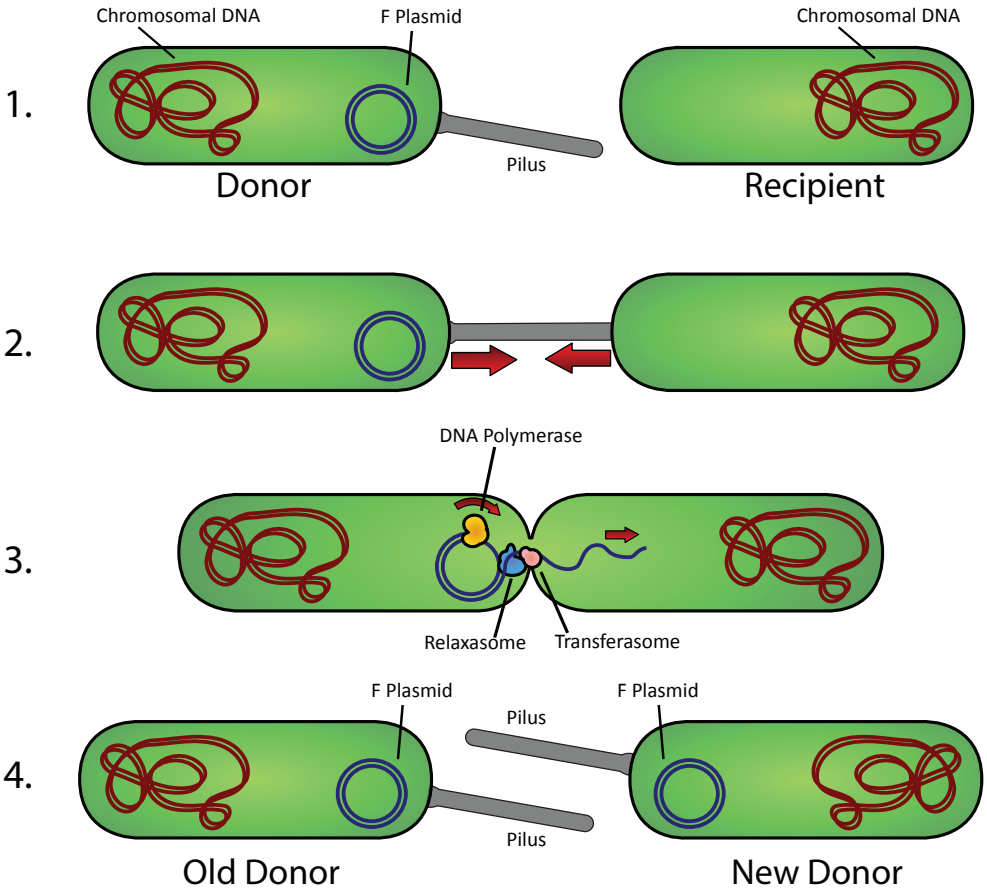

Figure 1. Replication (A) and conjugation (B)[18].

A Replication to two bacteria, both containing chromosomal DNA and plasmid.
B Conjugation.
1 Donor bacterium contains plasmid with ESBL.
2 Donor bacterium makes contact with bacterium without ESBL.
3 Transfer of the plasmid.
4 Both donor and recipient contain the ESBL encoding plasmid and can act as donor for other bacteria.

In a survey in the late 1980s in France 9% of the *Klebsiella* spp. were found to be ESBL-producers[12], whereas a screening of 14,000 Enterobacteriaceae in the UK between 1985 and 1987 identified only 3 ESBL-producing isolates[19]. Subsequently, in the 1990s TEM- and SHV-ESBLs were reported at an increasing rate, especially in association with nosocomial outbreaks. These were reported from all sorts of units, but these outbreaks were mostly seen in Intensive Care Units[20-22]. *K. pneumoniae* was the species most commonly expressing ESBLs at that time[23]. Comparative prevalences of ESBLs in other species of Enterobacteriaceae

were less well reported in the 1990s. Most of the outbreaks were due to patient-to-patient spread of ESBL-producing clones, but plasmid transfer between unrelated strains of different species was also reported[24,25].
Multiple surveys have shown that the highest rates for ESBL-producing *E. coli* and *Klebsiella* spp. are found in India and China (>60%), followed by East and Southeast Asia, Latin America and Southern Europe (>30%) and finally Australia, North America and Northern Europe (<30%)[26].

Since 2000 *E. coli* producing CTX-M beta-lactamases have emerged as an important cause of community-onset infections, predominantly urinary tract infections[27-29]. Nowadays, the most widespread CTX-M enzyme worldwide is CTX-M-15, which was first detected in *E. coli* in India in 2001[30]. CTX-M-15-producing *E. coli* is emerging worldwide, causing community-acquired, as well as hospital-acquired infections[31]. These infections seem to occur, more than in the past, in patients without healthcare-associated risk-factors[32]. The most successful clade of *E. coli* producing CTX-M-15 is identified as ST131 by multilocus sequence typing (MLST). This clade is the biggest contributor to the rising ESBL prevalence[33-36].

According to the European Antibiotic Resistance Surveillance system (EARSS) resistance to third-generation cephalosporins in blood culture isolates of *E. coli* and *K. pneumoniae* has increased also in Europe in the past years. This resistance is in 85-100% of cases due to ESBL production[37]. In general, resistance rates in Europe are the lowest in the Scandinavian countries and the Netherlands with resistance rates of around or even below 5% for *E. coli* and *K. pneumoniae* between 2008 and 2012 (Figure 2). The highest rates are found in Southern European countries like Italy, Greece and Portugal, as well as Poland with resistance rates of 15-25% for *E. coli* and of 50% to even over 70% for *K. pneumoniae*. As mentioned before, the CTX-M-types are the most prevalent ESBLs nowadays, followed by the TEM- and SHV-type. Other ESBL-types, such as PER, GES, IBC and certain OXA-types, have also been identified in European countries, though not frequently[38].

In the Netherlands antimicrobial resistance is monitored by the Infectious Diseases Surveillance Information System for Antibiotic Resistance (ISIS-AR), to which already over 26 laboratories were connected in 2008. This number is still increasing. With this information better insight is gained in the prevalences of detected ESBL-producing isolates. These data show ESBL positivity in 4-7% of the *E. coli* isolates and 5-9% in the *K. pneumoniae* isolates between 2008 and 2012 (Figure 3). In faeces, ESBL prevalences are much higher; around 30% for

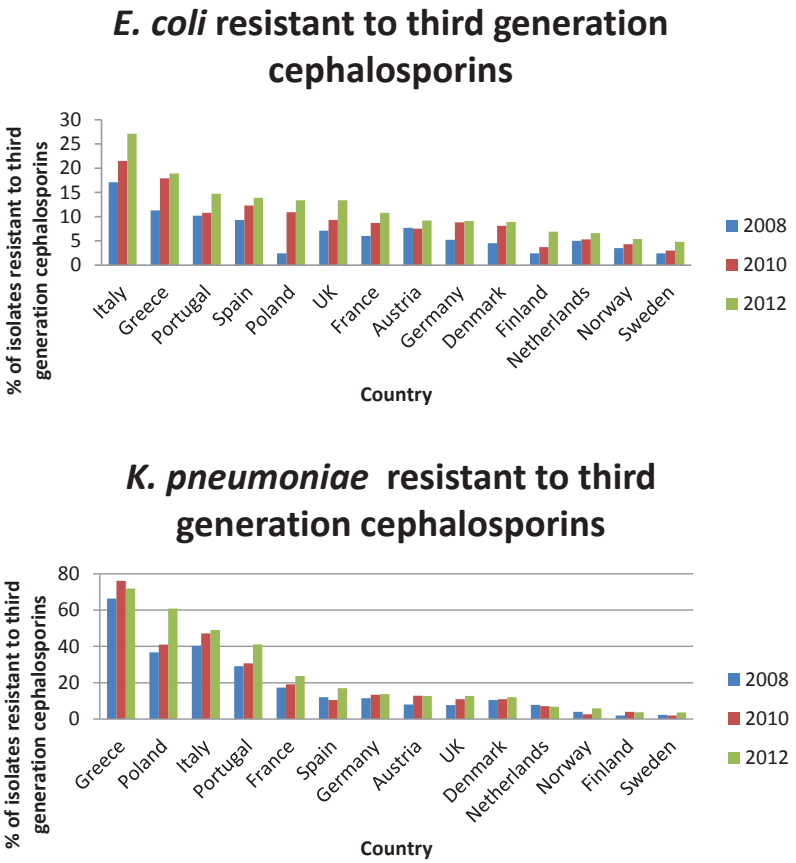

Figure 2. Resistance to third generation cephalosporins in E. coli and K. pneumoniae from blood cultures in European countries, according to EARSS data[39].

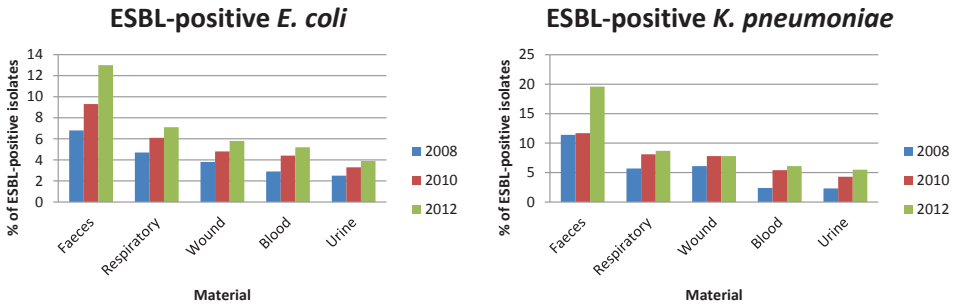

Figure 3. Percentage of ESBL-positivity for E. coli and K. pneumoniae in The Netherlands, according to ISIS-AR data[40].

E. coli and *K. pneumoniae*, which most likely results from selection bias (faeces cultures are mostly obtained from patients at risk of ESBL-carriage).
For the Netherlands, limited epidemiologic data is available. In 1997 less than 1% of the hospital isolates were found to be ESBL-producing isolates and no CTX-M-positive isolates were found[41]. In 2004 one hospital-based study reported an ESBL-prevalence of 7.8% with mainly CTX-M-enzymes, and another, also hospital-based study, reported an ESBL-prevalence of 2.1% for *E. coli*, 5.2% for *K. pneumoniae* and 2.4% for *K. oxytoca*, without a predominance of CTX-M-enzymes[42,43]. In 2008 the reported ESBL prevalence in nursing homes was 6%. No genotyping was performed in this study[44]. In 2009 a predominance of CTX-M enzymes was found in the Rotterdam region[45]. Between 2004 and 2009 the prevalence of ESBLs in uropathogenic *E. coli* isolates, collected at general practitioners, rose from 0.1% to 1%[46]. And in 2010, the prevalence of ESBL-producing isolates in patients visiting a general practitioner with gastrointestinal complaints or diarrhoea was 10.1%[47]. CTX-M-15 was found in 47% of the ESBL-producing isolates. Between 2009 and 2010 ESBL-producing isolates from three microbiology laboratories mostly produced ESBLs of the CTX-M-1 group (55.8%) or of the CTX-M-9 group (24.5%)[48]. No precise CTX-M-types are available for these isolates, as they were typed using micro-array (further explained under "Genotypic confirmation tests for ESBL-producing isolates").

Carriage and transmission-dynamics

The fact that ESBL genes were initially mainly present in *K. pneumoniae*, which typically causes hospital-acquired infections, might be one of the explanations for the almost exclusive detection in hospitals at that time. Besides, the high selective pressure by antibiotics in hospitals probably plays a central role in the acquisition, selection, persistence and transmission of ESBL-producing bacteria via two different routes. On the one hand patients will become more susceptible to acquisition of ESBL-producing bacteria after eradication of their antibiotic susceptible gut flora, and on the other hand antibiotics can select for ESBL-producing bacteria present in carriers, turning low-level carriers into high-level-carriers. Hereby the likelihood of spread is also being increased[49]. Besides, the increased colonisation pressure might increase the acquisition of ESBL-producing bacteria[50].
Previously, it was assumed that carriers of ESBL-producing bacteria would become non-carriers spontaneously after a few months, but more recent data suggest that some people carry these bacteria for a longer period of time, even longer than a year[51]. Herein they constitute a risk for themselves, but also for other people around them, as they can transmit their resistant bacteria to others. Conceptually, it would be ideal if we could completely eliminate the bacteria

from carriers, which is called eradication. For some multiresistant pathogens, like methicillin resistant *Staphylococcus aureus* (MRSA) this is an option to decrease the risk on infections and transmission. Unfortunately, for ESBL-producing bacteria, there is no effective method to eradicate (yet)[52,53].
The concept that acquisition of ESBL-producing bacteria occurs solely in the hospitals is superseded nowadays. ESBL-carriage and infections with ESBL-producing bacteria are more and more diagnosed in patients without previous healthcare contact. Slowly we learn more about the transmission dynamics of ESBLs, as continuously new reservoirs of ESBL-producing bacteria are identified. Besides in patients, ESBL-producing bacteria have also been identified in vegetables, meat, the environment (e.g. soil and rivers) and domestic and wild animals[54-59].
Similarities between the strains and plasmids found in humans and other reservoirs have been found, using methods offering a low to moderate resolution, which might infer that ESBL-producing bacteria were transferred from one compartment to another. Results from high-resolution methods, like whole-genome sequencing, though, do not confirm these assumptions. It seems too easy to conclude that one source outside humans is the major source for carriage of ESBL-producing bacteria in humans[60]. Although the relations between the different compartments still remain unclear, probably all compartments are interrelated, and humans will be source as well as recipient (Figure 4).

Detecting ESBLs

Humans can carry ESBL-producing bacteria in the gut, without signs of infection or other symptoms, which makes identification of carriers difficult. In case of carriage, ESBL-producing bacteria can be unexpectedly demonstrated in surveillance cultures, which are for example performed at ICU-admission. Most frequently, ESBL-producing bacteria are detected when culturing is performed in case of a (suspected) infection. If ESBL-producing bacteria migrate from the gut to other parts of the body they can cause infections. Logically, carriage of ESBL-producing bacteria will increase the risk of infection from such bacteria[61,62]. The signs of infection will not be different from those of an infection by an ESBL-negative strain. Therefore, methods are available to determine whether infection is caused by an ESBL-positive or negative strain.

In 2009 an ESBL detection strategy was established in a Dutch guideline, aiming at reliable phenotypic detection of ESBL-producing bacteria[63]. This guideline and the evaluation of the routine setting performance hereof will be discussed in chapter 2.

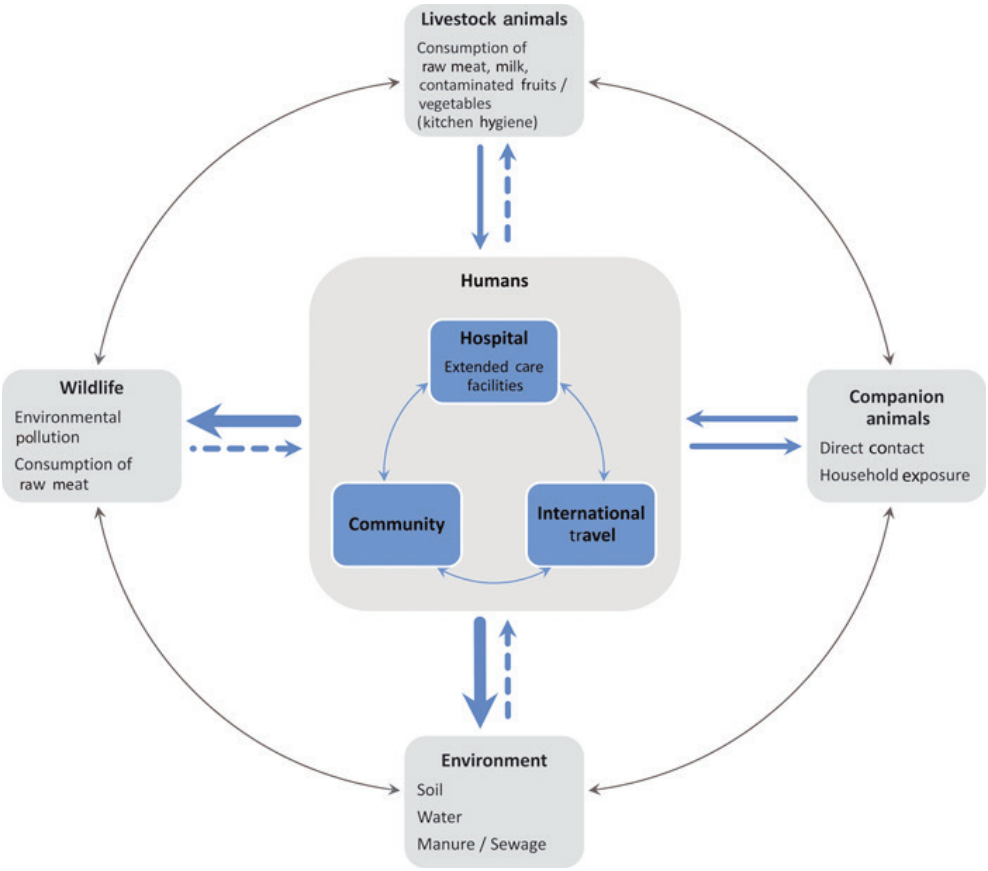

Figure 4. Possible transmission-pathways for ESBL-producing bacteria[60].

Susceptibility testing and species determination

In clinical practice automated systems (e.g., Vitek and Phoenix) and MALDI-TOF are most frequently used to perform species identification. Automated systems can also perform antibiotic susceptibility testing. Hereby the minimum inhibitory concentration (MIC) is measured. This is the minimal concentration of antibiotics that inhibits growth of bacteria. Susceptibility testing in most automated systems is based on broth dilution methods, using panels with fixed antibiotic dilutions. After incubation, interpretation of growth in presence of different concentrations of antibiotics is performed, whereby the MIC is being determined. The automated systems can also give an alarm for suspected ESBL-positivity of the culture, based on resistance patterns. Also non-automated methods to determine the antibiotic susceptibility are used and these include broth microdilution and disk diffusion.

Phenotypic confirmation tests for ESBLs

When an isolate, based on its antibiotic resistance profile or an alarm of the automated system, is suspected of ESBL production, confirmation is needed. These confirmation tests are usually performed using Etests or combination disks.

The ESBL-Etest uses a strip which is impregnated with a gradient of cephalosporins with and without clavulanic acid in order to demonstrate synergy of extended-spectrum cephalosporins and clavulanic acid. During incubation, antibiotic diffuses out of the strip into the agar and an elliptical bacterial growth inhibition zone is produced. The MIC can be read at the point at which the inhibition zone meets the strip. Whether isolates are ESBL producers is determined by calculation of the ratio of the inhibition zones with and without clavulanic acid (Table 1).

Table 1. Guidelines for interpretation of Etest ESBL[64].

ESBL	MIC ratio
Positive	CT≥0.5 and CT/CTL≥8 OR TZ≥1 and TZ/TZL≥8 OR PM≥0.25 and PM/PML≥8 OR "Phantom" zone or deformation of the CT, TZ or PM ellipse.
Negative	CT<0.5 or CT/CTL<8 AND TZ<1 or TZ/TZL<8
Non-determinable (ND)	CT>16 and CTL>1 AND TZ>32 and TZL>4 AND PM>16 and PML>4 OR When one strip is ESBL negative and the other ND.

CT= cefotaxime; CTL= cefotaxime + clavulanic acid
TZ= ceftazidime; TZL= ceftazidime + clavulanic acid
PM= cefepime; PML= cefepime + clavulanic acid
MIC= minimum inhibitory concentration; ND= non-determinable

Presence of a so-called phantom zone or deformation of the ellipse can also indicate presence of ESBLs. Especially in these cases interpretation of Etest results can be difficult. Another problem in interpreting Etest results is that the MIC values may be out of range, giving an inconclusive result (Figure 6).

In the combination disk method antibiotics are added to the agar using disks with and without clavulanic acid. The test is positive if the inhibition zone diameter is 5 mm larger with clavulanic acid than without (and the MIC for the isolate is >1 mg/L for the cephalosporin tested) (Figure 7).

Figure 5. A "rounded" phantom inhibition zone below CT indicative of ESBL.

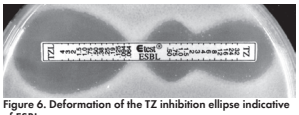

Figure 6. Deformation of the TZ inhibition ellipse indicative of ESBL.

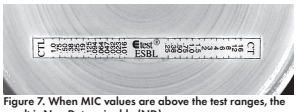

Figure 7. When MIC values are above the test ranges, the result is Non-Determinable (ND).

Figure 6. ESBL detection using Etest[64].

CT= cefotaxime; CTL= cefotaxime + clavulanic acid
TZ= ceftazidime; TZL= ceftazidime + clavulanic acid
MIC= minimum inhibitory concentration; ND= non-determinable

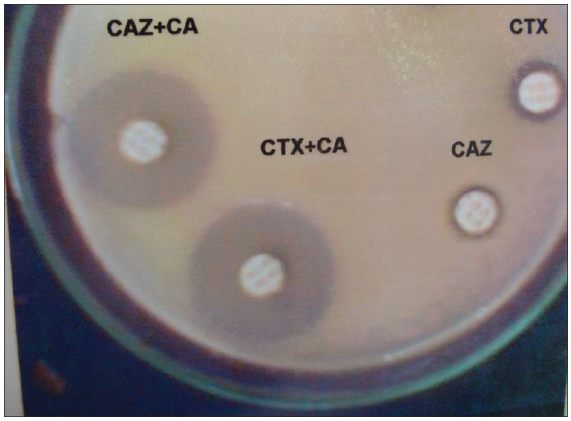

Figure 7. ESBL detection using combination disk[65].

CAZ= ceftazidime
CTX= cefotaxime
CA= clavulanic acid
A difference of ≥5 mm between the zone of inhibition of a single disc and in combination with clavulanic acid is indicative for ESBL-production.

Genotypic confirmation tests for ESBL positivity

Genotypic evaluation of ESBL producing bacteria can be important for surveillance purposes, in case of a suspected outbreak or for (epidemiologic) research. Genotypic analysis of ESBL genes is most often performed using microarray analysis or polymerase chain reaction (PCR) and sequencing.

In microarray two probes are ligated to each other when hybridized to a specific target sequence. One probe consists of a target-specific sequence and a primer for PCR and the other consists of a second target-specific sequence, a second primer for PCR. Hereby a collection of DNA molecules is generated, which are subsequently PCR amplified. The PCR products are detected by hybridization to a DNA microarray. Positive hybridization is detected by means of a label, which is incorporated in one of the PCR primers. ESBL-gene identification relies on a series of probes targeting DNA markers whose sequence is specific to these

enzymes and yields a unique microarray hybridization profile to identify and discriminate between the different ESBL-groups[66].

Typing of isolates

If bacteria from the same species and with similar resistance profiles are found in multiple patients in the hospital, the question is raised whether this is a result of clonal spread, especially when there is a clear epidemiologic link between the patients. This is for example the case when patients stayed on the same ward in the same period of time. To determine resemblance between isolates, not only in susceptibility and species, but also in higher resolution on genetic material, several isolate typing methods have been developed. Genotyping methods can be used to determine which ESBL genes are present or whether isolates are related to each other or not, by means of comparing the DNA of multiple isolates.

The most commonly used methods for the genotyping of isolates are multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Compared to phenotypic analysis of isolates, these methods are relatively expensive, but yield additional information on the relatedness of isolates.

In MLST fragments of (usually) seven species-specific housekeeping genes are sequenced, and a MLST number is being assigned to each new combination of sequences using an electronic database. Based on this number, relatedness of isolates can be determined, as isolates with the same sequence type are closer related than isolates with different numbers. This makes comparison between isolates across the world possible[67].

In PFGE restriction enzymes are used to cut specific sequences, present in the bacterial genome, generating a small number of (restriction) fragments. PFGE facilitates the differential migration of large DNA fragments through agarose gels by constantly changing the direction of the electrical field during electrophoresis. Thus large DNA pieces can be separated by PFGE. Hereby it is possible to compare bacteria.

An advantage of MLST over PFGE is that strains from all over the world can be compared. Besides, interpretation is easier. However, the discriminatory power of PFGE is higher, but PFGE results are harder to interpret.

DiversiLab typing is a standardized semi-automated typing system, based on rep-PCR, which amplifies naturally occurring repetitive extragenic palindromic (rep) sequences in the genome. The rep-PCR generates multiple bands that can be separated by electrophoresis. This method aims at reducing reproducibility problems due to variation in assay conditions. The DiversiLab analysis software allows comparison of individual amplification product patterns (peak patterns),

which enables easier interpretation of the patterns. The patterns can be stored in an online database and used for comparison.

Typing of plasmids

Beside strains, also plasmids can be typed. Plasmid-based replicon typing (PBRT) is most frequently used. Classification of plasmids is based on a phenomenon called plasmid incompatibility. Incompatibility is a manifestation of the relatedness of plasmids that share common replication controls. Incompatibility is defined as the inability of two related plasmids to be propagated stably in the same cell line; thus, only compatible plasmids can be rescued in transconjugants[68]. As plasmids of different incompatibility groups have different sites of replication, the different types can be distinguished by performing PCR (and amplification of its product) with these replicons as target region. This method gives a relatively low resolution, and does not relate to any genetic information present on the plasmid[69]. In addition, not all incompatibility groups can be detected.

In addition, plasmid MLST (pMLST) can be performed. Hereby, a higher resolution is provided, as conserved genes on plasmids are sequenced. As with 'isolate' MLST, a MLST-number can be assigned to the plasmid. Hereby, also differences within one PBRT-type can be demonstrated. However, pMLST is only available for a limited number of PBRT types.

Clinical considerations

Urinary tract infection is the second most common infectious disease patients present with in general practice, after the respiratory tract infections[70]. Worldwide about 150 million people are diagnosed with UTI each year[71]. More than 70% of the UTIs are caused by *E. coli*. The increase in ESBL-producing *E. coli* is also reflected in the prevalence of ESBL-producing *E. coli* in The Netherlands, which increased from 0.1% in 2004, to 1.0% in 2009[46]. Unfortunately, no reliable recent data for the Netherlands are available.

Beside the resistance to penicillin and cephalosporins, the frequent co-resistance to trimethoprim/sulfamethoxazole and quinolones raises special concern in urinary tract infections caused by ESBL-producing bacteria as delayed adequate therapy can significantly affect the course and outcomes of infections.

Another important infection, not because of its high incidence, but because of the big threat for patients' health is the bloodstream infection. Bloodstream infections caused by ESBL-producing Enterobacteriaceae are associated with increased rates of treatment failure, mortality and hospital costs[72,73]. As ESBL-producing bacteria are often resistant to antibiotics, frequently empirically

prescribed for bloodstream infections, a delay in administration of adequate antimicrobial therapy can occur[74-76]. This delay is among the most frequently reported risk factors for mortality in patients with bloodstream infections caused by ESBL-producing Enterobacteriaceae. There is no clear evidence that ESBL-producing bacteria by themselves are associated with worse outcomes[75,77].

With the detection-methods used in clinical microbiology today, it may take 1 to 3 days to identify presence of ESBL-producing bacteria in case of suspected carriage or infection. This has implications for empirical therapy in critically ill patients. Usually, patients with an infection are treated empirically, based on the likely pathogens and local resistance rates. Decisions concerning such treatment become more difficult if resistance rises, especially in critically ill patients. One response is to start with the broadest-spectrum empirical therapy and to de-escalate when the microbiology results are available. For this reason the increasing prevalence of cephalosporin-resistant Enterobacteriaceae forces towards directed and empirical use of carbapenems (imipenem, meropenem and doripenem), which are the only widely marketed beta-lactam antibiotics stable to ESBLs. The risk with this strategy is that de-escalation is frequently omitted. Besides, it is difficult to determine the appropriate response if patients do show signs of infection, but no pathogen is isolated. Both factors contribute to prolonged use of broad-spectrum antibiotics, increasing the selection of resistant pathogens.

Improvement could be gained by the development of new detection methods, decreasing detection-time, as this could decrease the use of the carbapenems. Besides, if we could predict patients at risk for carriage and/or infection with ESBL-producing bacteria, screening of these patients could take place. Increased knowledge of patients at risk and/or directed screening for carriage in risk groups, could support decision-making regarding empirical therapy.
Many factors have been proposed as potential risk factors for carriage and infection with ESBL-producing bacteria. These factors include patient factors, like age, gender, severity of illness, previous colonization with multiresistant bacteria and the presence of comorbidities, but also health-care associated factors, like use of antibiotics and immunosuppressants, indwelling devices, surgery, duration of hospitalisation and admissions in ICUs or long-term care facilities[78]. Lately, attention has been also called to factors like travel to high-prevalence countries and contact with animals or their products[79,80]. As mentioned in the section “Epidemiology of ESBLs” also here the large differences in populations and methods used makes comparisons between studies difficult. What is clear from the research performed until now, is that, unlike for example

MRSA in The Netherlands, it is difficult to identify risk groups for carriage of ESBL-producing bacteria. This complicates the battle against the increasing prevalences for ESBL-production.

Another option to decrease carbapenems use would be development of new antibiotics. For a long time very little progress is made in the development of antibiotics against Gram-negative bacteria. This is explained by multiple factors. First, it is technically difficult and laborious to discover new antibiotics[30,73], besides introduction of new antibiotics has become more restricted by increasing regulations[81], and new antibiotics are reserved as last resort antibiotics thereby severely limiting their use. Therefore, antibiotics are not a lucrative investment for pharmaceutical companies[75,76]. As a result new broad-spectrum antibiotics directed against Gram-negative bacteria are not to be expected in the near future. Therefore, all available options, like antibiotic stewardship and infection control, as well as improvement of the methods used in our microbiology laboratories, should be considered to prolong the safe use of existing antibiotics.

Since new antibiotics for treatment of infections with ESBL-producing bacteria are not to be expected soon, antibiotics considered inferior to carbapenems, are increasingly considered for treatment of infections. The associated co-resistance to other antibiotic classes than beta-lactams in ESBL-producing isolates limits therapeutic options. Besides to cephalosporins, ESBL-producing isolates are often resistant to fluoroquinolones, trimethoprim-sulfamethoxazole and most of the aminoglycosides, and many isolates are still susceptible to amikacin, nitrofurantoin, colistin, tigecyclin and fosfomycin[82]. Nitrofurantoin and fosfomycin might be appropriate for treatment of uncomplicated urinary tract infections with ESBL-producing isolates[83-85], while colistin[86,87], tigecyclin and amikacin may be still appropriate in case of other kinds of infections[88-90].

The efficacy of beta-lactam/beta-lactamase inhibitor combinations is uncertain. They might be effective when *in vitro* susceptibility is demonstrated, but experience with this treatment is still limited[91]. Therefore, the decision whether this treatment can be considered should depend on disease severity and the site of the infection[92].

Especially in patients suspected of a bloodstream infection, caused by an ESBL-producing pathogen, carbapenems should still be considered treatment of choice for the empirical treatment, but this may be altered as the prevalence of carbapenemase-producing strains rises. Some other antibiotics may provide appropriate treatment options and may, in some settings, be used as carbapenem-sparing antibiotics, but further research to these options is warranted[82,93].

Thesis objectives

The rising prevalence of ESBL-producing Enterobacteriaceae is a major threat to public health, as treatment options in case of infections are reduced. One of our main weapons in the battle against these ESBL-producing bacteria is rapid detection of carriage or infection of ESBL-producing bacteria, as this is crucial for appropriate infection control measures and decision making regarding (empirical) therapy in case of infections.
This thesis aims to provide deeper insight in the epidemioly op ESBL-producing bacteria in the Netherlands and the possibilities to detect patients carrying ESBL-producing bacteria quickly and accurately.

In chapter 2 we evaluated performance of the Dutch guideline for phenotypic detection of ESBLs in Enterobacteriaceae in the routine setting. The guideline recommends ESBL confirmation with Etest or combination disk for isolates with a positive ESBL screentest. Also, the performance of Etest and combination disk as ESBL confirmation tests in the clinical setting were compared.

In chapter 3 the accuracy of the Check-KPC ESBL microarray (Check-Points B.V., Wageningen, the Netherlands) as a confirmatory test of ESBLs was determined on randomly selected clinical isolates with a positive ESBL-screentest, as one of the conclusions of the study described in chapter 2 was that for *P. mirabilis* and *K. pneumoniae* phenotypic ESBL-detection is not reliable in clinical practice.

In chapter 4 we determined the population distribution of beta-lactamases conferring resistance in a cross-sectional sample of clinical isolates in the Netherlands, using the methods described in chapter 2 and 3.

In chapter 5 the hypothesis that poultry-associated ESBL genes (CTX-M-1 and TEM-52 genes), were predominant in the community, whereas the non-poultry-associated ESBL genes were predominant in hospitals, was tested. This hypothesis was based on two distinct compartments for the dynamics of ESBL-producing bacteria, one in the community fuelled by food contamination and one in hospitals fuelled by cross-transmission.

In chapter 6 we aimed to develop a prediction rule for carriage of ESBL-producing bacteria at hospital admission, as early detection could improve decision making regarding appropriate infection control measures and empirical therapy in case of infections.

In chapter 7 we determined the duration of ESBL carriage and investigated which factors influence carriage duration.

In chapter 8 we aimed to determine the ESBL-acquisition rate among household contacts of index-patients that acquired ESBL-carriage during hospitalization.

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Chapter 2

Multi-centre evaluation of a phenotypic ESBL detection guideline in the routine setting

T.N. Platteel[1,2], J.W. Cohen Stuart[2], A.J. de Neeling[3], G.M. Voets[2], J. Scharringa[2], N. van de Sande[3], A.C. Fluit[2], M.J.M. Bonten[2], M.A. Leverstein-van Hall[2,3], on behalf of the ESBL national surveillance working group.

1. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands.
2. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands.
3. Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

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Abstract

This study aimed to evaluate the routine setting performance of a guideline for phenotypic detection of extended spectrum beta-lactamases (ESBLs) in Enterobacteriaceae, recommending ESBL confirmation with Etest or combination disk for isolates with a positive ESBL screen test (i.e. cefotaxime and/or ceftazidime MIC>1 mg/L or an automated system ESBL warning).

Twenty laboratories submitted 443 Enterobacteriaceae with a positive ESBL screen test and their confirmation test result (74% *Escherichia coli*, 12% *Enterobacter cloacae*, 8% *Klebsiella pneumoniae*, 3% *Proteus mirabilis*, 2% *Klebsiella oxytoca)*. Presence of ESBL genes was used as reference test. Accuracy of local phenotypic ESBL-detection was 88%. The positive predictive value (PPV) of local screen tests was 70%, and differed per method (Vitek-2: 69%, Phoenix: 68%, disc diffusion: 92%), and species (95% *K. pneumoniae* - 27% *K. oxytoca*). A low PPV (3%) was observed for isolates with automated system alarm but third-generation cephalosporin MICs<2 mg/L. Local ESBL confirmation had a PPV and negative predictive value (NPV) of 93% and 90%, respectively. Compared with centrally performed confirmation tests, 7% of local tests were misinterpreted. Combination disc was more specific than Etest (91% versus 61%). Confirmation tests were not reliable for *P. mirabilis* and *K. oxytoca* (PPV 33% and 38%, respectively, although NPVs were 100%). In conclusion, performance of Etests could be enhanced by education of technicians to improve their interpretation, by genotypic ESBL confirmation of *P. mirabilis* and *K. oxytoca* isolates with positive phenotypic ESBL confirmation, and by interpreting isolates with a positive ESBL alarm but an MIC<2 mg/L for cefotaxime and ceftazidime as ESBL-negative.

Introduction

The prevalence of extended spectrum beta-lactamases (ESBLs) in Enterobacteriaceae is increasing worldwide[1]. Accurate detection of ESBLs is necessary for adequate antibiotic therapy, infection control precautions and surveillance purposes. In 2008, the Dutch Society for Medical Microbiology (NVMM) issued a guideline for phenotypic screening and confirmation of ESBLs in Enterobacteriaceae to standardize the method and to improve the accuracy. In contrast to the CLSI guideline for ESBL detection, the Dutch guideline also provides methods for phenotypic ESBL confirmation in Enterobacteriaceae with inducible chromosomal AmpC beta-lactamases, using an Etest or combination disc with cefepime and cefepime plus clavulanic acid.
The objectives of this study were to determine the accuracy of phenotypic ESBL detection in Dutch clinical laboratories using this guideline and to compare the performances of Etest and combination discs as ESBL confirmation tests in the clinical setting.

Materials and Methods

Guideline
The ESBL detection strategy in the Dutch guideline recommends a screening step and a confirmation step (Figure 1). Isolates with a ceftazidime MIC >1 mg/L and/or a cefotaxime MIC >1 mg/L determined by any MIC method fulfilling quality control criteria, or an ESBL alert in Phoenix (BD Diagnostics, Sparks, MD, USA) or Vitek-2 (BioMérieux Marcy l'Etoile, France) are considered screen positive. In centres using the disc diffusion method isolates were selected for ESBL confirmation in case of zones ≤20 mm with the Oxoid disks for ceftazidime or cefotaxime.
Confirmation of ESBL production is based on the detection of synergy between clavulanic acid and third generation cephalosporins. The recommended methods are the ESBL Etest (BioMérieux) or combination discs. The synergy between cephalosporins and clavulanic acid may be masked in isolates co-expressing an ESBL and AmpC beta-lactamase; therefore, the Enterobacteriaceae are divided into two groups, with a specific strategy for ESBL confirmation for each. Group I comprises species without inducible chromosomal AmpC beta-lactamases (*Escherichia coli, Klebsiella* spp., *Proteus mirabilis, Salmonella* spp. and *Shigella* spp.). ESBL production in these species is confirmed by demonstrating synergy between ceftazidime and/or cefotaxime and clavulanic acid. Group II comprises Enterobacteriaceae with inducible chromosomal AmpC beta-lactamases (*Enterobacter* spp., *Serratia* spp., *Providencia* spp., *Citrobacter freundii, Morganella morganii* and *Hafnia alvei*). ESBL confirmation in this group is based on synergy

between clavulanic acid and cefepime, a 4th generation cephalosporin that is hydrolysed by ESBLs, but generally not by AmpC beta-lactamases[2-4]. The results of the confirmation test are classified as positive, negative or out-of-range.

Isolates

From 1 February 2009 until 1 May 2009, 20 Dutch laboratories submitted all *E. coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis* and *Enterobacter* spp. with a positive ESBL screen test. For each isolate, participating laboratories provided information on the method and results of screening (Vitek, Phoenix, disc diffusion), ESBL confirmation results (combination disc or Etest), and the MICs of third-generation cephalosporins from automated systems for isolates with an ESBL alarm but an MIC of ceftazidime and cefotaxime < 2mg/L as determined in the reference laboratory. The first 25 non-repeat isolates of each laboratory (if available) were selected for further analysis. Isolates were excluded when: 1) there was evidence that another isolate was submitted than originally tested by the participating laboratory (defined as an eightfold or higher difference in MICs of the indicator-cephalosporins reported by the participating and tested by the central laboratory), or 2) the phenotypic test results of the participating laboratory were lacking.

ESBL detection

Phenotypic detection in the reference laboratory was performed by ESBL Etest (BioMérieux) or combination disc (ROSCO, Taastrup, Denmark). The MICs for ceftazidime and cefotaxime were determined using micro-broth dilution (Sensititre, TREK Diagnostic Systems, East Grinstead, UK).

The presence of an ESBL gene was determined using microarray analysis (Check-KPC ESBL, Check-Points B.V., Wageningen, the Netherlands), which detects the most prevalent CTX-M, TEM and SHV ESBL gene groups[5]. PCR and sequencing, using the same DNA batch as used for the microarray, was performed to determine the exact CTX-M, TEM and SHV-genes. Additional PCRs were performed to detect the presence of rare ESBL families such as PER, GES, and VEB beta-lactamase genes, as well as plasmid-borne AmpCs[6].

ESBL confirmation

The reference method for determining the presence of ESBL genes consisted of a step-wise procedure.

Step 1. If the results of the phenotypic confirmation tests reported by the participating laboratory, and the ESBL microarray results were both positive, the isolate was defined as ESBL-positive. If both were negative the isolates was defined as ESBL-negative.

Step 2. If the phenotypic result of the participating laboratory and the result of the microarray were discrepant, the phenotypic confirmation tests (Etest or combination disc, identical to the test performed in the participating laboratory) were repeated in the reference laboratory. If the result of the repeated phenotypic test confirmed the microarray result, the isolate was considered accordingly: either ESBL positive or negative.
Step 3. If there was a discrepancy between the array results and the repeated phenotypic confirmation test the isolates were tested with PCR and, if indicated, DNA sequencing. The results of the PCR and sequencing were considered the gold standard for the presence of an ESBL gene.

Statistics
Frequency data were analysed with the Chi-square test or Fisher's exact if appropriate, using SPSS 15.0

Results

Among the 443 isolates included, *E. coli* were the most prevalent (n=326; 74%), followed by *Enterobacter cloacae* (n=54; 12%), *K. pneumoniae* (n=37; 8%), *P. mirabilis* (n=15; 3%) and *K. oxytoca* (n=11; 2%). Based on micro-array and/or sequencing 312 (70%) isolates contained ESBL genes: 79% CTX-M (66% CTXM-1 group and 12% CTX-M-9 group, <1% other CTX-M groups), 6% TEM (5% TEM-3 group, 1% other TEM groups), 7% SHV (6% SHV-4 group, 1% other SHV groups), 6% a combination of these and 1% PER or GES.

ESBL-screening
Vitek-2 was used for ESBL-screening for 350 isolates (79%) in 16 laboratories, Phoenix was used for 68 isolates (15%) in three laboratories and the disc diffusion method (Oxoid, Basingstoke, UK) for 25 isolates (6%) in one laboratory. The overall positive predictive value (PPV) of local ESBL screening methods was 70% (312/443). The PPV of the Vitek-2 was 69% (243/350; 95%CI 64-74%), of the Phoenix 68% (46/68; 95%CI 56-78%) and of the disc diffusion method 92% (23/25; 95%CI 74-99%).
The PPV of ESBL screening - as locally performed - varied per species: 95% (35/37; 95%CI 81-99%) for *K. pneumoniae*, 76% (248/326; 95%CI 71-80%) for *E. coli*, 44% (24/54; 95%CI 32-58%) for *E. cloacae*., 13% (2/15; 95%CI 2-39%) for *P. mirabilis*, and 27% (3/11; 95%CI 9-57%) for *K. oxytoca* ($p<0.01$ for each species compared with *K. pneumoniae*). Per species, the false positive rate did not depend on the screening method (data not shown).
The PPV of the ESBL screen tests was influenced by the MIC of isolates to ceftazidime and cefotaxime. For the 40 isolates with cefotaxime and ceftazidime

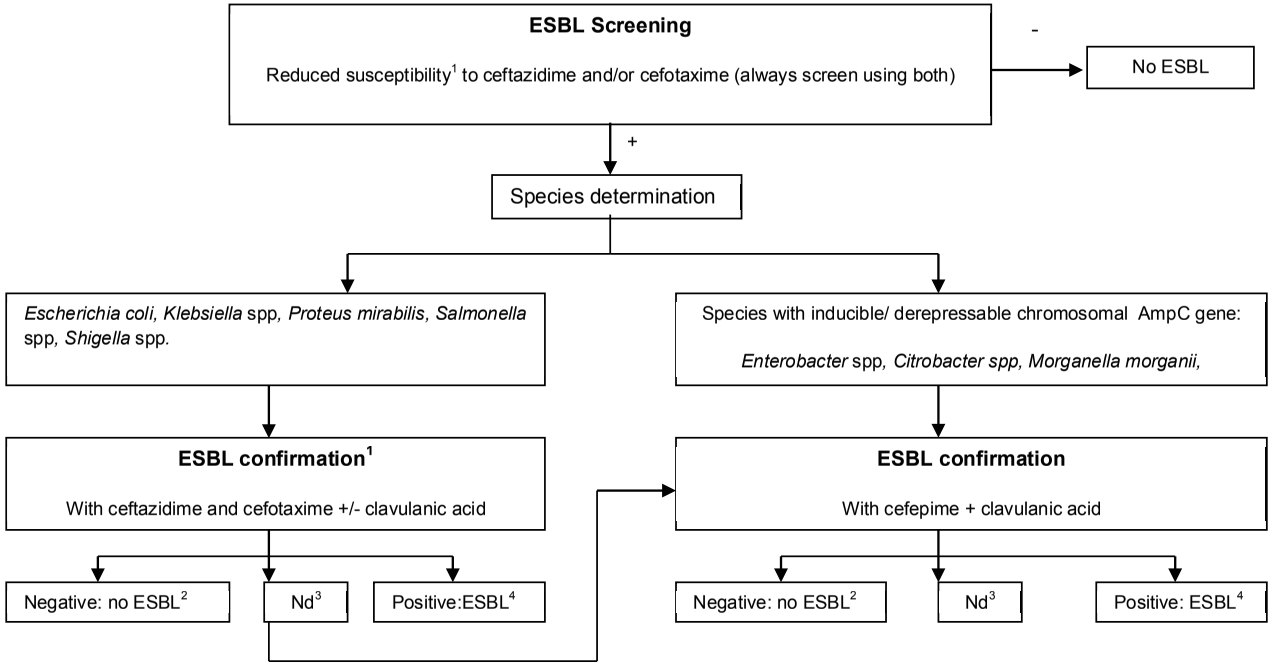

Figure 1. Extended spectrum beta-lactamase (ESBL) detection algorithm for Enterobacteriaceae according to the Dutch guideline for phenotypic ESBL detection.

1 If cefoxitin resistant, perform cefepime confirmation test.
2 Inhibitor resistant ESBL not excluded.
3 Nd= non-determinable= out of range (MIC> Etest strip or no inhibition zone)
4 The ESBL confirmation test may generate false-positive results in K1 beta-lactamase hyperproducing Klebsiella oxytoca. Exceptions are K. oxytoca isolates with high-grade resistance to ceftazidime and synergy between ceftazidime and clavulanic acid, which is indicative of ESBL-production.

MIC <2mg/L, as determined by broth microdilution in the reference laboratory, but an ESBL alarm of the automated system (*K. pneumoniae* 3%, *E. coli* 10%, *E. cloacae* 2%, *P. mirabilis* 33%, *K. oxytoca* 36%), the PPV was 3% (1/40), and the rate of false-positive confirmation tests in those isolates was 30% (12/40). If isolates had an MIC <2mg/L for ceftazidime and cefotaxime in the reference laboratory, this was also the case in the automated system of the participating laboratory, except for two of the 40 cases. Only one isolate (an *E. coli* with a cefotaxime MIC of 0.25 and ceftazidime MIC of 1 mg/L) contained an ESBL gene (CTX-M-1 group). For 403 isolates with a cefotaxime and/or ceftazidime MIC ≥ 2mg/L, the PPV of ESBL screening was 77% (311/403) and the rate of false-positive confirmation tests was only 3% (12/403) The odds ratio was 131.8, (95%CI 17.9-972.7) for comparison of PPVs and 0.07, (95%CI 0.03-0.17%) for false-positive rate.

ESBL confirmation

ESBL confirmation was performed with Etest in 282 isolates (64%; 13 laboratories), with combination disc in 135 isolates (30%; six laboratories) and with both Etest and combination disc in 26 isolates (6%; one laboratory). Overall sensitivity and specificity of phenotypic ESBL confirmation tests as performed by the local laboratories was 95% and 70%, respectively (PPV 93% and negative predictive value (NPV) 90%) (Table 1, Figure 2). The PPV of confirmation tests varied per species and was ≥ 95% for *E. coli*, *K. pneumoniae* and *E. cloacae,* but <40% for *K. oxytoca* and *P. mirabilis* (Table 2).

Although sensitivity, PPV and NPV of ESBL confirmation with Etest and combination discs, as performed in local participating laboratories, were comparable, specificity of the Etest was 59% (95%CI 48-68%) compared with 92% (95%CI 80-97%) for the combination disc (Table 1). In the one laboratory that confirmed 26 isolates with both confirmation tests, test characteristics were equal (sensitivity 100% (21/21), specificity 80% (4/5), NPV 100% (4/4) and PPV 95% (21/22)).

Discrepancy analysis

For 388 (88%) of 443 isolates the results of phenotypic confirmation tests, as performed by local laboratories and genotypic confirmation of presence or absence of ESBL genes were concordant. For 32 (7%) of the isolates, phenotypic results of the local laboratory were discrepant from phenotypic confirmation tests repeated in the reference laboratory as well as genotype confirmation (Figure 2, step 1). In six isolates (in four laboratories) presence of ESBL was missed because of misinterpretations of Etest (n=1) and combination discs (n=5). Eight isolates (in eight laboratories) were misclassified as ESBL-positive

Table 1. Comparison of extended spectrum beta-lactamase (ESBL) confirmation with Etest versus combination disc in the clinical setting.

	All isolates n=443* ESBL-pos n=312 ESBL-neg n=131	**Etest n=308 ESBL-pos n=221 ESBL-neg n=87**	**Combidisc n=161 ESBL-pos n=113 ESBL-neg n=48**	**p-value (Etest versus combidisc)**
Sensitivity	95% (296/312)	96% (212/221)	93% (105/113)	N.S.
Specificity	70% (92/131)	59% (51/87)**	92% (44/48)	P<0.001
PPV* **	93% (296/320)	91% (212/233)	96% (105/109)	N.S.
NPV* **	90% (92/102)	94% (51/54)	85% (44/52)	N.S.

* N=443 (Etest n=282, combination disc n=135, both Etest and combination disc n=26 isolates)
** The participating laboratories reported the ESBL Etest as off-range in 15 of 87 (17%) of the ESBL-negative isolates and as false-positive in 21 of 87 (24%) of the ESBL-negative isolates.
*** For calculation of the PPV and NPV the off-range Etests were not taken into account.
Pos, positive; neg, negative; NPV, negative predictive value; N.S., not significant; PPV, positive predictive value.

because of misinterpretations of seven Etests and one combination disc. Three of these eight isolates (38%) had MICs for cefotaxime and/or ceftazidime <2mg/L. Furthermore, there were 21 isolates (from nine laboratories) with non-determinable Etest results, of which 16 (76%) were ESBL-negative when the Etest was repeated in the central laboratory (Figure 2). Of those 16 isolates, two isolates harboured a plasmid AmpC, and 12 isolates were probably chromosomal AmpC producers or had decreased permeability, as the cefoxitin MICs were ≥16 mg/L (nine *E. coli*, two *E. cloacae,* and one *P. mirabilis*). For the last isolates, the guideline recommends ESBL confirmation with cefepime/cefepime plus clavulanic acid (Figure 1), but this was not performed in eight isolates (deviation from protocol).

For 23 (5%) of the 443 isolates, the discordance between ESBL phenotype and genotype was not the result of inappropriate protocol execution. Repeated testing according to protocol by the reference laboratory failed to confirm phenotypic ESBL-positivity in seven isolates. In six isolates clavulanic acid synergy was not observed with cefotaxime, ceftazidime or cefepime: in all six strains because of interference of AmpC with the synergy tests (three isolates plasmid AmpC, three chromosomal AmpC). Besides, in one isolate presence of ESBL genes was associated with MICs for cephalosporins <2 mg/L.

Sixteen isolates were phenotypically ESBL-positive according to the protocol, but ESBL genes were not detected in the array. Nine of those isolates had an MIC <2 mg/L for cefotaxime and ceftazidime, of which three *K. oxytoca* isolates, which may have been false-positive due to production of the chromosomal

Table 2. Performance of extended spectrum beta-lactamase (ESBL) confirmation in Enterobacteriaceae without (group I) and with inducible chromosomal AmpC beta-lactamase (group II) in the clinical setting.

	Group I (n=389)					**Group II (n=54)**
	E. coli **K. pneumoniae** **P. mirabilis** **K. oxytoca** **ESBL+** **n=248 (76%)**	**E. coli n=326**	**K. pneumoniae n=37** **ESBL+** **n=35 (95%)**	**P. mirabilis n=15** **ESBL+** **n=2 (13%)**	**K. oxytoca n=11** **ESBL+** **n=3 (27%)**	**E. cloacae (n=54)** **ESBL+** **n=24 (44%)**
Sensitivity	96% (276/288)	96% (238/248)	94% (33/35)	100% (2/2)	100% (3/3)	83% (20/24)
Specificity	65% (66/101)	69% (54/78)	1/2*	62% (8/13)	38% (3/8)	87% (26/30)
PPV	92% (276/299)	95% (238/251)	97% (33/34)	33% (2/6)	38% (3/8)	95% (20/21)
NPV	90% (66/73)	90% (54/60)	1/2*	100% (8/8)	100% (3/3)	90% (26/29)

* Only two ESBL-negative *K. pneumoniae* isolates were included, one of which was incorrectly reported as false-positive in the ESBL confirmation test.
NPV, negative predictive value; PPV, positive predictive value.

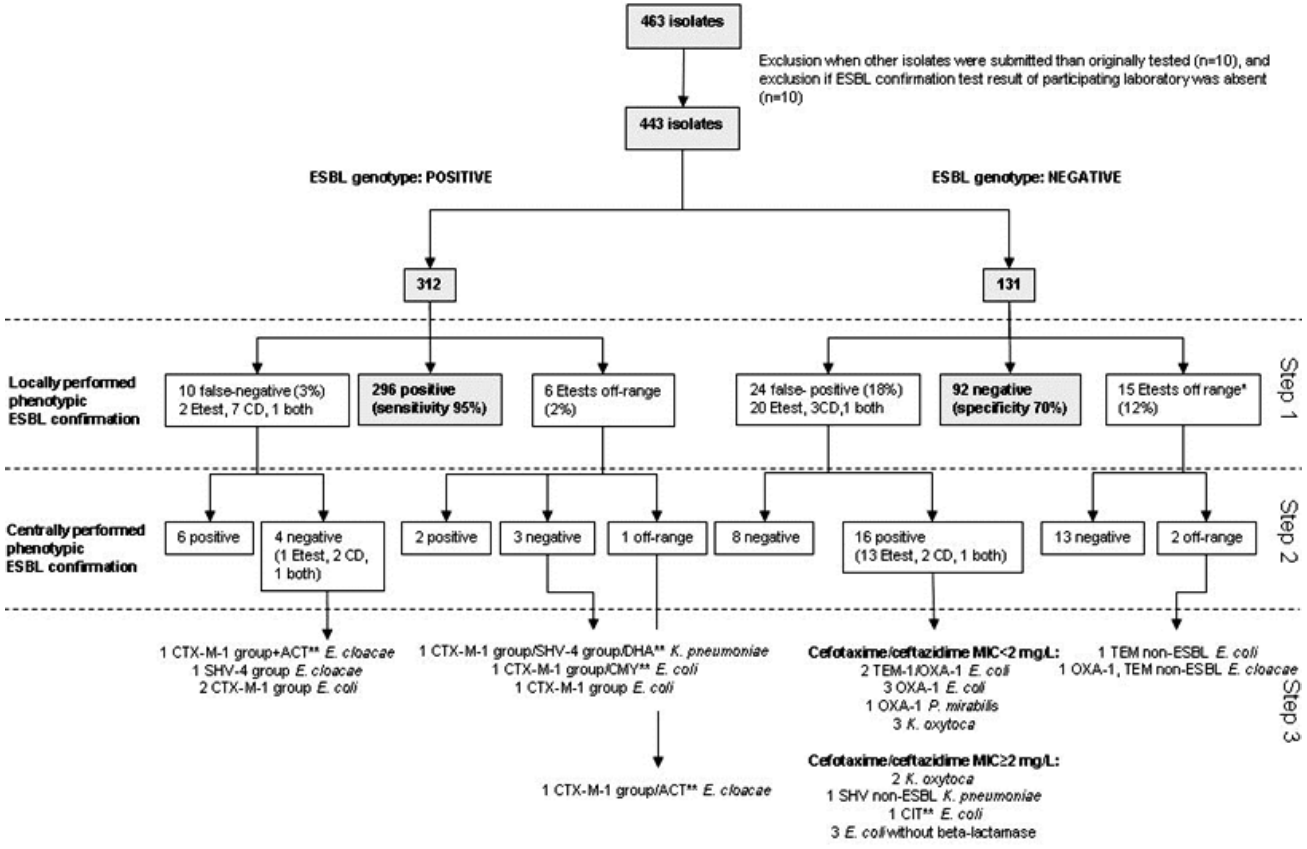

Figure 2. Diagram of inclusion and test results of isolates.

* In five isolates no cefepime/cefepime plus clavulanic acid Etest was performed.

** Plasmid-borne AmpC.

OXY (or K1) class A beta-lactamase, and six isolates contained an OXA-1 beta-lactamase gene. Of the seven remaining isolates, two were *K. oxytoca*, four *E. coli* isolates had no genetic substrate for the false positivity (1 CIT positive, the other three contained no beta-lactamases), and one *E. coli* isolate was SHV-1 positive, with a susceptibility pattern compatible with SHV-1 hyperproduction (reduced susceptibility to ceftazidime and amoxicillin-clavulanic acid, but susceptible to cefotaxime and cefuroxim)[7].

Therefore, we conclude that the guideline will have a maximum accuracy compared with the used genotyping methods of 94% (95% CI 92-96%) (Figure 2, step 2). Substitution of the misinterpreted local confirmation results with centrally performed test results yielded sensitivities of the Etest and combinations discs of 97% (95%CI 94-99%) and 97% (95%CI 92-99%), respectively, and specificities of 82% (95%CI 72-88%) and 94% (95%CI 82-98%), respectively.

Discussion

Based on the results from 20 clinical microbiology laboratories in the Netherlands we conclude that application of the Dutch national guideline for phenotypic ESBL detection resulted in correct interpretation of the ESBL-status in 388 (88%) of 443 isolates with a positive ESBL screen test. Of note, discordance between phenotypic testing and genotypic confirmation remained even after extensive retesting in the reference laboratory for 23 of the incorrect interpretations (5% of all isolates). Based on our findings we provide specific recommendations to further optimize phenotypic ESBL detection in routine microbiology diagnostics. These include genotypic ESBL testing for *P. mirabilis* and *K. oxytoca* isolates with a positive ESBL confirmation test, and to interpret isolates with MIC <2 mg/L for cefotaxime and ceftazidime (but considered as ESBL-positive in automated testing) as ESBL-negative.

The PPV of ESBL screening was 70% and depended on the method, the species, and the third-generation cephalosporin MICs. ESBL screening accuracy was comparable for the Vitek-2 and Phoenix method. Disc diffusion appeared more specific, but results were based on one laboratory and 6% of all isolates only. Per species, PPV ranged from 95% for *K. pneumoniae* to 13% for *P. mirabilis*. As a consequence, the diagnostic yield of ESBL confirmation tests is rather limited for *K. pneumoniae,* but definitely indicated for all other species. The accuracy of ESBL screening with automated systems correlated with the MIC for third-generation cephalosporins, as measured by broth microdilution. An extremely low PPV of screening (3%) was observed in isolates with an ESBL alarm of the automated system but third-generation cephalosporin MICs <2 mg/L.

The accuracy of ESBL confirmation tests also depended on the confirmation method, species, and the third-generation cephalosporin MICs. The test

characteristics of combination disc and Etest for ESBL confirmation, as performed by the participating laboratories, were generally comparable, but ESBL Etests were less specific than combination discs (59% versus 92%). This was because of non-determinable and false-positive Etest results. Most Etest results (86%) reported as off-range could not be confirmed in the reference laboratory, indicating problems with the interpretation of the ESBL Etests and/or lack of expertise in the clinical setting. This problem was not observed in previous experimental studies using the Etests as ESBL confirmation test[8,9]. These data indicate that education of the technicians may improve the accuracy of ESBL confirmation tests with 8% for Etest and 4% for combination disc results. It should be noted that the combination discs are cheaper than the Etest.

The PPV of the ESBL confirmation test in *E. coli, K. pneumoniae* and *Enterobacter* spp. was 95-97% versus 33-38% in *P. mirabilis* and *K. oxytoca*. However, a negative confirmation test result excluded ESBL production (NPV 100%, Table 2). Especially the low PPV for *P. mirabilis* is noticeable and, to our knowledge, not reported before. As four out of five false-positive phenotypic test results could not be reproduced in the reference laboratory these false-positive results suggest difficulties with the ESBL confirmation test interpretation, possibly due to swarming of the isolates. For *P. mirabilis* and *K. oxytoca* we recommend a genotypic ESBL detection method if the phenotypic ESBL confirmation test is positive.

The accuracy of the confirmation test was low in isolates with an ESBL alarm from the automated system and a third-generation cephalosporin MIC <2 mg/L. False-positive confirmation results were frequently obtained in such isolates. We, therefore, recommend that such isolates be interpreted as ESBL-negative and ESBL confirmation tests should not be performed. According to our findings, this strategy would not significantly reduce the sensitivity, but decrease the rate of false-positive test results with 50%.

Genetic analysis showed that six isolates without an ESBL genotype, but an ESBL-positive confirmation test in the reference laboratory and an MIC <2 mg/L for cefotaxime and ceftazidime, contained an OXA-1 gene (combined with a TEM-1 in two isolates). These six isolates showed decreased susceptibility to cefepime (median MIC 6 mg/L, range 1-8 mg/L) and a positive cefepime/cefepime plus clavulanic acid confirmation Etest result, in line with a recent report that expression of OXA-1 may lead to false-positive ESBL test results because of fourth-generation cephalosporin resistance without resistance to cefotaxime and ceftazidime[10].

In contrast to the CLSI guideline for ESBL detection, the Dutch guideline also provides an adequate method for phenotypic ESBL confirmation in Enterobacteriaceae with inducible chromosomal AmpC beta-lactamases, using

an Etest or combination disc with cefepime and cefepime plus clavulanic acid. Previous studies using cefepime-clavulanate disc combinations for confirmation reported a sensitivity of 88% and a specificity of 91% in a research setting[2,11]. Our findings demonstrate that application of the Dutch guideline is associated with equally high accuracy in daily clinical practice

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Chapter 3

Evaluation of a commercial microarray as a confirmation test for the presence of extended-spectrum beta-lactamases in isolates from the routine clinical setting

T.N. Platteel[1,2], J.W. Cohen Stuart[2], G.M. Voets[2], J. Scharringa[2], N. van de Sande[3], A.C. Fluit[2], M.A. Leverstein- Van Hall[2,3], on behalf of the ESBL national surveillance working group.

1. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands.
2. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands.
3. Centre for Infectious Disease Control (CIb), National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

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Abstract

Since the diagnostic characteristics of the Check-KPC ESBL microarray as a confirmation test on isolates obtained in a routine clinical setting have not been determined, we evaluated the microarray in a random selection of 346 clinical isolates with a positive ESBL screen test (MIC >1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek-2 expert system) collected from 31 clinical microbiology laboratories in the Netherlands in 2009. Using sequencing as the reference method the sensitivity of the microarray was 97% (237/245), the specificity 98% (97/99), the positive predictive value 99% (237/239) and the negative predictive value 92% (97/105).

Worldwide, the prevalence of extended-spectrum beta-lactamases (ESBLs) is increasing at an alarming rate[1]. For infection control precautions and the choice of adequate antibiotic therapy, accurate and rapid detection of ESBLs is important.

In Enterobacteriaceae, the most prevalent ESBL gene families are CTX-M, TEM and SHV[1]. For rapid detection of those ESBL families, a microarray system has been developed (Check-KPC ESBL, Check-Points B.V., Wageningen, the Netherlands)[2]. This system uses ligation-mediated amplification, combined with detection of amplified products on a microarray to detect the various CTX-M groups (CTX-M group 1, 2, 9, or combined 8/25) and the ESBL-associated single-nucleotide polymorphisms (SNPs) in TEM and SHV variants. The assay can not provide a Lahey type number for TEM and SHV genes (e.g. TEM-6 or SHV-2), but reports to which group they belong. Compared with phenotypic detection methods, this array system is faster (obtaining results within one working day) and provides information on the (combination of) TEM, SHV or CTX-M groups present, which may be used for epidemiologic or infection control purposes.

Evaluation of this microarray has been performed on collections of isolates expressing a wide variety of beta-lactamase genes[2-4]. High sensitivities (95-100%) and specificities (96-100%) were found in these studies. The aim of this study was to determine the accuracy of the Check-KPC ESBL microarray as a confirmatory test of ESBLs in the routine laboratory setting (i.e. on randomly selected clinical isolates with a positive ESBL screen test). Therefore 346 clinical isolates collected in a national ESBL surveillance study in the Netherlands were included. In this survey, 31 clinical microbiology laboratories collected all *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, and *Enterobacter* spp. isolates with a positive ESBL screen test according to the national guideline (MIC >1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek-2 expert system) from 1 February until 1 May 2009 (http://www.nvmm.nl/richtlijnen/esbl-screening-en-confirmatie). Of the 1418 collected isolates, the first 25 non-repeat isolates (one per patient) per participating laboratory were selected for genotypic analysis, resulting in a collection of 692 isolates. The accuracy of the microarray was evaluated on a computer-generated random sample of 50% of those isolates (n=346). There were no significant differences between the species distribution in the random sample and the total collection. As a reference test, we used the presence of ESBL genes determined by PCR and DNA sequencing on the same DNA batch as used for the microarray[2]. In case of presence of multiple TEM- and SHV-alleles, base calling for both alleles at positions in the sequence chromatogram that showed double peaks in the forward and reversed strand was resolved manually.

Microarray analysis was performed according to the instructions of the manufacturer, and interpreted using software version 20090508T164015R74 (Check-Points, Wageningen, the Netherlands). DNA isolation was performed using Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the instructions of the manufacturer. In the case of a positive ESBL phenotype according to the participating laboratory (confirmation was performed according to the national guideline using ESBL Etest (BioMérieux, Marcy l'Etoile, France) or combidisks with ceftazidime, cefotaxime and/or cefepime with and without clavulanic acid) and an ESBL-negative result of PCR and DNA sequencing for CTX-M, TEM and SHV ESBL genes, additional PCRs were performed to detect the presence of rare ESBL families such as PER, GES, and VEB beta-lactamase genes[5].

Statistical analyses were performed using SPSS 15.0 (IBM Inc., Armonk, NY, USA) and Microsoft Excel 2003 (Microsoft, Redmond, WA, USA).

Two of the 346 included isolates were excluded from analysis because DNA sequence results could not be obtained. Of the remaining 344 isolates, 75% were *E. coli* (n=257), 10% *K. pneumoniae* (n=35), 10% *Enterobacter cloacae* (n=33), 3% *P. mirabilis* (n=10) and 3% *K. oxytoca* (n=9). Based on PCR and sequencing, 245 isolates were ESBL positive and 99 ESBL negative. Among the 245 ESBL-positive isolates, in total 255 ESBL genes were identified: 209 CTX-M, 28 SHV, 16 TEM, 1 GES and 1 PER.

The sensitivity of the microarray for the detection of an ESBL was 97% (237/245), the specificity 98% (97/99), the positive predictive value 99% (237/239) and the negative predictive value 92% (97/105).

For 95% (228/239) of the isolates with an ESBL positive microarray result the outcome of the microarray was in accordance with the sequencing results. In Table 1 the discrepancies in the 11 isolates are specified.

A false-negative result was obtained in eight isolates. In six isolates, a CTX-M-1 group ESBL gene was not detected (four CTX-M-15/28 positive isolates, one CTX-M-1 positive isolate, and one CTX-M-22 positive isolate), even after repeating the test. These six represented 3% (6/182) of all CTX-M-1 group positive isolates in the collection (three *E. coli*, two *K. pneumoniae* and one *E. cloacae*). This finding is in contrast to previous studies, where only failures in the detection of TEM and SHV genes were reported, and worrisome since CTX-M-1 group enzymes, especially CTX-M-15/28, are the most prevalent ESBLs worldwide[6]. The reason is unknown, but may be explained by chance, since 74% (182/245) of the isolates in this collection harboured a CTX-M-1 group gene, a limited sensitivity of the CTX-M-1 group specific probe or a modification of the interpretation software resulting in an alteration of the detection limit. The other two false negative

isolates contained an ESBL gene not included in the design of the array (one PER and one GES producing isolate).

A false-positive result was obtained in two isolates containing a TEM-1 gene. However, in these isolates a TEM-17 and a TEM-19 group ESBL gene were identified by the array next to a non-ESBL TEM, and both had an ESBL-positive phenotype as determined by ESBL Etest. Therefore, these false-positive results may be explained by the limitation of using an unselective PCR and sequencing as the reference test, which failed to detect TEM ESBL genes in the presence of non-ESBL TEM genes, whereas the microarray system uses a selective amplification approach that detects these TEM ESBL genes accurately. Taking these characteristics of the reference test into account, the specificity and positive predictive value of the microarray would be 100% (99/99 and 237/237) and the negative predictive value 93% (99/107). There was one other isolate in which a TEM-19 group ESBL gene was identified by the array next to a non-ESBL TEM, while sequencing only detected a non-ESBL TEM. However, this discrepancy did not influence the test characteristics since the isolate possess also a CTX-M gene detected by both array and PCR.

A limitation of the study is that although the microarray was evaluated on isolates representative for the routine clinical setting the test was performed centrally. However, because of the simple and straightforward protocol of the microarray, no problems with the performance are to be expected in other laboratories. In conclusion, this study shows that the Check-KPC ESBL microarray is a rapid and accurate confirmation test for the presence of ESBL genes in the clinical setting. It may be used if a rapid result is required, if the phenotypic ESBL confirmation test is non-conclusive, for species in which phenotypic ESBL detection is difficult (e.g. *K. oxytoca)* due to production of other class A beta-lactamases or for infection control purposes (investigating a plasmid outbreak). Furthermore, the array is a high throughput diagnostic tool that is particularly well suited for epidemiological studies when testing a large number of isolates for the presence of ESBL.

Table 1. Comparison of DNA sequence and microarray results.

ESBL-genotype based on DNA-sequencing (n)	Isolates with concordant results n (%)	Isolates with discrepant results n (%)	Outcome sequencing*	Outcome microarray
Negative (99)	97 (98%)	2 (2%)	1 TEM-1 (non-ESBL)	1 TEM-17 group (ESBL)
			1 TEM-1 (non-ESBL)	1 TEM-19 group (ESBL)
CTX-M-family (199)	190 (95%)	9 (5%)	4 CTX-M 15/28 (CTX-M-1 group)	4 Negative
			1 CTX-M-1 (CTX-M-1 group)	1 Negative
			1 CTX-M-22 (CTX-M-1 group)	1 Negative
			1 CTX-M-65 (CTX-M-9 group)	1 CTX-M-1 group
			1 CTX-M-15/28 (CTX-M-1 group)	1 CTX-M-1 group, CTX-M-8/25 group
			1 CTX-M-15/28, TEM-1 (CTX-M-1 group, TEM non-ESBL)**	1 CTX-M-1 group, TEM-19 group
SHV-family (20)	18 (90%)	2 (10%)	1 SHV-12 (SHV-4 group)	1 SHV-2 group
			1 SHV-12 (SHV-4 group)	1 SHV-4 group, CTX-M-9 group
TEM-family (14)	13 (93%)	1 (7%)	1 TEM-19 (TEM-19 group)	1 TEM-3 group
Combination of genes (10)	7 (70%)	3 (30%)	1 CTX-M-1, SHV-12 (CTX-M-1 group, SHV-4 group)	1 CTX-M -1 group
			1 SHV-12, TEM-25 (SHV-4 group, TEM-19 group) 1	1 SHV-31 group, TEM-19 group
			CTX-M-15/28, SHV-12 (CTX-M-1 group, SHV-4 group)	1 CTX-M-1 group, SHV-2 group
Other ESBL genes (2)	0 (0%)	2 (100%)	1 PER-5	1 Negative
			1 GES-1	1 Negative

* Between brackets the array group to which the gene belongs is noted.

** Beside the noted CTX-M-gene, a TEM ESBL could not be confirmed and only a TEM non-ESBL was found by sequencing.

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Chapter 4

Population distribution of beta-lactamase conferring resistance to third-generation cephalosporins in human clinical Enterobacteriaceae in the Netherlands

Guido M. Voets[1*], Tamara N. Platteel[1,2], Ad C. Fluit[1], Jelle Scharringa[1], Claudia M. Schapendonk[1], James Cohen Stuart [1], Marc J.M. Bonten[1], Maurine A. Leverstein-van Hall [1,3] on behalf of the National ESBL Surveillance Working Group

1. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands
2. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands
3. National Institute for Public Health and the Environment, Bilthoven, the Netherlands

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Abstract

There is a global increase in infections caused by Enterobacteriaceae with plasmid-borne beta-lactamases that confer resistance to third-generation cephalosporins. The epidemiology of these bacteria is not well understood, and was, therefore, investigated in a selection of 636 clinical Enterobacteriaceae with a minimal inhibitory concentration >1 mg/L for ceftazidime/ceftriaxone from a national survey (75% *E. coli*, 11% *E. cloacae*, 11% *K. pneumoniae*, 2% *K. oxytoca*, 2% *P. mirabilis*). Isolates were investigated for extended-spectrum beta-lactamases (ESBLs) and *ampC* genes using microarray, PCR, gene sequencing and molecular straintyping (DiversiLab and multi-locus sequence typing (MLST)). ESBL genes were demonstrated in 524 isolates (82%); of which 442 (84%) belonged to the CTX-M family. Among 314 randomly selected and sequenced isolates, $bla_{\text{CTX-M-15}}$ was most prevalent (n=104, 33%), followed by $bla_{\text{CTX-M-1}}$ (n=48, 15%), $bla_{\text{CTX-M-14}}$ (n=15, 5%), $bla_{\text{SHV-12}}$ (n=24, 8%) and $bla_{\text{TEM-52}}$ (n=13, 4%). Among 181 isolates with MIC ≥16 mg/L for cefoxitin plasmid encoded AmpCs were detected in 32 and 27 were of the CMY-2 group. Among 102 *E. coli* isolates with MIC ≥16 mg/L for cefoxitin *ampC* promoter mutations were identified in 25 (25%). Based on DiversiLab genotyping of 608 isolates (similarity cut-off >98%) discriminatory indices of bacteria with ESBL and/or *ampC* genes were 0.994, 0.985 and 0.994 for *E. coli*, *K. pneumoniae* and *E. cloacae*, respectively. Based on similarity cut-off >95% two large clusters of *E. coli* were apparent (of 40 and 29 isolates) and 21 of 21 that were typed by belonged to ST131 of which 13 contained $bla_{\text{CTX-M-15}}$. Our findings demonstrate that $bla_{\text{CTX-M-15}}$ is the most prevalent ESBL and we report a larger than previously reported prevalence of *ampC* genes among Enterobacteriaceae responsible for resistance to third-generation cephalosporins.

Introduction

The increasing prevalence of plasmid-borne beta-lactamases in Enterobacteriaceae that confer resistance to third-generation cephalosporins is a world-wide problem. The most prevalent amongst these acquired beta-lactamases are the Ambler class A ESBLs of the CTX-M, TEM and SHV families[1]. These ESBLs are capable of hydrolysing penicillins, cephalosporins (except cephamycins), and monobactams and are inhibited by clavulanic acid[2]. An emerging class of β-lactamases are the plasmid-borne Ambler class C cephalosporinases (pAmpCs)[3]. AmpC enzymes are capable of hydrolysing penicillins, cephalosporins (although fourth-generation cephalosporins only weakly), and monobactams and are not inhibited by clavulanic acid[3]. The molecular epidemiology of these resistance mechanisms is largely unknown, as most large-scale molecular surveys were limited, either to certain species (e.g., *Escherichia coli* or *Klebsiella pneumoniae*), a specific environment (either hospital or general practice) or specimen type (e.g. urine or faeces)[4-9].

In the Netherlands the proportions of urine samples and blood cultures with *E. coli* (intermediate) resistant to third-generation cephalosporins increased from 2.6% and 2.6%, respectively in 2008 to 3.4% and 4.7%, respectively, in 2010[10].

The aim of this study was to determine the population distribution of beta-lactamase conferring resistance to third-generation cephalosporins in an unbiased, cross-sectional, large and nation-wide sample of clinical isolates in the Netherlands.

Materials and Methods

Isolates

From February 1. 2009 until May 1. 2009, 31 Dutch microbiology laboratories were asked to submit all isolates of *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis* and *Enterobacter cloacae* with a positive ESBL screen test (minimal inhibitory concentration (MIC)>1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek-2 expert system). The need for written consent of patients was waived by the ethical committee because of the retrospective nature of the study, the use of fully anonymized patient data only and because of the absence of any study related procedures. From each laboratory the first 25 consecutive isolates, if available, were included in this study, allowing for only one isolate per patient.

In a central laboratory screen tests were repeated using broth microdilution (BMD) (Merlin Diagnostic GmbH, Rüsselsheim, Germany) and only isolates with a confirmed positive test were included in this study. Susceptibility testing was performed for amikacin, cefotaxime +/- clavulanic acid, ceftazidime +/- clavulanic

acid, chloramphenicol, ciprofloxacin, fosfomycin, gentamicin, nitrofurantoin, piperacillin/tazobactam, tobramycin and trimethoprim/sulfamethoxazole using Sensititre microbroth dilution plates (TREK Diagnostic Systems, East-Grinstead, UK). MICs were interpreted according to EUCAST criteria.
For each isolate the following epidemiological data were collected: age (0-19, 20-59 and ≥60 years) and gender of the patient, specimen type (urine, faeces, wounds/skin, respiratory tract, blood and other (e.g., ascites, gynaecological cultures)) and institution (hospital (university, non-university), general practitioner (GP), or long term care facility (LTCF)). The participating laboratories are geographically dispersed over the Netherlands and represent a mixture of secondary and tertiary care hospitals, LTCFs and GPs. The 31 laboratories serve 58 hospitals, covering approximately 45% of all hospital beds in the Netherlands.

Molecular characterization of beta-lactamase genes
The presence of ESBL genes was determined by Check-KPC ESBL microarray analysis (Cat. No. 10-0018, CheckPoints, Wageningen, the Netherlands), which detects single nucleotide polymorphisms (SNPs) and reports the presence of TEM or SHV SNPs associated with an ESBL phenotype and specifies CTX-M groups (CTX-M group 1, 2, 9, or combined 8/25)[1]. As the assay cannot provide a type number for TEM, SHV and CTX-M genes (http://www.lahey.org/Studies), PCR and gene sequencing was performed for definite determination of ESBL genes as previously described[11-13]. From all screen-positive isolates a random sample of 314 isolates was taken for sequence-based confirmation of resistance genes. Isolates with a negative array result were first investigated using TEM, SHV, and CTX-M group-specific PCRs and, if negative, with multiplex PCRs for detecting other Ambler A class ESBL families (GES, PER and VEB)[14]. All PCR products were sequenced. In isolates with an AmpC phenotype (cefoxitin MIC ≥16 mg/L) the presence of pAmpC was determined by PCR and sequencing[14]. For *Enterobacter* spp., PCR results for plasmid ACT-1 and MIR-1,-2,-3 were not included because the primers used for these PCRs are based on primers that may also detect the chromosomal *ampC* of *Enterobacter* spp.[15]. If negative and no other β-lactamase was detected, the promoter of the chromosomal *ampC* of *E. coli* was sequenced to identify mutations associated with derepression[16-18]. For this PCR the following primers were designed: ECC-GS-F: GATCGTTCTGCCGCGTG and ECC-GS-R: GGGCAGCAAATGTGGAGCAA.

Isolate typing
E. coli, K. pneumoniae, K. oxytoca and *Enterobacter* spp. isolates were typed using DiversiLab (BioMérieux, Marcy l'Etoile, France)[19]. Representative *E. coli*

isolates from dominant patterns identified by DiversiLab were also analysed by multi-locus sequence typing (MLST) (http://mlst.ucc.ie/mlst/dbs/Ecoli). Discriminatory index calculations were performed using Ridom EpiCompare as previously described[19].

Statistical analysis

Statistical analysis (Mann-Whitney) was performed using SPSS 15.0 (IBM, Nieuwegein, the Netherlands). Associations were considered statistically significant in case of a p-value ≤ 0.02.

Results

ESBL and AmpC distribution

In the three-month study period, 1,427 ESBL screen-positive isolates were collected in the 31 participating laboratories. The first 25 isolates per laboratory, if available, comprised 723 isolates, of which 31 were excluded because of lack of viable cells or contamination with other strains, and 56 because positive screen tests could not be confirmed, leaving 636 isolates for further investigation: 479 *E. coli* (75%), 68 *E. cloacae* (11%), 67 *K. pneumoniae* (11%), 11 *K. oxytoca* (2%), and 11 *P. mirabilis* (2%). Sources and specimens of these isolates are listed in Table S1.

ESBL genes were detected in 524 of 636 isolates (82%): in 416 of 479 (87%) *E. coli,* in 64 of 67 (96%) *K. pneumoniae,* in 35 of 68 (51%) *E. cloacae*, in 6 of 11 (54%) *K. oxytoca,* and in 3 of 11 (27%) *P. mirabilis* (Table 1). Two ESBL genes were detected in 27 isolates (Table S2). Genes from the CTX-M-groups were detected most frequently (in 442 isolates), followed by SHV-genes (in 56 isolates) and TEM (in 46 isolates). Sequencing of 314 isolates revealed 16 CTX-M-variants, three SHV-variants, four TEM-variants, one GES-variant and one PER-variant (Table 2). Sequencing could not discriminate between CTX-M-15 and CTX-M-28 in 12 isolates. Nor could it discriminate between CTX-M-1 and CTX-M-61 in two isolates. Overall, CTX-M-15 was most prevalent, followed by CTX-M-1 and CTX-M-14 (Table 2).

In total 181 (28%) of 636 isolates had an AmpC phenotype; a cefoxitin MIC ≥16 mg/L (102 *E. coli* (56%), 63 *E. cloacae* (35%), 10 *K. pneumoniae* (6%), six *P. mirabilis* (3%)). A pAmpC gene was detected in 31 isolates: 24 of 102 (24%) *E. coli*, three of ten (30%) *K. pneumoniae* and in three of six (50%) *P. mirabilis*. These 31 isolates represented 5% of the 636 isolates (Table 3). Five different types of pAmpC beta-lactamases were identified: CMY-2-group, ACT-1, MIR-1/2/3, DHA-1, and ACT-

like. Sequencing did not allow for discrimination in the CMY-2 group, and MIR-1 and -2 and -3.
The remaining 36 *E. coli* isolates with AmpC-resistance phenotype and without either an ESBL or pAmpC were further investigated. Sequencing of the promoter region of the chromosomal *ampC* revealed different mutations that have been linked to resistance to third-generation cephalosporins in 25 isolates[16-18]. These mutations were found at the positions –1, –18,–42, –82, –88, and +58, and an insertion of an amino acid between the –10 and –35 region of the promoter. No mechanism was elucidated for the remaining 11 isolates.

Isolate Typing
All *Klebsiella* spp., *E. coli*, and *E. cloacae* isolates were analysed by DiversiLab (n=625), and seven isolates appeared non-typable. For the following analysis only isolates with a detectable ESBL- or *ampC* gene were included (n=608). When using a similarity >98% for pattern definition, 253 (53%) of 414 ESBL-producing *E. coli* had unique patterns, and cluster sizes ranged from two isolates (44 patterns) to 25 isolates (one pattern) (Table 4). The overall discriminatory index of ESBL-producing *E. coli* was 0.994 (95% confidence interval (CI) 0.991-0.996). When using a similarity of >95% two large clusters emerged, one of 40 isolates (comprising three patterns (n=22, n=14, n=4)) and one of 29 isolates (comprising four clusters (n=17, n=6, n=5, and n=1)). MLST typing of 21 (15 (71%) with CTX-M-15, four (19%) with CTX-M-1, one (5%) CTX-M-52, one (5%) with TEM-52) randomly selected isolates (11 and ten from the cluster of 43 and 30 isolates, respectively) revealed that all belonged to ST131.
Forty-one (64%) of 64 *K. pneumoniae* had unique patterns, and clusters of identical patterns ranged from two (n=4) to seven (n=1), and the discriminatory index was 0.985 (95% CI 0.969-1.000). All isolates of this cluster of seven contained a CTX-M-1 group ESBL and sequencing of three of these genes revealed CTX-M-15.
Forty-eight (71%) of 68 *E. cloacae* had unique patterns. There were eight clusters with two (n=6) or three isolates (n=2) and a discriminatory index of 0.994 (95% CI 0.989-0.999).
Inclusion of the 22 isolates without detectable ESBL or *ampC* genes did not change interpretation (data not shown).

Table 1. Identification of ESBL-groups as determined by ESBL array and PCR in 3rd generation cephalosporin resistant Enterobacteriaceae.

ESBL-group	E. coli N=479	E. cloacae N=68	K. pneumoniae N=67	K. oxytoca N=11	P. mirabilis N=11	All species N=636
CTX-M-1	301	16	48	3	2	370
CTX-M-2	2					2
CTX-M-8/25	2					2
CTX-M-9	59	11	1	1		72
SHV-2	2		4			6
SHV-4	23	14	11			48
SHV-31	1	1				2
TEM-3	28	1	2			31
TEM-4	1					1
TEM-5	2					2
TEM-17	2					2
TEM-19	10					10
GES				2		2
PER					1	1
No ESBL-gene detected	63	33	3	5	8	112

Note: 27 isolates contained 2 ESBLs

Table 2. Identification of ESBL β-lactamase genes in 3rd generation cephalosporin resistant Enterobacteriaceae.

ESBL-group	ESBL-gene	E. coli n=235 (75%)	E. cloacae n=33 (11%)	K. pneumoniae n=32 (10%)	K. oxytoca n=8 (3%)	P. mirabilis n=6 (2%)	Species n=314
CTX-M-1	CTX-M-1	47			1		48
	CTX-M-15	80	4	20			104
	CTX-M-15/28	9		3			12
	CTX-M-22	3		1			4
	CTX-M-79	2					2
	CTX-M Other	3				2	5
CTX-M-9	CTX-M-9	3	3				6
	CTX-M-14	15					15
	CTX-M-17	3					3
	CTX-M-27	4					4
	CTX-M Other	2					3
CTX-M-2	CTX-M-2	1					1
	All CTX-M Variants	172	7	24	1	2	206*
SHV-2	SHV-2	1		1			2

ESBL-group	ESBL-gene	E. coli n=235 (75%)	E. cloacae n=33 (11%)	K. pneumoniae n=32 (10%)	K. oxytoca n=8 (3%)	P. mirabilis n=6 (2%)	Species n=314
SHV-4	SHV-5			2			2
	SHV-12	13	8	3			24
	All SHV Variants	14	8	6			28*
TEM-3							
	TEM-52	13					13
TEM-5	TEM-12	1					1
TEM-19	TEM-19	1					1
	TEM-25	1					1
	All TEM Variants	17					16*
	GES-1				1		1
	PER-5					1	1
	Non-determinable	2	1		1		4
	None of the above genes detected	37	19	2	5	3	66

* In 10 isolates multiple ESBL-genes were detected.

Table 3. Presence of AmpC β-lactamase genes in isolates with a MIC ≥16 mg/L for cefoxitin.

AmpC-gene	E. coli n=102 (56%)	E. cloacae n=63 (35%)	K. pneumoniae n=10 (6%)	P. mirabilis n=6 (3%)	Species n=181
CMY-2 group	22		1	4	27
ACT-1	1				1
ACT-like *	1				1
MIR-1/2/3			1		1
DHA-1			1		1
Chromosomal	25	53 **			78
None of the above genes detected	53	10	7	2	72

* GenBank Number = EF125014.1, ** = presumed

Table 4. Number of clusters for each cluster size in DiversiLab using >98% similarity per species.

Species	DiversiLab cluster size										not	Discriminatory	
(n=isolates)	1	2	3	4	5	6	7	14	17	25	typable	Index	95% CI
E. cloacae (n=68)	48	6	2								2	0.994	0.989 - 0.999
E. coli (n=465)	253	44	14	2	2	1		1	1	1	2	0.994	0.991 - 0.996
K. pneumoniae (n=64)	41	4	2				1				2	0.985	0.969 - 1.000
K. oxytoca (n=11)	7		1								1	0.911	0.801 - 1.000

Association between β-lactamase gene and susceptibility

CTX-M-15 isolates were – on average – susceptible to 4.5 of 8 antibiotics tested, which was lower than isolates harboring TEM-52, CTX-M-1, or CTX-M-14 (mean susceptibility to 6.6, 6.0 and 5.7 antibiotics, respectively; p<=0.004 Mann Whitney U-test) and a similar co-resistance pattern as isolates harboring SHV-12 (Table 5). There were no significant associations between beta-lactamase genes and age, gender, specimen type, and institution (data now shown).

Table 5. Co-susceptibility according to EUCAST breakpoints in *E. coli* harboring the five most common ESBL genes.

Antibiotic	CTX-M-15 (n=77) (%, n)	CTX-M-1 (n=44) (%, n)	SHV-12 (n=7) (%, n)	CTX-M-14 (n=15) (%, n)	TEM-52 (n=12) (%, n)
Ciprofloxacin	9 (7)	57 (25)	43 (3)	60 (9)	53 (7)
Tobramycin	21 (16)	86 (38)	43 (3)	67 (10)	100 (12)
Trimethoprim/ sulfamethoxazole	35 (27)	25 (11)	14 (1)	53 (8)	33 (4)
Gentamicin	56 (43)	86 (38)	57 (4)	67 (10)	92 (11)
Amikacin	60 (46)	96 (42)	74 (5)	93 (14)	100 (12)
Chloramphenicol	69 (53)	64 (28)	29 (2)	33 (5)	75 (9)
Nitrofurantoin	99 (76)	96 (42)	100 (7)	100 (15)	100 (12)
Fosfomycin	99 (76)	98 (43)	100 (7)	100 (15)	100 (12)
Mean no. of co-susceptible antibiotics (range)	4.5 (2-8)	6.1 (3-8)	4.6 (3-7)	5.7 (3-8)	6.6 (5-8)

Discussion

The population structure of third-generation cephalosporin resistant Enterobacteriaceae in the Netherlands is characterized by predominance of *E. coli* with CTX-M-15 ESBL genes, a high level of bacterial genotypic diversity, although clusters of genotypes, often *E. coli* belonging to MLST131, were observed in individual laboratories. AmpC type resistance was observed in 53 *E. coli* isolates (11% of all *E.* coli) and resulted from pAmpC genes, mostly being bla_{CMY-2}, or *ampC* promoter mutations in equal frequencies.

The predominance of the CTX-M-gene family among the ESBL genes in Dutch isolates has also been observed in Belgium, France, Italy, Poland, Spain and Canada[6-8,20-22]. As in the Netherlands, CTX-M-15 was most prevalent within the

CTX-M family in all these countries, except in Poland and Spain were CTX-M-3 and CTX-M-14 were more prevalent[7,21].

E. coli ST131 carrying IncFII plasmids with CTX-M-15 is considered the most important disseminator of CTX-M-15 worldwide. Our finding of clusters of ST131 isolated within a short time-frame in single laboratories suggests the occurrence of clonal dissemination of ESBL-producing ST131 *E. coli*. In our study most of the ST131 *E. coli* contained CTX-M-15, and presence of this gene was associated with – on average – higher levels of resistance. Whether the clonal spread of ST131 occurs in health care-facilities or in the community remains to be determined as in the present study ST131 was in equal numbers obtained from samples submitted from health care institutes or by GPs. As such we agree with current literature that the origin ST131 remains unclear[23].

The high prevalence of CTX-M-1 and TEM-52 in *E. coli* in the Netherlands may result from food-borne exposure, as poultry and retail meat are frequently contaminated with *E. coli* harbouring these genes on identical plasmids as found in human isolates[24]. Similar findings (albeit with lower prevalence of TEM-52) have been reported from Belgium, where retail meat was also frequently contaminated with ESBL-producing *E. coli* and which country shares food distributors with the Netherlands, and north Italy[20,22]. In contrast, TEM-19 rather than TEM-52 is the most prevalent TEM beta-lactamase in Spain and Poland[7,21].

Five percent of *E. coli* and 4% of *K. pneumoniae* conferring resistance to third generation cephalosporins carried pAmpC beta-lactamases. Little is known about the epidemiology of CMY-group beta-lactamases. In Poland CMY-12 and CMY-15 were predominant in *E. coli* and in the UK and Ireland pAmpCs of the CIT group, which includes the CMY-2 group, and genes belonging to the FOX and ACC family were detected in *E. coli* and *K. pneumoniae*[7,25]. The source of CMY-2 is unknown. Although CMY-2 genes have been identified in poultry and poultry meat in Belgium, Spain and the Netherlands[26-28], more detailed studies are needed to demonstrate the relevance and frequency of gene or strain transmission between both reservoirs.

All *E. coli*, except eleven isolates, resistant to cefoxitin in which no pAmpC beta-lactamases or mutated promoters were detected, contained ESBL genes, which may explain their resistance phenotype. In the other eleven isolates the increased MIC for cefoxitin may have resulted from mechanisms not investigated in this study, e.g. porin mutations, alteration in the expression of efflux pumps and/or porins, and mutations in the target of β-lactamases.

In this study, derepressed chromosomal *ampC* genes and pAmpC beta-lactamase genes were equally prevalent in *E. coli.* This has also been observed in a French study[29], but a higher prevalence of derepressed chromosomal *ampC* genes was

detected in Belgium[30]. This difference could result from differences in selection of isolates.

In 636 isolates with phenotypic resistance to third-generation cephalosporins 551 ESBLs and 109 AmpCs (including 53 assumed chromosomal *ampC* genes in *E.* cloacae) were detected in 610 (96%) isolates. In the remaining 26 isolates (14 *E. coli*, five *K. oxytoca*, four *P. mirabilis*, and three *K. pneumoniae*) resistance may have been caused by mechanisms not investigated in this study, such as the presence of OXA genes, hyperproduction of chromosomal OXY genes in *K. oxytoca*, porin mutations, alteration in the expression of efflux pumps and/or porins, and mutations in the target of the β-lactamases.

Although we consider the selected isolates to be representative for the Netherlands, there may have been some selection bias as we did not adjust isolate selection on the size of the catchment populations of the different laboratories.

Our findings demonstrate that in the Netherlands $bla_{\text{CTX-M-15}}$ is the most prevalent cause of third-generation cephalosporin resistance in the Netherlands and that resistance due to either hyperproduction of chromosomal *ampC* or plasmid-borne AmpC beta-lactamases occurs more frequently than previously reported.

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Supplementary table 1. Material and provider of the isolates grouped by species.

	E. cloacae (n=68)	**E. coli (n=479)**	**K. oxytoca (n=11)**	**K. pneumoniae (n=67)**	**P. mirabilis (n=11)**
Material					
Ascites	3	5		1	
Blood	1	10		2	
Gynaecological		3		15	
Pulmonary	8	29	5		2
Rectum/faeces	12	58	3	18	
Urine	33	333	2	25	7
Wound/Abscess/Skin	11	39	1	5	2
Other/Unknown		2		1	
Provider					
Academic Hospital	1	23	2	14	1
Non-Academic Hospital	52	259	8	35	7
Long-term Care Facility	3	26		4	2
General Practitioner	10	147		12	1
Other/Unknown	2	24	1	2	

Supplementary table 2. Combinations of β-lactamases expressed in one isolate in the primary selection and random sample.

ESBL-ESBL combinations	Species	n	Beta-lactamase genotypes
CTX-M-1 gr. & CTX-M-9 gr.	E. cloacae	2	Not Typed (n=2)
	E. coli	1	CTX-M-15 & CTX-M-27 (n=1)
CTX-M-1 gr. & CTX-M-8/25 gr.	E. coli	1	CTX-M-15 (CTX-M-8/25-gr. Not Typed) (n=1)
CTX-M-1 gr. & SHV-4 gr.	E. cloacae	2	CTX-M-15 & SHV-12 (n=1)
			Not typed (n=1)
	E. coli	6	CTX-M-1/61 & SHV-12 (n=2)
			CTXM-15 & SHV-12 (n=2)
			Not typed (n=2)
CTX-M-9 gr. & SHV-4 gr.	E. cloacae	3	CTX-M-9 & SHV-12 (n=1)
			SHV-12 (CTX-M-9-gr. not typed) (n=1)
			Not typed (n=1)
	E. coli	1	CTX-M-9 & SHV-12 (n=1)
	K. pneumoniae	1	Not typed (n=1)
CTX-M-1 gr. & TEM-3 gr.	E. coli	2	CTX-M-1/61 & TEM-52 (n=1)
			Not typed (n=1)
CTX-M-1 gr. & TEM-19 gr.	E. coli	2	CTX-M-15 (TEM-19-gr. not typed) (n=1)
			TEM-19 (CTX-M-1-gr. not typed) (n=1)
CTX-M-1 gr. & TEM-4 gr.	E. coli	1	Not typed (n=1)
SHV-2 gr. & TEM-19 gr.	E. coli	1	Not typed (n=1)
SHV-31 gr. & TEM-3 gr.	E. cloacae	1	Not typed (n=1)
	E. coli	1	Not typed (n=1)
SHV-4 gr. & TEM-25 gr.	E. coli	1	SHV-12 & TEM-25 (n=1)

ESBL-AmpC combinations	Species	n	ESBL genotypes
CTX-M-1 gr. & CIT	E. coli	4	CTX-M-1 (CMY not typed) (n=1)
			CTX-M-15 (CMY not typed) (n=2)
			Not typed (n=1)
	K. pneumoniae	1	Not typed (n=1)
CTX-M-1 gr. & MIR	E. coli	1	CTX-M-15 & MIR-1/2/3 (n=1)
SHV-4 gr. & MIR	K. pneumoniae	1	MIR-1/2/3 (SHV-4-gr. not typed) (n=1)

ESBL-ESBL-AmpC combinations	Species	n	ESBL genotypes
CTX-M-1 gr. & SHV-4 gr. & DHA	K. pneumoniae	1	DHA-1

Chapter 5

Differences in antibiotic susceptibility of human E. coli with poultry-associated and non-poultry-associated extended-spectrum beta-lactamases

T.N. Platteel[1,2], M.A. Leverstein-Van Hall[2,3], J.W. Cohen Stuart[2], G.M. Voets[2], M.P. van den Munckhof[2], J. Scharringa[2], N. van de Sande[3], A.C. Fluit[2], M.J.M. Bonten[2], on behalf of the ESBL national surveillance working group.

1. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands.
2. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands.
3. National Institute for Public Health and the Environment (RIVM), Centre for Infectious Disease Control (CIb), Bilthoven, the Netherlands.

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Abstract

The concurrent presence of $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ genes on similar plasmids of *Escherichia coli* isolated from poultry, chicken meat and humans supports the occurrence of food-borne transmission of extended-spectrum beta-lactamase (ESBL) genes. ESBL-producing *E. coli* (ESBL-*E. coli*) are most frequently detected in hospitalised patients and are known to spread in healthcare settings. We hypothesised that poultry-associated (PA) ESBL genes are predominant in the community, where acquisition is fuelled by food contamination, whereas non-PA ESBL genes are predominant in hospitals, with acquisition fuelled by cross-transmission. Then, differences in antimicrobial selective pressure in hospitals and poultry would create differences in co-resistance between PA and non-PA ESBL-*E. coli*. We, therefore, determined the prevalence and co-resistance of PA and non-PA ESBL-*E. coli* in community-acquired and nosocomial urinary tract infections in humans and $bla_{CTX\text{-}M\text{-}1}$ and $bla_{TEM\text{-}52}$ isolates from poultry.

A total of 134 human ESBL-*E. coli* urine isolates were included in this study. Isolates containing $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$ were considered PA, with the remainder being non-PA. Also, 72 poultry ESBL-*E. coli* were included. Minimum inhibitory concentration (MIC) values were determined by broth microdilution.

The prevalence of PA ESBL genes in isolates obtained in general practice and hospitals was 28% versus 30% (n.s.). Human PA ESBL-*E. coli* were more frequently susceptible to ciprofloxacin (51% vs. 25%; p=0.0056), gentamicin (86% vs. 63%; p=0.0082), tobramycin (91% vs. 34%; p=0.0001), and amikacin (98% vs. 67%; p=0.0001), compared to human non-PA ESBL-*E. coli*.

PA ESBL-*E. coli* are not more prevalent in community-acquired than nosocomial urine samples, but more often susceptible to ciprofloxacin and aminoglycosides than non-PA ESBL-*E. coli*. This does not support the existence of different reservoirs of ESBL genes.

Introduction

Infections caused by Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) have become a healthcare problem of global proportions and have been associated with increased mortality, morbidity and healthcare costs. Although most of these infections are healthcare associated, community-acquired infections with ESBL-positive bacteria are increasingly being reported as well[1].

Recent findings demonstrated the genetic similarity between ESBL genes, plasmids and bacterial genotypes among *Escherichia coli* isolates from poultry, retail chicken meat and humans[2]. Poultry-associated (PA) ESBL genes were defined as ESBL genes found in poultry and poultry meat. Of the human isolates, 35% contained PA ESBL genes, which were mostly (86%) $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$. These genes also accounted for 77% and 75% of the ESBL genes in bacteria recovered from poultry and retail chicken meat, respectively. The same genes were identified in chicken meat as well as humans in a similar study, also in the Netherlands[3]. These findings suggest that bacteria harbouring these genes were acquired by humans through the food-chain. Yet, within health-care settings, the cross-transmission of ESBL-producing bacteria is considered an important mode of acquisition. These findings could indicate the existence of two distinct compartments for the dynamics of ESBL-producing bacteria; one in the community fuelled by food contamination and one in hospitals fuelled by cross-transmission. This would be supported if PA ESBLs would be especially prevalent among community-acquired infections, most notably urinary tract infections. Moreover, one would expect that bacteria harbouring PA ESBL genes differ from those considered to be hospital-acquired regarding co-resistance, due to differences in the antimicrobial selective pressure in human medicine and the poultry industry. For instance, aminoglycosides are used almost exclusively in hospitals. We, therefore, hypothesized that because of food-borne transmission of PA ESBL genes, prevalence among community-acquired and hospital-acquired infections and resistance patterns would differ between PA and non-PA ESBL-positive bacteria in humans.

Materials and methods

Isolates

From February 1. until May 1. 2009, 31 clinical microbiology laboratories collected all Enterobacteriaceae isolates with a positive ESBL screening test, according to the national guideline (Minimum inhibitory concentration (MIC) >1 mg/L for cefotaxime or ceftazidime or an ESBL alarm from the Phoenix or Vitek 2 expert system)[2,4]. Genotyping was performed on a random sample of 50% of the

first 25 non-repeat isolates (one per patient) per participating laboratory. ESBL-positive *E. coli* isolates, obtained from urine were selected for further analysis. Isolates with non-conclusive sequence results and isolates containing >1 ESBL gene were excluded, yielding 134 isolates.
CTX-M-1 and TEM-52 positive isolates were marked as PA; isolates containing other ESBLs were marked as non-PA[2]. For each isolate information on the setting in which it was obtained, the age and gender of the patient and the material was collected.
MICs for amikacin, ciprofloxacin, fosfomycin, gentamicin, nitrofurantoin, tobramycin and trimethoprim were determined with Sensititre microbroth dilution plates (TREK Diagnostic Systems, East-Grinstead, UK) for the human isolates and interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints.
In 2010, 98 raw chicken breasts were purchased in 12 stores in Utrecht, the Netherlands[2]. Per meat sample, 25 g was homogenised with 225 ml of peptone water in a stomacher. For qualitative culturing, an overnight pre-enrichment was performed with 225 ml of the homogenate. Phenotypic ESBL production was determined using Etests on all (n=163) isolates (BioMérieux, Marcy L'Etoile, Lyon, France). Sequencing of the ESBL gene was performed on a random 50% of the isolates (n=81). All TEM-52 and CTX-M-1 positive *E. coli* isolates were selected for further analysis (n=72). The MICs of the above mentioned antibiotics, except for fosfomycin, were determined by broth micro-dilution (Merlin, Bornheim-Hersel, Germany) and interpreted using EUCAST breakpoints.

Gene characterisation
The presence of ESBL genes was determined by TEM, SHV, and CTX-M group-specific polymerase chain reaction (PCR) assays. If negative, further investigation was performed by multiplex PCRs designed to detect Ambler class A ESBL families[5]. Gene sequencing was performed on ESBL-positive isolates.

Statistic Analysis
Chi-square or t-test (if applicable) was performed using Excel 2003 and SPSS 15.0.

Results

Of the 134 human isolates selected for the analysis; 60 were from samples submitted from general practice, 57 were from hospitalised patients and 17 were from an unknown origin or from outpatient departments. Forty-three isolates (32%) harboured CTX-M-1 or TEM-52 and were considered to be PA. Among the 60 isolates obtained in general practice, 17 were PA (28%), as were 17 of the

57 isolates obtained in the hospital (30%; n.s.). PA ESBL-positive isolates were more frequently obtained from women than non-PA ESBL-positive isolates (84% (36/43) vs. 63% (57/91), p=0.016).

Although isolates with PA genes were (as compared to bacteria with non-PA genes) more frequently susceptible to tobramycin (91% (39/43) for PA vs. 34% (31/91) for non-PA isolates; p<0.0001), ciprofloxacin (51% (22/43) vs. 25% (23/91); p=0.0056), gentamicin (86% (37/43) vs. 63% (58/91); p=0.0082) and amikacin (98% (42/43) vs. 67% (61/91); p<0.0001), it was not possible to predict the presence of PA ESBL types based on resistance patterns (Table 1). The proportions of ESBL-producing isolates susceptible to nitrofurantoin and fosfomycin were >95% and >90% for PA and non-PA ESBL-positive isolates, respectively. As the six resistant isolates (one isolate was resistant to nitrofurantoin as well as fosfomycin) were all derived in different laboratories, and the ESBL genes were diverse (two CTX-M-1, two CTX-M-15/28, one CTX-M-79 and one CTX-M-52), there were no signs that the isolates were clonally related.

ESBL-producing *E. coli* (ESBL-*E.* coli) derived from chicken meat were, as compared to human-derived ESBL-*E. coli*, more frequently susceptible to ciprofloxacin (86% (62/72) for chicken meat samples vs. 51% (22/43) for PA isolates from humans; p<0.0001) and to gentamicin (99% (71/72) vs. 86% (37/43); p=0.0108; Table 1).

Discussion

In this nationwide study, urinary *E. coli* isolates harbouring so-called PA ESBL-genes ($bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$) were as often detected in samples obtained in general practice as in isolates obtained in hospitals. This does not support the existence of two epidemiologically distinct patterns of ESBL transmission, with one being in the community fuelled by food contamination and the other in hospitals fuelled by cross-transmission. Yet, susceptibility patterns were different, with PA ESBL-*E. coli* being more often susceptible to ciprofloxacin, gentamicin, tobramycin and amikacin, which are antibiotics used relatively infrequently in the animal industry, compared to isolates harbouring non-PA genes.

Although our findings are based on a non-biased sample of isolates from a nationwide surveillance, there are some methodological limitations that deserve attention. There was no information about previous hospitalisations, creating a possibility of misclassification of nosocomial and community-acquired infections. Furthermore, although the distinction between PA and non-PA ESBL-genes, as used in this study, was based on the results of two independent studies[2,3], there is still the possibility of misclassification, as some genes may have been missed in animal-derived samples.

Table 1. Co-resistance in non-poultry vs. poultry-associated isolates *Escherichia coli* isolates obtained from urine.

Antibiotic	Non-poultry associated isolates Susceptibility (n); n=91	Poultry associated isolates Susceptibility (n); n=43	p-value non-poultry vs. poultry associated isolates	Poultry isolates Susceptibility (n); n=72	p-value poultry associated vs. poultry isolates
Tobramycin	34% (31)	91% (39)	<0,0001	99% (71)	n.s.
Ciprofloxacin	25% (23)	51% (22)	0,0056	86% (62)	<0.0001
Trimethoprim/ sulfamethoxazole	34% (31)	30% (13)	n.s.	39% (28)	n.s.
Gentamicin	63% (58)	86% (37)	0,0082	99% (71)	0.0108
Amikacin	67% (61)	98% (42)	<0,0001	99% (71)	n.s.
Nitrofurantoin	96% (87)	98% (42)	n.s.	100% (72)	n.s.
Fosfomycin	99% (90)	98% (42)	n.s.	-	

Naturally, these findings do not rule out a relevant role of food contamination and it is possible that PA ESBL genes, although originating in the community, have already spread widely in hospitals or are constantly instructed in hospitals by patients carrying these genes in their faecal flora at admission. The higher resistance prevalence for aminoglycosides and fluoroquinolones in PA ESBLs derived from human samples, as compared to those derived from poultry meat, supports some adaptation to human antibiotic use, since aminoglycosides and fluoroquinolones are hardly used in broilers[6].

As our study was based on a randomly selected part of a nationwide surveillance, the resistance patterns observed are likely to be representative of the current situation in the Netherlands. Proportions of ESBL-producing isolates susceptible to nitrofurantoin and fosfomycin were >95% and >90% for PA and non-PA ESBL-positive isolates, respectively. Since obtaining cultures in general practice is only advised in case of relapses and therapy failure, isolates at the population level in primary care will probably be even more often susceptible to these antibiotics than isolates included in this study. Current Dutch guidelines for general practitioners recommend nitrofurantoin, trimethoprim and fosfomycin as first, second and third choice of empirical therapy, respectively, for uncomplicated urinary tract infections. From the results of this study, we conclude that nitrofurantoin and fosfomycin are appropriate choices, even in case of ESBL-positive uropathogenic *E. coli*. Trimethoprim, though, seems less suitable in such cases. In two other Dutch surveys[7,8] (data collection 2003-2004 and 2004-2009, respectively) 77-81% of isolates from urinary tract infections in primary care were susceptible to trimethoprim, and 98-100% were susceptible to nitrofurantoin and fosfomycin[9,10]. The prevalence of ESBL-positive isolates in urinary tract infections in general practice in the Netherlands is still low, but increased from 0.1% in 2004 to 1% in 2009[9]. Based on these figures, fosfomycin and nitrofurantoin might be more suitable than trimethoprim as treatment for uncomplicated urinary tract infection in primary care. For complicated urinary tract infections, treatment based on the results of microbiological testing is considered standard care.

From this study, we can conclude that PA ESBL-*E. coli* have the same prevalence among ESBL-*E. coli* in community-acquired and nosocomial urinary tract infections, not supporting the existence of two different compartments. Yet, PA ESBL-*E. coli* were more susceptible to antibiotics than non-PA ESBL-*E. coli*, suggesting different levels of exposure to antibiotic pressure.

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Chapter 6

Predicting carriage with ESBL-producing bacteria at hospital admission; a cross-sectional study

T.N. Platteel[1,2], M.A. Leverstein-van Hall[3], J.W. Cohen Stuart[4], S.F.T. Thijsen[5], E.M. Mascini[6], B.C. van Hees[7], J. Scharringa[2], A.C. Fluit[2], M.J.M. Bonten[2]

1. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands
2. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands
3. Bronovo Hospital, Department of Medical Microbiology, Den Haag, and Diaconessenhuis, Leiden, the Netherlands
4. Medisch Centrum Alkmaar, Department of Medical Microbiology, Alkmaar, the Netherlands
5. Diakonessenhuis, Department of Medical Microbiology, Utrecht, the Netherlands
6. Rijnstate Hospital, Department of Medical Microbiology, Arnhem, the Netherlands
7. Gelre Hospitals, Department of Medical Microbiology and Infection prevention, Apeldoorn, the Netherlands

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Abstract

The prevalence of patients colonized with extended-spectrum beta-lactamase (ESBL)-producing bacteria increases, especially in long-term-care facilities (LTCFs). Identification of ESBL-carriers at hospital admission is relevant for infection control measures and antibiotic therapy for nosocomial infections. We aimed to develop a prediction rule for ESBL-carriage at hospital admission for patients admitted from home and LTCFs, and to quantify incidences of nosocomial infections caused by ESBL-producing bacteria.

The ESBL-carrier status was determined of patients admitted from LTCFs and from home settings in four hospitals in the Netherlands using peri-anal swabs obtained within 48 hours of admission. Risk factors for ESBL-carriage were assessed. Infections caused by ESBL-producing bacteria were identified retrospectively.

Among 1,351 patients, 111 (8.2%) were ESBL-carriers at admission; 50/579 (8.6%) admitted from LTCFs and 61/772 (7.9%) from home settings (p=0.63). Previous ESBL-carriage and previous hospital admission were risk factors for ESBL-carriage in multivariable analysis. The AUC of the ROC-curve of the model was 0.64 (95%CI 0.58-0.71). Presence of ≥1 risk factor (n=803, 59%) had sensitivity of 72%. Incidences of nosocomial infections caused by ESBL-producing bacteria were 45.5/10,000 and 2.1/10,000 admission-days for ESBL-carriers and non-carriers, respectively (p<0.05).

In conclusion, prevalence of ESBL-carriage at hospital admission was 8.2%, and was comparable among patients admitted from LTCF and home. A clinically useful prediction rule for ESBL-carriage at admission could not be developed. The absolute incidence of nosocomial infections by ESBL-producing bacteria was low, but higher among patients carrying ESBL-producing bacteria at the time of hospital admission.

Introduction

Infections due to extended-spectrum beta-lactamase (ESBL)-producing bacteria are increasing worldwide[1,2], and are often preceded by asymptomatic carriage, i.e. colonization[3]. Prevalence of ESBL-carriage in hospitalized patients and patients treated in long-term care facilities (LTCFs) have been reported to be as high as 27%[4,5], and the prevalence of ESBL-carriage in non-hospitalized subjects seems to be increasing[6-8].

Identification of ESBL-carriers at hospital admission is relevant for implementing appropriate infection control measures and selecting empirical antibiotic therapy in case of nosocomial infection[9].
Previous hospital stay, severity of illness, time in the ICU, intubation and mechanical ventilation, urinary or arterial catheterisation, previous exposure to antibiotics and urinary tract infections have been identified as risk factors for acquisition of ESBL-producing bacteria during hospital stay[10-13]. In addition, LTCF-residents are considered to have an increased risk of ESBL-carriage, because of the presumed high risk for acquisition and transmission of ESBL-producing bacteria in these settings, facilitated by antibiotic use, understaffing and failing infection control measures[14]. However, risk-factors for ESBL-carriage at the time of hospital admission have only been determined prospectively in unselected hospitalized patients in 2006[14].
We, therefore, prospectively determined the prevalence of ESBL-carriage in consecutively admitted patients coming from LTCFs and home settings, and aimed to develop a prediction rule for ESBL-carriage at hospital admission. In addition, the incidence of infections with ESBL producing bacteria was determined in patients identified as ESBL-carriers at hospital admission and non-carriers.

Methods

Setting and patients
This study was conducted in four hospitals (one tertiary care teaching hospital and three general teaching hospitals) in the Netherlands between January 2010 and December 2012. All patients admitted from LTCFs (nursing homes and rehabilitation facilities) to one of the surgery or general medicine wards were eligible for inclusion, as were patients admitted from home during three periods of nine weeks, at the beginning, middle and end of the study period in each hospital. Exclusion criteria were age <18 years, an expected hospital-stay <48 h, admission from another hospital and inability to fill-out the questionnaire and

hospital-stay. Hospital-acquired infections (HAI) caused by ESBL-producing bacteria were defined as presence of a clinical culture with ESBL-producing bacteria obtained more than 48 h after hospital admission that was treated by the clinician with antibiotics. Community-acquired infections (CAI) caused by ESBL-producing bacteria were defined as presence of a clinical culture with ESBL-producing bacteria obtained within 48 h after hospital admission that was treated by the clinician with antibiotics.

Statistical analysis

Statistical analyses were performed by Chi-square test or Fisher's exact test for categorical data, and Student's T-test or Mann-Whitney U-test were used for continuous data. Backward multivariate logistic regression analysis was conducted on variables significantly associated with carriage of ESBL-producing bacteria in univariate analysis, after exclusion of multicollinearity, as well as some other suspected important predictors. Variables with a p-value <0.10 were included in the model. The model's predictive ability was examined using the area under the receiver operating characteristics (ROC) curve. Calibration of the model was estimated by the Hosmer-Lemeshow statistic in which a p-value greater than 0.20 indicates adequate fit.

Missing data were replaced by multiple imputation (automatic method) before univariate analysis. To obtain a weighted score for the prediction rule, the regression coefficients of the predictive variables were rounded to the nearest number ending in .5 or .0, resulting in a weighted score.

Isolates which grew on the ESBL Brilliance plate, but that were not available for genotyping were assumed ESBL-positive. All analyses were performed using SPSS 20.

Results

In total 1531 patients were screened for ESBL carriage. Of these, 180 patients were excluded from the study because their cultures on both the MacConkey and the ESBL Brilliance agar were negative. Therefore, a total of 1,351 patients were included; 579 (42.9%) admitted from >100 different LTCFs and 772 (57.1%) from the community. Patient characteristics are depicted in Table 1. Populations admitted from home and LTCFs at the four sites did not differ significantly in gender or previous ESBL-carriage. In one hospital the mean age for patients from home was higher (60 vs. 62 vs. 64 vs. 76 years; p<0.01 for the last hospital as compared to the other hospitals), as for another hospital the mean age for patients from LTCFs was lower (74 vs. 81 vs. 82 vs. 86 years; p<0.01 for the first hospital as compared to the other hospitals). In one hospital, patients from home had less previous hospital admissions (19% vs. 26% vs. 34% vs. 34%; p<0.01 for

Table 1. Possible risk factors in ESBL-positive and –negative patients, admitted from home and LTCFs, and missing values before multiple imputation.

Characteristics		Patients admitted from home		Patients admitted from LTCFs	
		ESBL-negative N (%) n=711	ESBL-positive N (%) n=61	ESBL-negative N (%) n=529	ESBL-positive N (%) n=50
Sex	Female	388 (55)	28 (46)	338 (64)	23 (46)
	Male	323 (45)	33 (54)	191 (36)	27 (54)
	Missing	0 (0)	0 (0)	0 (0)	0 (0)
Age, mean years (SD)		64 (18)	67 (15)	82 (12)	81 (12)
Previous AB (1yr)	Yes	401 (56)	25 (41)	298 (56)	33 (66)
	No	307 (43)	36 (59)	229 (43)	17 (34)
	Missing	3 (0)	0 (0)	2 (0)	0 (0)
Previous IS (1yr)	Yes	113 (16)	14 (23)	110 (21)	7 (14)
	No	597 (84)	46 (75)	418 (79)	43 (86)
	Missing	1 (0)	1 (2)	1 (0)	0 (0)
Previous admissions (1yr)	Yes	268 (38)	33 (54)	208 (39)	26 (52)
	No	442 (62)	28 (46)	317 (60)	23 (46)
	Missing	1 (0)	0 (0)	4 (1)	1 (2)
Catheter	Yes	63 (9)	7 (11)	90 (17)	16 (32)
	No	647 (91)	54 (89)	435 (82)	34 (68)
	Missing	1 (0)	0 (0)	4 (1)	0 (0)
Previous surgery (1yr)	Yes	172 (24)	20 (33)	104 (20)	15 (30)
	No	538 (76)	41 (67)	421 (80)	35 (70)
	Missing	1 (0)	0 (0)	4 (1)	0 (0)
Previous travel (1yr)	Yes	234 (33)	16 (26)	30 (6)	2 (4)
	No	466 (66)	44 (72)	494 (93)	48 (96)
	Missing	11 (2)	1 (2)	5 (1)	0 (0)
Occupation with animal contact	Yes	13 (2)	1 (2)	1 (0)	1 (2)
	No	642 (90)	55 (90)	528 (100)	49 (98)
	Missing	56 (8)	5 (8)	0 (0)	0 (0)

Characteristics		Patients admitted from home		Patients admitted from LTCFs	
		ESBL-negative N (%) n=711	ESBL-positive N (%) n=61	ESBL-negative N (%) n=529	ESBL-positive N (%) n=50
Previous ESBL-carriage (1yr)	Yes	5 (1)	7 (11)	6 (1)	4 (8)
	No	706 (99)	54 (89)	523 (99)	46 (92)
	Missing	0 (0)	0 (0)	0 (0)	0 (0)

AB= use of antibiotics, IS= use of immunosuppressants

not having any relatives present to do so. The institutional regulatory board approved the study and considered the culture scheme as part of "usual care".

Study design

This was a prospective study. Peri-anal swabs, obtained within 48 h of admission, were inoculated on an ESBL Brilliance plate (Thermo Fisher Scientific, UK) to detect ESBL-producing strains and on MacConkey agar (Thermo Fisher Scientific, UK) as a control for adequate sampling. In case of no growth on both plates patients were excluded from analysis. Isolates obtained from the ESBL Brilliance plates were investigated by microarray analysis (Check-Points, Wageningen the Netherlands) for the presence of ESBL genes. DNA isolation was performed using Ultraclean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the instructions of the manufacturer.

Species identification was performed using MALDI-TOF (Bruker Daltonik, Bremen, Germany). Susceptibility was determined by disk diffusion (ROSCO, Taastrup, Denmark) for all antibiotics except for fosfomycin that was tested by Etest (BioMérieux, Marcy l'Etoile, France). Results were interpreted using EUCAST breakpoints.

A standardized questionnaire was used to collect information on use of antibiotics or immunosuppressants, surgical procedures, presence of indwelling devices, travel to foreign countries and work-related contact with animals in the year before admission. Patients were encouraged to mark "unknown" if they were uncertain whether they used antibiotics or immunosuppressants. In case patients declared use or were uncertain about use, this information was retrieved from their pharmacy records. Besides, the pharmacy records of 50 patients who reported no use were randomly checked.

Departments were instructed to keep records of the number of patients that were not approached or refused participation.

Microbiology databases were used to identify ESBL-carriage before admission, as well as presence of clinical cultures yielding ESBL-producing bacteria during

the first hospital as compared to the last two). Among populations admitted from a LTCF no statistical significant differences were observed in previous hospital admissions. For 71% of patients from LTCFs and 55% of patients from home information on use of antibiotics and immunosuppressants was retrieved from pharmacy records. Among the 50 patients checked that declared no use, only one had antibiotics in the year before admission and none in the six months before admission.
The prevalence of ESBL-carriage at hospital admission was 7.9% (n=60; range per hospital 5.4%-10.3%; n.s.) and 8.6% (n=51; range per hospital 5.3%-18.8%; n.s.) for patients admitted from home and LTCFs, respectively. There was no significant difference in ESBL prevalence between the four sites. The prevalence of 18.8% (6/32) in one hospital among patients from LTCFs resulted mostly from patients admitted from a single LTCF with a known high endemic prevalence of ESBL-carriage. Four of the six patients in this hospital, being ESBL-positive at admission, originated from this LTCF.
Species identification and susceptibility testing was available for 109 isolates from 97 patients. Susceptibility rates were for tobramycin 77%, gentamicin 85%, ciprofloxacin 62%, trimethoprim/sulfamethoxazole 35%, amikacin 100%, fosfomycin 84% and nitrofurantoin 77%.
Distribution of species and ESBL genes is shown in Table 2. No differences were observed in distribution of genes between patients admitted from home and LTCFs.

In univariate analysis eight potential risk factors for ESBL-carriage were identified. These together with age and patient origin (LTCF or community) were included in multivariate analysis after exclusion of multicollinearity, which yielded documented ESBL-carriage within one year before admission, hospital admission in the previous six months and male gender as associated with ESBL-carriage (Table 3). Twenty-two (2%) of 1,351 patients had been identified as ESBL-carriers in the 12 months before hospital admission, and eleven (50%) still were ESBL-carrier at hospital admission, yielding a sensitivity of 10% and positive predictive value of 50% for prior ESBL-carriage. The area under the ROC curve of the model based on these predictors was 0.64 (95%CI 0.58-0.71). The goodness of fit was adequate (Hosmer-Lemeshow statistic p-value=0.60).

A strategy of screening patients with at least two statistically significant risk factors (15% of all patients) would identify 31% of ESBL-carriers, and screening all patients with at least one risk factor (59% of all patients) would identify 72% of ESBL-carriers (Table 4). Thirty-one of the 111 patients carrying ESBL-

Table 2. Distribution of species and ESBL genes.

Species	Total n=109 n (%)	CTX-M-1 group n (%)	CTX-M-9 group n (%)	SHV-4 group n (%)	TEM-3 group n (%)	SHV-2 group n (%)	CTX-M-2 group n (%)	SHV-4 group & CTX-M-9 group n (%)
E. coli	74 (68)	48 (65)	11 (15)	8 (11)	6 (8)	1 (1)		
K. pneumoniae	14 (13)	10 (71)	2 (14)			2 (14)		
E. cloacae	13 (12)	3 (23)	7 (54)		1 (8)			2 (15)
C. freundii	3 (3)	2 (67)	1 (33)					
P. putida	2 (2)	2 (100)						
E. asburiae	1 (1)	1 (100)						
K. ascorbata	1 (1)						1 (100)	
M. morganii	1 (1)		1 (100)					
Total	109 (100)	66 (61)	22 (20)	8 (7)	7 (6)	3 (3)	1 (1)	2 (2)

Table 3. Predictors of ESBL-carriage at admission.

Variable	Univariate analysis OR (95%CI)	Multivariate analysis final model OR (95%CI)
Patient admitted from LTCF	1.11 (0.75-1.66)**	
Male	1.58 (1.06-2.36)*	1.49 (0.99-2.23)
Age (yr)	0.99 (0.98-1.01)**	
ESBL <1 yr	12.99 (5.49-30.47)*	11.35 (7.22-17.84)
Penicillins <6 mo	2.61 (1.54-4.44)*	
Cephalosporins <6mo	1.78 (1.17-2.70)*	
Fluorquinolones <6 mo	2.03 (1.26-3.28)*	
Macrolides <6 mo	1.54 (0.79-3.00)	
Carbapenems <6 mo	2.79 (0.69-11.25)	
Tetracyclins <6 mo	1.82 (0.91-3.64)	
Aminoglycosides <6 mo	2.02 (0.40-10.27)	
Sulfonamides/trimethoprim <6 mo	1.04 (0.49-2.22)	
Immunosuppressants <6 mo	1.27 (0.76-2.11)	
Admissions <6 mo	2.32 (1.55-3.47)*	2.13 (1.41-3.21)
External device	1.84 (1.12-3.03)*	
Surgery	1.57 (1.02-2.41)*	
Travel <1 yr	0.69 (0.40-1.18)	
Occupation with animalcontact	1.41 (0.34-5.83)	

* Variables included in multivariable analysis based on OR.

** Variables included in multivariable analysis based on evidence.

Table 4. Test-characteristics of screening based on risk factors.

Risk factors	Sensitivity	Specificity	PPV	NPV	% needed to screen
Individual risk factors					
Male gender	0.51	0.59	0.10	0.93	42
Previous admission	0.49	0.70	0.13	0.94	31
Previous ESBL-carriage	0.10	0.99	0.50	0.92	2
Number of risk factors					
≥1	0.72	0.42	0.10	0.94	59
≥2	0.31	0.87	0.17	0.93	15
≥3	0.054	1.00	0.75	0.92	1
Weighted score*					
≥1	0.72	0.42	0.10	0.94	59
≥2	0.51	0.70	0.13	0.94	32
≥3	0.33	0.86	0.17	0.94	16
≥6	0.10	0.99	0.50	0.92	2
≥7	0.09	1.00	0.67	0.92	1
≥8	0.07	1.00	0.67	0.92	1
≥ 9	0.05	1.00	0.75	0.92	1

PPV= positive predictive value, NPV= negative predictive value, TGC= third-generation cephalosporins.
*Weigthed score: male gender 1 pnts, previous admission 2 pnts, previous ESBL-carriage 6 pnts.

producing bacteria (28%) had none of the risk factors identified in multivariate analysis.
Of these thirty-one, 18 (58%) came from home and 13 from a LTCF. The median age was 82 years (IQR 66-89 yrs). Nine had travelled to foreign countries, but only four outside Europe (one to Korea and New Zealand, one to Aruba and Cuba and two to Turkey). One worked with animals.

Of the 1,351 patients included, 20 (1,5%) developed an infection with ESBL-producing bacteria: 15 a CAI and five a HAI. From the 111 patients identified as intestinal carrier at admission 13 (12%) developed a CAI and three a HAI (2%) with ESBL-producing bacteria. From the 1240 patients not identified as

intestinal carriers, two developed a CAI (0.2%) and two a HAI (0.2%) with ESBL-producing bacteria. The incidence densities of hospital-acquired infection with ESBL-producing bacteria were 45.5 (95%CI 9.4-132.8) and 2.1 (95%CI 0.26-7.7) per 10,000 admission-days for patients carrying and not-carrying ESBL-producing bacteria at hospital admission.
One patient without positive screenings cultures at admission acquired ESBL-positive cultures during hospitalization but was not treated.

Discussion

In this prospective, multicenter study of 1,351 patients the prevalence of ESBL-carriage at hospital admission was 8.2%, and was comparable among patients admitted from LTCFs and home settings. Despite the study size and detailed data collection it was not possible to develop a clinically useful prediction rule for ESBL-carriage at hospital admission. These findings underscore the widespread occurrence of ESBL-carriage and the difficulties for developing targeted screening strategies to identify ESBL–carriers. Incidence of nosocomial infections by ESBL-producing bacteria was higher in ESBL-carriers than in non-carriers.

The observed prevalence of ESBL-carriage of 8.2% is remarkably consistent with reported prevalences of 8.6% and 9.0% among healthy subjects in the Netherlands, with a mean age of 33 and 43 years respectively, screened before travel departure to high-risk areas between 2010 and 2012. Furthermore, the distribution of ESBL-genes was comparable to the reported distribution in clinical isolates from Dutch patients in 2009[15], suggesting that the molecular epidemiology of ESBL-producing Enterobacteriaceae has remained unchanged from 2009 to 2012.

In this study prior ESBL-carriage, hospital admission within the last 6 months and male gender were associated with ESBL-carriage, which confirms results from previous studies[11,16,17]. Although male gender has been identified as a risk factor for ESBL-carriage in previous studies as well, the biologic substrate remains unknown[14]. We could not confirm findings from two studies in Israel in which nursing home residence was a risk-factor for ESBL-carriage at the time of hospital admission[11,16]. Possibly, this is a result of the restrictive antibiotic policy in The Netherlands. Although antibiotic use in LTCFs is higher than in the community, this is still very low compared to other countries[18,19]. Neither could we confirm use of antibiotics, diabetes mellitus, connective tissue disease and liver failure as risk-factors[13]. Furthermore, numbers of patients with an

occupation involving animal contacts or that had travelled to so-called high-risk countries were too low in the current study to be identified as risk factor.

We could not develop a clinically useful prediction rule to identify patients with ESBL-carriage at the time of hospital admission. The obtained area-under-the-curve (AUC) of the identified risk-factors for ESBL-carriage at admission was 0.65 (95%CI 0.63-0.66). Increasing the positive predictive value towards clinically useful values would reduce the sensitivity to unacceptable low levels. Previous attempts in retrospective studies in single centers or single wards, and with fewer patients enrolled also failed to develop such a prediction rule[12,16,20]. This is possibly the result of heterogeneous transmission routes that include nosocomial, household and food contacts. Antibiotic use and host susceptibility add further complexity.

In absence of a useful prediction rule screening all patients at admission could be considered. However, there is currently no rapid detection tool available for ESBL-carriage, implying that in case of universal screening patients should either be pre-emptively isolated while awaiting culture results or treated without precautions until results are available, which is suboptimal for infection control. However, in an international study in Intensive Care Units, universal screening and isolation of detected carriers was not associated with a statistically significant reduction in ICU-acquired carriage with highly-resistant Enterobacteriaceae, as compared to isolation of detected carriers alone[21]. Neither do the results of this study support universal screening. Although the risk of HAIs with an ESBL-producing pathogen was significantly higher in patients with documented carriage of ESBL-producing bacteria at the time of hospital admission, the absolute risk of infection was low. In addition, three of the five patients with an HAI were not colonized with ESBL-producing bacteria at the time of hospital admission, and none of the observed infections could be considered invasive. Furthermore, pre-emptive isolation is probably not feasible in most settings. The association between ESBL-carriage at admission and a higher risk to develop a HAI with ESBL-producing bacteria, as compared to non-carriers, was also observed in patients receiving liver transplants in France[22]. Infections caused by ESBL-producing bacteria occurred in 45% and 4% of patients identified as ESBL-carriers and non-carriers before transplantation. In contrast, such an association was not found in neutropenic patients with haematologic malignancies, as the total number of patients with documented infection was only three[23].

Strengths of our study include the sample size of 1,351 patients, the multi-centre design, the inclusion of patients admitted to different hospital wards, the enrichment of patients from LTCFs and the protocolized data collection.
Study limitations include the potential for inclusion bias, as patients with inability to fill-out questionnaires were excluded and severely ill patients were probably more likely to refuse participation. Although departments were instructed to record how many patients were not approached or refused participation, this data appeared to be unreliable. Possibly, inclusion of comorbidities could have improved the prediction rule. Although, the ESBL-microarray has shown to match well to the prevalent ESBL-genes in the Netherlands, some ESBL-producing strains might have been missed, as not all ESBL-types can be identified by the ESBL-microarray[24]. Furthermore, the determination of hospital-acquired infections caused by ESBL-producing bacteria was a post-hoc analysis, based on retrospective chart review.

In conclusion, prevalence of ESBL-carriage at admission was 8.2%, without elevated risk for patients from LTCFs. A clinically useful prediction rule for ESBL-carriage at admission could not be developed. The incidence of nosocomial infections by ESBL-producing bacteria was higher in ESBL-carriers than in non-carriers but the absolute risk of an infection among ESBL-carriers was low.

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Chapter 7

Duration of colonization with extended-spectrum beta-lactamase producing bacteria following hospital discharge

T.N. Platteel[1,2], J.W. Cohen Stuart[3], M.A. Leverstein-van Hall[4], S.F.T. Thijsen[5], E.M. Mascini[6], B.C. van Hees[7], J. Scharringa[1], A.C. Fluit[1] R.C. Kloosterman[1] and M.J.M. Bonten[1]

1. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherland
2. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands
3. Medisch Centrum Alkmaar, Department of Medical Microbiology, Alkmaar, the Netherlands
4. Bronovo Hospital, Department of Medical Microbiology, Den Haag, and Diaconessenhuis, Leiden, the Netherlands
5. Diakonessenhuis, Department of Medical Microbiology, Utrecht, the Netherlands
6. Rijnstate, Department of Medical Microbiology, Arnhem, the Netherlands
7. Gelre Hospital, Department of Medical Microbiology and Infection prevention, Apeldoorn, the Netherlands

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Abstract

Introduction
Identification of patients colonized with extended-spectrum beta-lactamase-producing bacteria (ESBL-carriers) at hospital admission is relevant for infection control measures and selection of empiric antibiotic therapy in case of infection. It is unknown whether previously identified ESBL-carriers should be treated as suspected ESBL-carriers at hospital-admission. We, therefore, determined the duration of ESBL carriage and investigated which factors influence carriage duration.

Material and methods
We studied patients in which ESBL-producing bacteria were demonstrated in clinical cultures during hospitalization. From that point on, faecal samples were obtained during hospitalisation, and three, six, 12 and 18 months after discharge. ESBL-genes were characterized by PCR and sequencing, strain relatedness was based on DiversiLab typing and plasmid relatedness was based on PCR-based replicon typing (PBRT). The effects of ESBL type, species type, previous hospital admissions and antibiotic use on duration of ESBL carriage was determined.

Results
Among 49 patients included, the prevalence of ESBL carriage was 49% (95%CI 34-61%), 47% (95%CI 33-61%), 34% (95%CI 21-50%) and 29% (95%CI 16-47%) three, six, 12 and 18 months after hospital discharge. Six of 31 patients (19%; 95%CI 9-37%) were colonized with the same ESBL-producing strain for at least 18 months. CTX-M-15 was associated with a longer carriage duration than non-CTX-M-15 strains (OR 5.07; 95%CI 1.44-17.90). There was evidence of plasmid-transfer in 3/27 (11%) patients.

Conclusions
In 29% of ESBL-carriers, ESBL-producing bacteria could be identified at 18 months after discharge. For 19% of all carriers, this was uninterrupted ESBL-carriage. CTX-M-15 was associated with a longer duration of carriage.

Introduction

Infections due to extended-spectrum beta-lactamase (ESBL)-producing bacteria are increasing world-wide[1,2]. The duration of carriage after hospital discharge is an important determinant for the epidemiology of ESBL-producing bacteria, as this probably represents the duration of infectiousness and the duration of increased risk for infections with ESBL-producing bacteria. In the Netherlands, barrier precautions are recommended for all hospitalized patients with an ESBL-positive culture within the year before admission. However, data suggest that some patients remain colonized for longer than a year, and withholding of barrier precautions in ESBL-carriers with unknown carrier statues may reduce the overall effectiveness of infection control[3].

Most previous studies on the duration of ESBL-carriage were performed in outbreak situations with patients carrying similar strains of ESBL-producing bacteria, in specific patient groups, or were performed retrospectively[3-11]. One study prospectively assessed the duration of ESBL carriage in hospitalized patients, outpatients and patients from clinical practice, that all had clinical infections at the time of enrolment[12]. Aim of our study was to determine the duration of ESBL carriage after hospital discharge, in a non-outbreak situation and in an unselected group of hospitalized patients. In previous studies the duration of carriage was based on demonstrating the same ESBL-producing strain in time, but the frequency of plasmid-transfer between different strains within one patient was evaluated only once[12]. As an overestimation of the duration of carriage might occur when relatedness of ESBL-producing bacteria is only determined on the level of ESBL-type and strain-level, we also determined relatedness on plasmid-level.

Materials and methods

Population

Hospitalized patients with a newly recognized infection or colonization with ESBL-producing bacteria and an expected survival of at least a year were eligible for inclusion. The study was performed between July 2010 and October 2013.

Methods

Patients were identified as ESBL carrier by a clinical ESBL-positive culture. This culture could be obtained from every body-site and was performed on a clinical indication or for directed screening during hospitalization. This culture is referred to as the clinical culture. Only the first clinical culture was included in the analysis.

If the first two study cultures were ESBL-negative and no ESBL-producing bacteria were demonstrated in a clinical faeces culture, the patient was excluded from the analysis.
Besides, faeces cultures were performed before hospital discharge and three, six, 12 and 18 months after discharge to determine presence of intestinal ESBL carriage. These were the study cultures. Patients without study cultures during follow-up were excluded from the analysis.
All cultures were inoculated on an ESBL Brilliance plate (Thermo Fisher Scientific, UK) to detect ESBL-producing strains and on MacConkey agar (Thermo Fisher Scientific, UK) as a control for adequate sampling. Growth on MacConkey agar, but not on ESBL Brilliance plates, was defined as ESBL-negative; growth on ESBL Brilliance plates was defined as possible ESBL carriage. Isolates obtained from ESBL Brilliance plates were investigated by microarray analysis (Check-Points, Wageningen, the Netherlands) for the presence of ESBL genes. On ESBL-positive isolates PCR and DNA-sequencing were performed. The results were available for patients.

Relatedness of isolates was determined at the level of the ESBL-gene-, strain and plasmid. ESBLs were considered related if the ESBL-gene and plasmid incompatibility group were the same, according to PCR, sequencing and plasmid based replicon typing (PBRT). Strain relatedness was based on DiversiLab typing (BioMérieux, Marcy l'Etoile, France). Occurrence of ESBL-relatedness in absence of strain relatedness was considered as occurrence of within-host plasmid transfer.
To account for the possibility of false-negative culture results, ESBL carriage was supposed to be uninterrupted when a single culture was ESBL-negative was followed by an ESBL-positive culture with a related ESBL. The duration of uninterrupted ESBL carriage lasted from hospital discharge until the last follow-up culture with a related ESBL.
Strain relatedness was investigated using the last isolate during hospitalization and the last follow-up isolate with similar an ESBL gene. Isolates with similarities of >98% in DiversiLab typing were considered similar. All isolates with similarities of <98% were judged manually using the pattern overlay of the analysis tool in the software and could be judged as related or not related.
From sets of isolates with similarities of <98% and obtained from the same patient, plasmids were typed with PCR-based replicon typing (PBRT) after transformation, as previously described[13]. For sets of isolates with similarities of >98% PBRT was performed without transformation. If plasmid types were not similar PBRT and DiversiLab were also performed for more ESBL-producing isolates of that patient to determine the duration of carriage. If transformation

was unsuccessful after two attempts or if transformation for both was successful, but the plasmid type could not be determined, the plasmids were considered the same.

The proportion of patients carrying ESBL-producing bacteria per time point was based on the actual number of patients for which samples were available. The presence of determinants possibly affecting the duration of ESBL carriage were assessed using a standardized questionnaire at each time-point. Information on antibiotic use was derived from both pharmacy departments and community-based pharmacies. Use of cephalosporins, beta-lactam/beta-lactamase inhibitor combinations or penicillins were evaluated as a composite endpoint of antibiotics selective for ESBL-producing bacteria. Besides, the effect of the ESBL type, species, hospital admissions in the previous three months, and use of carbapenems were evaluated for their influence on the duration of ESBL carriage.

Statistical analysis

The effect of previous mentioned factors on the duration of carriage was evaluated using logistic regression with robust parameters and an added time-variable. All analyses were performed using the statistical software package SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Fifty-five patients were included. After exclusion of six patients for whom the first two clinical cultures were ESBL-negative and no ESBL-positive clinical faeces culture was available, yielding 49 patients for the analysis (Table 1). Twenty-seven (55%) patients were male and the median age was 49 years (IQR 37-69). Thirty-one patients harbored ESBL-producing *Escherichia coli*, 12 *Klebsiella pneumoniae*, five *Enterobacter cloacae* complex isolates and one *Citrobacter freundii*. In 23 (47%) patients bacteria of the clinical culture harbored CTX-M-15, seven CTX-M-1 (14%) and seven CTX-M-9 (14%). Other genes included SHV-12, CTX-M-14, CTX-M-55/79, CTX-M-27and SHV-2. Among *E. coli* 15 (60%) harbored CTX-M-15.

Completeness of follow-up data was 100% (n=49), 92% (n=45), 78% (n=38), 63% (n=31) at three, six, 12 and 18 months, respectively. ESBL-producing bacteria were identified in 23/49 (49%; 95%CI 34-61%) patients at three months after discharge, 21/49 (47%; 95%CI 33-61%) at six months after discharge, 13/38 (34%; 95%CI 21-50%) at 12 months and in 9/31 (29%; 95%CI 16-47%) at 18 months (Table 2).

Table 1. Baseline characteristics of included patients.

Characteristics		Patients (n=495) N (%)
Gender-male		27 (55)
Age, median (IQR)		49 (37-69)
Hospital-admission in previous 3 months		29 (59)
Use of carbapenems before study culture		11 (22)
Use of ESBL-selecting antibiotics before study culture		38 (78)
ESBL-gene at inclusion	**CTX-M-15**	23 (47)
	CTX-M-1	7 (14)
	CTX-M-9	7 (14)
	SHV-12	4 (8)
	CTX-M-14	3 (6)
	CTX-M-55/79	2 (4)
	SHV-2	2 (4)
	CTX-M-27	1 (2)
Species	***E. coli***	31 (63)
	K. pneumoniae	12 (24)
	E. cloacae	5 (10)
	C. freundii	1 (2)

Although all 49 patients had ESBL-positive clinical cultures during hospitalization and all had faecal samples investigated, intestinal carriage could not be demonstrated in the study culture during hospitalization in 15/49 patients (31%). Of these 15 patients, 12 (80%) had ESBL-positive clinical cultures during hospitalization. The remaining three had no clinical faeces cultures performed, but intestinal ESBL carriage was demonstrated three months after discharge.
Based on the typing data uninterrupted ESBL carriage after hospital discharge was demonstrated for at least three months for 27/49 (55%; 95%CI 41-68%) patients, for at least six months for 20/45 (44%; 95%CI 31-59%) patients, for at least 12 months for 11/38 (26%; 95%CI 17-45%) patients and for over 18 months for 6/31 (19%; 95%CI 9-37%) patients. Twenty-two of 27 (81%) patients with at least three months of uninterrupted ESBL carriage, carried the same strain according to DiversiLab data. Within-host plasmid transfer was assumed to have occurred in three of 27 (11%; 95%CI 3-29%) patients (once a similar ESBL

Table 2. Culture results for ESBL-carriers during hospitalization and after discharge.

Culture type	**Clinical culture**	**Study cultures**				
Culture moment	**During hospitalization**	**During hospitalization**	**3 months after discharge**	**6 months after discharge**	**12 months after discharge**	**18 months after discharge**
Included patients	49	49	49	45	38	31
ESBL-positive cultures	49 (100)	34* (69)	23** (47)	21*** (46)	13 (34)	9 (29)
Uninterrupted carriage		41 (84)	27 (55)	20 (44)	11 (29)	6 (19)
Carriers LTFU			0	1	4	2

Uninterrupted carriage: consecutive cultures harbored related ESBLs. A single culture could be ESBL-negative, but had to be followed directly by an ESBL-positive culture with a related ESBL.
LTFU: Lost to follow-up
* For three patients the first culture was missing, for two of them following cultures harbored ESBLs related to the clinical culture, for one following cultures were negative
** For two patients the second culture was missing; for both the previous and following cultures harbored related ESBLs.
***For one patient the third culture was missing, the previous culture was positive, the next negative and following cultures ESBL-positive with related ESBLs.

and plasmid in unrelated *K. pneumoniae* strains, once in unrelated *E. coli* strains and once in an *E. coli and K. pneumoniae* isolate). In two (7%) patients DiversiLab results were non-determinable.

Eight patients had new acquisitions of ESBL-producing bacteria, yielding an acquisition rate of 0.4 per 1000 days at risk (95%CI 0.1-0.6 per 1000 days at risk). In five patients only the ESBL-gene was the same, in one patient the strain type differed (though ESBL-gene and plasmid type were similar), and in one patient the ESBL-gene and plasmid type were equal and strains related. These last two patients had two and three ESBL-negative cultures before acquisition, respectively. Of the 18 patients lost-to-follow-up between month three and month 18, 11 were considered no longer colonized before lost to follow-up. The seven still colonized were all lost-to-follow-up due to death. One of these patients was censored after three months, four after six months and two after 12 months.

The median duration of carriage was 207 days (IQR 47-435) for those carrying CTX-M-15 ESBL 45 days (IQR 0-145; OR 5.07; 95%CI 1.44-17.90) for those carrying other genotypes. The duration of carriage was not significantly influenced by previous admissions (OR 0.78, 95%CI 0.18-3.30), use of antibiotics selective for ESBL-producing bacteria (OR 0.26, 95%CI 0.07-1.01), carbapenems (OR 2.51; 95%CI 0.40-15.93), or *E. coli* species (as compared to other bacteria) (OR 0.50, 95% CI 0.15-1.71).
One of 15 patients (7%) with at least two consecutive ESBL-negative cultures and with follow-up cultures thereafter available, had ESBL-producing bacteria identified after one year.

Discussion

The main findings of this study are that in 334of the patients ESBL carriage persisted for at least one year after hospital discharge, that there was evidence of within-host plasmid transfer in 11% of patients and that carriage with CTX-M-15 ESBL isolates was associated with a longer duration of carriage, irrespective of the species, use of antibiotics and hospitalization.

Few studies assessed the duration of ESBL carriage after hospital discharge. In two studies on carriage of CTX-M-15 positive outbreak strains, the mean duration of colonization was 7.5-12.5 months[3,4], whereas in two studies that included other ESBL types this was approximately six months[3,4,7,8]. Differences in the duration of carriage might be due to the number of proportion of CTX-M-15 positive isolates included in the studies, as results from our study suggest a longer duration of carriage for CTX-M-15 positive and *E. coli* isolates. CTX-M-15 *E. coli* frequently belongs to sequence type (ST) 131, of which the prevalence is increasing worldwide[14]. This increase might be partly explained by the prolonged duration of carriage. Sixty percent of the CTX-M-15 positive isolates in our study were *E. coli* and could well belong to ST 131, but this was not determined. CTX-M-15 represented 45% of the ESBLs, which is more than the 33% found in a Dutch surveillance study in 2009[15].
Titelman et al. determined the duration of faecal ESBL-carriage in patients with clinical infection caused by ESBL-producing bacteria, of which 75% were hospitlaized[12]. Carriage of ESBL-producing bacteria was observed in 43% of patients after 12 months, which is higher than in our study and also compared to other studies. This might be due to the selection of patients with infections. Also the percentage of plasmid-transfer of 28% was higher than in our study (11%). As patients remained colonized for a longer period of time, more transmission events could be observed. Besides, they found that prolonged carriage is associated with *E. coli* phylogroup B2. Phylogroup B2 is associated with

increased virulence and will therefore probably be more frequently identified in infections. Strength of our study is, that we assessed the duration of ESBL-carriage also by the performance of PBRT, to assess the plasmid incompatibility group. Besides, we aimed to collect long-term and concise follow-up data in an unselected group of hospital patients[16]. Isolates of three patients were probably part of a small outbreak. There was epidemiologic linkage between these patients, isolates contained similar ESBL-genes and plasmid types, and the strains showed >95% similarity according to DiversiLab data.
We assumed that patients were no longer colonized at their first ESBL-negative faeces sample, unless it was followed by an ESBL-positive sample, to account for the fact that cultures might be false-negative. Two patients acquired ESBL-producing bacteria with the same ESBL-gene and plasmid, after at least two ESBL-negative cultures. It is uncertain whether in these patients new acquisitions took place or cultures were false-negative. Probably, false-negative results most frequently occur when the amount of ESBL-producing bacteria in the sample does not reach the detection level.

In five patients ESBL genes were similar, but transformation of plasmids within one patient repeatedly failed or plasmids were non-typable. For these patients we assumed the plasmid-types to be similar, which may have overestimated the duration of carriage. In four of them, strains were related and in one unrelated. If we assume that these patients were no longer colonized before discharge, this would have some effect on the duration of carriage, especially in early follow-up, lowering percentages to 45% for carriage of at least three months, 38% for six months, 26% for 12 months and 16% for more than 18 months.
ESBL-producing bacteria were identified in 29% of patients 18 months after hospital-discharge. This is considerably higher than the 8% prevalence found in the Netherlands in patients at hospital-admission (Chapter 6), favouring extension of the period in which additional hygienic measures are undertaken at hospital-admission to at least 18 months. Though, almost 50% of patients were no longer colonized at three months after discharge, meaning that extension of the period in which additional hygienic precautions are performed would be unnecessary for most patients. To guarantee optimal infection control, but to keep costs and room capacity under control, determination of patients at risk for carriage for longer than a year could help.

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Chapter 8

Frequent carriage of ESBL-producing bacteria in household contacts of previously identified ESBL-carriers

T.N. Platteel[1,2], J.W. Cohen Stuart[3], M.A. Leverstein-van Hall[4], S.F.T. Thijsen[5], E.M. Mascini[6], B.C. van Hees[7], J. Scharringa[1], A.C. Fluit[1] R.C. Kloosterman[1] and M.J.M. Bonten[1]

1. University Medical Centre Utrecht, Department of Medical Microbiology, Utrecht, the Netherlands
2. SALTRO, Department of Medical Microbiology, Utrecht, the Netherlands
3. Medisch Centrum Alkmaar, Department of Medical Microbiology, Alkmaar, the Netherlands
4. Bronovo Hospital, Department of Medical Microbiology, Den Haag, and Diaconessenhuis, Leiden, the Netherlands
5. Diakonessenhuis, Department of Medical Microbiology, Utrecht, The Netherlands
6. Rijnstate, Department of Medical Microbiology, Arnhem, the Netherlands
7. Gelre Hospitals, Department of Medical Microbiology and Infection prevention, Apeldoorn, the Netherlands

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Abstract

Although high carriage rates with extended-spectrum beta-lactamase (ESBL)-producing bacteria have been reported among household contacts of patients discharged from hospital with ESBL-carriage (index-patients), within-household transmission rates have not been determined. We, therefore, aimed to determine the ESBL-acquisition rate among household contacts of index-patients that acquired ESBL-carriage during hospitalization.

Index patients had newly documented intestinal carriage with ESBL during hospitalization. Faecal samples were obtained from index patients and their household contacts before hospital discharge of the index patient (T0) and after three (T3), six (T6), 12 (T12) and 18 (T18) months after discharge of the index-patient. ESBL-genes were characterized by PCR and sequencing. Strain relatedness was based on interpretation of DiversiLab typing. Relatedness of plasmids was based on PCR-based replicon typing (PBRT).

There were 32 index-patients and 50 household contacts (median of 1 per index, interquartile range 1-2. At T0 16 of 50 household contacts (32%) were colonized with ESBL-producing bacteria. The total proportion of ESBL-carriage among household contacts remained ≥20% between T3 and T18. Among the 34 household contacts without ESBL-carriage on admission 11 acquired ESBL-carriage during follow-up, yielding an acquisition-rate of 1.8/1,000 days at risk.

In this study there was an unexpected high prevalence of ESBL-carriage among household members of patients that apparently acquired ESBL-carriage while being hospitalized. Among household members at risk for ESBL-colonization the observed acquisition rate was 1.8/1,000 days at risk. If these results are confirmed being a household member of an ESBL-carrier should be considered as a risk factor for ESBL-carriage.

Introduction

Infections due to ESBL-producing bacteria are increasing world-wide[1,2]. Identification of ESBL-carriers is relevant for infection control measures and empiric antibiotic therapy. In the Netherlands, patients with ESBL-producing bacteria identified in a microbiological culture within the year before admission or with recent hospitalization in a foreign hospital are considered at an increased risk for ESBL-carriage. This has consequences for empirical therapy and infection control measures at hospital admission. Being a household contact of a previously identified ESBL-carrier is currently not considered a risk factor. Yet, several studies have reported colonization rates of 20% in household contacts of ESBL-carriers[3-7]. In most of these studies index-patients belonged to specific patient groups, like children, patients involved in a hospital-outbreak or patients with community-acquired infections caused by ESBL-producing bacteria[4-8], and most studies were cross-sectional without follow-up data[4,6,7]. In only one study unselected hospitalized patients were studied with follow-up, but in this study, like in most others, the possibility of plasmid transfer of ESBL genes was not taken into account, as only clonal spread was investigated[3]. Furthermore, the colonization status of household contacts before hospital-discharge of index-patients was not assessed in any of the studies. Therefore, it remains unclear whether household contacts were already colonized before discharge of the index-patients or became colonized after discharge. In the first situation it is likely that spread in the household occurred before hospitalization of the index-patient via human-to-human transmission or via a third compartment, like companion animals or the environment[9]. The other scenario, in which household members acquire ESBL-carriage from the index patient after hospital discharge, would allow specific preventive measures for transmission. However, the need for such interventions depends on the within-household acquisition rate.

Aims of this study were to determine, first, the acquisition rates of ESBL-producing bacteria in household contacts, and, second, the prevalence of ESBL-carriage in household contacts before hospital discharge of the index-patient.

Material and methods

Population

This study was performed in a cohort of ESBL-carriers and their household contacts (Chapter 7). Hospitalized patients with a newly recognized infection or colonization with ESBL-producing bacteria and an expected survival of at least one year were eligible for inclusion, along with their household contacts. *Household contacts* were defined as persons who shared the same household

with the index patient on a regular basis. The study was performed between July 2010 and January 2013.

Methods

Faecal samples were obtained from index-patients and household contacts before hospital discharge (T0) and three (T3), six (T6), 12 (T12) and 18 (T18) months after discharge of the index-patient. The analyses included all index patients and their household contacts if at least one set of follow-up cultures was available and if at least one of the first two follow-up cultures of the index patients grew ESBL-producing bacteria. For household contacts follow up was discontinued if the index patient was lost to follow-up.

Cultures were inoculated on an ESBL Brilliance plate (Thermo Fisher Scientific, UK) to detect ESBL-producing strains and on MacConkey agar (Thermo Fisher Scientific, UK) as a control for adequate sampling. Growth on MacConkey agar, but not on ESBL Brilliance plates, was defined as ESBL-negative; growth on ESBL Brilliance plates was defined as possible ESBL-carriage. Isolates obtained from ESBL Brilliance plates were investigated by microarray analysis (Check-Points, Wageningen, the Netherlands) for the presence of ESBL genes. ESBL-positive isolates underwent PCR and DNA-sequencing and species identification using MALDI-TOF (Bruker Daltonik, Bremen, Germany). Participants were informed about their ESBL-carriage status.

To determine whether ESBL-producing bacteria, demonstrated in household contacts, were related to those of the index-patients, relatedness on gene-, strain- and plasmid-level was determined. ESBL-carriage was considered related if the ESBL-gene and plasmid incompatibility group were the same, according to PCR, sequencing and plasmid based replicon typing (PBRT). If bacterial strains were related, according to DiversiLab typing, clonal spread was assumed. If not, plasmid transfer was assumed.

Relatedness of bacterial isolates was determined with DiversiLab (BioMérieux, Marcy l'Etoile, France), which was performed on the first isolated cultured of the household contact with the same ESBL-gene as identified from the index-patient and on the previous ESBL-positive culture of the index patient. Isolates with similarities of >98% were considered similar. All isolates with similarities of <98% were judged manually using the pattern overlay of the analysis tool in the software and could be judged as related or not related.

For isolates with similarities of <98% plasmids were typed with PBRT, after transformation, as previously described[10]. On isolates with similarities of >98% in DiversiLab typing, PBRT was performed without transformation of

the isolates. If transformation was repeatedly unsuccessful or if plasmid types could not be determined, plasmids were considered the same.

Acquisition rates among household contacts were determined for ESBL-producing bacteria related to those of the index-patient for the period that the index-patient remained colonized, and also for the period that the index-patient had lost ESBL-carriage. Acquisition rates were also determined for ESBL-producing bacteria that were unrelated to those of the index-patient. Acquisition of an ESBL-producing isolate was assumed to have occurred between the first culture of a household contact with the ESBL-producing isolate and the previous culture without.

Statistical analysis
All analyses were performed using the statistical software package SPSS version 20.0 (SPSS Inc, Chicago, IL, USA).

Results

Baseline characteristics.
There were 32 index-patients, who had 51 household contacts, of which 50 were included. Seventeen index patients had complete follow-up, 4 had follow-up till T12, 7 till T6 and 4 till T3. All household contacts had the same duration of follow-up as their corresponding index patient. For index-patients loss to follow-up occurred because death. For two household contacts T3 culture results were missing.
The mean age was 57 years (interquartile range (IQR) 40-70) for index-patients and 48 years (IQR 21-65 for household contacts (Table 1). Eighteen index patients (56%) and 25 household contacts (50%) were male. The median number of household contacts per index-patient was 1 (IQR 1-2). Most household contacts (n=26 (52%)) were partner of the index-patient, 10 (20%) were a parent, eight (16%) were a child and six (12%) were sibling.
Twenty-one index-patients (66%) were colonized with ESBL-producing *Escherichia coli* at T0, as were 13 of 50 (26%) household contacts. Three household contacts (6%) were colonized with non-*E. coli* ESBL-producing bacteria. The colonized household contacts were from 13 families. CTX-M-15 was the most prevalent ESBL-gene detected at T0, both for colonized index-patients (n=18, 56%) as for household contacts (n=10, 63%).

In 31 of 50 (62%) household contacts ESBL-producing bacteria were demonstrated at some time during follow up; in 19 of 26 (73%) partners, 6 of 10 (60%) parents,

Table 1. Baseline characteristics.

Characteristics		Index-patients (n=32) N (%)	Household contacts (n=50) N (%)
Gender-male		18 (56)	25 (50)
Age, median (IQR)		57 (40-70)	48 (21-65)
No of household contacts, median (IQR)		1 (1-2)	
ESBL-type at inclusion	**CTX-M-15**	18 (56)	10 (20)
	CTX-M-1	4 (13)	1 (2)
	CTX-M-9	3 (9)	1 (2)
	SHV-12	3 (9)	0 (0)
	CTX-M-14	1 (3)	1 (2)
	CTX-M-55/79	2 (6)	1 (2)
	SHV-2	1 (3)	1 (2)
	TEM-19	0 (0)	1 (2)
	ESBL-negative	0 (0)	35 (69)
Relation to index patient	**Partner**		26 (52)
	Parent		10 (20)
	Child		8 (16)
	Sibling		6 (12)
Species	***E. coli***	21 (66)	13 (81)
	K. pneumoniae	7 (22)	3 (19)
	E. cloacae	3 (9)	
	C. freundii	1 (3)	

5 of 8 (63%) children and 1 of 6 (17%) siblings. At T3 16 household contacts (32%) were colonized with ESBL-producing bacteria, and this was 20% at T6, 23% at T12 and 24% at T18 (Table 2).

Relatedness of ESBLs for household contacts and index-patients.
In 13 of the 16 (81%) household contacts colonized with ESBL-producing bacteria at T0, ESBLs were related to those of the index-patient. These 13 household contacts originated from 11 households. Of the 11 index-patients the ESBL-producing bacteria were obtained within three days after hospital-admission

Table 2. Culture results for household contacts of index-patients, during hospitalization and after discharge of the index-patient.

Culture moment	During hospitalisation	3 months after discharge	6 months after discharge	12 months after discharge	18 months after discharge
Included families	32	32	28	21	17
Colonized index patients	32	23	18	11	5
Included household contacts	50	50	45	31	25
ESBL-positive cultures	16 (32)	16* (32)	10 (20)	7 (23)	6 (24)
ESBLs related to the index patient	13 (26)	13 (26)	8 (18)	5 (16)	6 (24)
Acquisitions of ESBLs related to those of index-patient	0	5	4	1	1

* 2 cultures were missing.

in six, without negative culture results since admission. In these patients it is, therefore, not excluded that these patients were already colonized at the time of hospital admission. No faeces cultures were performed within three days after hospital-admission for the other five index-patients. Of the strains with related ESBLs, 12 were clonally related *E. coli* isolates and the other strain was an unrelated *Klebsiella pneumonia* isolate. Nine household contacts were partners, three parents and one a sibling of the index-patient. These 12 household contacts were assumed not to be at risk anymore for acquisition of ESBLs related to those of the index-patient.

During follow-up, 11 of the remaining 37 household contacts acquired carriage with ESBLs related to those of the index-patient, yielding an acquisition rate of 1.8 per 1000 days at risk (95%CI 0.9-3.1 per 1000 days at risk). In all cases the index-patient was still colonized at the time that acquisition was demonstrated in the household contact. In eight of these 11 (73%) acquisitions, the isolate of

the household contact was clonally related to the isolate of the index-patient (in five *E.* coli and in three *K. pneumoniae*). For the other three, the same ESBL- and plasmid-type were identified in two unrelated *E. coli* isolates, an *E. coli* and *K. pneumonia* isolate and an *E. coli* and a *Pseudomonas putida* isolate for the index-patient and the household contact, respectively.

Acquired ESBL-types were CTX-M-15 (n=6; 55%), CTX-M-1 (n=3; 27%) and CTX-M-14 (n=2; 18%). The acquisition rate was not significantly different for CTX-M-15 isolates, as compared to isolates with other ESBL-types (data not shown). Acquisition took place in 9/15 (60%) partners and 2/8 (25%) children at risk.

No new acquisitions of bacteria with related ESBLs were identified in household contacts in the 3670 days at risk when the index-patient was not colonized anymore. Unrelated ESBLs were identified in household-contacts at a rate of 0.3 per 1000 days at risk (95%CI 0.1-0.7 per 1000 days at risk).

Discussion

Results from our study show that 62% of household contacts were colonized with ESBL-producing bacteria during the 18 months follow-up and that 32% of household contacts were already colonized with ESBL-producing bacteria before hospital discharge of the index-patients. Even a year after discharge of the index-patient, 23% of household contacts were colonized with ESBL-producing bacteria and most ESBLs were related to those of the index-patient. During follow-up, household contacts acquired isolates with ESBLs related to those of the index-patient at a rate of 1.8 per 1000 days at risk. No acquisitions of related ESBLs took place when the index-patient was no longer colonized.

For the 13 household contacts that were colonized with related ESBLs at the time of inclusion, it is unlikely that their carriage resulted from transmission from the index-patient to the household contact, during hospital-stay of the index-patient. A more logical explanation would be, that the index-patient was already colonized with the ESBL-producing bacteria before hospital-admission. This is supported by the fact that from six of the 11 index-patients ESBL-positive cultures were already obtained within three days after hospital-admission. In the remaining five patients no faeces cultures were performed within three days after hospital-admission. If colonization was indeed already present before hospital-admission, transmission within the household, could have occurred from human to human, or from other sources, like the environment, food or a companion animals[9]. The fact that at inclusion especially ESBL-*E. coli* were isolated in index-patients and household contacts, fits the concept of community-spread, as ESBL-*E. coli* is frequently acquired in the community[11]. Also after hospital-discharge new acquisitions could result from direct transmission from

index-patients to household contacts, but other reservoirs might have been part of the transmission-route as well.

As no new acquisitions of isolates with related ESBLs were observed in susceptible household-contacts when the index-patient was no longer colonized, an important role for the index-patient is expected in the risk to acquire ESBL-producing bacteria. This is reflected in the acquisition rates of 1.8/1000 days for the colonized period of the index patient and 0.0/1000 days at risk for the decolonized period. Household contacts have a higher risk to acquire an ESBL related to those of the index-patient than an unrelated ESBL (1.8/1000 days at risk versus 0.3/1000 days at risk). The data on one hand show that ESBL-transmission in households does not solely depend on new introductions of ESBL-producing bacteria in the household, as a result of acquisition by index-patients in the hospital, and that many household contacts are already colonized before discharge of the index-patient. On the other hand, the importance of index-patients in the transmission dynamics of ESBL-producing bacteria has been shown, as no new acquisitions of related ESBLs were identified when the index-patient was no longer colonized. Though, the household contacts might well play an important role in the transmission dynamics as well. It is likely that recolonizations were not identified in our study when the period that a person in the household was not colonized was short. It is possible that index-patient and household contact repetitively transmit their ESBL-producing bacteria within the household, causing that people (and possible other sources) within the household stay intermittently colonized over a long period of time.

In previous studies related ESBLs were found in 17-32% of household contacts[3-8], which is comparable to results of our study. Strengths of our study are the assessment of the colonization status in household contacts before hospital-discharge of the index-patient and the protocolled data collection with culturing of index-patients and household contacts on fixed time-points. Besides, we were the only to take the possibility of plasmid transfer among different Enterobacteriaceae species into account by the performance of transformations and PBRT[12].

When ESBLs were similar, but transformation of plasmids within one patient repeatedly failed or plasmids were non-typable, we assumed the plasmid-types to be similar. This could have resulted in overestimation of the presence of related ESBLs for index-patients and household contacts. If present, this overestimation will have minor effect on the results. In that case the 26% household contacts with related ESBLs at inclusion would be lowered to 22% and the acquisition rate for related ESBLs during the colonized period of the index patient would be lowered from 1.8/1000 days at risk to 1.6/1000 days at risk. Included patients had to be hospitalized for at least a few days, as the first study culture in household

contacts had to be performed when the index-patient was still hospitalized. As a result, selection might have taken place, including index-patients from a probably relatively sick population, which is also reflected in the high death-rate. It is questionable whether this in itself would have any effect on the acquisition-rate in household-contacts. Though, if direct transmission between index-patient and household contact is assumed, closer contact between index-patient and household contacts as a result of an increased need for help in sick index-patients, might have favored the occurrence of transmission-events. A more detailed insight in the transmission-dynamics within the household could have been gained if other possible compartments of ESBL-producing bacteria, like companion animals, would have been assessed for ESBL-carriage as well.

During follow-up at least 20% of household contacts carried ESBL-producing bacteria at every time point. These prevalences are considerably higher than the 8% prevalence, found at hospital admission in the Netherlands (Chapter 6). Therefore, household contact of known ESBL-carriers could be considered at an increased risk for ESBL-carriage regarding empirical therapy, like previous identified carriers and patients recently hospitalized in foreign countries.
The increased prevalence could also be an argument to introduce isolation and culturing for ESBL-carriage at hospital-admission for household contacts of known ESBL-carriers. Though, it is questionable whether benefits of screening outweigh the costs of screening and isolation, difficulties in identification of household contacts of known ESBL-carriers at hospital-admission and the probably low frequency at which household member will be hospitalized. Especially as in general ESBL-*E. coli* does not spread easily in hospitals, although for ST131 ESBL-*E. coli* this situation seems to be different[13,14]. It is questionable whether interventions to prevent ESBL-transmissions from index-patients to household contacts, after hospital-discharge of the first, will have great effect, as most household contacts were already colonized before discharge of the index-patient.

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Chapter 9

Summary and discussion

Introduction

Bacteria have threatened our health since ages. As we seemed to win the battle against bacteria with the use of penicillin since the Second World War and the development of new antibiotics from that time on, the bacteria strike back. Prevalences of multiresistant strains are increasing and even the first pan-resistant strains have been detected. This thesis is dedicated to one group of multiresistant bacteria, the extended-spectrum beta-lactamase (ESBL-) producing Enterobacteriaceae.

Bacteria of the Enterobacteriaceae family (e.g. *Escherichia coli* and *Klebsiella pneumoniae*) are commensals, but can also be pathogenic, causing urinary tract infections, pneumonias and intra-abdominal infections, not rarely leading to sepsis.
ESBLs are able to hydrolyze penicillin, cephalosporins and monobactams and are inhibited by beta-lactamase inhibitors. ESBL-producing bacteria often possess mechanisms to resist antibiotics of other classes than beta-lactams as well, further narrowing therapeutic options.
Patients frequently carry ESBL-producing Enterobacteriaceae without signs of infection, mostly in the gut. ESBL-producing bacteria will, in general, not cause much harm when they stay in the gut, but after migration to other parts of the body, they can cause infections. In contrast to, for instance, methicillin resistant *Staphylococcus aureus* (MRSA), it is impossible to eradicate ESBL-producing bacteria by antibiotic treatment as a measure to prevent carriage, as there are currently no effective methods without major drawbacks available[1,2].
There are signs that some patients remain carriers for a long period of time, even longer than a year[3]. As a result, they constitute a risk for themselves, and, as these strains can be transmitted, also for others in their surroundings.

Generally, patients with signs of infection will be treated empirically. In principal, cephalosporins are first choice of empirical therapy in hospitalized patients with sepsis. With rising resistance rates, the risk of failure of this therapy increases and forces towards increased use of carbapenems, especially in critically ill patients. Drawback of these antibiotics is that they can only be administered intravenously. Besides, as a result of increased use of carbapenems, also the resistance rates for carbapenems are emerging world-wide. In the Netherlands, luckily, this is still a rare phenomenon (0.1% in *K. pneumoniae* and 0.2% in *Enterobacter cloacae* in 2013)[4]. For infections due to carbapenem-resistant bacteria therapeutic options are scarce. Besides, no new effective antibiotics are to be expected soon.

In addition, infections with ESBL-producing bacteria are associated with an increased morbidity and mortality, primarily as a result of delayed administration of adequate treatment in the time that it takes to identify these strains[5-7].
If whether an infection is probably caused by ESBL-producing or non-ESBL producing bacteria could be determined faster and more accurate, not only carbapenem use could be decreased, but also patients could receive adequate treatment faster, possibly lowering morbidity and mortality. This goal can not only be reached by improvement in terms of rapidity and accuracy of the methods used for ESBL-detection, but also by identification of patients at an increased risk of ESBL carriage and infection, even before an infection occurs. Another solution to decrease carbapenem use can be found in the development of new antibiotics, or use of antibiotics which are already available, but assumed inferior to carbapenems for the treatment of infections with ESBL-producing bacteria, like colistin, tigecycline, fosfomycin and nitrofurantoin[8]. These antibiotics might be sufficient in case of (mild) infections. Use of these antibiotics can in that case decrease the carbapenem use without harming patients.

Aim of the research performed for this thesis was to provide deeper insight in the epidemioly op ESBL-producing bacteria in the Netherlands and the possibilities to detect patients carrying ESBL-producing bacteria.

Phenotypic detection of ESBLs

In chapter 2 we evaluated the routine setting performance of the Dutch guideline for phenotypic detection of ESBLs in Enterobacteriaceae recommending ESBL confirmation with Etest or combination disk for isolates with a positive ESBL screentest.
In this study 443 isolates from 20 Dutch clinical microbiology laboratories were included. PCR and sequencing were used as golden standard for the presence of ESBL-genes. We concluded that in 88% of cases the correct ESBL-status was assigned to isolates in daily practice. The positive predictive value of the screentest was on average 70%, but was dependent on the method, the species and the minimum inhibitory concentration (MIC) for the third-generation cephalosporins. Especially for *K. pneumoniae* the predictive value of a positive screentest was high with 95%. Performing a confirmation test was therefore of limited additional value in this species.
The positive predictive value of the confirmation tests varied from 95% to 97% in *E. coli*, *K. pneumoniae* and *Enterobacter* spp, but was only 33% to 38% in *Proteus mirabilis* and *Klebsiella oxytoca*.
The ESBL-Etest was less specific as compared to the combination disk (59% versus 92%) for ESBL confirmation. This was especially due to non-determinable

and false-positive outcomes of the ESBL-Etest. The first is probably a result of problems with interpretation of the test in clinical practice. False-positives especially occurred in *K. oxytoca* and *P. mirabilis* isolates. For *K. oxytoca* this is probably due to K1-hyperproduction and for *P. mirabilis* due to swarming of the isolates.

Whereas the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Testing (EUCAST) recommended ESBL confirmation before 2009, the guidelines for the detection of ESBLs have undergone a paradigm shift in 2009 and 2010[9,10]. The breakpoints for most cephalosporins and aztreonam were lowered (from 8 mg/L for ceftazidime and cefotaxime, to 1 mg/L and 4 mg/L, respectively) and confirmatory testing of ESBL production with clavulanic acid was not mandatory anymore, though still advised for epidemiological or infection control purposes. Rationale for these decisions lies in the fact that the MIC is considered a better predictor of outcome than the resistance mechanisms present in bacteria. However, caution is warranted, as the evidence that infections, caused by ESBL-producing bacteria, are responding as well to treatment with cephalosporins as their ESBL-negative counterparts is not conclusive yet[11]. Besides, to desert the detection and reporting of the presence of ESBLs will lead to a decrease in local epidemiologic knowledge, which is very important for infection control.

Genotypic detection of ESBLs

In chapter 3 the accuracy of the Check-KPC ESBL microarray as a confirmatory test for ESBLs was determined on randomly selected clinical isolates with a positive ESBL screentest. Aim of this study was to determine the accuracy of the Check-KPC ESBL microarray as a confirmatory test of ESBLs in the routine clinical setting. Besides, the microarray might be a useful ESBL-detection tool for isolates in which phenotypic ESBL-detection is not reliable in clinical practice, which are, as we could conclude from chapter 2, *P. mirabilis* and *K. oxytoca*. Hereto, a sample of the clinical isolates with a positive ESBL screentest from the evaluation of the Dutch guideline for ESBL detection, as described in chapter 2, was used.

Using PCR and sequencing of the ESBL-gene as the reference method, the sensitivity of the microarray was 97% (237/245), the specificity 98% (97/99), the positive-predictive value 99% (237/239) and the negative-predictive value 92% (97/105). In 3% (6/182) of all CTX-M-1 group positive isolates in the collection a false-negative result was obtained by microarray. This finding has not been reported in other studies testing this microarray and may be explained by chance, since 74% (182/245) of the isolates in this collection harboured a

CTX-M-1 group gene, a limited sensitivity of the CTX-M-1 group specific probe or by a modification of the interpretation software resulting in an alteration of the detection limit[12,13].

Genetic confirmation methods, such as ESBL-microarray, could be considered for implementation in the routine diagnostic laboratory. The microarray can be useful in a number of situations. First, when it is necessary, for example in clinical practice, to obtain a fast result in case of assumed ESBL-production of an isolate. Research from Fishbain et al. showed that performing microarray on blood cultures could reduce the notification time by 18 to 20 hours[14]. As a delay in adequate antibiotic therapy in case of sepsis might worsen the outcome of patients, swift detection of ESBL-producing bacteria is very relevant in these cases[15].

Second, the microarray is a good solution in case of non-determinable Etest results in phenotypic ESBL detection and for isolates for which the positive predictive value of the phenotypic confirmation tests is low, like in *P. mirabilis* and *K. oxytoca*. Genetic confirmation of *K. oxytoca* has also been advised by Polsfuss et al[16]. Also in this study a diagnostic flowchart for the detection and confirmation of ESBL-producing Enterobacteriaceae was evaluated. They give no advice on genetic confirmation in *P. mirabilis*, but this might be due to the low number of ESBL-producing *P. mirabilis* isolates in their study. It might even be cost-effective to genotypically confirm all isolates, as the costs of isolation of patients falsely marked as ESBL-producing could be saved[17]. The biggest effect though, will be reached for species in which ESBL-confirmation often gives false-positive results. It is doubtful whether it would save costs in species in which ESBL-detection is reliable, like *K. pneumoniae*, *E. coli* and *E. cloacae*.

Third, as the microarray provides information on the ESBL-gene, it can also be used for infection control purposes, for example in case of a suspected outbreak with an ESBL-producing strain. As for example ESBL-genes are present beside carbapenemase genes, they might be difficult to detect and ESBLs in these isolates might therefor remain unidentified as part of an outbreak[18]. As only ESBL-groups can be determined by microarray, further determination of isolates is always necessary if they are suspected to be part of an outbreak. Besides, to guarantee the reliability of the ESBL-microarray in the future, monitoring of the local epidemiology will be crucial, as not all ESBL genes can be detected by microarray. If the ESBL genes that can be detected by ESBL-microarray correspond with the local epidemiology, the microarray is also useful as a high-throughput tool for epidemiologic studies, if large numbers of strains have to be tested for ESBL production. This has been proven with its implementation in the SMART study[19].

Population distribution of beta-lactamases in the Netherlands

In chapter 4 we determined the population distribution of beta-lactamases conferring resistance against third generation cephalosporins, in a cross-sectional sample of clinical isolates in the Netherlands, using the methods described in chapter 2 and 3.

We demonstrated that resistance for third-generation cephalosporins in Enterobacteriaceae in the Netherlands results mainly from CTX-M-15 ESBL genes in *E. coli*. Of all ESBLs, 33% was CTX-M-15 (104/314) and of all included isolated, 25% of all isolates was *E. coli*, positive for CTX-M-15. All 15 randomly selected CTX-M-15 *E. coli* isolates belong to sequence type (ST)131, according to multilocus sequence typing (MLST). ST131 is an emerging, disseminated lineage of virulent *E. coli* and is associated with CTXM-15[20]. The high prevalence of CTX-M-15 positive isolates is not unique for the Netherlands and is observed in most other European countries, North America, the Middle East and India, whereas CTX-M-14 is most common in China, Southeast-Asia and Spain, and CTX-M-2 in Argentina, Israel, and Japan[21]. Though, also in these countries, a shift towards the predominance of CTX-M-15 seems to take place[22].

CTX-M-15 isolates were –on average– susceptible to 4.5 of 8 antibiotics tested, which was lower than isolates harboring TEM-52, CTX-M-1, or CTX-M-14 (mean susceptibility to 6.6, 6.0 and 5.7 antibiotics, respectively; $p<=0.004$). To our knowledge this has not been reported elsewhere.

The most prevalent ESBLs of the SHV- and TEM families were SHV-12 and TEM-52. Where SHV-12 is also prevalent in other countries, TEM-52 and CTX-M-1 are more prevalent in the Netherlands then in other countries. CTX-M-1 is even the second most prevalent ESBL in the Netherlands. This ESBL type has not been reported frequently in clinical studies outside the Netherlands.

Hypothesis that CTX-M-1 and TEM-52 spread in the community

It is hypothesized that the relatively high prevalence of CTX-M-1 and TEM-52 results partly of spread of ESBL-producing strains via food, as these ESBL-types are especially prevalent in poultry.

This hypothesis was based on a study from Leverstein-van Hall et al and a study from Overdevest et al[23,24]. In the first study the distribution of ESBL genes, plasmids and strain genotypes in *E. coli* obtained from poultry and retail chicken meat in the Netherlands was determined. Six ESBL genes were defined as poultry associated: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{SHV-2}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-20}}$, and $bla_{\text{TEM-52c}}$, as they were frequently identified in poultry. Subsequently, the proportion of *E. coli* isolates with poultry-associated ESBL genes, plasmids and strains was quantified in a representative sample of clinical isolates. Isolates were analyzed using ESBL-microarray, sequencing of ESBL genes, PCR-based

replicon typing of plasmids (PBRT), plasmid multi-locus sequence typing (pMLST), and strain genotyping (MLST). A total of 35% of the human isolates contained poultry associated ESBL genes and 19% contained poultry-associated ESBL genes located on IncI1 plasmids that were genetically indistinguishable from those obtained from poultry and poultry meat. Of these ESBL genes, 86% were $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-52}}$ genes, which were also the predominant genes in poultry (78%) and retail chicken meat (75%). Of the retail meat samples, 94% contained ESBL-producing isolates of which 39% belonged to *E. coli* genotypes also present in human samples. The proportion of human isolates for which the ESBL-gene, plasmid and strain was similar to those identified in poultry was 75% for CTX-M-1 and 33% for TEM-52. The conclusion of the authors was that these findings are suggestive for transmission of ESBL genes, plasmids and *E. coli* isolates from poultry to humans, most likely through the food chain. Similar results were found in the second study, using PCR and sequencing and MLST[24]. However, newer data, using the more sensitive whole genome sequencing failed to confirm the evidence for recent transmission of ESBL-producing *E. coli* strains from poultry to humans. These data did show big resemblance between plasmids encoding ESBL-production in poultry and humans, suggesting plasmid transfer between humans and poultry (M. de Been et al, submitted). The direction of this transmission can not be distinguished.

In chapter 5 we tested the hypothesis that poultry-associated ESBL genes, here defined as CTX-M-1 and TEM-52 genes, were predominant in the community, whereas the non-poultry-associated ESBL genes were predominant in hospitals. Ground for this hypothesis was the concept of two distinct compartments for the dynamics of ESBL-producing bacteria, one in the community fuelled by food contamination and one in hospitals fuelled by cross-transmission.
The results of the more recent study of de Been et al. has no effect on the hypothesis that CTX-M-1 and TEM-52 are poultry-associated. Also in case of transmission of CTX-M-1 and TEM-52 encoding plasmids from poultry to humans, isolates positive for CTX-M-1 and TEM-52 would be expected to be predominant in the community, if this transmission had recently taken place.
The two separate compartments could not be demonstrated in this study, as the so-called poultry-associated ESBL genes were as prevalent in *E. coli* urine cultures from general practice, as from the hospitals. These data do not entirely exclude relevant transmission from food, as it is possible that poultry-associated ESBLs, although they originate from the population, are already widely spread in the hospitals or are continuously introduced in the hospitals by patients.

The resistance patterns differed between the poultry-associated and non-poultry associated isolates with higher susceptibility for aminoglycosides and fluoroquinolones in the CTX-M-1 and TEM-52 positive isolates from humans. These antibiotics are hardly used in animals. In both groups there were high susceptibility rates for nitrofurantoin and fosfomycin, which is beneficial for treatment of uncomplicated urinary tract infections, as they are first and second choice empirical treatment for urinary tract infections according to the Dutch guideline "urinary tract infections" for general practitioners. This guideline has been revised in 2013. Until this revision, trimethoprim was considered second choice in case of uncomplicated urinary tract infections, which has now been replaced by fosfomycin, as a result of rising resistance rates for trimethoprim. Fosfomycin is not only more suitable than trimethoprim, based on resistance rates for Dutch urinary tract *E. coli* isolates in general, but also based on the co-resistance rates found in the ESBL-producing urinary *E. coli* isolates included in this study[25]. This suggests that treatment failure in uncomplicated urinary tract infections as a result of ESBL-production in *E. coli*, is not expected frequently.

Prediction of ESBL-carriage at hospital admission

For chapter 6 we aimed at the development of a prediction rule for carriage of ESBL-producing bacteria at hospital admission, as early detection could improve decision-making regarding appropriate infection control measures and empirical therapy in case of infections.

As long-term care-facility (LTCF-)residents are considered at an increased risk of ESBL carriage, we put extra emphasis on these patients by enrichment of patients from LTCFs[26]. Besides, we determined the incidence of nosocomial infections in carriers and non-carriers of ESBL-producing bacteria at hospital admission.

In this prospective multicenter study, including 1,351 patients, the prevalence of ESBL carriage at hospital admission was 8.2%, and was comparable among patients admitted from LTCFs and home settings. Using multivariate analysis documented ESBL-carriage within 1 year before admission (adjusted OR=11.35; 95%CI 7.22-17.84), hospital admission in the previous 6 months (adjusted OR=2.13; 95%CI 1.41-3.21) and male gender (adjusted OR=1.49; 95%CI 0.99-2.23) were identified to be significantly associated with ESBL-carriage.

Despite the study size and the detailed data collection it was not possible to develop a clinically useful prediction rule for ESBL carriage at hospital admission. Probably this is due to the fact that ESBL-producing bacteria are not only acquired by patients with healthcare associated risk factors but also by healthy individuals without any known risk factors. A strategy of screening patients with at least two statistically significant risk factors (15% of all patients)

would identify 31% of all ESBL-carriers. Screening all patients with at least one risk factor would identify 72% of ESBL-carriers but 59% of all patients that are admitted to the hospital, including all males, should be screened to achieve this.

Duration of ESBL-carriage after hospital discharge

As mentioned in chapter 6, previously documented ESBL-carriage was identified as the most important risk factor for ESBL-carriage at hospital-admission. In the Netherlands, barrier precautions are recommended for all hospitalized patients with an ESBL-positive culture within the 12 months before admission, to prevent spread to other patients. If prevalences in previous colonized patients remain high after a year, extension of the 12 month-period should be considered[27].
Therefore, aim of the study described in chapter 7 aimed to determine the duration of carriage of ESBL-producing bacteria from the moment of discharge from the hospital.
For this study the colonization-status of 49 ESBL-carriers from hospitalization to a maximum of 18 months after discharge was determined. Results of this study showed that in 34% (95%CI 21-50%) of the patients identified as ESBL carrier during hospitalization, carriage of ESBL-producing bacteria could be demonstrated 12 months following discharge, and in 29% (95%CI 16-47%) at 18 months. This was a result of uninterrupted carriage of the same ESBL in 26% (11/38; 95%CI 17-45%) of all patients at 12 months and in 19% at 18 months (11/42; 95%CI 9-37%). From these findings, we can conclude that ESBL-carriers frequently remain colonized for longer than a year and that this is often with the same strain. New acquisitions of ESBLs took place in ten patients during the whole period of follow-up with a rate of 0.4/1000 days at risk. Carriers of ESBL-producing bacteria positive for CTX-M-15 remained carrier for a significant longer time, than carriers of other ESBL-types, irrespective of use of antibiotics and hospitalizations and irrespective of the species (207 vs 45 days; adjusted OR 5.07; 95%CI 1.44-17.90). This longer duration of carriage for isolates positive for CTX-M-15 might partly explain the increasing prevalence of CTX-M-15 world-wide[21].

Prevalence of ESBL-carriage in household contacts of ESBL-carriers

In chapter 8 we determined the prevalence of ESBL-carriage in household contacts of known ESBL-carriers (index-patients). This prevalence was determined before as well as after discharge of index-patients. Besides, the acquisition-rate of ESBL-producing bacteria in household contacts was determined.
Hospitalized patients with documented intestinal colonization with ESBL-producing bacteria were included before hospital discharge, along with their household contacts. Faecal samples were obtained before hospital discharge and

three, six, 12 and 18 months after discharge of the index-patient. To determine whether ESBLs in household contacts were related to those of the index-patient, relatedness of the ESBLs bacteria was determined at the level of the ESBL-gene, strain and plasmid. ESBLs were defined as related when at least the ESBL-gene and the plasmid incompatibility group, according to PBRT, were the same. For this study 32 index-patients and their 50 household contacts were followed-up for a maximum period of 18 months. Results show that in 62% of household contacts ESBL-producing bacteria could be demonstrated at some moment during follow up. Thirty-two percent of household contacts were at inclusion colonized with ESBL-producing bacteria, as this was ≥20% at every time-point during follow-up. At every time-point ESBLs, related to those of the index-patient were identified in at least 16% of household contacts. The proportion of household contacts colonized, is considerably higher than the 8% carriage at hospital admission observed in chapter 6, placing household-contacts of previously identified ESBL-carriers at an increased risk for carriage.
During follow-up the acquisition-rate of ESBLs, related to those of the index-patients was 1.8/1000 days at risk, as compared to 0.3/1000 days at risk for acquisition of unrelated ESBLs.

Screening of patients at risk for ESBL-carriage

According to the Dutch guideline patients with ESBL-positive cultures within 12 months before hospital-admission and patients who have been hospitalized in a foreign country for at least 24 hours in the previous two months, should be screened for ESBL-carriage at hospital admission. Results of the studies performed for this thesis, suggest that some improvement of the guideline might be possible.
Unfortunately, a useful prediction rule for ESBL-carriage at hospital-admission could not be developed, but from chapter 6 and 7, though, some recommendations can be distilled. In absence of a useful prediction rule, it could be considered to screen and pre-emptively isolate all admitted patients, awaiting culture results. This is probably not feasible, as most hospitals will fall short in single room capacity and expenses, even though use of genetic information, like microarray, could limit the detection time and therefore the duration of isolation.
In chapter 7, it has been shown that in about one-third of previous identified ESBL-carriers, ESBL-producing bacteria could be identified a year after discharge, which is approximately four times as high as the 8% prevalence found at hospital-admission. This prevalence remained high until the end of follow-up at 18 months. Also in household contacts of known ESBL-carriers the ESBL-prevalence was high. As shown in chapter 8, the prevalence was three

times as high as found at hospital-admission until 18 months after discharge of the index-patient.

Beside these two risk groups, previous research has shown that also patients who recently travelled to foreign countries are also at an increased risk for ESBL-carriage with an acquisition rate of 24%[28]. The duration of colonization was shorter in this group, as only 17% was still colonized 6 months after return[29].

For all three groups, ESBL-prevalences are that high that screening at hospital admission should be considered. Though, also other factors play a role.

First, it is questionable whether identification of all ESBL-carriers for infection control is strictly necessary, as transmissions within the hospital seem not to occur frequently (5.6 per 1000 exposure days for ESBL-*E. coli* and 13.9 per 1000 exposure days for ESBL-*K. pneumonia*) and detection of ESBL-producing bacteria not linked to an index-patient in the hospital seems to occur as often as nosocomial transmission events[30]. In all three risk groups mentioned above, the most frequently identified species was *E. coli*, for which the lowest transmission rate was found.

Second, as shown in chapter 6, the absolute risk of nosocomial infections with ESBL-producing bacteria is low (45.5 and 2.1 per 10,000 admission-days for patients carrying and not-carrying ESBL-producing bacteria at hospital admission) and most infections were not invasive. Previous research showed that the rate of ESBL-bacteremia in The Netherlands was also low with 4.8 per 100,000 patient days in 2007[31].

Therefore, it is questionable whether universal screening for carriage of ESBL-producing bacteria would improve infection control and morbidity and mortality for nosocomial infections significantly.

Although no data on absolute numbers are available, one can hypothesize that absolute number of additional screening and isolation will not be too high if above mentioned risk groups would be screened at hospital admission. Previous carriers are already screened at hospital admission up to 12 months after the last ESBL-positive culture. Besides, for travelers, Paltansing et al. only advise screening of patients who travelled to Asia until six months after return, which group will not be too big[29]. Therefore, it could well be cost-effective to screen previously identified ESBL-carriers and household contacts up at least to 18 months at hospital-admission and to screen patients who recently travelled to high risk countries until six months after return, but further research hereon would be necessary.

Isolation of patients

With increasing resistance rates and, as a result, possible upcoming problems to isolate all ESBL-carriers (or suspected carriers), it could be considered to only

isolate ESBL-carriers in specific cases. A possibility would be to isolate carriers of ESBL-producing *K. pneumonia* and not carriers of ESBL-producing *E. coli*, as ESBL-*E. coli* seems to spread less easy in hospitals as compared to ESBL-*K. pneumoniae*[30]. Another approach could be to isolate all carriers of ESBL-*K. pneumonia* and those carrying ESBL-*E. coli* isolates which are thought to spread easily and are pathogenic, like ST131 ESBL-*E. coli*[32]. Drawback of the strategy to isolate *K. pneumoniae* and ST131 ESBL-*E. coli*, is that it requires genotypic analysis of *E. coli* isolates, which is time-consuming and costly.

The future

In the future, resistance rates will probably continue to rise. This will not only apply to ESBL-producing bacteria, but also to, for example, carbapenemase-producing strains. This will challenge us, in our search for new antibiotic therapies and (re-)use of older antibiotics, and we will be forced to frequently evaluate empirical therapy.

In an attempt to prevent the increasing resistance rates, it would be profitable if international rules would be established regarding (over-the-counter) use of antibiotics. But, not only antibiotic-use in human health-care might favor rising resistance rates. Also in veterinary practice there is a lot to gain in terms of reduction of antibiotic use and prescription on indication. First steps in The Netherlands have been taken by aiming at a reduction of antibiotic-use in farming with 70% in 2015 and restriction of use of certain antibiotic classes like colistin, beta-lactams, fluoroquinolones and aminoglycosides. The antibiotic use has already been decreased with 50% in farming between 2009 and 2013[33]. In the coming years we will hopefully see its effect on resistance rates. Up to now, it remains unclear what the effect of presence of ESBL-producing bacteria in the environment is on resistance rates in humans and what the transmission dynamics look like. Hopefully, more of this will be unraveled in the coming years. Better understanding of the transmission-dynamics might play a key role in the prevention of further dissemination of ESBL-producing bacteria.

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Dutch Summary

Nederlandse samenvatting

Introductie

Bacteriën bedreigen onze gezondheid sinds jaar en dag. Waar we met het gebruik van penicilline sinds de Tweede Wereldoorlog en de ontwikkeling van nieuwe antibiotica sindsdien de strijd tegen bacteriën leken te winnen, slaan ze terug. Het voorkomen van multiresistente bacteriën neemt toe. Dit proefschrift is gewijd aan een groep multiresistente bacteriën, de extended-spectrum beta-lactamase (ESBL-)producerende Enterobacteriaceae.
Gram-negatieve bacteriën uit de familie van de Enterobacteriaceae (met name *Escherichia coli* en *Klebsiella pneumoniae*) kunnen onder andere urineweginfecties, pneumonieën en intra-abdominale infecties veroorzaken, met niet zelden een sepsis tot gevolg.
Extended-spectrum beta-lactamase kan door bacteriën geproduceerd worden. Deze enzymen kunnen penicillines, cephalosporines en monobactams hydrolyseren en daarmee de effectiviteit van deze middelen verminderen. Gegevens voor ESBL-productie liggen op een stukje ringvormig DNA, een plasmide. Bacteriën kunnen, als zij zich vermenigvuldigen dit plasmide ook aan hun nazaten doorgeven. Tevens kunnen zij dit plasmide doorgeven aan andere bacteriën van de familie van Enterobacteriaceae. Op dit plasmide ligt vaak niet alleen ESBL-productie gecodeerd, maar ook resistentie voor andere soorten antibiotica, wat het aantal therapeutische opties verder verkleint.

Vaak zijn patiënten alleen drager van ESBL-producerende bacteriën in de darm, zonder tekenen van infectie. Zolang deze bacteriën in de darm blijven, zullen ze over het algemeen weinig problemen veroorzaken. Echter, bij migratie naar een ander deel van het lichaam kunnen deze bacteriën infecties veroorzaken (met name in de urinewegen, wonden en longen). In tegenstelling tot bijvoorbeeld MRSA is het bij ESBL-producerende bacteriën niet mogelijk eradicatie toe te passen en dragerschap op te heffen, omdat hiervoor (nog) geen effectieve methode bestaat zonder forse nadelen.
Waar eerder werd aangenomen dat ESBL-dragers deze bacteriën na een aantal maanden vanzelf deze bacteriën kwijt raken, zijn er aanwijzingen dat sommigen deze bacteriën zelfs langer dan een jaar bij zich kunnen dragen. Hierdoor vormen zij een risico voor zichzelf en, omdat deze stammen ook kunnen worden overgedragen naar anderen, ook voor anderen in de omgeving.
Over het algemeen worden patiënten met tekenen van een infectie empirisch behandeld. In het geval van ernstige infecties, zoals een sepsis, zijn cephalosporines in principe het middel van eerste keuze. Met de toename van resistentie door onder andere ESBL-productie, neemt ook de kans op het falen van deze therapie toe. Hierdoor worden we gedwongen om vaker carbapenems te gebruiken, met name in ernstig zieke patiënten. Een nadeel van

deze antibiotica is dat ze alleen maar intraveneus toegediend kunnen worden. Daarnaast neemt ook de resistentie tegen deze middelen wereldwijd toe als gevolg van een toename in het gebruik. In Nederland komt dit gelukkig nog maar weinig voor (0,1% resistentie voor carbapenems in *K. pneumoniae* en 0,2% in *Enterobacter cloacae* in 2013). Voor infecties met carbapenem-resistente bacteriën zijn de therapeutische opties beperkt. Daarnaast worden er korte termijn geen nieuwe antibiotica verwacht[1,2].
Infecties met ESBL-producerende bacteriën zijn geassocieerd met een toename in de morbiditeit en mortaliteit, met name ten gevolge van vertraging in het toedienen van adequate therapie[3-5].
Als we sneller betrouwbaar zouden kunnen bepalen of een infectie wordt veroorzaakt door ESBL-producerende bacteriën, zou niet alleen het gebruik van carbapenems kunnen worden teruggedrongen, maar zouden patiënten ook sneller adequaat behandeld kunnen worden, wat mogelijk de mortaliteit en morbiditeit verlaagt.
Dit doel kan niet alleen behaald worden door ESBL-producerende bacteriën sneller en betrouwbaarder te detecteren in het laboratorium, maar ook door patiënten met een verhoogde kans op ESBL-dragerschap en –infectie tijdig te identificeren, zelfs voordat een infectie optreedt. Door de ontwikkeling van nieuwe antibiotica, of gebruik van antibiotica die al beschikbaar zijn, zoals colistine, tigecycline, fosfomycine en nitrofurantoine zouden het carbapenem-gebruik kunnen terugdringen[6]. Mogelijk zouden deze antibiotica toereikend zijn in het geval van (milde) infecties, waardoor carbapenems gespaard kunnen worden zonder patiënten te schaden.

Doel van het onderzoek wat is uitgevoerd voor dit proefschrift was een dieper inzicht te verkrijgen in de epidemiologie van ESBL-producerende bacteriën in Nederland en de mogelijkheden om snel en accuraat patiënten te identificeren die drager zijn van ESBL-producerende bacteriën.

Phenotypische detectie van ESBLs
In hoofdstuk 2 werdde Nederlandse richtlijn voor phenotypische detectie van ESBLs in Enterobacteriaceae in de dagelijkse praktijk geëvalueerd. Deze richtlijn adviseert ESBL-confirmatie met Etest of combinatiedisk voor isolaten met een positieve ESBL screentest. Ook werden de resultaten van gebruik van de Etest en combinatiedisk als ESBL confirmatietest in de klinische setting vergeleken.
Uit deze studie konden we concluderen dat in de dagelijkse praktijk aan 88% van de isolaten de juiste ESBL-status werd toegekend. De positief voorspellende waarde van de screentest was gemiddeld 70%, maar dit was afhankelijk van de methode, de species en de minimale remmende concentratie (MRC) voor de derde

generatie cephalosporines. Met name in *K. pneumoniae* was de voorspellende waarde van een positieve screentest hoog met 95% en het uitvoeren van een confirmatietest was hierin dan ook van minimale toegevoegde waarde.
De positief voorspellende waarde van de confirmatietesten varieerde van 95% tot 97% voor *E. coli*, *K. pneumoniae* en *Enterobacter* spp, maar was slechts 33% tot 38% voor *Proteus mirabilis* en *Klebsiella oxytoca*. Voor *P. mirabilis* en *K. oxytoca* adviseren we dan ook een genotypische ESBL detectiemethode als de phenotypische ESBL confirmatietest positief is. Mede naar aanleiding hiervan is gesteld dat overwogen zou moeten worden om genetische confirmatiemethoden, zoals de ESBL-microarray, te implementeren in de routine van de diagnostische laboratoria. Mogelijk zou het kosten-effectief zijn om alle isolaten genotypisch te confirmeren, aangezien de kosten van ziekenhuishygiënische maatregelen, zoals isolatie, in patiënten die ten onrechte aangewezen worden als ESBL-positief, zouden kunnen worden bespaard[7].
De ESBL Etest was minder specifiek dan de combinatiedisk (59% versus 92%) voor ESBL confirmatie. Dit was met name ten gevolge van niet-conclusieve uitkomsten en vals-positieve uitslagen van de Etest.

Genotypische detectie van ESBLs
Voor hoofdstuk 3 werd de accuratesse van de Check-KPC ESBL microarray als confirmatietest van ESBLs bepaald op willekeurig geselecteerde klinische isolaten met een positieve ESBL-screentest. Het doel was om de nauwkeurigheid te bepalen van de Check-KPC ESBL microarray als confirmatietest van ESBLs in de klinische setting. Zoals besproken in hoofdstuk 2 zou daarnaast de microarray een goed instrument kunnen zijn voor de ESBL-detectie in isolaten waar de phenotypische ESBL-detectie niet betrouwbaar is gebleken in de klinische praktijk.
De prestaties van de microarray waren goed, met een sensitiviteit van 97%, specificiteit 98%, positief-voorspellende waarde van 99% en negatief-voorspellende waarde van 92%. In 3% van alle CTX-M-1 groep positieve isolaten in de collectie werd een vals-negatief resultaat afgegeven door de microarray.

Uit dit onderzoek concludeerden wij dat genetische confirmatiemethoden, zoals de ESBL-microarray geïmplementeerd zouden kunnen worden in de routine van diagnostische laboratoria. Inmiddels is de ESBL-microarray dan ook toegevoegd als een optie voor confirmatie in de Nederlandse richtlijn voor ESBL-detectie.
Gebruik van de microarray zou in een aantal situaties nuttig kunnen zijn. Ten eerste, wanneer het nodig is om snelle uitslag te hebben van mogelijke ESBL-productie in een stam. Ten tweede kan de microarray een goede oplossing zijn in het geval van niet te beoordelen uitslagen van de Etest bij phenotypische

ESBL-detectie en voor isolaten waarvan bekend is dat de positief voorspellende waarde van de phenotypische confirmatie laag is, zoals in *P. mirabilis* en *K. oxytoca*. Ten derde, aangezien de microarray ook informatie verschaft over het ESBL-gen, zou hij ook gebruikt kunnen worden voor ziekenhuishygienische doeleinden, bijvoorbeeld in het geval van de verdenking op een uitbraak met een ESBL-producerende stam. Tot slot kan hij om deze reden ook gebruikt worden voor epidemiologische studies.

De distributie van beta-lactamases in de Nederlandse populatie
Voor hoofdstuk 4 werd de distributie van beta-lactamases die resistentie tegen derde-generatie cephalosporines veroorzaken in Nederland bepaald. Er werd aangetoond dat resistentie voor derde generatie cephalosporines in Enterobacteriaceae vooral veroorzaakt wordt door CTX-M-15 ESBL-genen in *E. coli.* Alle willekeurig geselecteerde CTX-M-15 *E. coli* behoorden tot sequence type (ST) 131, volgens multilocus sequence typing (MLST). ST 131 is een zich snel verspreidend type virulente *E. coli* en is geassocieerd met CTX-M-15[8]. Het frequente voorkomen van isolaten met CTX-M-15 wordt in de meeste Europese landen, Noord-Amerika, het Midden-Oosten en India gezien[9].
CTX-M-15 positieve isolaten waren resistent voor meer soorten antibiotica, buiten de cephalosporines, dan isolaten met andere ESBL-types. De meest voorkomende ESBLs van de SHV- en TEM families waren SHV-12 en TEM-52. Waar SHV-12 in het buitenland ook frequent wordt beschreven, is dit voor TEM-52 niet het geval. Dit geldt ook voor CTX-M-1, de op een na meest frequente ESBL in Nederland.

Hypothese dat CTX-M-1 en TEM-52 zich vooral in de gemeenschap verspreiden
Doordat CTX-M-1 en TEM-52 niet alleen frequent in mensen, maar ook in pluimvee worden aangetroffen, zou een aanwijzing kunnen zijn dat ESBL-producerende bacteriën zich via het voedsel kunnen verspreiden. Eerdere studies hiernaar wezen in de richting van overdracht van ESBL-genen, plasmides en *E. coli* stammen tussen mensen en kip, meest waarschijnlijk via de voedselketen[10,11].

Voor hoofdstuk 5 werd de hypothese getest dat kip-geassocieerde ESBL-genen, gedefinieerd als de CTX-M-1 en TEM-52 genen, vooral voorkomen in de gemeenschap, terwijl de niet-kip-geassocieerde ESBL-genen vooral in het ziekenhuis worden aangetroffen. De achtergrond van deze hypothese is het concept van twee afzonderlijke compartimenten waar ESBL-producerende bacteriën in circuleren. Eén in de gemeenschap, gevoed door besmetting via voedsel en één in de ziekenhuizen, gevoed door kruisoverdracht.

De scheiding tussen twee compartimenten kon met deze studie niet worden aangetoond, aangezien de zogenoemde kip-geassocieerde ESBLs even vaak werden aangetoond in *E. coli* urinekweken, afgenomen in huisartsenpraktijken, als in ziekenhuizen. Desalniettemin sluiten deze gegevens relevante overdracht vanuit voedsel niet geheel uit, aangezien het zou kunnen dat kip-geassocieerde ESBLs, zelfs als ze in eerste instantie geïntroduceerd zijn in de gemeenschap, zich inmiddels al wijd verspreid hebben in de ziekenhuizen, of continu in ziekenhuizen ingebracht worden door patiënten.
De resistentiepatronen tussen de kip-geassocieerde en niet-kip-geassocieerde isolaten verschilden, met grotere gevoeligheid voor met name aminoglycosides en fluoroquinolonen in de kip-geassocieerde stammen in mensen. Deze antibiotica worden nauwelijks gebruikt in de veeteelt. In beide groepen was er een grote gevoeligheid voor nitrofurantoine en fosfomycine, de middelen van eerste keuze ter behandeling van ongecompliceerde urineweginfecties in Nederlands volgens de richtlijn van het Nederlands huisartsengenootschap. Dit betekent dat ook in geval van een ongecompliceerde urineweginfectie met ESBL-producerende bacteriën, de therapie die voor de huisarts eerste keuze is over het algemeen effectief zal zijn.

Het voorspellen van ESBL-dragerschap bij ziekenhuisopname
Aangezien vroege detectie van ESBL-dragerschap van waarde zou kunnen zijn in de keuze voor empirische therapie in het geval van infecties en van belang is voor bepalen of aanvullende ziekenhuishygienische maatregelen nodig bij ziekenhuisopname, was het doel van hoofdstuk 6 het ontwikkelen van een predictieregel voor dragerschap van ESBL-producerende bacteriën bij ziekenhuisopname. Hiernaast werd bepaald hoe vaak in het ziekenhuis infecties optreden met ESBL-producerende bacteriën bij zowel mensen die bij opname al drager zijn van deze bacteriën, als bij niet-dragers.
Ongeveer 8% van de mensen die werden opgenomen in het ziekenhuis bleken drager te zijn van ESBL-producerende bacteriën. Vastgesteld ESBL-dragerschap in het jaar voor opname, ziekenhuisopnames in de afgelopen zes maanden en het mannelijk geslacht bleken geassocieerd te zijn met ESBL-dragerschap bij de huidige ziekenhuisopname. Helaas was de voorspellende waarde van deze factoren laag. Bij screenen van alle patiënten met minimaal twee risicofactoren zou 31% van de ESBL-dragers geïdentificeerd worden bij opname, terwijl daarvoor 15% van de patiënten bij opname gescreend zou moeten worden. Screenen van alle patiënten met maar één risicofactor zorgt voor detectie van 72% van de ESBL-dragers, maar daarvoor zou 59% van alle patiënten bij ziekenhuisopname gescreend moeten worden, waaronder alle mannen. Waarschijnlijk is de slechte voorspelbaarheid van ESBL-dragerschap te wijten

aan het feit dat ESBL-producerende bacteriën ook opgelopen kunnen worden door de gezonde populatie, zonder duidelijke risicofactoren.

Duur van ESBL-dragerschap na ontslag uit het ziekenhuis
Eerder vastgesteld ESBL-dragerschap bleek in hoofdstuk 6 de belangrijkste risicofactor voor ESBL-dragerschap bij ziekenhuisopname te zijn. In Nederland worden isolatiemaatregelen geadviseerd voor alle patiënten die worden opgenomen in het ziekenhuis en in de 12 maanden voor opname een ESBL-positieve kweek hadden, om verspreiding naar andere patiënten tegen te gaan. Als patiënten vaak langer gekoloniseerd blijven dan een jaar, zou overwogen moeten worden om de periode van 12 maanden te verlengen[12]. Daarom was het doel van de studie voor hoofdstuk 7 om de duur van ESBL-dragerschap te bepalen na ontslag uit het ziekenhuis. Patiënten werden hiervoor tot 18 maanden na ontslag uit het ziekenhuis vervolgd.
Het bleek dat ESBL-dragers vaak langer dan 12 maanden gekoloniseerd blijven met ESBL-producerende bacteriën en dat dit vaak met dezelfde stam is. Dragers van een stam, positief voor CTX-M-15 bleven gemiddeld langer drager dan patiënten van een andere stam (mediaan 207 dagen versus 45 dagen). Deze langere duur van dragerschap, zou voor een deel de toename in CTX-M-15 positieve stammen wereldwijd kunnen verklaren[9].

ESBL-dragerschap in huisgenoten van ESBL-dragers
In hoofdstuk 8 bepaalden wij het voorkomen van ESBL-dragerschap in huisgenoten van de bekende ESBL-dragers uit hoofdstuk 7 (index-patiënten). Ook de huisgenoten werden voor 18 maanden vervolgd.
Resultaten lieten zijn dat in 62% van de huisgenoten op een zeker moment tijdens het vervolg ESBL-producerende bacteriën konden worden aangetoond. Tijdens de opname van de index-patiënt was dit in 32% van de huisgenoten het geval en tijdens elk meetpunt was dit in minimaal 20% van de huisgenoten het geval. Op elk meetpunt waren in minimaal 16% van alle huisgenoten de ESBLs gelijkend aan die van de index-patiënt. Aangezien het deel huisgenoten van bekende ESBL-dragers aanzienlijk hoger is dan de 8% dragerschap bij ziekenhuisopname van de gemiddelde populatie, lijken huisgenoten een verhoogd risico op ESBL-dragerschap te hebben.
Index-patiënten verkregen gemiddeld 1.8 ESBLs gerelateerd aan die van de index-patiënt per 1000 risicodagen, voor ongerelateerde ESBLs waren dit er 0.3 per 1000 risicodagen.

Screening van patiënten met een verhoogd risico op ESBL-dragerschap

Volgens de Nederlandse richtlijn moeten patiënten die in de 12 maanden voor ziekenhuisopname een ESBL-positieve kweek hadden en patiënten die in de afgelopen twee maanden voor minimaal 24 uur in een buitenlands ziekenhuis opgenomen zijn geweest worden gescreend op ESBL-dragerschap bij ziekenhuisopname. Resultaten van de onderzoeken die zijn verricht voor dit proefschrift, suggereren dat enige verbetering van de richtlijn mogelijk zou kunnen zijn.

Helaas kon een bruikbare predictieregel voor ESBL-dragerschap bij ziekenhuisopname niet worden ontwikkeld, echter, uit hoofdstuk 6 en 7 konden wel enkele aanbevelingen worden gehaald. In afwezigheid van een bruikbare predictieregel zou overwogen kunnen worden om alle patiënten bij opname te screenen op dragerschap en te isoleren. Dit is echter waarschijnlijk niet haalbaar, gezien het tekort aan eenpersoons kamers in de meeste ziekenhuizen en de kosten.

In hoofdstuk 7 is aangetoond dat ongeveer een derde van de eerder geïdentificeerde ESBL-dragers, een jaar na ontslag nog steeds als ESBL-drager konden worden geïdentificeerd, wat ongeveer drie keer zo hoog is als de 8% ESBL-dragerschap bij ziekenhuisopname. Deze prevalentie bleef hoog tot het einde van de follow-up na 18 maanden. Ook in huisgenoten van bekende ESBL-dragers, was het ESBL-dragerschap bijna drie keer zo hoog als de prevalentie bij ziekenhuisopname tot 18 maanden na ontslag van de index-patiënt.

Naast deze twee risicogroepen, heeft eerder onderzoek aangetoond dat patiënten die recent in het buitenland zijn geweest ook een verhoogd risico (24%) op ESBL-dragerschap hebben[13]. De duur van dragerschap was korter in deze groep met slechts 17% kolonisatie 6 maanden na terugkomst.

Voor al deze drie groepen zijn de ESBL-prevalenties dermate hoog, dat screening bij ziekenhuisopname overwogen zou moeten worden. Echter, ook andere factoren spelen een rol. Ten eerste is het de vraag of identificatie van alle ESBL-dragers noodzakelijk is, aangezien overdracht in het ziekenhuis niet vaak lijkt voor te komen[14]. Ten tweede, zoals aangetoond in hoofdstuk 6, is het absolute risico op nosocomiale infecties met ESBL-producerende bacteriën laag en waren de meeste infecties niet invasief. Om deze reden is het discutabel of universele screening voor ESBL-dragerschap de verspreiding van ESBL-producerende bacteriën in het ziekenhuis kan verminderen en de morbiditeit en mortaliteit voor nosocomiale infecties significant zou verminderen.

Bekende dragers worden reeds gescreend bij ziekenhuisopname tot 12 maanden na de laatste ESBL-positieve kweek. Mogelijk zou het kosteneffectief kunnen zijn om naast bekende ESBL-dragers, ook hun huisgenoten te screenen tot 18 maanden na ontslag uit het ziekenhuis en patiënten die recent naar hoog-risico

gebieden zijn gereisd in de afgelopen zes maanden. Deze patiënten zouden dan ook geïsoleerd moeten worden bij ziekenhuisopname. Verder onderzoek hiernaar is noodzakelijk.

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Lieve Alke, nu het boekje klaar is, is er eindelijk tijd om samen aan een nieuw hoofdstuk te beginnen!

Curriculum vitae

Tamara Platteel

Curriculum vitae

Tamara Nadine Platteel was born on September 12th in Nijmegen, the Netherlands. She grew up in Deventer, Bathmen and Beekbergen and graduated from the Gymnasium Apeldoorn in 2002. That same year she started her medicine study at the University of Utrecht. During this study she already gained some experience in epidemiologic research doing epidemiologic research on breast cancer in the Julius Center as an optional subject.
After obtaining her medical degree in September 2009, she immediately started the work described in this thesis at the department of Medical Microbiology of the University Medical Center Utrecht, under supervision of Prof. Dr. Marc Bonten, Dr. James Cohen Stuart and Dr. Maurine Leverstein-van Hall. She combined her PhD project with the general practitioner vocational training at Utrecht University. During this combined programme she also started with the master's programme Clinical Epidemiology at the Utrecht University Graduate School of Life Sciences in 2013.
At present she is about to obtain her master's degree and she is in her second year of general practitioner vocational training.