Prevalence and spread of antibiotic resistant microorganisms in a cross border region

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Aan ons pap en mam

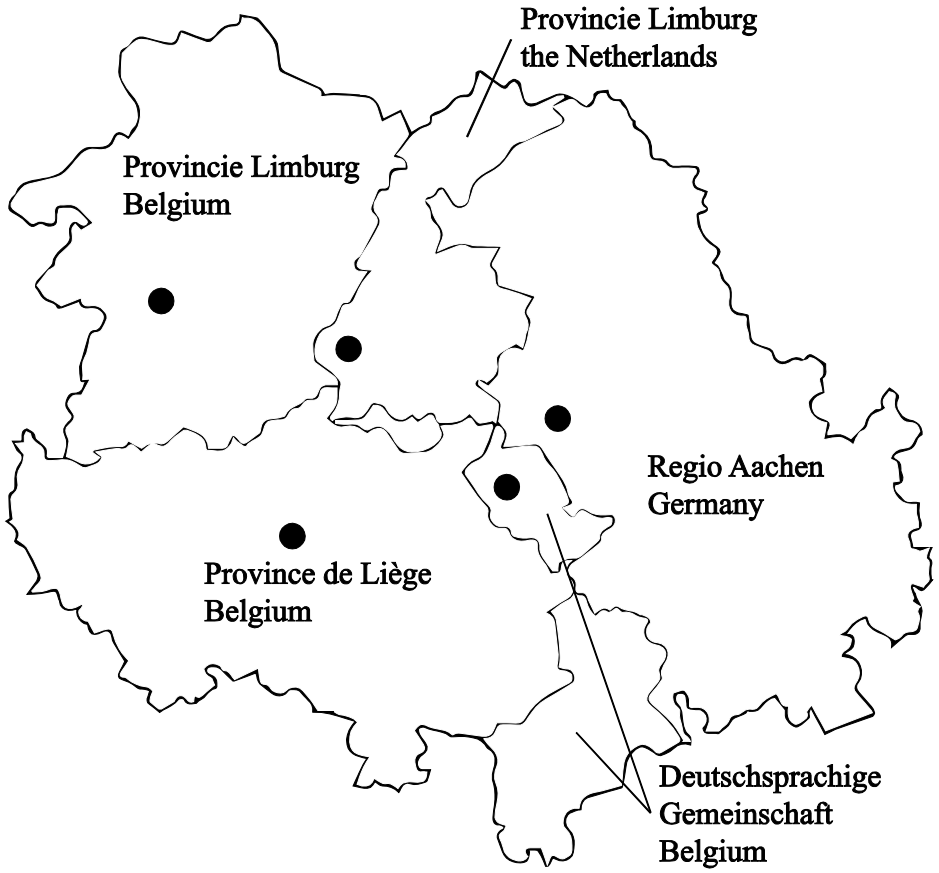
Contents

Abbreviations

AMC	amoxicillin-clavulanic acid
AMO	amoxicillin
ATC	anatomical therapeutic chemical classification system
ATCC	American type culture collection
AZI	azitromycin
B	Belgium
BURP	based upon repeat pattern
BURST	based upon related sequence types
CAZ	ceftazidime
CA-MRSA	community-associated MRSA
CC	clonal complex
CIP	ciprofloxacin
CFA	clonal frame analysis
CFM	cefixime
CFX	cefotaxime
CLI	clindamycin
CTX-M	cefotaxime-Munich
CXM	cefuroxime
DDD	defined daily dosage
DI	diversity index
ERY	erytromycin
ESBL	extended spectrum beta-lactamase
EUCAST	European committee on antimicrobial susceptibility testing
FDR	false discovery rate
FEP	cefepime
FUC	fusidic acid
G	Germany
GEN	gentamicin
GP	general practice
HA-MRSA	hospital associated MRSA
ICU	intensive care unit
IMP	imipenemase
IPM	imipenem
KPC	*Klebsiella pneumoniae* carbapenemase
L	Limburg
MDR	multi drug resistant
MEM	meropenem
MIC	minimal inhibitory concentration
MLST	multi locus sequence typing
MLVA	multi locus variable number tandem repeat analysis
MRSA	methicillin resistance *Staphylococcus aureus*
MSSA	methicillin susceptible *Staphylococcus aureus*

MUMC	Maastricht University Medical Centre
MUP	mupirocin
NDM	New Delhi metallo-beta-lactamase
NH	nursing home
NIT	nitrofurantoin
NL	the Netherlands
NOR	norfloxacin
NT	not tested
OXA	oxacillin
OXA	oxacillinase
PBP	penicillin binding protein
PCR	polymerase chain reaction
PEN	penicillin
PFGE	pulsed field gel electrophoresis
PIP	piperacillin
QUI	quinolones
SCC*mec*	Staphylococcal cassette chromosome
SHV	sulfhydryl variable
spa	staphylococcal protein A gene
spa-CC	*spa* clonal complex
ST	sequence type
SXT	trimethoprim-sulfamethoxazole
TAZ	piperacillin-tazobactam
TEM	Temoneira
TOB	tobramycin
TRIM	trimethoprim
UPGMA	unweighted pair group method with arithmetic averages
URO	urology services
UPEC	uropathogenic *Escherichia coli*
UTI	urinary tract infection
VIM	Verona integron-encoded metallo-beta-lactamase
WHO	world health organization

Map of the Euregion Meuse-Rhine



Chapter 1

General introduction
Aim & Outline of the thesis

Commensal microbiota

Only when in uterus is every child presumed sterile. This will change at the start of delivery and will never change back again[1-3]. Each human being carries with him or her approximately 10^{14} bacteria in the intestines alone, outnumbering our own human cells by at least ten fold[1]. All these bacteria put together are our commensal microbiota or commensal flora but the make up and amount of bacterial species differ per site on the human body. For example the most abundant flora of the skin consists of *Staphylococcus epidermidis*[4], while these are *Bacteroides* spp. in the colon[1]. Moreover, the colon is also the location with the highest number of colonizing bacteria[5].
Commensalism is a symbiotic relationship between two species in which the symbiont benefits, but the host does neither gain nor lose from the relationship[4]. However, this is not completely true for the human microbiota, since the host does benefit but is sometimes harmed too. By their number commensal microorganisms outcompeed pathogenic microorganisms and many bacteria produce or breakdown nutrients which are vital in maintaining human health. The bacteria are also necessary for priming the host's immune system[1, 3-6].
Although, our commensal microbiota is mostly of value to us, it is also for a small part made up of potentially pathogenic micro-organisms. These include *Staphylococcus aureus*, *Escherichia coli* and other Enterobacteriaceae including *Klebsiella pneumoniae*[4, 5]. These micro-organisms are prevalent causes of many infections, nosocomial and community acquired.

Staphylococcus aureus

Staphylococcus aureus is a potentially pathogenic, facultatively anaerobic, Gram positive commensal of the skin and the mucosa, that was first described in 1880 by Sir Alexander Ogston[7]. Although, these bacteria can be cultured from multiple body sites, the anterior nares are the prime location of colonization[8]. *S. aureus* can cause community acquired and nosocomial infections ranging from mild skin and soft tissue infections, like erysipelas and cellulitis, to severe and life-threatening infections, such as necrotizing pneumonia, bacteraemia and endocarditis[7].
Persistent carriers of *S. aureus* (12-30% of the population) are more at risk for an infection with *S. aureus* than intermittent carriers (16-70% of the population) and non-carriers (16-69%) since *S. aureus* infections are often caused by the patient's own commensal strains[8-10].

Escherichia coli

Escherichia coli is a potentially pathogenic, facultatively anaerobic, Gram negative commensal of the intestinal microbiota[5] and was first described in 1885 by Theodor Escherich[11].

E. coli is one of the facultative anaerobic species in the human gut microbiotica, which mainly consists of obligatory anaerobics, such as *Bacteroides* spp., *Clostridium* spp., bifido-, eu- and fusobacteria[1].
E. coli is a prevalent causative agent of both intra- and extra intestinal infection. In particular, the extra intestinal infections are mainly caused by the host's own *E. coli* strains[12, 13]. Extra intestinal infections with *E. coli* range from mild self-limiting cystitis to severe urosepsis and meningitis[14]. Whether a certain *E. coli* isolate causes an intra or extra intestinal infection is mainly determined by its combination of virulence factors, which are usually encoded for by multiple genes located on pathogenicity islands[15-17]. Extra intestinal *E. coli* bacteria usually express many adhesins and iron acquisition systems to survive outside the intestines, while intra intestinal (diarrhea-causing) *E. coli* express specific secretions systems to inject toxins into the host cells[17].

Klebsiella pneumoniae

Klebsiella pneumoniae is an opportunistic potentially pathogenic, facultatively anaerobic, Gram negative bacteria and was first described in 1883 by Carl Friedländer and named after the German bacteriologist Edwin Klebs[18]. This bacteria is, unlike *E. coli*, also common in the environment[19]. Infections caused by *K. pneumoniae* include but are not limited to urinary and respiratory tract infections. The type of infection is just as with *E. coli* dependent on the strain's specific virulence factors[19]. In patients with lowered host defense it can also cause severe infections including bacteraemia[14].

Antibiotic treatment

The suitability of an antibiotic for the treatment of an infectious disease is determined mostly by the site of infection, the causative bacteria and the resistance of this bacterium to certain antibiotics. For many infections, often treatment is initiated empirically. These include amongst others urinary tract infections (UTIs) and skin and soft tissue infections. Empiric therapy means treatment with antimicrobial agent(s) for an infection for which the causative agent(s) and the antimicrobial resistance are unknown. Therefore, to make an appropriate choice the probable causative agent and the anticipated susceptibility to antimicrobial agents should be taken into account.
For urinary tract infections the most prevalent bacterial agents include *E. coli*, *Enterococcus* spp., *Proteus mirabilis*, *Staphylococcus saprophyticus*, *Klebsiella pneumoniae* and *Enterobacter cloacae*[20-22]. However, distribution of the species differs per patient population. For example: *E. coli* is by far the number one causative agent in every patient population but *S. saprophyticus* is more often isolated from young and *K. pneumoniae* from elderly general practice (GP) patients[21] Bacteria such as *Proteus* spp. and *Enterobacter* spp. but also *Klebsiella* spp. are observed more among hospitalized patients and nursing home residents[20, 23]. Urinary tract infections range from mild self-limiting cystitis to prostatitis and pyelonephritis and can lead to

urosepsis. These infections remain one of the most common indications for antibiotic treatment[24, 25].
For skin and soft tissue infections the most prevalent bacterial agents include *S. aureus* and *Streptococcus pyogenes*. These infections are generally mild, such as impetigo or cellulitis, but can be more severe (necrotizing fasciitis) and can be accompanied with signs of systemic involvement[26]. If a patient presents with a skin and soft tissue infection with mild symptoms, often the topical application of the antimicrobial agent can be effective[27].

Antibiotic agents

Antibiotic is a derivative of a Greek word meaning against life. An antibiotic is a chemical substance produced by one species that is life threatening to another species. Throughout the centuries antimicrobial substances have been used to treat infections but renewed search for such agents started with the acceptance of the germ theory of disease[28]. One of the first agents, still to be used today, was discovered by Sir Alexander Fleming in 1928[29, 30]. However, it was not until 1942 that this substance, penicillin, could be manufactured in high amount[29, 30]. This was the start of the development of many new agents: the era of antibiotics.
Ideally antibiotics should be specific and only inhibit bacterial cell processes and should not damage the host cells (selective toxicity). However, antibiotics do not differentiate between pathogenic and non-pathogenic bacteria[3, 31] resulting in a disturbance of the commensal flora, however, the degree of disturbance is dependent on the antibiotic used[31].
The following antibiotics agents are often but not solely used for the treatment of urinary tract infections (UTIs) and, skin and soft tissue infections. These include nitrofurantoin, fosfomycin, the fluoroquinolones and amoxicillin-clavulanic acid for the UTIs and flucloxacillin, the macrolides, clindamycin and fusidic acid for the treatment of *S. aureus* infections

Nitrofurantoin
This antimicrobial agent has been available since 1953. After influx into the bacterial cell, nitrofurantoin first has to be activated by the microbial nitroreductases before it inhibits several bacterial enzymes and thereby interferes in the DNA and RNA synthesis, carbohydrate metabolism and other metabolic processes[32].
Nitrofurantoin is active against most Gram negative and Gram positive bacteria. However, *Pseudomonas* spp, *Serratia* spp. and *Proteus* spp. are intrinsically resistant[32, 33]. Approximately 40% of the nitrofurantoin is excreted unchanged via the kidneys. The highest concentration in the urine will be reached after 4-5 hours. This high concentration in the urine and the low tissue penetration deem this agent only suitable for uncomplicated cystitis[32, 34]. Potential severe side effects include polyneuropathy among the elderly and those with renal impairment, and lung- or hepatotoxicity[32].

Fosfomycin

Fosfomycin is an antibiotic, which has been available for many years and is mainly used for the treatment of uncomplicated cystitis. Fosfomycin has a broad antibacterial activity including both Gram positive (e.g. *S. aureus* and *E. faecalis*) and Gram negative bacteria (e.g. Enterobacteriaceae and *Pseudomonas aeruginosa*).

Fosfomycin is an agent that interferes in the cell wall synthesis by inhibiting the formation of N-acetylmuramic acid, a precursor of peptidoglycan[35, 36]. It also decreases the adhesion of the bacteria to the bladder epithelium[36]. After a single dose, the urine concentration of this agent is high and may exceed 2000mg/L. Fosfomycin also has a good tissue penetration and overall adverse effects are minor[37]. Worldwide resistance to fosfomycin has remained low[21, 38] and fosfomycin retains activity against ESBL producing strains[35, 36]. Unfortunately, the use of fosfomycin is only indicated for the treatment of uncomplicated UTI and thus not used for other infections, despite its promising characteristics.

(Fluoro)quinolones

Quinolones are broad spectrum antibiotics prescribed both in the hospital[23] and in the extramural setting, especially in long-term care facilities[39-41]. They are prescribed for a wide range of infections, caused by both Gram positive (for example: the fluoroquinolones levofloxacin and moxifloxacin) and/or Gram negative bacteria (for example: the fluoroquinolones ciprofloxacin and norfloxacin)[42].

Quinolones have been available since 1962 and lots of analogues have been developed since then including the fluoroquinolones. Their mode of action is the inhibition of DNA gyrase and topoisomerase IV. These enzymes are required for the coiling/uncoiling and (de)catenating of the DNA strand during replication[42-44].

Amoxicillin-clavulanic acid and flucloxacillin

These agents are part of the beta-lactam group. Amoxicillin-clavulanic acid is a combination of a beta-lactam antibiotic with a beta-lactamase inhibitor and flucloxacillin is resistant to penicillinase.

Beta-lactam antibiotics inhibit the formation of the peptidoglycan layer by inhibiting the transpeptidase or Penicillin Binding Protein (PBP), which cross links the different peptidoglycan layers of the bacterial cell wall[45].

Amoxicillin-clavulanic acid is a broad spectrum antibiotic prescribed for various infections, mild and severe and is also used for a complicated UTI[46]. Flucloxacillin, a narrow spectrum antibiotic, is mainly used for the treatment of *Staphylococcus aureus* infections[46]. Both agents are prescribed in intra- and extramural settings[23].

Macrolides and clindamycin

The macrolides are a group of antibiotics that act by inhibiting the protein synthesis by blocking the attachment of the next tRNA molecule and the ribosomal translocation at the 50S subunit of the bacterial ribosomes[47]. Both the macrolides and clindamycin are mainly used to battle Gram positive infections[48] but these agents are not active to facultative anaerobic Gram negatives, such as the Enterobacteriaceae[33].

Fusidic acid

Fusidic acid is an antibiotic that inhibits bacterial growth, mainly bacteriostaticly, by binding to elongation factor G on the ribosome and thereby preventing the bacterial protein synthesis to continue[49]. Fusidic acid is active against Staphylococci, Corynebacteria and Gram positive anaerobes. The Enterobacteriaceae are resistant and the activity to streptococci and enterococci is limited[49]. Although, fusidic acid is available both in systemic as topical formulation[46], in the Netherlands the use of systemic fusidic acid is very low[50] while the topical use is much higher[51]. It is mostly used as a topical agent to treat skin and soft tissue infections[51]. Overall the tolerability of fusidic acid is high with few side effects[27].

Antibiotic resistance

There are several mechanisms for bacterial resistance to antibiotic agents. They can be divided in four groups: 1) alteration of the drug target site, 2) inactivation of the drug, 3) decreased permeability or influx to reduce the drug uptake, 4) increased efflux of the drug[52]. The main resistance mechanisms and the genes encoding the mechanisms of resistance for the above mentioned antibiotics are described on the following pages.

Methicillin resistant *S. aureus* (MRSA)

MRSA was first described in 1961 only 2 years after the introduction of methicillin[53], a penicillinase resistant penicillin. The resistance to methicillin is encoded by the *mecA* gene, which codes for the PBP2a. This is an altered PBP and, therefore, the affinity of the drug to this target is decreased and the peptidoglycan synthesis remains uninterrupted[54]. The *mecA* gene is located on a mobile genetic element, the staphylococcal cassette chromosome, the SCC*mec*[55, 56], which contains several elements besides the *mecA* gene. These include amongst others: 1) the *ccr* gene complexes, responsible for the integration or excision of the cassette from the genome, 2) genes responsible for regulation of *mecA* transcription (*mecI, mecR1*), 3) associated insertion sequences and 4) the so called junkyard (J) regions[54]. In *S. aureus* 11 types of SCC*mec* cassettes have been described so far, each with its own variants[57-59]. SCC*mec* I to V have been observed most often. SCC*mec* IV and V only encode resistance to methicillin while SCC*mec* I, II and III also encode for resistance to several other antibiotic classes including the macrolides, aminoglycosides and fluoroquinolones, due to integrated plasmids or transposons in the cassette[54]. In the mid nineties MRSAs with SCC*mec* IV and V were isolated from healthy persons with no apparent risk factors for MRSA acquisition or contact with healthcare and were, therefore, classified as community associated MRSA. This in contrast to the hospital associated MRSAs, which carry mostly SCC*mec* II and III, and are isolated from patients at healthcare facilities with more risk factors and co morbidities[53, 54]. However, during recent years CA-MRSA isolates have also been isolated from hospital admitted patients[60]. Thus, the strict division between the two groups of MRSAs is fading.

Beta lactamases and extended spectrum beta-lactamases (ESBLs)

Beta-lactamases are enzymes produced by many bacteria both Gram positive and negative, aerobic and anaerobic[61]. These enzymes hydrolyze the C-N bond in the beta-lactam ring of the beta-lactam antibiotics, which makes them inactive[45, 52, 62]. The first beta-lactamase was described before the introduction of penicillin[63]. However, since their first discovery the development of new beta-lactam antibiotics and the "development" of new beta-lactamases, especially by Enterobacteriaceae have kept in pace. Nowadays the increasing prevalence of new beta-lactamases, i.e. extended spectrum, AmpC and inhibitor resistant beta-lactamases and carbapenemases, is a point of concern[64-66].

Beta-lactamase enzymes can be classified based on their molecular or functional characteristics. Based on molecular characteristics the TEM, SHV, CTX-M and *Klebsiella pneumoniae* carbanemase (KPC) belong to class A, New Delhi metallo-beta-lactamase (NDM), imipenemase (IMP) and Verona integrated metallo-beta-lactamase (VIM) to class B, the AmpC to class C and oxacillinase (OXA) to class D[65, 67]. The functional classification according to Bush et al. divides beta-lactamases into 3 groups. Group 1 includes the AmpC and group 3 the metallo beta-lactamases including the NDM carbapenemases. Group 2 is subdivided in 12 subgroups (i.e. 2a, 2b, 2be, 2ber 2br, 2c, 2ce, 2d, 2de, 2df, 2e and 2f) of which 2f and 2df include the other carbapenemases[68].

Resistance genes encoding for beta-lactamases are mostly located on plasmids or some on transposons, but these genes can also be located in the chromosomal DNA[69, 70].

Due to the relatively high prevalence of ESBLs and other beta-lactamases and their impact on treatment outcome it is essential to detect beta-lactamases rapidly to prevent the too long continuation of inappropriate antibiotic therapy[71, 72] and to start infection control measures as soon as possible.

Both phenotypic and genotypic tests for the detection of beta-lactamases have been developed. Phenotypic tests include: a combination disk diffusion test or an Etest with 3rd gen cephalosporines with and without clavulanic acid and micro broth dilution for ESBLs and, a modified Hodge test and carbapenemase inhibition tests for the detection of carbapenemases[73]. However, these tests all require overnight incubation and this means a delay in initiation of the appropriate antibiotic therapy.

Fast and simple tests to determine the presence of a beta-lactamase a day earlier than the described phenotypic tests are available[74, 75]. These are biochemical tests based on the *in vitro* hydrolysis of cefotaxime and imipenem. However, the characterization of the type of ESBL is not possible with these tests. Molecular detection of the different ESBL genes with PCR is an alternative and many PCR assays have been developed[76-79] but this type of detection is costly and not available in every laboratory. Also, due to the high number of resistance genes it is hard to detect all of them in one assay. New diagnostic tests such as micro-arrays might be able to overcome this problem[80].

Quinolone resistance

Due to the wide antibacterial spectrum and oral availability of the quinolones, they have been extensively used over the years[81]. This, together with misuse or unnecessary use, is probably the main reason for increased resistance worldwide[50, 82, 83]. Resistance

to quinolones is mediated by alterations in the drug target and decrease of the drug inside the cell due to decreased influx or increased efflux.
Alteration of the drug targets i.e. DNA gyrase (catalyses of the negative supercoiling of DNA) and topoisomerase IV (decatenating of daughter replicons) are encoded by *gyrA*, *gyrB* and *parC*, *parE*, respectively[43]. These alterations are mostly chromosomally encoded[69]. Although, a comparable mechanism but plasmid-mediated, encoded by the *qnrA*, *qnrB* and *qnrS* genes, has been described[69]. The qnr proteins can bind to the DNA gyrase and this binding protects the bacteria from the activity of the quinolones[69]. Decreased uptake can be accomplished through alterations of the membrane permeability, usually due to decreased expression of porins, mostly OmpF (one of the two major outer membrane porins of *E. coli*)[42, 43]. Increased efflux is regulated by overexpression of efflux pump systems[42, 43]. Another mechanism of resistance is encoded by the aminoglycoside acetyltransferase (aac('6)-Ib-cr), which is capable of acetylating not only the aminoglycosides but also ciprofloxacin and norfloxacin[69]. The prevalence of this mechanism is still low[84].

Resistance mechanism of the other described antibiotic agents
Nitrofurantoin: Bacterial resistance to this agent was and still is very low[85], which is in contrast to the relative high prevalence of resistance to other agents. The mechanism of resistance is probably due to the capacity of bacteria to stop the activation of the nitrofurantoin by nitroreductase[86]. This has a fitness cost for the bacteria[86] and resistant bacteria are thus easily outcompeted by other susceptible bacteria.
Fosfomycin: Till today several mechanisms of resistance have been described, which include: 1) decreased drug uptake, 2) inactivation of fosfomycin or 3) modification of the target site[35]. These mutations have a biological cost for the bacteria[35, 36] and, therefore, resistant bacteria are not often found.
Macrolides and clindamycin: Resistance to the macrolides is often facilitated through methylation of the drug target (encoded by the *erm* genes), which also results in (inducible) cross resistance with clindamycin, or alteration of the drug target through mutation[48]. Other mechanisms are efflux of the drug or drug inactivation[48].
Fusidic acid: The main resistance mechanism for this antibiotic is the alteration of the drug target site, mediated through a mutation in the coding gene e.g. *fusA*, or the acquisition of the *fusB* gene (mostly plasmid mediated). However, the underlying mechanism for *fusB* is still unknown. *FusB* is the predominate mechanism of resistance in the resistant European clone[49, 51].

Risk factors for resistance

The number of factors associated with increase of antibiotic resistance or the acquisition of a resistant strain is high. The most important risk factors are antibiotic use, living in a nursing home, contact with husbandry and travelling to endemic countries. However, several other risk factors have been described, which include amongst others the presence of invasive devices or foreign materials such as urinary catheters, co-morbidities such as skin diseases, poor functional status, wounds and diabetes[87].

Antibiotic use

The use of antibiotics is being considered as the main risk factor for the development or increase of antibiotic resistance. In countries with a higher use of antibiotics the prevalence of resistance is higher compared with countries with lower use of antibiotics[82]. Several studies observed a higher, prior use of antibiotics among patients with a resistant strain compared with those without a resistant strain[88-90]. Decrease of antibiotic use and antibiotic stewardship have been described to decrease or control antibiotic resistance[91].

Nursing homes

Nursing home (NH) residents are often considered a reservoir for resistance[87, 92, 93]. In many countries resistance among NH residents is high[93-95], due to amongst others the high prevalence of risk factors for acquiring a multi drug resistant strain[40, 96]. Also, the enclosed living environment of the residents facilitates the spread of resistant strains.

Husbandry

Contact with reservoirs of multi resistant microorganism such as husbandry is also a risk factor[97], since use of antibiotics among those animals is high[98]. Persons who have regular contact with these animals are at risk for acquiring one of their resistant strains[99, 100], but these resistant strains can also potentially spread to other humans via consumption meat [101, 102].

International travel

Due to international travel the spread of resistant strains is facilitated. Travellers visiting a country with a high prevalence of multi drug resistance have, especially when admitted to a hospital or when visiting a health care facility during their stay, a risk for acquiring a resistant strain[103, 104]. Also, visitors from a country with a high prevalence of resistance play a role in the spread of resistant strains in a country with low prevalence of resistance[105]. This potential spread of resistant strains applies not only for travelling to different continents but also to cross border movements in a border region.

Cross border health care

Everywhere around us the world is becoming more globalized and this definitely includes health care. In the European Union borders start to fade giving the European citizens the opportunity to cross borders at will and to appreciate all that Europe has to offer. This should also include the health care facilities. Therefore, a proposal was made by the European commission to aid and stimulate cross border health care[106].
Patients should be free to seek medical care not only in their own country but also in the other European countries[107]. However, differences between countries, such as variation in prevalence of antibiotic resistance, hamper patient mobility[108]. Patients moving across the border could have negative experiences due to differences in antibiotic resistance and infection control policies, for example: there is a risk of potential over or under treatment, risk of acquisition or spreading of resistant strains

and possible screening and isolation for (suspected) carriage of a resistant strain[109]. Therefore, after primary problems in cross border health care such as differences in health care, financing and assurance systems have been dealt with by the governments, the other dilemmas, such as the problems caused by differences in antibiotic resistance, have to be addressed[110]. This can be done by improving the cooperation between health care facilities in the different countries regarding infectious diseases, antibiotic policies and infection control policies.

Euregion Meuse-Rhine

The Euregion Meuse-Rhine consists of five subregions: Provincie Limburg (Midden and Zuid Limburg) in the Netherlands, Provincie Limburg, Province de Liège and Deutschsprachige Gemeinschaft in Belgium and Regio Aachen in Germany. In this region of 10793 km^2 live approximately 3.9 million people, which is a population density of approximately 360 people per square kilometer. In this region are approximately 60 general and academic hospitals[111]. The Euregion Meuse-Rhine is also a region with many cross border movements for example for working or educational, recreational and health care purposes[110, 111].

An issue related to cross border healthcare is the potential spread of resistant bacteria, such as *E. coli* and *S. aureus*. According to the EARS-net 2010 report the prevalence of MRSA in hospitals was 20.5%, 20.9% and 1.2% in Belgium, Germany and the Netherlands, respectively[83]. Resistance to third generation cephalosporines among *E. coli* was 5.2%, 8.4% and 5.1% and the resistance to the fluoroquinolones was 21.5%, 24.8% and 13.6% in Belgium, Germany and the Netherlands, respectively[83]. These differences in prevalence of resistance hamper free access to health care facilities on the other side of the border. For example: the infection control policy of the Netherlands only allows access to health care facilities after negative screening for MRSA[109].

Previous research has demonstrated that some MRSA clones occur and spread predominantly in a regional health care cluster[112, 113]. This suggests that spread of these clones can be interrupted by an intervention at the health care institutions at a regional level. In a cross border region this urges for cross border agreement of infection control policies and increase of cooperation not only nationally but also Euregionally, between the countries. Exchange of knowledge and experience will improve patient care and patient safety in the subregions of this Euregion[108].

Spread of resistance

Ever since the introduction of antibiotics, counter mechanism have been developed and adapted by bacteria. Due to globalization and increase of international and intercontinental travel these resistant bacteria spread across the globe[105].

MRSA

Just a few years after the introduction of penicillin, the first penicillin resistant strain was isolated. At the introduction of methicillin, in 1959, penicillin resistance was already around 80% and has remained high until this day[54, 114, 115]. Shortly, after the

introduction of methicillin, a resistant *S. aureus* (MRSA) strain was isolated in Europe. The prevalence of MRSA has increased over the years and spread across the world[54]. The origin of MRSA is not proven but suggested most is that the SCC*mec* was introduced into several *S. aureus* lineages (multi clone theory) instead of the SCC*mec* acquisition of just one strain as the ancestor of all MRSA clones[116-118]. Typing methods have shown that globally several MRSA clones are epidemic and most of those clones have also been isolated in the Netherlands, Belgium and Germany. The most prevalent include: ST45-MRSA-IV (Berlin clone), ST5-MRSA-II (New York/Japan or Rhine Hesse clone), ST5-MRSA-IV (Pediatric clone), ST8-MRSA-IV (EMRSA-2/6 or USA300 if PVL-positive) and ST22-MRSA-IV (EMRSA-15)[119-121]. The overall prevalence of MRSA is variable between and within countries and between patient populations. Overall, the prevalence of MRSA is higher in hospitals and nursing homes compared with the community[83, 92, 122].

Beta-lactamases

Like the Gram positive *S. aureus*, the Gram negatives developed resistance mechanisms to combat the antibiotics. The first penicillinase (also a beta-lactamase) producing *E. coli* was already observed before the introduction of penicillin[63] and this property has rapidly spread to other species. With the introduction of the first cephalosporines treatment options increased but this was also more or less the start of the emergence of more broad spectrum beta-lactamases (TEM and SHV), which were capable of hydrolyzing first generation cephalosporines. These beta-lactamases were already widespread among *E. coli* isolates in the early 1970s[62]. However, with the introduction and increased use of beta-lactamase resistant cephalosporines (third generation cephalosporines) newer beta-lactamases, i.e. the extended spectrum beta-lactamases (ESBL), were isolated[62, 123]. Since then many ESBLs have been detected. One mutation in the DNA sequence of the beta-lactmase gene leads to a new different ESBL, which is amongst others the reason for the explosive increase in prevalence of ESBLs. At present the most prevalent ESBL types belong to the CTX-M class together with TEM and SHV types[123]. The spread of these enzymes is nowadays mostly through transfer of plasmids and other mobile genetic elements[69].

In the Euregion Meuse-Rhine CTX-M-15, CTX-M-1, CTX-M-14, SHV-12, TEM-52 are common among *E. coli* isolates[102, 124]. An example of the global spread of the ESBLs is the UTI causing *E. coli* O25:H4-ST131[125-127]. This strain has been associated with the increased prevalence of CTX-M-15[125], is able to pick up new resistance properties easily and has been isolated in most European countries. ST131 strains carry genes encoding for multiple beta-lactamases including ESBLs, fluoroquinolones resistance and recently a carbapenemase gene have been described[127, 128].

Carbapenemases are a more recent problem among antibiotic resistant bacteria. These mostly plasmid mediated beta-lactamases confer resistance to the antibiotics of last resort: the carbapenems. The types most prevalent are: KPC, VIM, IMP, NDM and OXA[65, 129], which are not yet very frequently found in Western Europe. Infections with carbapenemase producing isolates can often be traced back to hospital admittance in countries where those carbapenemases are endemic, such as India and Pakistan[130]. The production of carbapenemases is a point of concern as: 1) their prevalence is

increasing, 2) the number of alternative treatment options is limited and 3) the pipeline of new antibiotic drugs is almost empty. If the prevalence of carbapenemase producing isolates increases, this could lead to a situation comparable to the time before the antibiotics, the pre penicillin era.

Typing of *Staphylococcus aureus* and *Escherichia coli*

To analyze a possible outbreak or spread of a certain (antibiotic resistant) strain, several typing techniques (sequence based and band based) have been developed to establish a possible relatedness between the isolates. Some techniques can be used for multiple species, such as pulsed field gel electrophoresis (PFGE), multi locus sequence typing (MLST) and multi locus variable number tandem repeat analysis (MLVA), while others are species specific, such as *spa* typing (*S. aureus*) and SCC*mec* typing (MRSA).
The choice of typing method is dependent on 1) the research question (local outbreak or epidemiological study), 2) the discriminatory power, 3) interlaboratory comparability, 4) hands-on time, 5) time-to-results and 6) costs of the method. For an outbreak investigation PFGE, MLVA and *spa* typing are usefull, but in (large) epidemiological studies MLST and SCC*mec* and *spa* typing are more widely used, but also MLVA could be an option.

PFGE
Pulsed field gel electrophoresis, a band based method, is performed by lysing the bacteria and digesting the bacterial DNA with infrequently cutting restriction enzymes such as *SmaI* for *S. aureus* and *XbaI* for *E. coli* and *K. pneumoniae*. The DNA fragments are then separated using gel electrophoresis for a "DNA fingerprint"[131, 132]. This fingerprint can be compared with the fingerprints of other isolates to determine relatedness according to criteria set by Tenover et al[133]. PFGE is a laborious method with high discriminatory power but the interlaboratory reproducibility is low[134], also due to the lack of one single protocol. Until now PFGE is still often used, mostly to analyse local outbreaks.

MLST
Multi locus sequence typing is a sequence based method. For this typing method the DNA sequences of seven or eight house keeping genes are determined. These genes are chosen specifically for each species[135, 136]. With these sequences, allelic polymorphism can be determined. The DNA sequences of these seven genes form a sequence type, which can be analysed with the international MLST database and can be clustered into clonal complexes with eBURST. MLST is, like PFGE, a labor intensive and expensive method[134]. However, the MLST results are, due to the use of an international database and a standardized protocol, easily and reliably comparable between laboratories. MLST is considered the gold standard for large epidemiological studies but is not useful to determine hospital outbreaks, due to a slightly lower discriminatory power compared to PFGE and MLVA[134].

MLVA

Multi locus variable number tandem repeat analysis is a newer method and is compared with MLST less expensive and less labor intensive with a higher discriminatory power[134]. For this analysis the number of tandem repeats is determined for seven or eight loci in the genome of the target bacteria. The loci differ per species. This method can be performed with conventional PCR and gel electrophoresis (band based method)[137], which requires large size of the repeats. Another option includes a real time PCR with fluorescent labeled products after which the product size is determined with an automated DNA sequencer[134]. The accurately determined number of repeats can be entered in a database, which makes interlaboratory comparison and clustering of related strains manageable. Since sequence analysis is not necessary with this method, it is less costly than the MLST method.

***Spa* typing**

Spa typing is, just as MLST, a DNA sequence based approach. The target is one locus: the polymorphic repeat region of the *Staphylococcus* protein A gene[138]. This method can be used to determine both hospital outbreaks of *S. aureus* as well as clonal relatedness in epidemiological studies. Due to the single locus scheme this method is less expensive and laborious. There is also an international database available to compare the *spa* typing data[139] and with algorithm Based Upon Repeat Pattern (BURP) it is also possible to cluster these *spa* data, which has good concordance with MLST and PFGE[140].

SCC*mec* typing

The SCC*mec* type of a MRSA can be determined with a PCR either by targeting different loci in the different SCC*mec* complexes or by determining the structure of the *mec* complex and de *ccr* genes. This can be done with a conventional or a real time PCR and with or without a multiplex assay[56, 141-146]. However, due to the high number of major and minor SCC*mec* variants, it is not possible to detect all types and their variants with any of those methods. A method such as a micro-array might be able to resolve this issue. SCC*mec* typing is used in epidemiological studies to determine the genetic background of MRSA clones.

Conclusion

S. aureus and *E. coli* are both part of the human commensal flora. Infections caused by these bacteria are prevalent in the community, nursing homes and hospitals, and often require antibiotic treatment. The prevalence of antibiotic resistance determines, amongst others, which antibiotics are appropriate as empiric treatment choice for these infections. Since the introduction of antibiotics, bacteria have developed different ways to combat these agents and have become resistance. Some of these mechanisms of resistance have spread between bacteria and across the globe.

In the past especially the multi drug resistant Gram positive bacteria, such as MRSA, have been a point of concern but more recently, the “neglected” Gram negatives have now emerged as a problem. In particular, since new types of beta-lactamases are

being detected with the ability to resist even the antibiotic agents of last resort. Cross border movements, such as in the Euregion Meuse-Rhine and international travel are important factors in the spread of these resistant strains. Therefore, action should be taken to control the increasing prevalence of antibiotic resistance and to prevent a situation comparable to the time before the antibiotics, when infections could not be treated.

Aim & and outline of the thesis

Differences in prevalence of resistance between and within countries and between different populations affect cross border movement and pose a challenge for medical doctors for appropriate empiric antibiotic treatment. Also, patients crossing the border can potentially carry bacteria with a variety of antibiotic resistance genes and might contribute to the spread of resistant strains.
The prevalence of antibiotic resistance can be determined for a wide range of micro-organisms but the main focus of this thesis was to evaluate the antibiotic resistance of two potential pathogenic commensals in a cross border region: 1) *E. coli*, the most prevalent causative agent of UTIs, 2) *S. aureus*, a primary cause of skin and soft tissue infections. Both types of infections are amongst the most common bacterial infections especially among GP patients and NH residents.
The aims of this thesis were to assess the prevalence and spread of resistance among *S. aureus* and *E. coli* isolated from the community, nursing home residents and hospitalized patients in the border region "the Euregion Meuse-Rhine". Antibiotic resistance surveillance studies provide important information on the prevalence and spread of antibiotic resistance and are necessary for the selection of empirical therapy and infection control policies at a local level.

In **Chapter 1**, the general introduction, the characteristics of *E. coli*, *S. aureus* and *K. pneumoniae* will be discussed. Also, several antibiotic agents, resistance mechanisms and spread of resistance are reviewed. The risk factors of resistance and the impact of a cross border region are described. Finally, different typing methods are discussed.
The prevalence of resistance of *E. coli* isolates collected from patients attending the urology services in the Euregion Meuse-Rhine is analyzed and discussed in **chapter 2**. These are patients, often with recurrent and complicated urinary tract infections. Due to the relatively high use of a small number of antibiotics among those patients, the resistance is expected to be higher than among GP patients.
The nursing home residents are another population where the prevalence of antibiotic resistance is assumed to be relatively high. They are vulnerable and often have several risk factors for the acquisition of a resistant strain. The antibiotic resistance of *E. coli* and the antibiotic use among nursing home residents in Limburg, a province in the Southern part of the Netherlands, are discussed in **Chapter 3**.
The spread of resistant strains among different populations and across the border is a point of concern. This spread is also a potential threat for acquiring an infection with such a resistant strain. Therefore, the spread of ESBL producing and other multi drug resistant *E. coli* isolates collected from four patient populations (GP patients, NH residents, Urology and ICU patients) in the entire Euregion Meuse-Rhine was investigated with MLST and PFGE (**Chapter 4**).
Nursing home residents are often regarded a reservoir for MRSA and other multi drug resistant strains, therefore, **Chapter 5** describes the resistance of *S. aureus* including the prevalence of MRSA among nursing home residents in the province of Limburg, the Netherlands and the regions of Euskirchen and Daun, Germany.

Cross border health care and the potential threat of difference in antibiotic resistance for a country with low resistance, such as the Netherlands, is often discussed. In **Chapter 6** the potential influence of the higher prevalence of resistance in Belgium and Germany on the border region of Limburg was determined by comparing the antibiotic resistance and population structure of *S. aureus* in the province of Limburg with the other provinces in the Netherlands.
In **Chapter 7** the data on the antibiotic resistance, the prevalence of ESBL producing isolates and the implications on empiric treatment for *K. pneumoniae* isolated from urology and ICU patients over a 12 year period are presented. These bacteria are, even more than *E. coli*, known for their multi drug resistance.
In **Chapter 8**, the results of the previous chapters together with implications for antibiotic treatment, future perspectives and additional recommendations will be discussed.

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Chapter 2

Antimicrobial resistance and spread of multi drug resistant *Escherichia coli* isolates collected from nine urology services in the Euregion Meuse-Rhine

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Abstract

We determined the prevalence and spread of antibiotic resistance and the characteristics of ESBL producing and/or multi drug resistant (MDR) *Escherichia coli* isolates collected from urine samples from urology services in the Euregio Meuse-Rhine, the border region of the Netherlands (n=176), Belgium (n=126) and Germay (n=119).

Significant differences in resistance between the three regions were observed. Amoxicillin-clavulanic acid resistance ranged from 24% in the Netherlands to 39% in Belgium (p=0.018), from 20% to 40% (p<0.004) for the fluoroquinolones and from 20% to 40% (p=0.018) for the folate antagonists. Resistance to nitrofurantoin was less than 5%. The prevalence of ESBL producing isolates varied from 2% among the Dutch isolates to 8% among the German ones (p=0.012) and were mainly CTX-M 15. The prevalence of MDR isolates among the Dutch, German and Belgian isolates was 11%, 17% and 27%, respectively (p<=0.001 for the Belgian compared with the Dutch isolates). The majority of the MDR and ESBL producing isolates belonged to ST131.

This study indicates that most antibiotics used as first choice oral empiric treatment for UTIs (amoxicillin-clavulanic acid, fluoroquinolones and folate antagonists) are not appropriate for this purpose and that MDR strains such as CTX-M producing ST131 have spread in the entire Euregion. Our data stress the importance of ward specific surveillance to optimize empiric treatment. Also, prudent use of antibiotics and further research to alternative agents are warranted.

Introduction

The increase of antimicrobial resistance is a major concern worldwide. Surveillance studies showed an increase in resistance for both Gram positive and Gram negative bacteria[1], including *Escherichia coli*, the most prevalent causative agent of urinary tract infections (UTIs)[1-4]. Moreover, the prevalence of extended spectrum beta-lactamase (ESBL) and/or carbapenemase producing multi drug resistant *E. coli* clones increased not only in the hospitals but also in the general population[1-4]. For the treatment of UTIs antibiotics are often prescribed empirically. An incorrect empiric choice is, in combination with the high prevalence of UTIs, a serious risk factor for the increase of antibiotic resistance among uropathogenic *E. coli*[5] and favors the spread of resistant clones such as *E. coli* ST131[6, 7]. Differences in the level of antibiotic resistance and the prevalence of ESBL producing strains exist between health care institutions within and between countries[1, 8, 9]. Since micro-organisms do not recognize national borders, cross border spread is likely to occur. This is also the case in the Euregion Meuse-Rhine (EMR), the border region of the Netherlands, Belgium and Germany. This Euregion is a densely populated area with considerable cross-border movement and cooperation between health care institutes[10]. Differences in antibiotic resistance and antibiotic treatment protocols as wel as in infection control policies pose a serious risk for patient transfer between health care institutions within and between countries. Current data of prevalence of antibiotic resistance including the prevalence of ESBL producing *E. coli* strains will guide physicians in their choice of adequate empiric treatment. Since such ward specific resistance data for patients visiting the urology services are hardly available we conducted a surveillance to determine the prevalence of (multi drug) resistant *E. coli* isolates including ESBL producing strains collected from patients visiting a urology service in the Euregion Meuse-Rhine.

Materials and Methods

Ethics statement

All bacterial isolates in this study were collected and analysed anonymously. Therefore, consent from the patient was not required and ethical approval was waivered. This is in agreement with the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies and the policy of the Medical Ethics Committee of het Maastricht University Medical Centre (MUMC).

Bacterial Isolates

During a 6 month collection period between 2009 and 2011 unique, unrelated, non duplicate, consecutive isolates from urine samples from patients attending the urology services were collected

A total of 421 *E. coli* isolates were included in this collection: 176, 126 and 119 from the Dutch, Belgian and German urology services, respectively. The following hospitals participated in the study: MUMC – Maastricht, Atrium Medical Centre – Heerlen and

Viecuri Medical Centre – Venlo in the Netherlands, General Hospital Vesalius – Tongeren, Jessa Hospital – Hasselt and Centre Hospitalier Universitaire – Liège in Belgium, and Hermann-Josef Hospital – Erkelenz, St. Antonius Hospital – Eschweiler and St. Marien Hospital – Düren in Germany. Only one isolate per patient was included. Clinical data were not available. The isolates were collected and identified at the local laboratories according to standard microbiological methods, stored and sent to the MUMC for susceptibility testing.

Antibiotic susceptibility testing

Quantitative susceptibility testing was performed using a broth microdilution with Mueller-Hinton II cation-adjusted broth (Becton-Dickinson, Sparks, MD, USA) and micro titre plates with freeze-dried antibiotics (MCS Diagnostics BV, Swalmen, the Netherlands). The minimal inhibitory concentration (MIC) was defined as the lowest concentration showing no growth after 18 hours of incubation at 35ºC. *E. coli* ATCC 35218 and ATCC 25922 were used as control strains. The MIC data were analysed using breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[11]. Intermediate results were considered resistant. Multi drug resistance (MDR) was defined as resistance to three or more classes of antibiotics excluding the broad spectrum penicillins without a beta-lactamase inhibitor.

Molecular characterization of beta-lactamases

Putative ESBL producing isolates (i.e. MIC for cefotaxime or ceftazidime ≥2mg/L) were confirmed as an ESBL producer with a combination disk diffusion test[12] and characterized for the presence of TEM, SHV and/or CTX-M beta-lactamases with a micro-array (Check-points, Wageningen, the Netherlands). Based on these results bla_{TEM} or $bla_{CTX\text{-}M}$ were amplified with PCR and specific primers[13, 14]. After purification (Spin PCRapace, Invitek, Berlin, Germany) automated sequencing was performed with the 3730 DNA analyzer with BigDye Terminator v1.1 (Applied Biosystems, Forster City, CA, USA).

Pulsed field gel electrophoresis (PFGE) and multi locus sequence tying (MLST)

All MDR isolates and ESBL producing isolates were further analyzed with PFGE[15]. All isolates with a indistinguishable or closely related pulsotype[16] and all ESBL producing isolates were analyzed with multi locus sequence typing (MLST) using the scheme specified at the University College of Cork *E. coli* MLST web site[17].

Empiric treatment

The appropriateness of amoxicillin-clavulanic acid, piperacillin-tazobactam, cefuroxime, ceftazidime, cefixime, ceftibuten, ciprofloxacin, nitrofurantoin, trimethoprim-sulfamethoxazole and gentamicin for empiric treatment was determined. These agents are considered representatives to other antibiotics in their class. As criterion for appropriateness of empiric treatment for complicated UTI, a 10% resistance cutoff value was used[18]. If the prevalence of resistance for a specific agent was higher than 10%, this agent was considered not appropriate choice for empiric treatment.

Statistical analysis

A Pearson's chi square test or Fisher's exact test was performed to determine statistically significant differences of resistance between the three different countries (subregions) of the Euregion Meuse-Rhine (PASW software, version 18.0, IBM, Armonk, NY, USA). A modified false discovery rate (FDR) method developed by Benjamini and Yekutieli was used as correction for multiple testing[19]. A p-value <0.05 was considered statistically significant.

Results

Significant differences in prevalence of antibiotic resistance between the three regions in the Euregion were found for several antimicrobial agents tested including the fluoroquinolones, and folate antagonists (Table 1).

Table 1: Antimicrobial resistance among (%) *E. coli* isolates

Antibiotic agent	NL n=176	B n=126	G n=119	NLvsB p-value	NLvsG p-value	BvsG p-value
Amoxicillin	48	60	49	-	-	-
Amoxicillin-clavulanic acid	24	39	27	0.018	-	-
Piperacillin	43	56	50	-	-	-
Piperacillin-tazobactam	3	9	1	-	-	0.014
Cefuroxime	5	17	10	<0.004	-	-
Cefotaxime	3	10	8	-	-	-
Ceftazidime	3	10	7	-	-	-
Cefixime	7	13	8	-	-	-
Ceftibuten	5	8	5	-	-	-
Cefepime	2	7	5	-	-	-
Ciprofloxacin	20	37	29	0.007	-	-
Norfloxacin	24	44	33	<0.004	-	-
Levofloxacin	19	37	29	<0.004	-	-
Moxifloxacin	20	39	32	<0.004	-	-
Nitrofurantoin	5	5	2	-	-	-
Trimethoprim	22	39	32	0.011	-	-
Trimethoprim-sulfamethoxazole	21	36	31	0.018	-	-
Amikacin	0	4	0	0.043	-	-
Gentamicin	5	6	12	-	-	-
Tobramycin	6	9	13	-	-	-

NL = the Netherlands, B = Belgium and G = Germany, - = not significant

Overall, resistance was highest among the Belgian isolates and lowest among the Dutch strains. Amoxicillin-clavulanic acid resistance ranged from 24% in the Netherlands to 39% in Belgium (p=0.018). Resistance to piperacillin-tazobactam was still low (1-9%) with the highest prevalence of resistance among the Belgian isolates (p=0.014). Among the Belgian isolates resistance to cefuroxime, cefotaxime, ceftazidime was higher compared with resistance among the Dutch isolates (p<0.004, p=0.097 and p=0.097, respectively). The prevalence of resistance to the fluoroquinolones ranged from 20%

among the Dutch isolates to 40% among the Belgian ones (p<=0.007). Significant difference in resistance between the Dutch isolates (20%) and Belgian isolates (40%, p=0.018) was observed for the folate antagonists. Resistance to the carbapenems was not demonstrated.
Putative ESBL producing isolates were found in 10, 14 and 9 isolates among the Dutch, Belgian and German isolates, respectively. ESBL production was confirmed for 20 isolates (i.e. 3, 8 and 9) resulting in a prevalence of ESBL producing isolates of 1.7%, 5.5% and 7.6% among the Dutch, Belgian and German isolates, respectively (p=0.043 for the Dutch and German isolates). The confirmed ESBLs were mainly CTX-M 15 (Table 2).

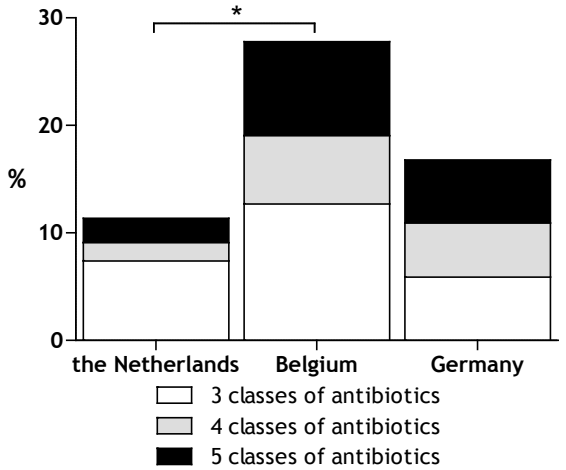
Table 2: Number and identification of ESBL producing isolates

	Netherlands	Belgium	Germany
CTX-M 1	0	2	1
CTX-M 14	1	1	0
CTX-M 15	2	1	8
CTX-M 55/79	0	2	0
TEM 52	0	1	0
Total	**3 (1.7%)**	**7 (5.5%)**	**9 (7.6%)***

* Significantly higher than among isolates from the Netherlands (p<0.05)

MDR was observed in 74 out of 421 isolates. The prevalence of the MDR isolates ranged from 11% (n=20) in the Dutch subregion to 27% (n=34) in the Belgian subregion (p<=0.001) (Figure 1).

Figure 1: Prevalence of multi drug resistance of *E. coli*



* = p<0.05, ** = p<=0.001

The PFGE pulsotypes of 24 isolates of these 74 MDR isolates were found more than ones. *E. coli* ST 131 strain was the most prevalent one (63%) of which 53% was an ESBL producer divided over 3 different pulsotypes (A, C and D, Table 3). This ST was demonstrated in all three subregions. The second most prevalent pulsotype (B) (13%) consisted of 2 STs, where ST1394 is a single nucleotide polymorphism of ST393. Among the ESBL producing isolates ST131 was the also the most prevalent ST (47%).
Only pulsotype A was demonstrated in all three subregions. The other pulsotypes were only observed in one subregion.

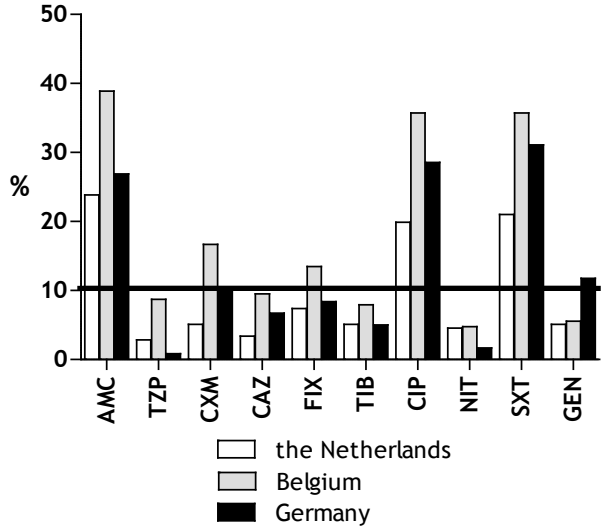
For empiric treatment of a complicated UTI, the prevalence of resistance should not exceed 10%. Taken into account this cutoff value several antibiotic agents are no longer appropriate for (oral) empiric treatment (Figure 2). These include amoxicillin-clavulanic acid, the fluoroquinolones and the folate antagonist in all three subregions and also some cephalosporines in the Belgian subregion. However, piperacillin-tazobactam and several third generation cephalosporines are still appropriate, which applies also for nitrofuratoin, although its pharmacokinetic properties do not support its use for a complicated UTI.

Table 3: Pulsotypes and STs of multi drug resistant and/or ESBL producing isolates

Pulsotype	ST (CC)	n	n ESBL	ESBL Types
A	131	10	5	CTX-M 14, 15
B	393 (31)	2	0	
	1394	1	0	
C	131	3	1	CTX-M 15
D	131	2	2	CTX-M 15
E	624	2	0	
F	162 (469)	2	2	CTX-M 55/79
G	88 (23)	2	0	
ESBL singletons	10 (10)	1		CTX-M 1
	744 (10)	1		CTX-M 1
	131	1		CTX-M 15
	23 (23)	1		TEM 52
	88 (23)	1		CTX-M 1
	224	1		CTX-M 15
	964 (405)	1		CTX-M 15
	2509	1		CTX-M 14
	648	1		CTX-M 15

ESBL singletons: ESBL strains with a pulsotype of which no similar type has been demonstrated among the other typed isolates.
ST = sequence type, CC = clonal complex, n = amount

Figure 2: Antibiotic resistance and suitability of antibiotics for empiric treatment



Antibiotic resistance for the different antibiotics among the three groups of isolates compared with a 10% resistance cutoff to decide whether an antibiotic agent is suitable for empiric treatment. AMC = amoxicillin-clavulanic acid, TZP = piperacillin-tazobactam, CXM = cefuroxime, CAZ = ceftazidime, FIX = cefixime, TIB = ceftibuten, CIP = ciprofloxacin, NIT = nitrofurantoin, SXT = trimethoprim-sulfamethoxazole and GEN = gentamicin

Discussion

This study provides current data of the antimicrobial resistance of *E. coli* isolates collected from nine urology services in the Euregio Meuse-Rhine. We found significant differences in resistance between the Dutch, Belgian and German isolates. Although, overall the prevalence of resistance was highest among the Belgian isolates and lowest among the Dutch ones, the prevalence of ESBL producing isolates was highest among the German isolates.

More importantly, this study also indicates that most antibiotics used as first choice oral empiric treatment for UTIs (amoxicillin-clavulanic acid, fluoroquinolones, trimethoprim-sulfamethoxazole)[20] are not appropriate for this purpose since the prevalence of resistance exceeds 10%[18]. Previously, it has been demonstrated that the prevalence of antimicrobial resistance from invasive clinical isolates was higher in Belgium and Germany than in the Netherlands[1]. but has, to our knowledge, not been described for isolates from the urology services. This study also demonstrates that the globally emerging *E. coli* ST131[6, 7, 21, 22] was prevalent in this entire population and also frequently produced an ESBL.

The *E. coli* isolates were collected from urine samples from patients of both gender and all ages visiting the urology services in the participating hospitals of this cross border multi centre surveillance study to prevent selection bias. Since all isolates were tested in one centre, the results were not influenced by differences in methodology.

Unfortunately, additional patient and clinical data were not available. Since we cannot specify the underlying illness of the patients, this should be taken into consideration when initiating empiric treatment.

Comparing the resistance rates of *E. coli* from the EARSS annual report 2009, which consist mainly of isolates from invasive disease[1], and this study, the prevalence of resistance was comparable, except for resistance to the fluoroquinolones. This was higher among the urology isolates (11% vs. 19-20% for the Dutch isolates, 20% vs. 37-45% for the Belgian isolates and 23% vs. 29-33% for the German isolates, Table 1). These differences might be related to the high prescribing rate of fluoroquinolones at the urology services[8, 23]

Moreover, differences in antibiotic use[24] are also the most likely reason for the differences in resistance between the three countries in this study, although, differences in patient population can also play a role.

Compared with prevalence of resistance among general practice (GP) patients[2, 3, 9, 25] the resistance in this study was much higher. This is probably due to the more frequent antibiotic use among urology patients compared with GP patients, the higher prevalence of urinary tract comorbidities and the use of catheters, which might select for resistant strains[5, 26, 27]. Compared to a previous study among urology isolates the Dutch isolates in the present study showed a lower prevalence of resistance for the folic acid antagonists and higher resistance for the quinolones[8]. Taken into account that only antimicrobial agents with a resistance level of 10% or less are suitable for empiric therapy[18], fluoroquinolones, broad spectrum penicillins and folate antagonist are not an appropriate choice. This applies for the whole Euregion. Alternatives agents are limited and include nitrofurantoin, which is not used for complicated UTIs because of poor tissue penetration[28], piperacillin-tazobactam, which cannot be administered orally, and the third generation cephalosporines. However, high use of cephalosporines could increase the selection and persistence of ESBL producers and should be kept to a minimum[29]. Therefore, further research to potential alternatives, such as fosfomycin, is essential.

During the last years (multi drug) resistant strains have become a major healthissue. National and international surveys show an increase in nosocomial and community acquired infections involving ESBL and/or carbapenemase producing isolates[1, 2, 30, 31]. Also, globally the MDR and often ESBL producing *E. coli* ST131 clone is emerging[6, 7, 21, 22]. An infection with these isolates can often not be treated with penicillins, cephalosporines, fluoroquinoles and folate antagonists[20, 32]. Consequently, adequate antibiotic therapy is delayed because of inadequate empirical therapy, which affects the patient outcome negatively[33]. In this study the percentage of ESBL producing isolates was less than 8% in all three subregions, with the lowest prevalence of ESBL producers among the Dutch isolates. Most ESBL producers contained the ESBL type CTX-M, which is prevalent[21, 22, 29]. The high prevalence of ST131 among the tested isolates is a point of concern, since this clone can easily acquire more resistance traits[6, 7, 21] and will become a major health risk since it is infesting itself in our population. However, current prevalences of (multi drug) resistance do not justify alteration of the treatment protocols to empiric therapy with carbapenems. Nevertheless, continuous ward specific surveillance is necessary to monitor changes in

antibiotic resistance MDR strains. Moreover, further research is indicated to find alternative antibiotic agents of which fosfomycin is a possibility. This agent has high tissue penetration, maintains active against ESBL producing isolates[34], but is until now not registered for complicated UTI. Therefore, further research into the usefullness of fosfomycin for treatment of this type of infection is warranted.

Concluding, ward specific surveillance of antimicrobial resistance is important to monitor resistance over time on a regional, national and international level since we demonstrated significant differences in antibiotic susceptibility between the three subregions in the Euregion Meuse-Rhine. We also found a high prevalence of *E.coli* ST131 and CTX-M type ESBLs, suggesting a spread of this clone in the entire Euregion.

Due to the high prevalence of resistance many antibiotics including amoxicillin-clavulanic acid, the fluoroquinolones and the folate antagonists are no longer suitable for empiric treatment of complicated UTI, therefore, further research to other possible agents is needed.

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Chapter 3

Prevalence and spread of multi drug resistant *Escherichia coli* isolates among nursing home residents in the southern part of the Netherlands

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Abstract

Objectives
Empiric antibiotic treatment should be based on recent surveillance data. Therefore, we conducted a surveillance of (multi drug) resistance of *Escherichia coli* and antibiotic use among Dutch nursing home (NH) residents. Pulsed-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) were used to describe the spread of multi drug resistant strains.
Desgin
Observational study
Setting
Five NHs in the southern part of the Netherlands
Participants
337 NH residents from both somatic and psycho-geriatric wards
Measurements
The prevalence and spread of antibiotic resistance and multi drug resistant *E. coli* isolates collected from urine samples and antibiotic use among the NH residents were investigated.
Results
A total of 208 *E. coli* isolates were collected from 308 urine samples.
Resistance to amoxicillin-clavulanic acid was 23% and resistance to ciprofloxacin was 16%. Resistance to trimethoprim-sulfamethoxazole was 19%, while nitrofurantoin resistance was less than 1%. Multi drug resistance was observed in 28 of the 208 isolates (13%). Several isolates showed a similar PFGE pulsotype and MLST type. ST131 was the most prevalent (48%) and was demonstrated in all NHs and with four different pulsotypes. Consumption of antibiotics for systemic use was 64.4 DDD/1000 residents/day. Amoxicillin-clavulanic acid was most frequently prescribed (20.92 DDD/1000 residents/day), followed by the quinolones (14.8 DDD/1000 residents/day).
Conclusion
We observed a high prevalence of antibiotic resistance and antibiotic use. In particular, the use of and resistance to fluoroquinolones is concerning. Due to the high prevalence of resistance many agents are no longer suitable for empiric treatment. *E. coli* ST131, which has also been demonstrated in this study, poses a potential risk to this vulnerable population.
We have clearly demonstrated that the resistance among NH residents is different from elderly living at home and hospitalized patients and with the emergence of resistant strains, such as ST131, NHs are a potential reservoir for multi drug resistant bacteria.

Introduction

Urinary tract infections (UTIs) are one of the most prevalent bacterial infections among nursing home (NH) residents[1]. This can vary from an asymptomatic or relatively mild cystitis to urosepsis and can have severe consequences for the residents overall condition[2, 3].
UTIs are a common indication for empiric antimicrobial treatment[4]. Approximately 20-60% of all used systemic antibiotics among NH residents are prescribed for a UTI[5]. Optimal empiric antibiotic treatment should be based on recent surveillance data. Unfortunately, for nursing homes these data are hardly available and the choice for empiric treatment is usually based on surveillance data from hospitals. This might result in a relatively high use of inappropriately prescribed broad-spectrum antibiotics, of which especially the use of fluoroquinolones is a point of concern[4]. These agents are often used due to their favourable pharmacokinetics and antibacterial spectrum, but resistance can be rapidly acquired, limiting the use of these antimicrobials[6]. Overall, the high use of antibiotics contributes to an increase of antimicrobial resistance and a more prevalent carriage of extended spectrum beta-lactamases (ESBLs)[7, 8], such as the multi drug resistant ESBL producing *Escherichia coli* sequence type (ST) 131[9, 10]. Although, antibiotic use is considered as one of the main risk factors for emergence of and colonization with antibiotic resistant isolates[7], other risk factors including poor functional status, presence of wounds or foreign materials are also very prevalent among NH residents[5, 11, 12]. Infections with (multi drug) resistant pathogens have been associated with higher morbidity, mortality and costs due to delayed adequate antibiotic treatment[13, 14].
UTIs are often caused by the resident's own commensal microorganisms[15, 16]. Therefore, actual data of resistance of the commensal flora will guide physicians in making an appropriate choice for empiric therapy. Since current resistance data for *E. coli*, a commensal and the most prevalent causative agent of UTIs[17, 18], are not available for NH residents in the Netherlands, we performed a surveillance in five NHs in the southern part of the Netherlands. Moreover, spread of multi drug resistant isolates, such as ST131, between and within NHs was investigated with multi locus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE).

Methods

Five NHs in the province of Limburg in the southern part of the Netherlands agreed to participate in this project. All psycho-geriatric and somatic residents of these NHs were eligible for participation but only those residents who signed a consent form (either by themselves or their legal representative) were included in the study.
This study was approved by the medical ethics committee of the Maastricht University Medical Centre (MUMC). We received a total of 336 consent forms (Table 1). Due to withdrawal of consent, faecal incontinence, moving or death of a resident, 29 residents could not be sampled. In each NH the samples were collected in one week in the period between February 2010 and June 2011.

Bacterial isolates

Urine samples were collected from all included, asymptomatic residents and used to inoculate a uricult (Biomérieux, Marcy l'Etoile, France) A uricult or dipslide is a semi quantitative microbial culture method, which has a two sided paddle in a protective vial with on one side a cystine lactose electrolyte deficient (CLED) agar and on the other side a MacConkey agar.

If a resident suffered from urine incontinence the uricult was inoculated by pressing the paddle onto the incontinence pads. The inoculated uricults were sent to the laboratory at the MUMC and incubated at 35ºC for 18 hours. The putative *E. coli* colonies were identified using standard microbiological methods. Samples were analysed anonymously, therefore, clinical data were not available.

Quantitative susceptibility testing

Antimicrobial susceptibility testing was performed using a broth microdilution with Mueller-Hinton II cation-adjusted broth (Becton-Dickinson, Sparks, MD, USA) and micro titre plates with freeze-dried antibiotics (MCS Diagnostics BV, Swalmen, the Netherlands). *E. coli* ATCC 35218 and ATCC 25922 were used as control strains. The following antimicrobial agents were tested: amoxicillin, amoxicillin-clavulanic acid, ciprofloxacin, gentamicin, nitrofurantoin, norfloxacin, trimethoprim and trimethoprim-sulfamethoxazole. The minimal inhibitory concentration (MIC) data were analysed using clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[19]. Amoxicillin-clavulanic acid resistant isolates were further tested for resistance to ceftazidime and ESBL production was confirmed for isolates with a ceftazidime MIC ≥2mg/L using a combination disk diffusion test according to guidelines of the Dutch society for medical microbiology[20]. Multi drug resistance was defined as resistance to three or more of the tested antimicrobial classes.

Molecular characterization

The ESBL positive isolates were further analyzed for the presence of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ with PCR and specific primers[21-24]. Automated sequencing was performed with the 3730 DNA analyzer with BigDye Terminator v1.1 (Applied Biosystems, Forster City, CA, USA). All multi drug resistant isolates and ESBL producers were analyzed with PFGE[25] and MLST[26, 27].

Use of antimicrobial agents

Data on the use of antibiotics were collected from each NH from the in the period of one year prior to the collection of the urine samples. These data were collected anonymously and could not be linked to an individual resident. These data were collected using the ATC/DDD classification protocol as defined by the WHO Collaborating Centre for Drug Statistics Methodology[28]. The defined daily dose (DDD) is the assumed average dose per day for a drug used for its main indication in adults. In this study the DDD are expressed as DDD per 1000 residents per day. The DDD is a unit of measurement and does not necessarily reflect the recommended or prescribed daily dose[28].

Statistical analysis

To determine significant differences in prevalence of resistance, a Pearson's chi-square or Fisher's exact test was performed (PASW-software 18.0, IBM, Armonk, NY, USA). A p-value <0.05 was considered statistically significant.

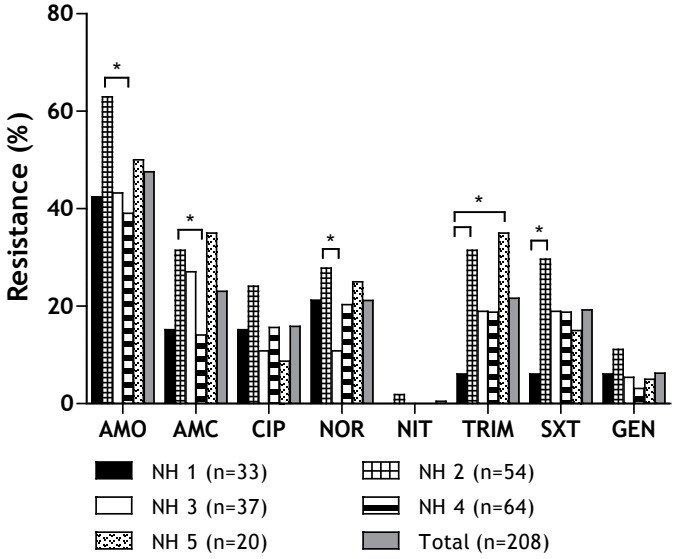
Results

In total 208 *E. coli* isolates were collected ranging from 20-64 isolates per NH from 308 urine samples from residents of the five NHs (Table 1).

Table 1: Overview of included residents and collected samples per nursing home

Nursing home	Eligible residents	Included residents (%)	Collected samples	Number of *E. coli* isolates
1	130	40 (31)	38	33
2	180	84 (47)	70	54
3	329	56 (17)	55	37
4	282	109 (39)	103	64
5	104	51 (49)	42	20
Total	**1025**	**337 (33)**	**308**	**208**

Figure 1: Antibiotic resistance of *E. coli* in the five nursing homes



AMO = amoxicillin, AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, NOR = norfloxacin, NIT = nitrofurantoin, TRIM = trimethoprim, SXT = trimethoprim-sulfamethoxazole, GEN = gentamicin, NH = nursing home, * = p<0.05

The prevalence of resistance is shown in Figure 1. Amoxicillin-clavulanic acid resistance varied from 14% to 31% (p=0.023). Ciprofloxacin ranged from 9% to 24% (p=0.056) and resistance to norfloxacin ranged from 11% to 28% (p=0.042). Resistance to trimethoprim ranged from 6% to 35% (p=0.010) and from 6% to 30% (p=0.008) for trimethoprim-sulfamethoxazole. Nitrofurantoin resistance was less than 1%. Overall resistance was highest among residents from NH2. ESBL production was observed in 1 isolate from NH4 (<1%) which was ST38 and CTX-M 15 positive.

Multi drug resistance was observed in 28 of the 208 isolates (13%) and ranged from 9% in NH4 to 22% in NH2. All of these isolates were resistant to norfloxacin and amoxicillin. 68% was resistant to ciprofloxacin and 43% to amoxicillin-clavulanic acid and 86% to the folate antagonists (Table 2). Among these isolates ST131 was the most prevalent ST (n=14, 50%) and was observed in each NH but with three different pulsotypes each of which was present in one or three NHs. ST69 (n=5) was demonstrated in three NHs. The other STs had only one pulsotype and were demonstrated in one NH only (Figure 2).

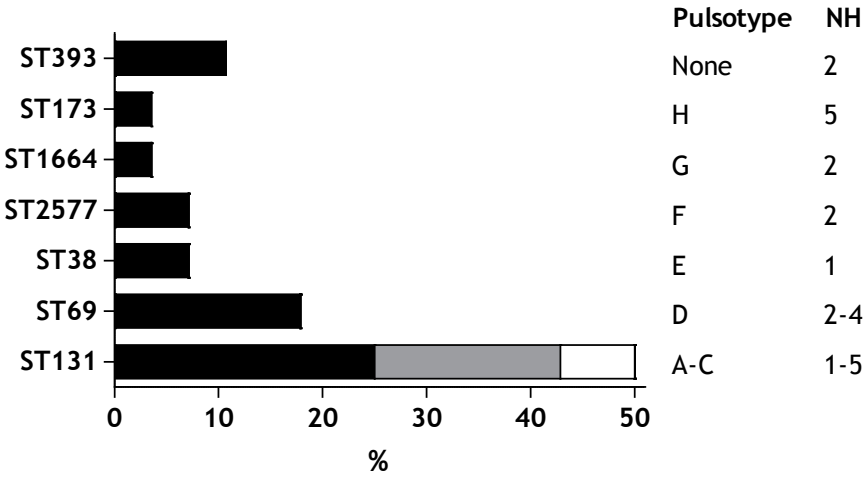
Table 2: Overview of the multi drug resistant isolates

Antibiotic resistance combinations	n
AMO/NOR/GEN	2
AMO/NOR/TRIM/SXT	2
AMO/NOR/GEN/TRIM/SXT	2
AMO/CIP/NOR/TRIM/SXT	4
AMO/CIP/NOR/GEN/TRIM/SXT	6
AMO/AMC/NOR/TRIM	1
AMO/AMC/NOR/TRIM/SXT	2
AMO/AMC/CIP/NOR/GEN	2
AMO/AMC/CIP/NOR/TRIM/SXT	6
AMO/AMC/CIP/NOR/GEN/TRIM/SXT	1
Total	**28**

This table shows to which antibiotics the multi drug resistant isolates were resistant
AMO = amoxicillin, AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, NOR = norfloxacin, TRIM = trimethoprim, SXT = trimethoprim-sulfamethoxazole, GEN = gentamicin, n = number of isolates.

The average consumption of antibiotics for systemic use in the five NHs was 64.4 DDD per 1000 residents per day ranging from 52.81 to 81.56 DDD/1000 residents/day (Table 3). Antibiotics in class J01CR were most frequently prescribed (20.92 DDD/1000 residents/day), followed by the quinolones (14.8 DDD/1000 residents/day) and tetracyclines (11.13 DDD/1000 residents/day). Antibiotic use of antimicrobials in class J01C ranged from 21.6 to 41.4 DDD/1000 residents/day, 81% of antibiotic use in this class could be attributed to the use of amoxicillin-clavulanic acid. Antibiotic use of antimicrobials in class J01E ranged from 1.4 to 5.9 DDD/1000 residents/day. The majority (i.e. 71%) could be attributed to the use of trimethoprim, which is solely prescribed for the treatment of UTI. Use of antimicrobials in class J01M, mainly ciprofloxacin and norfloxacin, ranged from 9.8 to 23.4 DDD/1000 residents/day. Antibiotic use of antimicrobials in class J01X ranged from 1.6 to 12.2 DDD/1000 residents/day of which 98% was nitrofurantoin.

Figure 2: Spread of isolates with related pulsotype and MLST sequence among the nursing homes



This figure describes the distribution of ST among the multi drug resistant isolates, the number of pulsotypes per ST and in which NHs these STs were observed. ST = sequence type, NH = nursing home

Table 3: Consumption of antibiotics for systemic use in DDD/1000 residents/day

ATC group	Therapeutic group	NH 1 n=130	NH 2 n=180	NH 3 n=329	NH 4 n=282	NH 5 n=104	Total
J01AA	Tetracyclines	4.87	6.74	11.08	20.97	0.08	11.13
J01CA	Penicillins with extended spectrum	0.94	0.34	3.93	0.14	2.06	1.69
J01CE	Beta-lactamase sensitive penicillins	4.68	2.99	0.12	0.00	0.33	1.19
J01CF	Beta-lactamase resistant penicillins	6.94	1.07	2.27	0.51	0.70	2.01
J01CR	Combination of penicillins incl. beta-lactamase inhibitors	19.86	17.50	18.01	20.59	38.28	20.92
J01DC	2nd gen. cephalosporines	3.31	2.05	0.00	0.00	0.00	0.78
J01DD	3rd gen. cephalosporines	0.00	0.00	0.12	1.84	1.52	0.67
J01EA	Trimethoprim and derivates	1.98	0.10	3.24	0.09	11.74	2.53
J01EE	Combinations of sulfonamides and trimethoprim incl derivates	3.90	1.61	0.83	1.28	0.00	1.40
J01FA	Macrolides	0.00	0.34	0.23	1.38	0.49	0.56
J01FF	Lincosamides	0.20	1.48	0.67	3.06	2.13	1.56
J01MA	Fluoroquinolones	9.83	14.25	10.61	23.37	12.00	14.80
J01XE	Nitrofuran derivates	2.50	9.82	1.59	4.47	12.22	5.02
J01	Antibacterials for systemic use	59.02	58.94	52.81	77.60	81.56	64.41

DDD = defined daily dose, ATC = anatomical therapeutic chemical classification system

Discussion

This study provides the current situation of antibiotic resistance and spread of multi drug resistant *E. coli* isolates in NHs in the southern part of the Netherlands. Resistance to the folate antagonists, amoxicillin-clavulanic acid and fluoroquinolones was mostly more than 20%, and these agents are, therefore, no longer suitable for empiric treatment of UTI[29]. The only exception is nitrofurantoin since resistance was only sporadically observed. Multi drug resistance was demonstrated among 13% of the isolates of which ST131 was the most prevalent type. This ST was observed in all NHs. The antibiotics most often prescribed were penicillins in combination with a beta-lactamase inhibitor and fluoroquinolones, which is in line with the high prevalence of resistance to these agents.

Bias of the results between the nursing homes due to differences in methodology was prevented by applying the same method of sampling and analysis of the urine samples for all NHs. Unfortunately, this study was performed anonymously and, therefore, detailed clinical data were not available and linkage of the antibiotic use to an individual resident was not possible.

An up to date overview of antibiotic resistance is necessary for a suitable empiric antibiotic treatment policy. However, if such an overview is not available other inappropriate data are used. Resistance data from (elderly) general practice (GP) patients were different from the data presented here and are, therefore, not suitable as basis for empiric antibiotic choice for NH residents. The prevalence of resistance to amoxicillin, amoxicillin-clavulanic acid and the quinolones was lower among elderly GP patients[30]. This suggests that age alone is not a risk factor for antibiotic resistance but that other factors contribute to increase of resistance. So, the difference in resistance between NH residents and GP patients might be due to the high use of antibiotics among the NH residents[31]. In particular, the high use of quinolones among NH residents compared with the GP patients[31], which is due to this agent's oral availability, favourable pharmacokinetics and the broad spectrum antibacterial activity. On the other hand, quinolone resistance was higher among the NH residents compared with hospitalized patients[31] while antibiotic use was higher among the hospitalized patients[31]. This suggests that other factors are also playing a role in the observed differences in resistance between the elderly GP patients and hospitalized patients, and the NH residents. Factors such as a chronic, worse physical status of the NH residents are also of importance. In turn, this could also explain the differences in antibiotic resistance between the participating NHs that cannot be attributed to differences in antibiotic use. Overall antibiotic use among the participating NH residents in this study could be ranked average/high compared with NH residents in other European countries[32].

With regard to initiation of empiric treatment with a specific antimicrobial agent, resistance should not exceed 20% for an uncomplicated UTI and should not exceed 10% for a complicated UTI[29]. Based on the resistance data, nitrofurantoin could be an appropriate agent for both uncomplicated and complicated UTI. However, since the tissue penetration of nitrofurantoin is very low[33], this agent is not suitable for the treatment of complicated UTI. Taken into account the 20% and 10% cutoff value,

amoxicillin-clavulanic acid, ciprofloxacin, norfloxacin, trimethoprim and trimethoprim-sulfamethoxazole are not appropriate anymore for treatment of complicated UTI but some agents might be appropriate for empiric treatment of uncomplicated UTI. This was, however, NH dependent. An alternative option for oral empiric treatment includes fosfomycin which, up till now, is not registered for the treatment of complicated UTI despite promising characteristics i.e. low resistance[30], high tissue penetration and activity against ESBL producing isolates[34].

Another point of concern is the global increase of resistance and spread of multi drug resistant, ESBL and/or carbapenemase producing strains. Infections with these bacteria are more difficult to treat as the number of adequate antimicrobial treatment options is limited. Globally, the *E. coli* ST131 strains are often multi drug resistant, produce ESBLs, have many virulence traits and cause infections such as, UTIs and bacteraemia[9, 10, 35]. The same applies for ST69[35, 36]. Both ST131 and ST69 strains were found among the NH residents in all participating NHs, as was ST405, another previously reported resistant ST[36]. A concerning property of these strains, especially ST131[9], is the high prevalence of resistance to the often prescribed fluoroquinolones, which was also demonstrated in this study. Fortunately, the resistant isolates observed in our population were no ESBL producers and they were also resistant to fewer antibiotics than other previously reported multi drug resistant strains[9]. We did demonstrate, however, that especially ST131 has spread into the NH population. This ST can also easily pick up plasmids with resistance traits and the number of plasmid relicons carrying resistance genes is high as is the number of antibiotic gene combinations on those plasmids[37].

Continuous surveillance to monitor this potential upcoming problem is important, as are prudent infection control measures and antibiotic use to prevent and control the spread of these antibiotic resistant strains. Maybe, the preventive use of cranberry tablets or lactobacilli could decrease empiric antibiotic use[38, 39].

Conclusion

In this study we observed a high prevalence of antibiotic resistance but a low prevalence of ESBL production among *E. coli* isolates collected from urine samples from NH residents. Antibiotic use was also relatively high. Especially the use of and the resistance to fluoroquinolones is a point of concern. Due to this prevalence of resistance many antibiotics including amoxicillin-clavulanic acid, the folate antagonists and the fluoroquinolones, are no longer suitable for empiric treatment and, therefore, oral treatment options have become limited. Moreover, *E. coli* STs globally emerging as multi drug resistant strains have also been demonstrated in this study and pose a potential risk to this vulnerable population, since the risk of treatment faillure would increase. We clearly demonstrated that the resistance among NH residents is different from elderly living at home and hospitalized patients and that with the emergence of multi drug resistant strains worldwide, such as ST131, NHs are a potential reservoir for antibiotic (multi drug) resistance. Therefore, continuous surveillance of antimicrobial resistance in nursing homes is needed to monitor resistance overtime and the increase

of multi drug resistant strains and should be continued to be able to adapt treatment protocol if necesary. Also, antibiotic treatment should be iniated with care and adequate infection control measures should be applied.

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Chapter 4

Prevalence and spread of multi drug resistant *E. coli* including ST131 in different patient populations in the Euregion Meuse-Rhine

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submitted

Abstract

Objective
To determine the prevalence and genetic background of resistant *E. coli* isolates collected from general practice patients, and nursing home residents, intensive care unit (ICU) and urology services patients in the Euregion Meuse-Rhine.
Methods
A total of 1651 *E. coli* isolates were collected from residents of the Euregion. Susceptibility testing was performed with broth microdilution. The genetic background was determined using PFGE and MLST, and analyzed with eBURST and clonal frame analysis.
Results
The prevalence of resistance varied significantly between the four populations. Amoxicillin-clavulanic acid resistance was highest among the ICU isolates (40%). Ciprofloxacin resistance was highest among the urology isolates (27%). Approximately 10% of the *E. coli* isolates was multi drug resistant and/or an ESBL producer. The most prevalent ESBL type was CTX-M 15. In total, 47 different STs were observed and ST131 was the most prevalent one. PFGE analysis of the ST131, 393 and 88 isolates suggested the spread of isolates belonging to the same PFGE group in the entire Euregion.
Conclusion
E. coli ST131 was the most prevalent ST in our Euregional study. The emergence of resistant strains might be a precursor for an increasing prevalence of resistance. To control the spread of these resistant strains adherence to infection control policies and implementation of an antibiotic stewardship program, based on the results of antibiotic resistance surveillance, is essential. In this way we could observe a shift in the prevalence of resistance and the genetic structure of the *E. coli* population and act accordingly.

Introduction

Since the introduction of antibiotics bacteria have developed ways to resist these agents, which urged for the development of new antibiotics. Now we have come to a time were there are even bacteria resistant to our last resort agents[1, 2]. The prevalence of these multi, extensively or pan drug resistant bacteria is increasing and they are spreading across the globe[3-6].

Previous research has shown that the spread of resistant bacteria is mostly within one health care network[7] and that health care institutions should not be considered as an individual centre but as parts of a network[8]. In a border region, such as the Euregion Meuse-Rhine, the region between Belgium, Germany and the Netherlands, we are confronted not only with the spread of resistant bacteria within the three countries, but also with cross border movement, including for health care purposes[9, 10]. This will facilitate the spread between the three countries and prevalence of resistant bacteria, since bacteria can potentially spread from one country to another.

Escherichia coli is part of the commensal gut microbiota and a prevalent cause of infections, in particular, urinary tract infections (UTI)[11]. It is well known that *E. coli* can carry a range of mobile genetic elements such as plasmids with different antibiotic resistance genes[12], such as *E. coli* with sequence type (ST) 131. This is a globally spreading clone often with many resistance and virulence genes[6] (including genes encoding for extended spectrum beta-lactamases (ESBL)).

UTI infections are often treated empirically. The first step for optimal empiric treatment is to make the right antibiotic choice: the right drug, dose, duration and minimize development of resistance[13]. This choice needs to be based on actual antibiotic resistance data phenotypically and preferably also genotypically. As the current situation in terms of genetic background of *E. coli* isolates and the potential spread of specific *E. coli* clones in the Euregion Meuse-Rhine is still unknown while this was investigated for other bacteria, such as *S. aureus*[14], we performed the present study. We determined the prevalence of (multi drug) resistant *E. coli* and the genetic background of these isolates collected from general practice (GP) patients, and nursing home (NH) residents, intensive care unit (ICU) and urology services (URO) patients in the Euregion. The isolates were characterized with quantitative susceptibility testing, multi locus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE). The obtained data will enable us to investigate the prevalence and spread of multi drug resistant (MDR) and/or extended spectrum beta-lactamase (ESBL) producing *E. coli* strains in the border region, the Euregion Meuse-Rhine.

Materials and Methods

Bacterial isolates and susceptibility testing

Between March 2009 and May 2012 a total of 1651 *E. coli* isolates were collected (Table 1) from urine samples from patients attending the URO services, GP patients and NH residents (colonization only for NH residents), and from various clinical samples from ICU patients. The samples were collected from 10 hospitals, 17 nursing

homes and 54 GPs in the Euregion Meuse-Rhine (i.e. in the Province of Limburg and the Province of Liège in Belgium, the Province of Limburg in the Netherlands and the Regions Aachen, Düren, Euskirchen, Heinsberg and Vulkaneifel in Germany).
The hospital samples and German GP samples were analyzed at the local laboratory, stored and sent to the Maastricht University Medical Centre (MUMC). The samples from the NH residents and the other GP samples were used to inoculate a dipslide (Biomérieux, Marcy l'Etoile, France) and were sent to the MUMC for isolation and identification. Quantitative susceptibility testing was performed in the MUMC using a broth microdilution with Mueller-Hinton II cation-adjusted broth (Becton-Dickinson, Sparks, MD, USA) and micro titre plates with freeze-dried antibiotics (MCS Diagnostics BV, Swalmen, the Netherlands). *E. coli* ATCC 35218 and ATCC 25922 were used as control strains. The minimal inhibitory concentration (MIC) breakpoints were defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[15]. MDR was defined as resistance to three or more of the tested antimicrobial classes: amoxicillin-clavulanic acid, fluoroquinolones, folate pathway antagonists, nitrofurantoin and gentamicin.
ESBL production among the URO and ICU isolates was confirmed for isolates with a ceftazidime and or cefotaxime MIC ≥2mg/L using a combination disk diffusion test according to the guidelines of the Dutch society for medical microbiology[16] and for the NH and GP isolates ESBL production was confirmed for isolates with an amoxicillin-clavulanic acid MIC ≥8mg/L.

ESBL characterization
The ESBL positive isolates were characterized for the presence of TEM, SHV and/or CTX-M beta-lactamases with a micro-array (Check-points, Wageningen, the Netherlands). Based on these results bla_{TEM}, bla_{SHV} or bla_{CTX-M} were amplified with PCR and specific primers[17-20].
Automated sequencing was performed with the 3730 DNA analyzer with BigDye Terminator v1.1 (Applied Biosystems, Forster City, CA, USA).

MLST
The MDR isolates and ESBL producers were analyzed with MLST using the scheme by Wirth et al.[21] specified at the University College of Cork *E. coli* MLST web site[22].
The Based Upon Related Sequence Types (eBURST) algorithm was used to analyse the allelic profiles and define clonal complexes[23]. The default settings of the eBURST V3 software (http://eburst.mlst.net/) were used.
Clonal frame analysis (CFA) software (version 1.1)[24] was used to construct a majority rule consensus tree. Ten runs were computed with the default settings (-x 50000, -y 50000, -z 100) after which a 50% consensus tree and network representations were constructed with the graphical user interface.

PFGE
Based upon the MLST results and the antibiotic resistance pattern, isolates belonging to ST393 and ST88, and a selection of the isolates belonging to ST131 were further analyzed with PFGE using the XbaI restriction enzyme[25]. For the ST131, at least 50% of

the isolates per population per country with one of three resistance patterns were selected for PFGE, which were: resistance to amoxicillin-clavulanic acid and ciprofloxacin combined with resistance to gentamicin and/or trimethoprim-sulfamethoxazole.
The PFGE profiles were analyzed with BioNumerics v.6.0 (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated from the cluster analysis of Dice similarity indices with 0.5 optimization and 2.0% tolerance based on the unweighted pair group method with arithmetic averages (UPGMA). Isolates were considered to belong to the same PFGE group if their Dice similarity index was ≥85%[26].

Statistical analysis
A Pearson's chi square test or Fisher's exact test was performed to determine statistically significant differences of resistance between the three different countries of the Euregion Meuse-Rhine and between the four different populations in this study (PASW-software, version 18.0, IBM, Armonk, NY, USA). A modified false discovery rate (FDR) method developed by Benjamini and Yekutieli was used as correction for multiple testing[27]. A p-value <0.05 was considered statistically significant. Diversity of the groups of isolates regarding the STs was determined with Simpson's Index of diversity[28].

Results

Bacterial isolates and antibiotic resistance
A total of 1651 E. coli isolate were collected (Table 1). The number of isolates per countries ranged from 488 to 639 isolates and the number of isolates per population category from 421-597 isolates except for the ICU isolates with a total of 180. Approximately 20% of those ICU isolates were collected from urine samples.

Table 1: The collected isolates per patient population and country

Unit	NL	B	G	Total
GP	184	194	75	453
NH	209	152	236	597
ICU	70	52	5	180
URO	176	126	119	421
Total	639	524	488	1651

NL = the Netherlands, B = Belgium, G = Germany, GP = general practice, NH = nursing home, ICU = intensive care unit, URO = urology services.

The prevalence of antibiotic resistance of the collected isolates is shown in Table 2. The prevalence of resistance was comparable for the three countries. Only for amoxicillin-clavulanic acid and ciprofloxacin the prevalence of resistance was significantly higher in Belgium than in the Netherlands i.e. 27% and 23% versus 21% and 16%, respectively. However, the prevalence of resistance varied significantly between the different populations. Overall the GP isolates showed the lowest prevalence of resistance. The ICU isolates had the highest prevalence of resistance for amoxicillin,

both alone and in combination with clavulanic acid (61% and 40%, respectively). Norfloxacin and ciprofloxacin resistance was significantly higher among the NH (27% and 22%), ICU (21% and 18%) and URO isolates (32% and 27%) compared with the GP isolates (12% and 8%). The prevalence of resistance to both folate pathway inhibitors was comparable among the GP (23% and 22%) and NH (22% and 20%) isolates and significantly higher among the ICU (33% and 30%) and URO (29% and 28%) isolates. The resistance to gentamicin and nitrofurantoin was low, the highest prevalence of resistance to these antibiotics was observed among the URO isolates, 7% and 4%, respectively.

Table 2: Prevalence of resistance in % among all collected isolates

Unit	NL	B	G	GP	NH	ICU	URO
AMO	46	50	43	39	43	61$^{+\dagger}$	49$^{+\dagger}$
AMC	21	27*	26	17	22	40$^{+\dagger\S}$	29$^{+\dagger}$
CIP	16	23*	20	8	22^{+}	18^{+}	27$^{+\ddagger}$
NOR	20	26	25	12	27^{+}	21^{+}	32$^{+\ddagger}$
TRIM	24	27	24	23	22	33$^{+\dagger}$	29†
SXT	22	26	23	22	20	30†	28†
GEN	5	6	8	4	6	6	7
NIT	2	1	0	0	0	0	4$^{+\dagger}$
Total (n)	639	524	488	453	597	180	421

NL = the Netherlands, B = Belgium, G = Germany, GP = general practice, NH = nursing home, ICU = intensive care unit, URO = urology services, AMO = amoxicillin, AMC = amoxicillin-clavulanic acid, CIP = ciprofloxacin, NOR = norfloxacin, GEN = gentamicin, TRIM = trimethoprim, SXT = trimethoprim-sulfamethoxazole, NIT = nitrofurantoin.

* = significantly higher compared with NL

$^{+}$ = significantly higher compared with GP, † = significantly higher compared with NH

‡ = significantly higher compared with ICU, § = significantly higher compared with URO

MDR and/or ESBL producing isolates

The overall prevalence of MDR and/or ESBL producing isolates was comparable for the three countries with the highest prevalence among the Belgian isolates (14%). Comparing the different populations the URO isolates showed the highest prevalence of MDR (14%) and the GP isolates (6 %) the lowest. The prevalence was for the NH (11%) and ICU (14%, range from 2% to 21%) isolates.

Table 3: The multi drug resistant and/or ESBL producing isolates per patient population and country

Unit	NL	B	G	Total
GP	5 (3)	12 (6)	8 (11)	25 (6)
NH	21 (10)	16 (11)	26 (11)	63 (11)
ICU	10 (11)	12 (21)	4 (2)	26 (14)
URO	18 (10)	31 (21)	19 (13)	68 (16)
Total	54 (8)	71 (14)	57 (12)	182 (11)

NL = the Netherlands, B = Belgium, G = Germany, GP = general practice, NH = nursing home, ICU = intensive care unit, URO = urology services. The percentage of all collected isolates (Table 1) is indicated between the parentheses.

Table 4: Different antibiotic resistance patterns per country and per patient population among the multi drug resistant and/or ESBL producing isolates

AB resistance	Total	NL	B	G	GP	NH	ICU	URO
AMC/QUI/GEN/SXT/NIT	1	0	0	1	0	0	0	1
AMC/QUI/SXT/GEN	38 (12)	7 (3)	17 (4)	14 (5)	6	12 (4)	5 (3)	15 (5)
AMC/QUI/SXT/NIT	4	0	4	0	0	0	0	4
AMC/QUI/SXT	72 (8)	24 (1)	31 (3)	17 (4)	7	27 (2)	11 (3)	27 (3)
AMC/QUI/GEN	21 (3)	3	7 (2)	11 (1)	6	10 (2)	1 (1)	4
AMC/QUI/NIT	2	2	0	0	0	0	0	2
AMC/SXT/GEN	3	2	1	0	1	0	1	1
AMC/SXT/NIT	1	0	1	0	0	0	0	1
QUI/SXT/GEN	14 (1)	10 (1)	1	3	2	8	1	3 (1)
QUI/SXT/NIT	2	1	0	1	0	0	1	1
QUI/GEN/NIT	1 (1)	1 (1)	0	0	0	0	0	1 (1)
AMC/QUI	3 (3)	2 (2)	1 (1)	0	0	1 (1)	1 (1)	1 (1)
AMC/SXT	3 (3)	1 (1)	1 (1)	1 (1)	0	1 (1)	2 (2)	0
QUI/SXT	7 (7)	0	1 (1)	6 (6)	0	3(3)	1 (1)	3 (3)
SXT/GEN	1 (1)	0	0	1 (1)	0	0	0	1 (1)
AMC	1 (1)	0	1 (1)	0	0	0	0	1 (1)
QUI	6 (6)	1 (1)	4 (4)	1 (1)	1(1)	1(1)	2 (2)	2 (2)
SXT	2(2)	0	1(1)	1(1)	2(2)	0	0	0
Total	182 (48)	54 (10)	71 (18)	57 (20)	25 (3)	63 (14)	26 (13)	68 (18)

AB = antibiotic, NL = the Netherlands, B = Belgium, G = Germany, GP = general practice, NH = nursing home, ICU = intensive care unit, URO = urology services, AMC = amoxicillin-clavulanic acid, QUI = fluoroquinolones, GEN = gentamicin, SXT = folate pathway antagonists, NIT = nitrofurantoin. The amount of ESBLs is indicated between the parentheses.

A total of 182 isolates (Table 3) were included for MLST of which 48 were ESBL producers. 23 of those ESBLs were not MDR.

The most prevalent patterns were resistance to: 1) amoxicillin-clavulanic acid, the fluoroquinolones and folate pathway inhibitors (n=72), 2) amoxicillin-clavulanic acid, the fluoroquinolones, folate pathway inhibitors and gentamicin (n=38), and 3) amoxicillin-clavulanic acid, the fluoroquinolones, and gentamicin (n=21). Only 11 (6%) isolates were resistant to nitrofurantoin, most of them were from the URO except for one ICU isolate (Table 4).

The most prevalent ESBL type was CTX-M 15 (n=34). Other ESBL types were CTX-M 1 (n=5), CTX-M 2 (n=1), CTX-M 14 (n=2), CTX-M 55/79 (n=2), TEM 52 (n=2) and SHV 12 (n=1). ESBL producing isolates were mostly found most among the ICU isolates (n=13 out of 180) and URO (n=18 out of 427). One isolate (NH) carried two ESBL genes i.e. CTX-M 15 and SHV 12.

MLST

Among the 182 MDR and/or ESBL producing isolates 47 different STs were found (Table 5). ST131 was observed in 74 out of 182 samples (41%) and in 10 out of the 12 groups of isolates (Table 5) and was overall the most prevalent ST followed by ST393 (n=10) and ST88 (n=9). The other STs were demonstrated one to seven times. Among them was one new ST (ST3088) and 2 new alleles i.e. *fumC*430 (ST3186) and *gyrB*316 (ST3187) were found.

All but one of the ST131 isolates were resistant to the fluoroquinolones and 54 were resistant to the folate pathway antagonists.

Nitrofurantoin resistant isolates had 10 different STs from 9 clonal complexes (CCs). Only one isolate (ST964) was an ESBL producer (CTX-M 15). The other ESBL producing isolates belonged in 53% of the cases to ST131 (n=25) of which 23 were CTX-M 15. The other ESBLs were found mostly among isolates with CC88 (n=6), CC58 (n=4) and CC10 (n=3, Table 6).

The diversity of the STs was highest among the GP and ICU samples compared with the NH samples (Table 5).

Table 5: Overview of the observed STs per patient population and country

	NL (56)	**B** (70)	**G** (54)	**DI** (95%CI)
GP (22)	69, 393, 405, 428, 1642	10, 73, 131(6), 156, 167, 405, 2538	34, 69, 88, 131(2), 167(2), 1914	0.944 (0.884-1)
NH (60)	23, 38, 69(2), 73, 131(11), 393(2), 1664, 2577(2)	88, 131(15)	46, 56, 58, 73, 80, 131(14), 354, 398, 453(2), 648, 744, 1411	0.717 (0,596-0.838)
ICU (25)	23, 99, 131(2), 393(2), 399(2), 1642, 3187	93, 131(2), 354, 393(2), 410(2), 685, 1011, 1394, 3186	58, 73, 410, 453	0.977 (0.949-1)
URO (68)	58, 69, 88, 131(4), 162, 167, 410, 453, 603, 648(3), 964, 2509, 3088	10, 23, 58(2), 69(2), 88(6), 95, 101, 131(9), 162(2), 167, 453, 617(2), 648(2), 744	10(2), 131(9), 393(3), 533, 624(2), 1011, 1394	0.912 (0.860-0.963)

NL = the Netherlands, B = Belgium, G = Germany, DI = diversity index, CI = confidence interval, GP = general practice, NH = nursing home, ICU = intensive care unit, URO = urology. The number of isolates is indicated between parentheses, if not indicated the ST was detected only once.

eBURST and CFA analysis

eBURST analysis of the isolates showed 7 groups and 27 singletons (Figure 1). The largest group with predicted founder ST10 contained 6 different STs. ST131 could not be assigned to any group. We also performed an eBURST analysis of the entire E. coli MLST database (up to ST3190) to investigate the clonal complex the isolates in our study population belonged to (Table 6).

Most of the STs belonged to CC10 and CC58 (former CC155[22]), two isolates (ST1914 and 2538) could not be assigned to a CC.

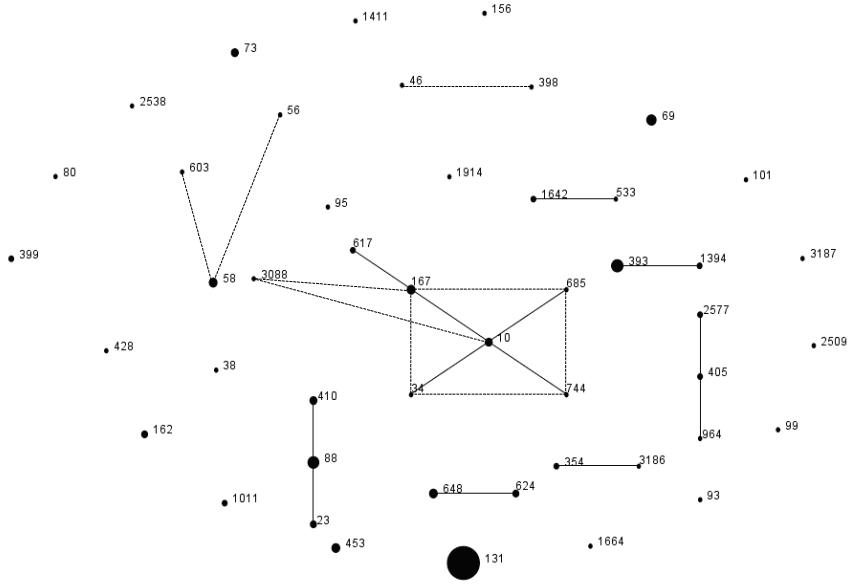
Table 6: STs per CC

CC	ST	CC	ST
10	10 (2), 34, 46, 93, 167, 398, 617, 685 (1), 3088	156	156
14	1411	354	354, 3186
38	38 (1)	399	399 (2)
58	56, 58 (1), 162 (2), 533, 603, 1642, 2509 (1)	405	405, 964 (1), 2577
69	69, 393, 1394	428	428
73	73	641	453 (1)
80	80	648	624, 648 (2)
88	23 (2), 88 (1), 410 (3), 1664	1011	1011
95	95	1611	99 (1)
101	101, 3187	-	1914, 2538
131	131 (21)		

The number of ESBL producing isolates is indicated between the parentheses.

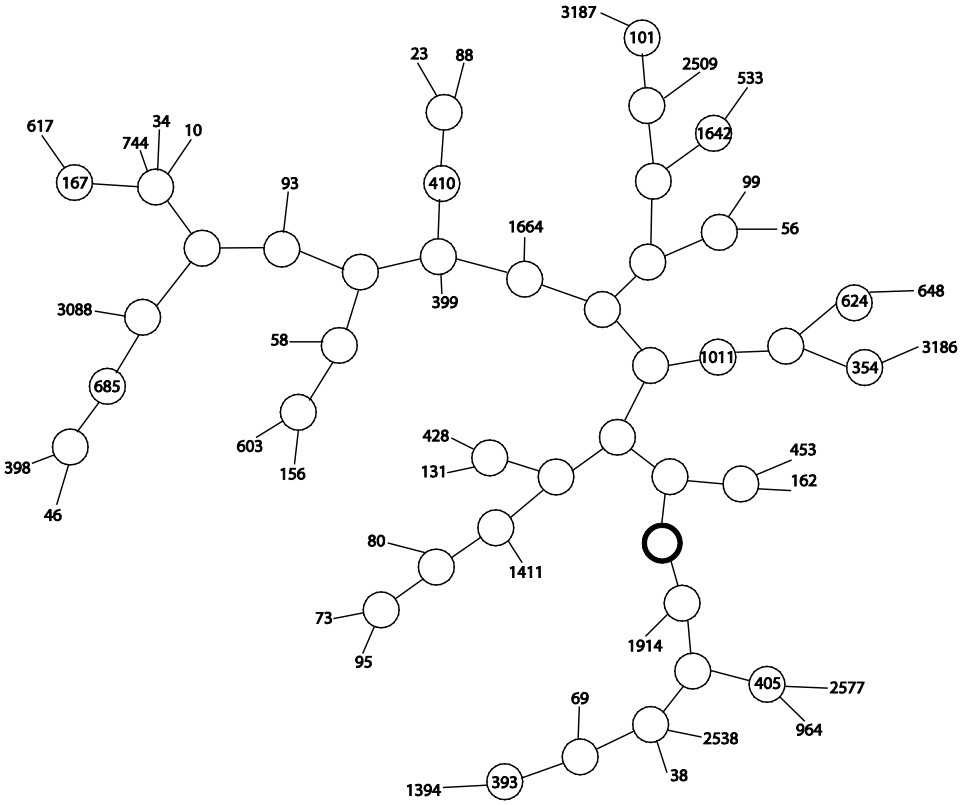
The results of the eBURST clustering and the clonal frame analysis were quite similar (Figure 2). STs that were clustered into one CC with eBURST were also quite closely related according to the CFA. The main exception was ST162, which was in CC58 according to eBURST, but according to the clonal frame analysis this ST was more distantly related to the other STs in CC58. Also, in CC58, ST162 was also far removed from the founder ST58 (eBURST). ST131 could according to the eBURST analysis not be clustered to any of the other demonstrated STs and was not placed as a node in the consensus tree with the CFA.

Figure 1: eBURST



eBURST diagram: Each dot represents a ST and the size of the dot correlates with the frequency this ST was found. The single locus variants and double locus variants are represented by the normal and dotted lines, respectively. The distance between the different dots does not reflect their relatedness

Figure 2: Clonal frame analysis



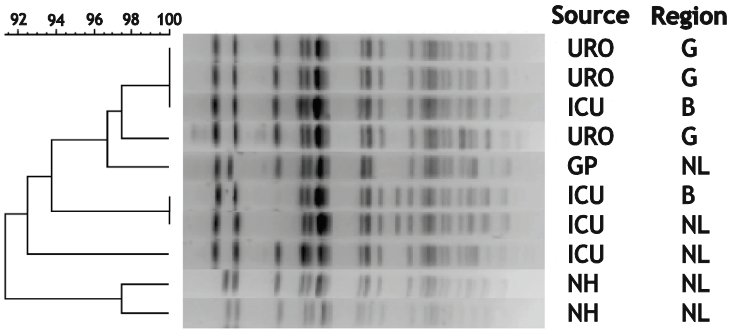
Network representation of ten 50% consensus trees. The analysis included all demonstrated STs (n=47). The ancestral node (unknown) is demonstrated with a bold border.

PFGE

All isolates belonging to ST393 and ST88 were further typed with PFGE. The isolates with ST393 had comparable PFGE pulsotypes (Dice ≥92%, Figure 3). The ST88 isolates had more diverse pulsotypes (Dice ≥68%, Figure 4).

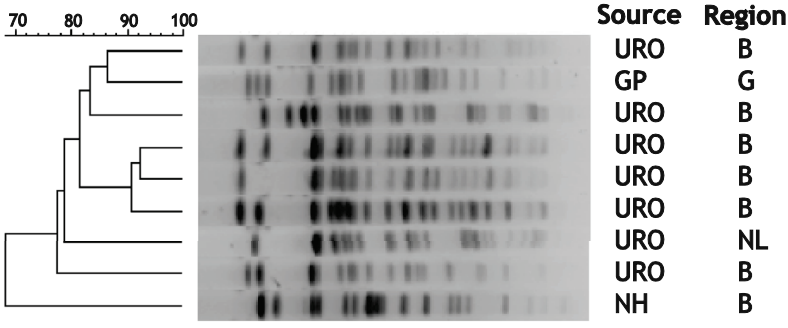
PFGE analysis demonstrated that among the ST131 isolates, several groups of isolates with a comparable pulsotype were found in the entire Euregion. Among these isolates a group of 13 (Figure 5: lane 17-29) was found with a high similarity (>84%), which were mostly isolates collected from the NHs in all three countries. A second group of 12 isolates (Figure 5: lane 1-12, Dice >82%) was demonstrated among the four population in the three countries.

Figure 3: PFGE dendrogram ST393



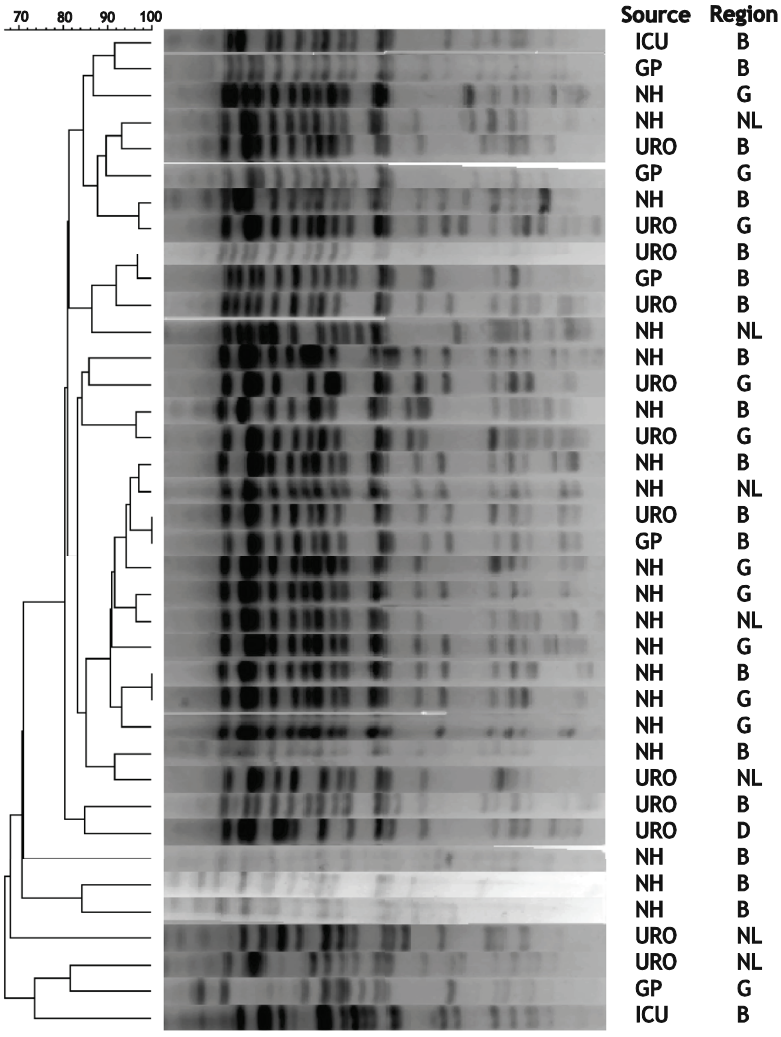
UPGMA tree of PFGE ST393 *E. coli* isolates

Figure 4: PFGE dendrogram ST88



UPGMA tree of PFGE ST88 *E. coli* isolates

Figure 5: PFGE dendrogram ST131



UPGMA tree of PFGE ST131 *E. coli* isolates

Discussion

This study shows that in the Euregion Meuse-Rhine among all the collected *E. coli* isolates from GP patients, NH residents, ICU and URO patients the prevalence of resistance was in the same order of magnitude in the three countries, but varied significantly between the four populations. Approximately 10% of the *E. coli* isolates was MDR with the highest prevalence among the URO isolates. In total, we observed 47 different STs of which ST131 was the most prevalent one. PFGE analysis of isolates belonging to the top three (ST131, 393 and 88) suggested the spread of isolates belonging to the same PFGE group in the entire Euregion. The results of the clustering via eBURST and CFA of all typed isolates were similar.
The strengths of this study were that isolates were collected from four populations in three different countries and to our are knowledge this is the first study to address the issue of cross border spread among *E. coli* The quantitative susceptibility of all isolates, MLST and PFGE was performed in the same laboratory to prevent bias because of difference in methodology. Weaknesses of the study were that only resistant isolates were included for MLST typing. Therefore, this study does not reflect the entire *E. coli* population. For ICU patients it was difficult to collect sufficient samples. Therefore, other clinical samples besides urine samples were included. This could give a higher diversity in genetic background of the collected isolates.

Overall there were only significant differences in prevalence of resistance between the three countries in this study for amoxicillin-clavulanic acid and ciprofloxacin. These results are not in line with the higher prevalence of resistance in Belgium and Germany compared with the Netherlands as described by EARS-Net[29]. The difference might be explained by the inclusion of GP and NH isolates in our study, since EARS-Net only included invasive clinical isolates from hospitalized patients. Similar observations were described for *S. aureus* isolates i.e. the prevalence of resistance of *S. aureus* from nasal carriers among GP patients in nine European countries was comparable[30] whereas among hospitalized patients the resistance differed significantly[29]. In our study we did observe significant differences in resistance between the URO isolates of the three countries (data not shown), which have been described previously[31].
There were significant differences in prevalence of resistance between the four populations included in this study, which were comparable with previous reports. Cullen et al. described a lower prevalence of resistance among community samples compared to urology and nosocomial samples and a higher resistance to ciprofloxacin among urology compared to nosocomial samples[32]. Xie et al. described a higher prevalence of MDR *E. coli* among nursing home residents compared with elderly in the community[33]. These differences in prevalence of resistance between the populations were probably caused by differences in the overall antibiotic use, which is low among GP patients compared with the other groups, and the use of specific antibiotics, such as the fluoroquinolones among the nursing home residents[34-37].

Although we demonstrated *E. coli* isolates with a variable genetic background, the high prevalence of *E. coli* ST131 (39%) is undeniable. The emerging prevalence of ST131 has

already been described before[38], but with this study we demonstrate a high prevalence of this ST in different populations both in the hospitals and in the community in the entire Euregion Meuse-Rhine, with the highest prevalence among NH residents and URO patients. An explanation for this might be the high use of fluoroquinolones among these populations. Van der Bij et al. described a potential influence of the restricted empirical use of ciprofloxacin on a decreased prevalence of ST131[39]. We hypothesize that a high use of this agent might increase the prevalence of ST131.

PFGE analysis demonstrated that among the ST131 isolates, several groups of isolates with a comparable pulsotype were found in the entire Euregion. Worrying was the occurrence of comparable ST131 isolates in the NHs. This suggested the emergence, spread and endurance of resistant bacteria among NH residents. The second group of isolates, with a high similarity demonstrated among the four populations in the three countries, supported a spread of ST131 clones throughout the entire Euregion. The same results were found for ST393 and to a lesser extend ST88. Thus, also, other non-ST131 clones have spread among our study populations in the Euregion.
The ST393 isolates, according to our PFGE results, were very similar (Dice ≥92%) and seemed to have pulsotypes comparable to those reported by Blanco et al[40]. Maybe this group is another emerging clone in the Euregion, though still less prevalent than ST131 and less diverse, based on the high similarity index.
The success in terms of spread of the ST131 clone in comparison to other STs might be the acquisition of multiple resistance genes and the presence of specific virulence factors[38].
The presence of ST131 isolates with different pulsotypes might suggest that this ST is diversifying and evolving, but this was not supported with MLST. We did not find any single locus variants (SLVs) of ST131 among the MDR isolates, although, the *E. coli* MLST database[22] already contains SLVs of ST131.
Overall, the MDR *E. coli* population in this study showed a high number of different STs but lower than among non-selected uropathogenic *E. coli* (UPEC) isolates[26, 41]. This suggests that only a limited number of STs have acquired multiple antibiotic resistance genes, while other STs did not and are still susceptible.
In our study the diversity of STs was highest among the ICU isolates, which could be due to the inclusion of various clinical samples, only 20% was collected from a urine sample. The higher diversity among the GP patients might be attributed to the low prevalence of MDR *E. coli*.

Most ESBL producing isolates, both ST131 and non-ST131, were CTX-M 15, which is in line with previous studies[26, 41, 42]. Reason for the dissemination is the location of $bla_{\text{CTX-M 15}}$ on very efficient mobile genetic elements, which are encoded on frequently encountered plasmids[38]. The other ESBL producers had a very diverse genetic background and were not associated to one CC.

Overall, the increasing prevalence of (MDR) resistant and/or ESBL producing bacteria is worrisome. As was suggested by Van der Bij et al. restrictions in the use of antibiotic seem to have an effect on the genetic background of the *E. coli* population[39] and a

meta-analysis by Davey et al. supports the theory that limiting antibiotic use reduces the prevalence of resistant Gram negatives[43]. Therefore, it is essential to implement antibiotic stewardship programs to optimize the use of antibiotics and thereby contribute to control the prevalence of resistance, and subsequently improve patient outcome[13].

Conclusions

We described the genetic background of resistant E. coli strains in the Euregion Meuse-Rhine. ST131 was most prevalent the three countries and in all four included populations, especially in the nursing homes. The emergence of resistant strains, such as E. coli ST131 and to a lesser extent ST393, might be a precursor for a further increasing prevalence of resistance. It is, therefore, essential to prevent and control the spread of these resistant strains by adherence to empiric antibiotic treatment protocols, infection control policies and antibiotic stewardship programs, which are based on the results of antibiotic resistance surveillance studies and mapping of the genetic background of E. coli. In this way it is possible to observe a shift in the prevalence of resistance and the genetic structure of the E. coli population and act accordingly.

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Chapter 5

Antibiotic resistance, population structure and spread of *Staphylococcus aureus* in nursing homes in the Euregion Meuse-Rhine

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submitted

Abstract

Objective
To determine the spread of *Staphylococcus aureus* within and between nursing home (NH) residents in the Euregion Meuse-Rhine, a cross border region of the Netherlands and Germany, we investigated the prevalence of antibiotic resistance, genetic background and population structure of both methicillin susceptible *S. aureus* (MSSA) and methicillin resistant *S. aureus* (MRSA) isolates.
Methods
A total of 245 *S. aureus* isolates were collected from NH residents in the Euregion. Susceptibility testing was performed with broth microdilution. The genetic background was determined using *spa* typing, SCC*mec* typing, PFGE and MLST.
Results
Differences in prevalence of resistance between the German and Dutch NH isolates were observerd for the macrolides (15% versus 2%, p=0.003), clindamycin (15% versus 0%, p=0.003) and ciprofloxacin (34% versus 25%). The macrolide and ciprofloxacin resistance varied between the NHs in both countries.
The MRSA prevalence was 3.5% and <1% among the German and Dutch NH residents (p=0.005). The German MRSAs, isolated in 7 out of 10 NHs, belonged to ST22-MRSA-IV or ST225-MRSA-II.
Spa-CCs 015 and 002 were most prevalent among the German MSSAs and *spa*-CCs 024 and 1716 among the Dutch ones.
Conclusions
The antibiotic resistance of MSSA and the MRSA prevalence were significantly higher among the German NH residents. Spread of two MRSA clones was observed within and between the German NHs, but not between the Dutch and German NHs. Differences in prevalence of resistance and prevalence of MRSA between NHs on both sides of the border warrant continuation of surveillance at a local level.

Introduction

Staphylococcus aureus is a commensal and potential pathogenic microorganism, which causes infections ranging from mild skin and soft tissue infections to severe necrotizing pneumonia, endocarditis and sepsis[1]. Since the introduction of penicillin and methicillin, *S. aureus* isolates resistant to these antibiotics have been isolated[2]. Resistance to methicillin among *S. aureus* is mediated by the presence of the staphylococcal cassette chromosome *mec* (SCC*mec*). This includes the *mecA* or *mecC* gene, encoding for a substitute penicillin-binding-protein (PBP), namely PBP2a for which beta-lactam antibiotics have a lower affinity compared with the native PBP[2]. Infections with a methicillin resistant *S. aureus* (MRSA) are, therefore, more difficult to treat and cause higher morbidity, mortality and have higher healthcare costs[3, 4].

Nursing home (NH) residents have a high risk for colonization and infection with antibiotic resistant bacteria including MRSA due to a relatively high prevalence of risk factors (e.g. wounds, co morbidities, use of medical devices and antibiotics, and previous hospital admission[5-7]). Therefore, NH residents are often considered a reservoir for MRSA, which could lead to outbreaks in NHs[8, 9] and spread from the NHs to hospitals and to the general population.

The spread, genetic background and population structure of MRSA and MSSA isolates within and between NHs has not often been investigated especially not in a cross border region.

In Germany the prevalence of MRSA is higher than in the Netherlands[10] and this difference in prevalence might pose a risk to the population of the border region (the Euregion Meuse-Rhine) and especially to a frail population such as the NH residents. Resistant bacteria might spread within and between NHs and cross the border. The spread of resistance in a cross border region has already been investigated for hospitals in the Euregion, but not for the NHs[11, 12] and also little is known about the population structure of MSSA isolates among NH residents on both sites of the border. Therefore, this study investigated the prevalence of antibiotic resistance, genetic background, population structure and potential spread of *S. aureus* including MRSA among NH residents in the Dutch-German border region.

Methods

Residents and bacterial isolates

Six NHs in the province of Limburg in the southern part of the Netherlands and ten NHs in the regions of Euskirchen (in North Rhine-Westphalia) and Daun (in Rhineland-Palatinate) in Germany participated in this study. All psycho-geriatric and somatic residents of these NHs were eligible for participation but only those residents (or their legal representatives) who signed a consent form were included. This study was approved by the medical ethics committee of the Maastricht University Medical Centre. We received 727 signed consent forms (Table 1) but due to withdrawal of consent, transfer to a hospital or other NH or passing away, 34 residents were

excluded, resulting in a final NH population of 693 residents. In each NH the swabs were collected on one day in the period July 2009 to June 2011.
Nasal swabs (Copan Diagnostics, Brescia, Italy) were taken from the anterior nostrils and analyzed for the presence of *S. aureus* in one laboratory (Maastricht University Medical Centre) using: 1. Culture on a colistin nalidixic acid agar plate (Becton Dickinson, Sparks, MD, USA) and 2. enrichment in nutrient broth (Oxoid, Hampshire, UK) with 6.5% NaCl followed by culture on oxacillin resistance screening agar (Oxoid). The enrichtment method was included to increase the detection of MRSA isolates. Putative *S. aureus* isolates were identified using a catalase and coagulase (tube) test.

Quantitative susceptibility testing
Quantitative susceptibility testing was performed using broth microdilution with Mueller-Hinton II cation-adjusted broth (Becton-Dickinson, Sparks, MD, USA) and microtitre plates with freeze-dried antibiotics (MCS Diagnostics BV, Swalmen, the Netherlands). Antibiotic susceptibility testing for fusidic acid and mupirocin was performed with a disk diffusion test on Mueller Hinton II agar plates (BD) and antibiotics tablets (fusidic acid 10µg and mupirocin 10 µg, (Rosco, Taastrup, Denmark). Zone diameters of >20mm for fusidic and >15mm for mupirocin were considered susceptible[13, 14]. *S. aureus* ATCC 29213 was the control strain for the susceptibility testing. The clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[13] were used. A disk-diffusion test was performed on all macrolide resistant but clindamycin susceptible isolates to test for inducible macrolide, lincosamide and streptogramin B (MLS-B) type resistance to clindamycin[15].

Genetic characterization
Oxacillin resistant *S. aureus* isolates were analyzed for the presence of the *mecA* gene using a real-time PCR assay as described previously[16]. Amplification of the *spa* locus, followed by sequencing, was performed on all MSSA and MRSA isolates[17, 18]. The *spa* types were clustered into *spa*-clonal complexes (*spa*-CCs) using the algorithm based upon repeat pattern (BURP) with the Ridom StaphType version 2.2.1. software package (http://www.ridom.de). The *spa* types with <6 repeats were excluded from the analysis and *spa* types were clustered if the cost was <5, to prevent the formation of too large and non specific *spa* clusters. After applying the BURP algorithm the associated MLST clonal complexes (CCs), were allocated through the Ridom SpaServer (http://spaserver.ridom.de)[19]. MRSA isolates were typed with MLST with primers as described by Enright et al.[20] and modified by Deurenberg et al[12]. The allelic profiles were allocated through the database on http://s.aureus.mlst.net. SCC*mec* typing and pulsed field gel electrophoresis (PFGE) was performed on all MRSA isolates as described previously[21, 22]. The PFGE profiles were analyzed according to the criteria by Tenover et al.[23]

Statistical analysis
Significant differences in antibiotic resistance and population structure between the Dutch and German isolates were calculated using the Pearson's chi-square test or Fisher's exact test (PASW-software, version 18.0, IBM, Armonk, NY, USA). A modified

false discovery rate (FDR) method developed by Benjamini and Yekutieli was used as correction for multiple testing[24]. A p-value of <0.05 was considered statistically significant. The diversity of the *spa* types was calculated with Simpson's index of diversity[25].

Results

S. aureus carriage

In total 245 (range: 1-33 per NH) *S. aureus* isolates were collected from 693 (range: 11-95 per NH) nasal swabs from residents of the 16 participating NHs (Table 1). The prevalence of *S. aureus* carriage was 30% and 39% (p=0.032) among the Dutch and German NH residents, respectively.

Table 1: Overview of included residents and collected samples per nursing home

NH	Eligible residents	Included residents (%)	Collected samples	Collected isolates	Prevalence in %
NL 1	130	40 (31)	39	10	26
NL 2	180	84 (47)	75	29	35
NL 3	329	56 (17)	50	13	26
NL 4	282	103 (37)	94	33	32
NL 5	104	53 (51)	53	18	30
NL 6	50	25 (50)	21	6	29
NL total	1075	361 (34)	332	109	30
G 1	118	39 (33)	33	11	33
G 2	74	19 (26)	17	7	41
G 3	110	30 (27)	24	15	63
G 4	134	63 (47)	54	31	52
G 5	44	24 (55)	23	6	26
G 6	112	51 (46)	46	21	41
G 7	139	78 (56)	77	29	36
G 8	182	31 (17)	28	9	32
G 9	79	11 (14)	11	1	9
G 10	103	21 (20)	20	6	30
G total	1095	367 (34)	333	136	39
Total	2170	728 (34)	665	245	35

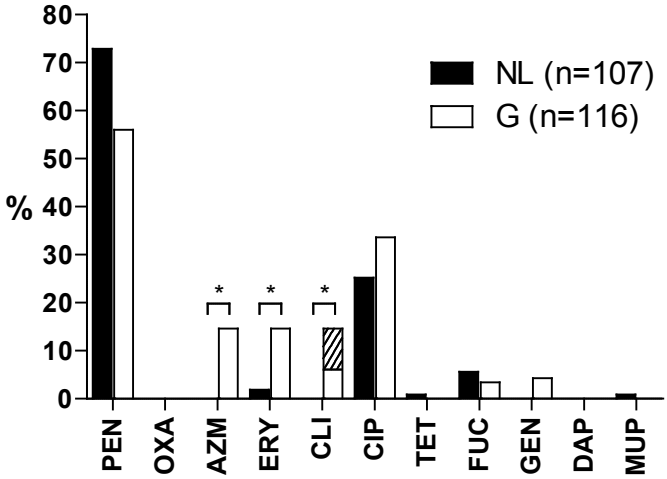
NL = the Netherlands, G = Germany

Antibiotic resistance of the MSSA isolates

Differences in prevalence of resistance among the MSSA isolates from the NHs in the Netherlands (NH-NL) and the NHs in Germany (NH-G) were observed (Figure 1). Resistance to erythromycin was higher among NH-G (15%) compared with NH-NL (2%, p=0.003), as was resistance to clindamycin (7%), which was absent among NH-NL (0%, p=0.097). Ten NH-G isolates were inducible MLS-B type resistant to clindamycin, which resulted in a total prevalence of clindamycin resistance of 15% (versus 0% among NH-NL, p=0.003). Differences in resistance, although not significantly, were demonstrated for ciprofloxacin: 25% versus 35%, and fusidic acid: 6% versus 3%, for the Dutch and German isolates, respectively. The prevalence of resistance to the other tested

antibiotics was either low (<5%) (i.e. gentamicin, tetracycline, daptomycin and mupirocin) or not observed (i.e. vancomycin, linezolid and co-trimoxazole) among both NH-NL and NH-G. For erythromycin and ciprofloxacin the prevalence of resistance varied between NH-NL and NH-G but also between the different NHs in each country (Figure 2).

Figure 1: Antibiotic resistance of the MSSA isolates

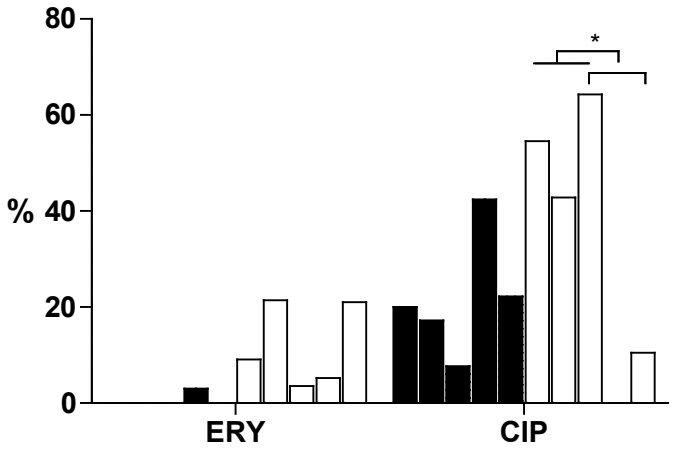


NL = the Netherlands, G = Germany, PEN = penicillin, OXA = oxacillin, AZM = azithromycin, ERY = erythromycin, CLI = clindamycin, CIP = ciprofloxacin, TET = tetracyclin, FUC = fusidic acid, GEN = gentamicin, DAP = daptomycin, MUP = mupirocin. The marked bar is inducible MLS-B type resistance to clindamycin, * $p<0.05$.

MRSA prevalence and genetic background

The prevalence of MRSA was higher among the German NH residents (6%, 19 out of 333 residents) compared with the Dutch NH ones (<1%, 2 out of 332 residents, $p<0.001$). One German NH resident carried two MRSA strains. The MRSA isolates were isolated in 7 out of 10 German NHs, but 50% of the MRSA isolates were found in one NH (G7). The prevalence of MRSA among the remaining German NHs was 3.9% (10 out of 256 swabs), which was compared with NH-NL still significantly higher ($p=0.005$). All but one of the German MRSA isolates were resistant to the macrolides, clindamycin (including four isolates of the inducible MLS-B type) and ciprofloxacin, while the Dutch MRSA isolates were only resistant to beta-lactam antibiotics.

Figure 2: Differences in antibiotic resistance among the participating nursing homes



Variation in antibiotic resistance among the MSSA isolates. Each bar represents one NH, the black bars the Dutch NHs and the white bars the German NHs. Only NHs with 10 or more collected isolates were included in the figure. ERY = erythromycin, CIP = ciprofloxacin, * $p<0.05$.

The German MRSA isolates consisted of two clones i.e. ST22-MRSA-IV (EMRSA-15 or Barnim clone, n=4), found in 3 out of 10 NHs, and ST225-MRSA-II (Rhine-Hesse or New York/Japan clone, n=16), observed in 6 out of 10 NHs. In two NHs (G4 and G6) both clones were present (Table 2). The ST22-MRSA-IV isolates had *spa* type t032, while the ST225-MRSA-II isolates had *spa* type t003 (n=13) and t151 (n=3). The four ST22-MRSA-IV isolates were indistinguishable or closely related using PFGE, which also applied for the 16 ST225-MRSA-II isolates (Figure 3). The two Dutch isolates were ST22-MRSA-IV with *spa* type t223 and ST7-MRSA-IV with *spa* type t097 (Table 2).

Table 2: Genetic background of the MRSA isolates

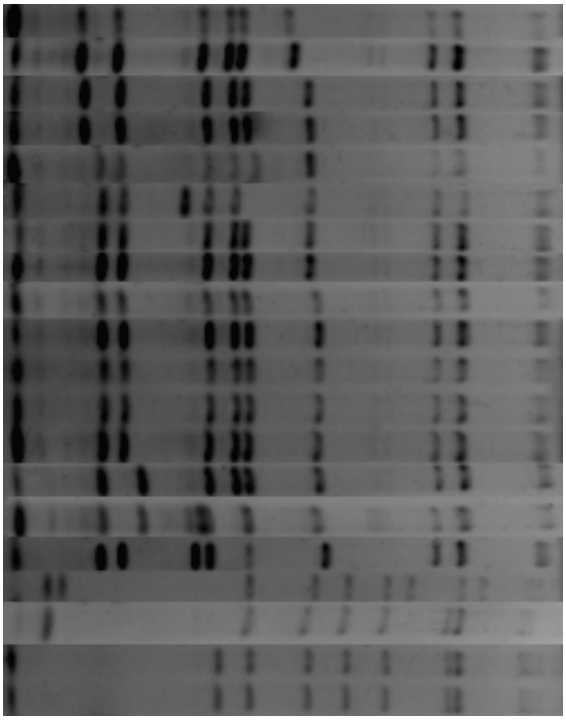
n	NL/G	ST	SCC*mec*	*spa* type	NHs
16	G	225	II	t003, t151	G 3,4,5,6,7,10
4	G	22	IV	t032	G 2,4,6
1	NL	22	IV	t223	NL 2
1	NL	7	IV	t097	NL 3

The column NHs indicates the NHs in which the MRSA were isolated.
NL = the Netherlands, G = Germany

***Spa* typing and BURP**

A total of 83 *spa* types were demonstrated among the 237 MRSA and MSSA isolates (8 isolates were not typable). A total of five *spa* types were demonstrated among the 22 MRSA isolates (Table 2) and among the 215 MSSA isolates a total of 82 *spa* types were detected (Table 3). Two of these *spa* types were not described previously (i.e. t10475 and t10476). The most prevalent *spa* types were t008 (n=22), t091 (n=10) among the

Figure 3: PFGE profiles of the German MRSA isolates



	NH	*spa*
1	G10	t003
2	G7	t003
3	G7	t003
4	G7	t151
5	G7	t151
6	G7	t003
7	G7	t003
8	G7	t151
9	G6	t003
10	G4	t003
11	G5	t003
12	G7	t003
13	G7	t003
14	G7	t003
15	G3	t003
16	G5	t003
17	G2	t032
18	G6	t032
19	G4	t032
20	G4	t032

The pulsotypes in lane 1-16 belong to the ST225-MRSA-II clone and in lane 17-20 to the ST22-MRSA-IV clone.

Dutch isolates and t003 (n=14) among the German ones. The diversity of the *spa* types was not significantly lower among the Dutch NH isolates (0.939, 95%CI 0.910-0.970) compared with those from Germany (0.968, 0.954-0.983).
The 82 *spa* types among the MSSA isolates were clustered into 11 clusters of which four had no founder. *Spa*-CCs 015 (p=0.017) and 002 (p=0.045) were most prevalent among the German MSSA isolates while among the Dutch MSSA isolates *spa*-CCs 024 (p<0.003) and 1716 were most prevalent (Table 3). Of all MSSA isolates, 146 isolates contained a *spa* type which could be associated to a MLST ST or CC using the Ridom SpaServer. There was no significant difference in the number of isolates with a *spa* type associated to a MRSA or MSSA related CC between the German and Dutch isolates, being 69% and 68%, respectively.

Table 3: Composition of *spa*CCs from the nursing home MSSA isolates per country

***spa*CC**	***spa* types**	**associated ST (CC)***	**n (%) NL isolates**	**n (%) G isolates**	**p**
015	t015, t031, t050, t095, t157, t230, t302, t583, t589, t630, t861, t1238, t1460, t5529, t8223, t9070	30, 45	12 (11)	29 (26)	0.017
1716	t084, t091, t254, t279, t393, t796, t1119, t1204, t1716, t2119	7, 15, 18	18 (17)	15 (14)	-
021/012	t012, t021, t122, t276, t2864, t7576	30	9 (9)	9 (8)	-
002	t002, t003, t010, t509, t548, t1227	5, 225, 231 (5)	6 (6)	18 (16)	0.045
040	t040, t065, t130, t266, t1281, t5834	45, 46	7 (7)	3 (3)	-
005	t005, t032, t449, t1862, t1863	22, 23, 60 (22)	1 (1)	8 (7)	-
024	t008, t024, t701	8, 247, 250, 254 (8)	24 (23)	4 (4)	<0.003
No founder	t127, t177	1, 3	6 (6)	0 (0)	0.038
No founder	t056, t087	101	3 (3)	2 (2)	-
No founder	t100, t10475	9	1 (1)	1 (1)	-
No founder	t476, t608	-	0 (0)	4 (4)	-
Singletons	t011, t151, t689, t1312, t1705, t1827, t2050, t9557, t9647, t10476	-	7 (7)	7 (6)	-
	t156	12	0 (0)	1 (1)	-
	t209	109 (9)	0 (0)	1 (1)	-
	t216	59	1 (1)	1 (1)	-
Excluded	t111, t457, t643, t929, t1011, t2207, t2614, t4384	-	6 (6)	3 (3)	-
	t026	45, 47 (45)	4 (4)	4 (4)	-
Total			105	110	

NL = the Netherlands, G = Germany, * = associated via the Ridom SpaServer

Discussion

In this study we demonstrated a significantly higher prevalence of *S. aureus* among the NH-G compared with NH-NL as was the prevalence of MSSA isolates resistant the macrolides, clindamycin and ciprofloxacin ($p<0.05$). Between the NHs in both regions we also observed variations in prevalence of resistance. The prevalence of MRSA was significantly higher among NH-G compared with NH-NL. The German MRSA isolates belonged to two major globally spread clones[2], suggesting spread of isolates within the German NHs. The population structure of the MSSA isolates was significantly different between NH-NL and NH-G, but the prevalence of isolates with a *spa* type associated to a MRSA related CC was comparable.

The strength of the study was that the same method of sampling and analysis was applied for all samples from all NHs. The results were, therefore, not influenced by differences in methodology. The weakness was that in this study 30% (14-56%) of all eligible residents were included and additional clinical data were not available. However, due to the unselected inclusion of residents this study still provides useful data on prevalence and spread of antibiotic resistance of *S.aureus*, population structure and genetic background. Although, this study was performed in a period of two years, we do believe that this timedifference does not explain the significant differences in prevalence of resistance between NH-NL and NH-G.

Previous studies have reported a wide range of *S. aureus* carriage among NH residents (23.9-43%)[26-28]. Although we observed a significant difference in carriage between the Dutch and German NH residents, the percentages were within the range mentioned in those studies.

For several antibiotics (the macrolides, clindamycin and ciprofloxacin) the prevalence of resistance among the MSSA isolates was higher among the German NH isolates compared with the Dutch ones. Flucloxacillin and clindamycin are often used as oral empiric treatment for a *S. aureus* infection[29]. Due to the high prevalence of resistance for clindamycin (including the inducible MLS-B type, 15%) among the German NHs the appropriateness of this agent for empiric treatment is debatable but flucloxacillin remains an appropriate choice. The prevalence of resistance among MSSA isolates and the prevalence of MRSA varied between the NHs (Figure 2), which might imply that there were differences in 1) antibiotic policies by the NHs, 2) prevalence of risk factors among the NH residents and/or 3) adherence to infection control policies by the NH staff. Therefore, it is very important that local resistance data should be made available and be used as a guiding tool for the choice of empiric treatment.
Several oral agents still had a low prevalence of resistance. These include fusidic acid, although resistance for this agent has also increased and was already 6% among the Dutch NH isolates. These isolates were probably not related to the epidemic European fusidic acid-resistant impetigo clone (EEFIC), which was prevalent among general practice patients, since their *spa* types were different from those reported for the EEFIC[30]. Other alternatives are trimethoprim-sulfamethoxazole and tetracycline[31],

since (almost) no resistance was detected. Another potential agent may be fosfomycin [32], since animal experiments have demonstrated the efficacy of this agent in *S. aureus* infection [33]. However, more research on humans regarding the systemic concentration and clinical efficacy is warranted.

MRSA is a point of attention due to, amongst others, the differences in prevalence[34]. The prevalence of MRSA among hospital patients has been demonstrated to be much lower in the Netherlands compared with Germany[10]. Our study confirmed that this difference also applies for NHs. Due to a possible outbreak of MRSA in one German NH (9 out of 77 swabs all with the same MRSA clone, 12%) we hypothesize that the prevalence of 3.5% that was demonstrated in the remaining NHs is a better reflection of the MRSA prevalence among German NH residents in a non-outbreak situation. This outbreak, however, does confirm the spread of MRSA within German NHs.
The prevalence of MRSA in the NHs in both countries was in agreement with previous reports [35-37], and was low in both countries compared to NH residents in other countries (22% in the UK and 16% in the USA)[5, 38]. Since the source of infection is mostly the resident's own commensal (nasal) flora[39, 40], the German NH residents are likely more at risk for an infection with MRSA than the Dutch NH residents. The MRSA prevalence among the German NH residents was high compared with the general German population, where prevalence of MRSA was much lower (<1%)[41].
The high prevalence of two MRSA clones in the German NHs compared with the Dutch NHs, demonstrated spread of these clones within the German NHs and suggested spread between NHs. Spread directly from one NH to another seems unlikely since few residents transfer between NHs, but a common factor might be the hospitals. Although we do not have data concerning previous hospital admissions spread via the hospitals to the NHs and vice versa could be considered a revolving door mechanism.
The MRSA isolates that were observed in this study were, all but one, globally reported including in the Netherlands and Germany[2, 11, 42, 43]. Both MRSA clones found among the German NH residents had an antibiotic resistance pattern which was in accordance with previous reports from the Robert Koch institute (i.e. co-resistance to the macrolides and fluoroquinolones) and are frequently found in Germany[42, 44]. However, among the Dutch isolates, we observed one MRSA with ST7. This is an unexpected finding since MRSAs usually have a ST belonging to the MRSA associated CCs (e.g. CC1, CC5, CC8, CC22, CC30 and CC45)[2]. There are only sporadic reports of MRSA isolates belonging to a MSSA associated CC[45, 46], which could be explained by the fact that mainly *S. aureus* lineages associated to the abovementioned CCs have the ability to attain and maintain a SCC*mec*.

Both the German and Dutch NH populations of MSSA showed a variety of *spa* types, but there were some significant differences in the distribution of the *spa*CCs. This does not apply to the number of isolates with a *spa* type associated to a MRSA related CC. The current data suggest a higher prevalence of MSSA isolates with CC1 and 8 in the Netherlands and with CC5, 22, 30 and 45 in Germany. .

Previous research has demonstrated that bacteria spread mostly within one health care cluster[47]. The differences in *spa*-CCs between the Dutch and German nursing homes suggest that there is no spread between those groups of NH residents.
The prevalence of MSSA isolates with a MRSA associated CC *spa* type (68% and 69%) was higher than among isolates collected from GP patients (52%)[16] but comparable to those from the ICU patients (62%)[19]. The relatively high antibiotic use[48-50] and the living environment of the residents might be responsible at least in part for this finding.

Conclusion

The prevalence of antibiotic resistance among MSSA isolates and the prevalence of MRSA was significantly higher among German NH residents compared with Dutch NH residents. Our results demonstrated a spread of two MRSA clones, each with several comparable pulsotypes, within and between German nursing homes. The population structure of the MSSA isolates, determined with *spa* typing, was significantly different between the Dutch and German NH isolates suggesting an absence of spread cross the border between these two groups of isolates in the two countries. Due to the increasing prevalence of resistance, the differences in resistance between NHs and the spread of MRSA isolates continuation of surveillance and implementation of infection control measures at a local NH level are important.

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Chapter 6

Is living in a border region a risk for a high prevalence of resistance?

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Abstract

This study assessed the differences in antimicrobial resistance and population structure of *Staphylococcus aureus* isolated from general practice (GP) patients and nursing home (NH) residents in the province of Limburg (near the border with Germany and Belgium) in comparison with those obtained in the remaining provinces of the Netherlands.

A total of 617 and 418 *S. aureus* isolates were isolated from 2691 and 1351 nasal swabs from GP patients and NH residents, respectively. Quantitative antibiotic susceptibility testing was performed using a broth microdilution method. Putative MRSA isolates were tested for the presence of the *mecA* gene and *spa* typing was performed on all *S. aureus* isolates.

No significant differences in prevalence of resistance were found between the two groups of GP isolates but the isolates from the NH residents showed a lower resistance for trimethoprim-sulfamethoxazole (p=0.003) in Limburg province compared with the remaining provinces in the Netherlands.

Among the isolates from NH residents in Limburg province the prevalence of *spa*-CC 084 was higher (p=0.003) and that of *spa*-CC 002 lower (p=0.01) compared with isolates from NHs in the remaining provinces of the Netherlands.

We observed no differences in resistance and population structure between *S. aureus* isolates from GP patients in Limburg and the remaining provinces of the Netherlands and only a few differences were observed between the NH populations. There was no higher prevalence of resistance among the GP and NH isolates from Limburg compared with the remaining provinces.

Introduction

In Europe, almost one third of its population lives in a border region. In the Netherlands one of those border regions is the province of Limburg, which has a border with Belgium and Germany of 351 km and a border with two other Dutch provinces (i.e. Noord-Brabant and Gerlderland) of only 113 km[1]. Limburg is also part of the Euregion Meuse-Rhine, a region with intensive cross border traffic (4% of all jobs in Limburg is fulfilled by Belgian and German citizens) and cross border patient mobility due to the (free) access to health care facilities on both sides of the border[2-4].
Staphylococcus aureus is a frequent causative agent of community and hospital acquired infections varying from minor skin and soft tissue infections to invasive infections like bacteraemia and endocarditis[5]. In many European countries the antibiotic resistance of *S. aureus* is increasing[6] and consequently an optimal empiric antibiotic choice for the treatment of *S. aureus* infections becomes more challenging. In particular, the prevalence of methicillin resistant *S. aureus* (MRSA) is a point of concern. In the Netherlands the prevalence of MRSA is still low[7], which can at least in part be attributed to the national antibiotic policy and the infection control guidelines (including the "search and destroy" protocol). However, in recent years several reports mentioned MRSA outbreaks in Dutch nursing homes (NHs)[8-11]
The prevalence of antibiotic resistance and MRSA is higher in Belgium and Germany than in the Netherlands [6] with a prevalence of MRSA in hospitals of 20.5%, 20.9% and 1.2%, respectively[6]. It is to be expected that these resistant isolates could spread more easily and could lead to a higher prevalence of resistance in the province of Limburg than to the other provinces in the Netherlands due to cross border traffic and access to healthcare across the border[2]. The differences in antibiotic resistance between these countries hamper patient mobility because of the risk of acquisition or spread of resistant isolates.
There is no information available whether residents of the province of Limburg have a higher prevalence of resistance among *S. aureus* isolates and whether these isolates have a population structure more at risk for acquiring the *mecA* gene encoding for resistance to methicillin than those in the remaining provinces of the Netherlands. Therefore, we evaluated the antibiotic resistance and population structure of *S. aureus* isolates from general practice (GP) patients and NH residents in the province of Limburg in comparison with those of the remaining provinces of the Netherlands.

Methods

Study population and isolation of *S. aureus*
In 2005, a total of 2691 nasal swabs (Amies agar gel swabs without charcoal 108C, Copan Diagnostics, Brescia, Italy) were taken from the anterior nostrils from GP patients with no apparent signs of infection from 10 GPs in Limburg province (GP-L) and 19 GPs in the remaining provinces in the Netherlands (GP-NL). The nasal swabs were analyzed as described previously[12]. In 2009 and 2010 a total of 1351 nasal swabs were collected from NH residents from six NHs in the province of Limburg (NH-L) and

from 24 NHs in other provinces of the Netherlands (NH-NL, Table 1). The swabs were analyzed with the same methods as the GP swabs[12]. Informed consent was obtained from all participants.

Quantitative susceptibility testing

Quantitative susceptibility testing was performed using broth microdilution with Mueller-Hinton II cation-adjusted broth (Beckton-Dickinson, Sparks, MD, USA) and microtitre plates with freeze-dried antibiotics (MCS Diagnostics BV, Swalmen, the Netherlands). *S. aureus* ATCC 29213 was used as a control strain. The breakpoints for resistance were according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[13]. Antibiotic susceptibility testing for fusidic acid and mupirocin was performed with a disk diffusion test with Mueller Hinton II agar plates (BD) and antibiotics tablets (fusidic acid 100 µg and mupirocin 10 µg, Rosco, Taastrup, Denmark). Zone diameters of >27mm for fusidic acid and >15mm for mupirocin were considered susceptible[14, 15]. Oxacillin resistant isolates were analyzed for the presence of the *mecA* gene using a PCR assay as described previously[12].

Typing of the *spa* locus

Amplification of the *spa* locus, followed by sequencing, was performed as described previously[16, 17]. The *spa* types were clustered into *spa*-clonal complexes (*spa*-CCs) using the Based Upon Repeat Pattern (BURP) algorithm with the Ridom StaphType software version 2.2.1 (http://www.ridom.de). The *spa* types with <6 repeats were excluded from the analysis and *spa* types were clustered if the cost was <5, to prevent the formation of too large and non specific *spa* clusters. After applying the BURP algorithm the associated multi locus sequence typing (MLST) clonal complexes (CCs) were allocated through the Ridom SpaServer (http://spaserver.ridom.de).

Statistical analysis

Significant statistical differences in antibiotic resistance were calculated using the Pearson chi-square test or a Fisher's exact test (PASW-software, version 18.0, IBM, Armonk, NY, USA). A modified false discovery rate (FDR) method developed by Benjamini and Yekutieli was used as correction for multiple testing[18]. A p-value <0.05 was considered statistically significant. The diversity of the groups of isolates regarding the *spa* types was determined with Simpson's Index of diversity[19].

Results

Prevalence of *S. aureus* carriage

The prevalence of *S. aureus* nasal carriage was 23%, 247 isolates out of 1096 swabs and 370 isolates out of 1595 swabs, among both GP-L and GP-NL, respectively (Table 1). The prevalence among the NH residents was 31%, 100 isolates out of 291 swabs, in NH-L and 29%, 318 isolates out of 1060 swabs, in NH-NL.

Table 1: Overview of collected swabs and *S. aureus* isolates

Unit	GP-L	GP-NL	NH-L	NH-NL	Total
No. of swabs	1096	1595	291	1060	**4042**
No. of isolates	247	370	100	318	**1035**
Prevalence	23%	23%	31%	29%	**26%**
No. of *spa* types	130	163	39	114	**329**

GP-L: general practice patients in the province of Limburg, GP-NL: general practice patients in the remaining provinces of the Netherlands, NH-L: nursing home residents in the province of Limburg, NH-NL: nursing home residents in the remaining provinces of the Netherlands.

Prevalence of antibiotic resistance

The antibiotic susceptibility patterns of the isolates are shown in Table 2. Unfortunately, for further analysis 22 of the *S. aureus* isolates could not be cultured from the frozen stock.

No significant differences in resistance were demonstrated between GP-L and GP-NL. Resistance to linezolid, trimethoprim-sulfamethoxazole, vancomycin, gentamicin and mupirocin was either not observed or less than 1%. Four isolates were resistant to oxacillin (MIC 4 or 8 mg/L) but none of these isolates harboured the *mecA* gene. These isolates had the following *spa* types: t062, t127, t224 and t1702.

Among the NH isolates the prevalence of resistance was lower for trimethoprim-sulfamethoxazole (p=0.003) and clarithromycin(p=0.093) from NH-L (0% and 2%, respectively) compared with NH-NL (9% and 8% respectively). All but one of the trimethoprim-sulfamethoxazole resistant isolates harboured *spa* t064. Six isolates (two in NH-L and four in NH-NL) were resistant to oxacillin (MIC 8 mg/L to >64 mg/L), carried the *mecA* gene and had the following *spa* types: t002, t037, t091, t223, t740 and t2164. Resistance to vancomycin, gentamicin, mupirocin and linezolid was either not demonstrated or less than 1%.

Table 2: Antibiotic resistance among *S. aureus* isolates collected from GP patients and NH residents

Antimicrobial agent	GP-L (247)	GP-NL (348)	NH-L (100)	NH-NL (318)
Oxacillin	1%	1%	2%	1%
Claritromycin	7%	5%	2%	8%
Clindamycin	1%	0%	0%	2%
Ciprofloxacin	1%	2%	24%	34%
Fusidic acid	6%	6%	6%	4%
Tetracyclin	3%	5%	2%	2%
Trimethoprim-sulfamethoxazole	0%	0%	0%*	9%*

GP-L: general practice patients in the province of Limburg, GP-NL: general practice patients in the remaining provinces of the Netherlands, NH-L: nursing home residents in the province of Limburg, NH-NL: nursing home residents in the remaining provinces of the Netherlands.
* significant difference between the two groups of NH isolates.

Distribution of *spa* types and BURP analysis

A total of 329 *spa* types were found. The most prevalent *spa* types among all isolates were t008 (6.6%), t002 (5.8%) and t091 (5.1%). The other *spa* types accounted for 0.1% to 4.1% each. Among the GP-L isolates *spa* types t012 (6.5%), t091 (5.7%) and t002 (4.0%) were most prevalent and among the GP-NL isolates t091 (5.5%), t008 (4.9%) and t012 (4.9%). Among the NH-L isolates *spa* types t008 (22%), t091 (10%) and t026 (7%) were most prevalent and among the NH-NL isolates t002 (9.8%), t064 (8.2%) and t008 (6.6%).

The *spa* types clustered into 16 *spa*-CCs (Table 3). Three clusters had no founder and 33 *spa* types (3.3%) could not be clustered in a *spa*-CC and were classified as singletons. 70 isolates (6.9%) were excluded from the analysis since these *spa* types consisted of less than five repeats and 11 isolates were not typable.

Overall, the percentage of isolates belonging to each *spa*-CC was similar for GP-L and GP-NL and for NH-L and NH-NL. The only difference was observed the NH isolates: the prevalence of *spa*-CC 084 (7%) was higher (p=0.003) and that of *spa*-CC 002 (17%) was lower (p=0.01) among isolates collected from NH-L compared with those from NH-NL.

There was also variation in diversity of the *spa* types among the NH isolates: 0.929 (95%CI 0.896-0.962) and 0.971 (95%CI 0.963-0.978) for the NH-L and NH-NL isolates, respectively.

Table 3: Distribution of *spa*-CCs among isolates collected from GP and NH patients

***spa*-CC**	**Isolates (%)**	***spa* (%)**	**GP-L (%)**	**GP-NL (%)**	**NH-L (%)**	**NH-NL (%)**
spa-CC 012	299 (30)	100 (30)	73 (30)	110 (32)	29 (29)	87 (27)
spa-CC 084	124 (13)	27 (9)	37 (15)	47 (14)	18 (18)*	22 (7)*
spa-CC 002	105 (10)	26 (8)	19 (8)	26 (7)	5 (5)*	55 (17)*
spa-CC 024	126 (12)	19 (6)	15 (6)	25 (7)	23 (23)	63 (20)
spa-CC 078	36 (4)	19 (6)	17 (7)	14 (4)	1 (1)	4 (1)
spa-CC 127	66 (7)	18 (5)	12 (5)	26 (7)	6 (6)	22 (7)
spa-CC 166	35 (3)	15 (5)	11 (4)	15 (4)	0 (0)	9 (3)
spa-CC 005	30 (3)	13 (4)	5 (2)	14 (4)	2 (2)	9 (3)
spa-CC 159	17 (2)	10 (3)	7 (3)	9 (3)	0 (0)	1 (0)
spa-CC 216	27 (3)	8 (2)	7 (3)	4 (1)	1 (1)	15 (5)
spa-CC 160	12 (1)	6 (2)	6 (2)	5 (1)	0 (0)	1 (0)
spa-CC 364	9 (1)	4 (1)	4 (2)	4 (1)	0 (0)	1 (0)
spa-CC 1045	5 (0)	3 (1)	2 (1)	1 (0)	1 (1)	1 (0)
No founder 1	3 (0)	2 (1)	0 (0)	3 (1)	0 (0)	0 (0)
No founder 2	2 (0)	2 (1)	1 (0)	1 (0)	0 (0)	0 (0)
No founder 3	3 (0)	2 (1)	2 (1)	1 (0)	0 (0)	0 (0)
Singletons	33 (3)	23 (7)	8 (3)	15 (4)	1 (1)	9 (3)
Excluded	70 (7)	32 (10)	19 (8)	25 (7)	11 (11)	15 (5)
Not typable	11 (1)	0 (0)	2 (1)	3 (1)	2 (2)	4 (1)
Total	**1013**	**329**	**247**	**348**	**100**	**318**

GP-L: general practice patients in the province of Limburg, GP-NL: general practice patients in the remaining provinces of the Netherlands, NH-L: nursing home residents in the province of Limburg, NH-NL: nursing home residents in the remaining provinces of the Netherlands.
* significant difference between the NH-L and NH-NL isolates.

The *spa* types were associated to a MLST ST or CC via the Ridom SpaServer (Table 4). No significant differences were found between the GP-L and GP-NL isolates (56% and 61%, respectively) and the NH-L and NH-NL isolates (73% and 79%, respectively)

Table 4: Composition of the *spa*-CCs

***spa*-CC**	***spa* types**	**MLST CC (ST)**
spa-CC 012	t004, t012, t015, t018, t019, t021, t031, t034, t037, t040, t050, t065, t069, t073, t074, t095, t096, t102, t116, t122, t138, t230, t238, t266, t274, t275, t276, t300, t302, t318, t331, t338, t370, t404, t406, t483, t505, t571, t576, t583, t589, t620, t630, t631, t706, t740, t772, t822, t840, t861, t908, t937, t950, t1130, t1149, t1238, t1239, t1281, t1504, t1510, t1574, t1827, t1932, t2077, t2135, t2143, t2208, t2209, t2210, t2239, t2254, t2269, t2275, t2387, t2479, t2489, t2496, t2539, t2540, t2541, t2544, t2548, t2557, t2561, t2566, t2568, t2572, t2610, t2659, t2674, t2682, t2821, t2864, t4441, t4905, t5834, t7110, t7126, t7143, t7147	30,45
spa-CC 084	t084, t085, t091, t094, t252, t279, t346, t348, t360, t393, t491, t547, t774, t796, t853, t867, t1243, t1363, t1716, t1943, t2074, t2543, t2556, t2567, t2616, t5875, t7134	7,15
spa-CC 002	t001, t002, t010, t041, t062, t067, t179, t242, t306, t311, t389, t447, t509, t539, t548, t688, t837, t1215, t1340, t2070, t2164, t2212, t2491, t2542, t2724, t6160	5
spa-CC 024	t008, t024, t064, t104, t190, t197, t334, t377, t530, t648, t701, t711, t846, t1171, t2041, t3060, t3802, t4299, t5279	8
spa-CC 078	t056, t078, t081, t087, t150, t258, t353, t436, t469, t660, t775, t814, t1102, t1312, t1541, t1671, t1898, t2039, t2078	25, 101
spa-CC 127	t114, t127, t177, t189, t224, t267, t286, t359, t376, t591, t1236, t1407, t1787, t2500, t2569, t2612, t2819, t7123	1
spa-CC 166	t089, t136, t153, t166, t240, t369, t884, t1014, t2038, t2071, t2073, t2080, t2547, t2854, t7162	
spa-CC 005	t005, t060, t223, t474, t790, t1433, t1629, t2570, t2618, t2681, t5485, t5926, t7156	22
spa-CC 159	t159, t171, t272, t284, t408, t645, t659, t738, t2213, t2820	121
spa-CC 216	t172, t216, t471, t1293, t2079, t2488, t3527, t4303	59
spa-CC 160	t156, t160, t213, t771, t1702, t3938	12
spa-CC 364	t364, t493, t2492, t2680	
spa-CC 1045	t099, t100, t1045	9
No founder 1	t148, t2016	
No founder 2	t186, t729	(88)
No founder 3	t246, t2495	
Singletons	t106, t344, t587, t818, t878, t1362, t1406, t2050, t2075, t2076, t2490, t2494, t2558, t2559, t2573, t2615, t2617, t5874, t7108, t7132	(20)
	t164	(59)
	t199	(109)
	t209	
Excluded	t059, t118, t287, t362, t502, t524, t535, t605, t643, t808, t929, t1152, t1200, t1209, t1456, t1509, t2176, t2207, t2211, t2246, t2365, t2383, t2493, t2571, t2611, t2613, t2614, t2853, t4386	
	t026	45
	t233	(59)
	t386	(1)

Overview of the *spa* types in every *spa*-CC and the associated MLST clonal complex (CC) or sequence type (ST), associated via the Ridom Spaserver.

Discussion

In this study we observed no difference in prevalence of resistance between the *S. aureus* isolates from GP-L and GP-NL but a significantly lower prevalence of resistance to trimethoprim-sulfamethoxazole for the isolates from NH-L compared with those from NH-NL. Significant differences in prevalence of *spa*-CC 084 and 002 were also found for the NH isolates. These few differences do not support the hypothesis that the cross border traffic between the province of Limburg, Belgium and Germany led to a higher prevalence of resistance and/or a population structure more at risk for acquiring the *mecA* gene among the residents of Limburg compared with the residents in the remaining provinces of the Netherlands.
The strength of the study is that all samples were analyzed at the same laboratory with the same methods. Bias due to differences in methods can, therefore, be excluded. The study has also some weaknesses: the number of NH-L isolates was lower compared to the other groups of isolates. Also, clinical data was not available and although there was a difference in sampling period between the GP and NH isolates, we do not expect that this could explain the differences in prevalence of resistance observed[20].

The 23% prevalence of nasal colonization with *S. aureus* among both groups of GP patients was in agreement with previous reports[21], which also applies for the 29% and 31% prevalence among the NH resident populations. Previous studies reported 23.9-43% [22-24].
No differences in antibiotic resistance were found between isolates collected from the two groups of GP patients, but between the two groups of NH residents a difference in trimethoprim-sulfamethoxazole resistance was observed. The difference observed might be due to differences in antibiotic use. NH residents are a frail population with a high use of antibiotics and more risk factors for acquiring and retaining a more resistant *S. aureus* isolate[22, 25]. However, among GP patients and NH residents resistance to trimethoprim-sulfamethoxazole was rare despite there relatively high use[26] and further studies seem warranted. The 27 resistant isolates (out of 1013) were found in 13 different NHs and all but one harbored *spa* type 064, which is a *spa* type associated with MLST CC 8, a MRSA associated MLST CC.
Quinolone resistance was higher among the NH residents compared with the GP patients. Higher use of quinolones in NHs is probably a main reason for the development of higher resistance[27, 28]. However, the differences in resistance could also be attributed to a different living environment, higher age, co morbidities and indwelling devices[23].
Among the isolates from the GP patients the four oxacillin resistant isolates were classified as borderline oxacillin resistant *S. aureus* (BORSA). Isolates like these have been described before, but mostly in a clinical setting[29-31]. The clinical relevance of these isolates remains questionable.

From our resistance data we may conclude that the spread of resistant isolates from Belgium and Germany to the Province of Limburg is not higher than to other Dutch provinces. However, a different population structure of the *S. aureus* isolates (i.e. isolates with a more stable genomic environment for the SCC*mec* cassette e.g. CC 1, CC5, CC8, CC22, CC30 and CC45[32]) in the province of Limburg might be an early warning for a country-to-country spread.
With BURP all *spa* types were allocated in *spa*-CCs. The only significant difference was found for *spa*-CC 084 and *spa*-CC 002 between the NH groups. Overall *spa*-CC 012 was quite large and could be associated to two very different MLST CCs i.e. CC 30 and 45. With more strict BURP settings (cost <4) this *spa*-CC could be divided into a few smaller clusters of which *spa*-CC 012, associated to MLST CC 30, and *spa*-CC 015, associated to MLST CC 45, were the largest (74% of all isolates in this group).
There was a difference in prevalence of isolates associated with a MRSA associated MLST CC between the groups from the NHs but this can be attributed to the differences in *spa*-CCs described above.
The diversity of the *spa* types from the GP isolates was comparable with previous reports[33, 34]. The lower diversity of the *spa* types among the NH isolates might be due to the enclosed living environment, transmission within the NHs and higher antibiotic use. These factors combined with the lower number of participating NHs in Limburg might explain the lower diversity among the NH isolates in Limburg.

In conclusion we observed only minor differences in antibiotic resistance and population structure between isolates collected from GP patients and NH resident in Limburg and the remaining parts of the Netherlands. We may conclude that cross border traffic in the Euregion Meuse-Rhine (between Belgium, Germany and the province of Limburg in the Netherlands) does not result in differences in prevalence of resistance and population structure between Limburg and the remaining provinces in the Netherlands. However, the impact of cross border traffic might be quite different in larger countries or perhaps that globalization has a greater influence on the antibiotic resistance[35] and population structure of *S. aureus* of the entire Dutch community than the location of the province of Limburg on this province alone. Therefore, surveillance at a local and international level is warranted, to keep informed as to changes in prevalence of resistance over time.

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Chapter 7

A 12 year (1998-2009) antibiotic resistance surveillance of *Klebsiella pneumoniae* collected from intensive care and urology patients in 14 Dutch hospitals

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Abstract

Objectives
We evaluated the changes in antibiotic resistance from 1998-2009 of *Klebsiella pneumoniae* isolated from the intensive care unit (ICU) and urology service of 14 Dutch hospitals and the consequences for empirical therapy.
Methods
Quantitative antibiotic susceptibility testing of *K. pneumoniae* was performed in a central laboratory using a broth microdilution. Breakpoints were as defined by the European Committee on antimicrobial susceptibility (EUCAST). The prevalence of extended spectrum ß-lactamase (ESBL) and carbapenemase producing isolates was determined.
Results
A significant increase of resistance among ICU isolates was observed for ceftazidime (4.2-10.8%), ciprofloxacin (5.8-18.5%) and trimethoprim-sulfamethoxazole (11.9-23.1%), and for cefuroxime (2.8-7.9%) and trimethoprim-sulfamethoxazole (13.5-27.8%) among urology isolates. Among ICU isolates the prevalence of ESBLs increased significantly from 2% to 8%. Carbapenemase production was not demonstrated. The prevalence of multidrug-resistance increased and has been 12% or more since 2004. Among urology isolates multidrug-resistance was highest in 2009 at 7.4%. Overall resistance was significantly higher among ICU isolates.
Conclusions
We observed an increase of resistance among ICU and urology isolates and an increased prevalence of ESBLs among ICU isolates. Carbapenemase production was not demonstrated. A regular update of empiric treatment protocols based on actual surveillance data is justified.

Introduction

The worldwide increase of antibiotic resistance among Gram negative bacteria including *Klebsiella pneumoniae* is alarming.[1] In particular the emergence of ESBL producing bacteria is troublesome.
According to Dutch guidelines, empiric treatment of first choice for bloodstream infections is a ß-lactam antibiotic (broad-spectrum penicillin-ß-lactamase inhibitor combination or 3rd generation cephalosporins) mostly in combination with an aminoglycoside.[2] However, the choice of therapy is challenging because the prevalence of *K. pneumoniae* isolates resistant to those antibiotics is increasing.[1, 3] Therefore, surveillance of antibiotic resistance is needed to determine the prevalence of resistance and to make an appropriate empiric antibiotic choice.
This study describes the development of resistance over the past 12 years among *K. pneumoniae* isolates from the ICU and urology services of 14 hospitals in the Netherlands as well as the consequences for empirical treatment of first choice especially in bloodstream and urinary tract infections (UTI).

Materials and Methods

Isolates
K. pneumoniae isolates from 14 Dutch hospitals were collected from 1998 to 2009, as part of the annual intramural surveillance of the Dutch Working Party on Antibiotic Policy.[3] The isolates were collected from the ICU and the urology service. ICU isolates were collected from various clinical samples, the urology isolates were derived from urine samples. Only one isolate per patient was included. Clinical data was not available.
The isolates were identified at the local laboratories, stored and sent to one central laboratory for susceptibility testing. Two laboratories functioned as central laboratory: that of the University Medical Centre St. Radboud Nijmegen (1998-2001) and that of the Maastricht University Medical Centre (since 2002).

Susceptibility testing
Quantitative susceptibility testing was performed using a broth microdilution with Mueller-Hinton II cation-adjusted broth (Becton-Dickinson, Sparks, MD, USA). Microtitre plates with freeze-dried antibiotics were obtained from MCS Diagnostics BV (Swalmen, the Netherlands). The minimal inhibitory concentration (MIC) was defined as the lowest concentration showing no growth after 18 hours of incubation at 35ºC. *Escherichia coli* ATCC 35218 and ATCC 25922 were used as control strains. The MIC data were recorded, stored and re-evaluated for this study using breakpoints defined as by the EUCAST.[4] Intermediate results were considered resistant.
Multidrug-resistance was defined as resistance to three or more classes of antibiotics.
Seven isolates from one hospital were presumably part of a local outbreak of a multi resistant *K. pneumoniae* strain at the ICU in 2002. PFGE confirmed this presumption. Therefore, only the first isolate from this outbreak was included.

Confirmation of ESBL and carbapenemase producing isolates

Confirmation of ESBL production was performed as described.[5] Confirmation of carbapenemase production was performed on isolates with a meropenem MIC≥0.5mg/L and/or imipenem MIC≥2mg/L using the modified Hodge test.[6]

Statistical analysis

The logistic regression analysis was performed to determine statistically significant trends during the study period. A p-value of <0.05 was considered statistically significant.

Results

A total of 1,578 isolates were collected: 720 from the ICUs and 858 from the urology services.

Table 1: The trends in antimicrobial resistance of *K. pneumoniae* strains isolated from 14 Dutch ICUs

Antimicrobial agent	%R '98	%R '09	p-value	OR	95% CI
AMC	16.7%	26.1%	0.063	1.053	0.997-1.111
TAZ	8.0%	15.2%	0.068	1.068	0.995-1.145
CXM	8.0%	15.5%	0.058	1.070	0.998-1.148
CAZ	4.2%	10.8%	*0.040	1.097	1.004-1.198
CFM	3.5%	11.2%	0.072	1.093	0.992-1.204
FEP	3.5%	8.1%	0.096	1.088	0.985-1.202
CTX	NT	7.9%	0.453	1.057	0.914-1.224
IPM	0.0%	0.0%	0.272	0.447	0.106-1.882
MEM	0.0%	0.0%	0.685	1.150	0.585-2.260
CIP	5.8%	18.5%	*0.001	1.126	1.047-1.211
GEN	9.9%	11.7%	0.634	1.017	0.948-1.092
SXT	11.9%	23.1%	*0.016	1.075	1.014-1.141
AMC with GEN	7.6%	7.8%	0.954	1.002	0.923-1.088
TAZ with GEN	3.2%	7.1%	0.141	1.081	0.974-1.199
CAZ with GEN	2.9%	6.4%	0.172	1.079	0.967-1.204
CTX with GEN	NT	6.2%	0.409	1.073	0.908-1.267
CIP with GEN	3.7%	9.3%	0.065	1.092	0.994-1.200

AMC = amoxicillin/clavulanate, TAZ = piperacillin/tazobactam, IPM = imipenem, MEM = meropenem, CXM = cefuroxime, CAZ = ceftazidime, CFM = cefixime, FEP = cefepime, CTX = cefotaxime, CIP = ciprofloxacin, GEN = gentamicin, SXT = trimethoprim/sulfamethoxazole, NT = not tested. Quantitative susceptibilty testing of cefotaxime commenced in 2002. Percentages of resistance are those calculated by the logistic regression analysis. * = significant increase of resistance

ICUs

An increase in resistance was observed for most antibiotics among ICU isolates (Table 1). Trend-analysis showed a significant increase for ceftazidime, ciprofloxacin and trimethoprim-sulfamethoxazole (Table 1). ESBL production was demonstrated in 34 of 45 putative isolates; prevalence rose from 2% in 1998 to 8% in 2009 (p=0.026, odds ratio (OR) 1.141 (1.016-1.282)) of all ICU isolates. Carbapenemase production was not demonstrated.

Considering empiric treatment options of first choice; resistance to amoxicillin-clavulanate with gentamicin remained stable around 8%, while resistance to the other antibiotic combinations increased slightly (p>0.05)(Table 1). The prevalence of multidrug-resistant isolates in ICUs fluctuated between 0-23%. However, since 2004 the prevalence has been at least 12%.

Table 2: The trends in antimicrobial resistance of *K. pneumoniae* strains isolated from 14 Dutch Urology Services

Antimicrobial agent	%R '98	%R '09	p-value	OR	95% CI
AMC	9.0%	15.1%	0.084	1.055	0.993-1.120
TAZ	2.2%	5.2%	0.134	1.086	0.975-1.210
CXM	2.8%	7.9%	*0.033	1.105	1.008-1.212
CAZ	0.8%	2.7%	0.185	1.115	0.949-1.309
CFM	0.5%	2.7%	0.091	1.172	0.975-1.408
FEP	0.3%	2.1%	0.074	1.210	0.981-1.492
CTX	NT	7.9%	0.616	1.069	0.823-1.388
IPM	0.0%	0.0%	0.467	0.861	0.575-1.289
MEM	0.0%	0.0%	0.521	0.824	0.456-1.489
CIP	5.1%	9.3%	0.137	1.060	0.982-1.144
GEN	1.0%	3.2%	0.157	1.111	0.960-1.287
SXT	13.5%	27.8%	*0.001	1.086	1.034-1.140
AMC with GEN	0.2%	1.9%	0.111	1.225	0.954-1.573
TAZ with GEN	0.4%	0.3%	0.998	1.000	0.722-1.385
CAZ with GEN	0.1%	1.3%	0.168	1.262	0.906-1.758
CTX with GEN	NT	1.1%	0.519	1.141	0.765-1.702
CIP with GEN	0.1%	1.2%	0.204	1.228	0.894-1.686

AMC = amoxicillin/clavulanate, TAZ = piperacillin/tazobactam, IPM = imipenem, MEM = meropenem, CXM = cefuroxime, CAZ = ceftazidime, CFM = cefixime, FEP = cefepime, CTX = cefotaxime, CIP = ciprofloxacin, GEN = gentamicin, SXT = trimethoprim/sulfamethoxazole, NT = not tested. Quantitative susceptibilty testing of cefotaxime commenced in 2002. Percentages of resistance are those calculated by the logistic regression analysis. * = significant increase of resistance

Urology services

An increase of resistance for most antibiotics was observed among isolates from the urology services, which was significant for cefuroxime and trimethoprim-sulfamethoxazole (Table 2). The prevalence of ESBL producing isolates increased from 0% in 1998 and to 2% in 2009 (p=0.101, OR 1.462 (0.955-1.803)). Carbapenemase production was not demonstrated. Prevalence of resistance to the different antibiotic combinations increased over time and ranged in 2009 from 1.1% to 1.9%, except for

resistance to the combination of piperacillin-tazobactam with gentamicin, which remained stable at 0.3-0.4% (Table 2). Multidrug-resistance rose to 7.4% in 2009.

ICU vs. urology isolates
Trend-analysis showed that antimicrobial resistance was significantly higher among ICU isolates except for the resistance to carbapenems, which was the same in both groups, and resistance to trimethoprim-sulfamethoxazole, which was higher in urology isolates but not significantly.

Discussion

We observed a significant increase in resistance to ceftazidime, trimethoprim-sulfamethoxazole and ciprofloxacin among isolates from the ICUs. Among these isolates the increase in prevalence of ESBL producing isolates was significant too. Likewise, we observed a significant rise in resistance to cefuroxime and trimethoprim-sulfamethoxazole among urology isolates. Overall the resistance levels of *K. pneumoniae* isolates from ICUs were significantly higher than resistance of isolates from the urology services. The increase in resistance to most antibiotics was in accordance with the global increase in antibiotic resistance.[1, 3]

ICUs
The ICU is a ward with a relatively high use of broad-spectrum antibiotics. Empiric treatment of first choice is usually a combination of a beta-lactam antibiotic with an aminoglycoside.[2] In 2009, resistance levels to these antibiotic combinations varied from 6% to 9%. Although a resistance level with an upper limit of 10% is considered suitable for empiric treatment,[7] from a clinical point of view a resistance level of ≤5% would be more appropriate for more severe illnesses. Whether a combination of agents is suitable for empiric therapy also depends on the prevalence of *K. pneumoniae* as the causative agent of an infection.
An alarming finding is the increase of resistance to 3rd generation cephalosporins and the increasing prevalence of ESBLs. Recently, Sturm et al.[8] described a ESBL-prevalence of 5,2% in unselected *K. pneumoniae* isolates. In our study, the prevalence of putative and confirmed ESBLs in *K. pneumoniae* was approximately the same as in Germany (8.2% in 2002) and France (5.% in 2002), but higher than in the Scandinavian countries and lower than many other countries in Southern Europe (28% in Italy 2002).[1] This is very likely due to the lower use of cephalosporins in the Netherlands.[3]

Urology services
The urology service is a ward with a high use of specific antibiotics. In particular fluoroquinolones, trimethoprim-sulfamethoxazole, cefuroxime and amoxicillin-clavulanate (with gentamicin) are often prescribed as empiric treatment of first choice.[2] Nevertheless, resistance to amoxicillin-clavulanate and trimethoprim-sulfamethoxazole was more than 10% and those antibiotics should not be used as empiric treatment for UTIs with *K. pneumoniae*.[7] Resistance to other beta-lactam

antibiotics was, in contrast to the antibiotics of first choice, much lower. Notably, also the prevalence of ESBLs (2%) was still low. The increase in resistance for fluoroquinolones was not significant. Nevertheless the resistance of *Escherichia coli* to ciprofloxacin increased to 15%-20% and, therefore, empiric treatment with fluoroquinolones is not recommended for a UTI.[3, 7]

Overall the resistance was higher in ICU isolates compared with urology isolates. This is probably due to the high use of broad-spectrum antibiotics: 132 defined daily doses (DDD) /100 patient days at the ICUs while the overall use in hospitals is 58.7 DDD/100 patient days.[3] This is in line with the generally accepted belief that antibiotic use is the main factor for the emergence of antibiotic resistance.[9]
Unfortunately, clinical data (e.g. patient characteristics and source of isolation) was not available. However, strengths of the study were that it was conducted over several years, the isolates were collected from 14 hospitals and quantitative susceptibility testing was performed in a central laboratory.
From our study we concluded that antibiotic resistance is increasing and that the prevalence of resistance was significantly higher at the ICU. Therefore, a regular update of empiric treatment protocols based on actual surveillance data is justified.

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Chapter 8

General discussion
Summary
Samenvatting

Worldwide the prevalence of resistance is increasing[1], which is mostly caused by the high use of antibiotic agents[2, 3]. The high prevalence of resistance complicates the optimal empirical antibiotic choice. When choosing an antibiotic agent for empirical treatment the expected prevalence of resistance should be less than 10% to 20%, depending on the severity of the infection[4]. Therefore, current antibiotic resistance surveillance data are needed to make an optimal empiric choice.
These data must be obtained from the target patient population: i.e. data from hospitals are not suitable for general practice patients or nursing home residents or vice versa.
In this thesis we focused not only on different populations in the Netherlands, Germany and Belgium but we also wanted to know the influence of living near the border with Germany and Belgium on the prevalence of resistance. The reason is that the prevalence of resistance of these both countries is higher than in the Netherlands. We hypothesize that cross border traffic (for health care, social or economical reasons) influences the prevalence of resistance in the province of Limburg.
The antibiotic surveillance was carried out in the Netherlands and in the Euregion Meuse-Rhine (consisting of the provincie Limburg in the Netherlands, the provincie Limburg, the province de Liège and the Deutschsprachige Gemeinschaft in Belgium, and the Regio Aachen in Germany). The main focus of this thesis was to evaluate the prevalence of antibiotic resistance in a cross border region. Differences in antibiotic resistance between the three countries hamper cross border health care[5].
We determined the prevalence of antibiotic resistance in different patient populations: general practice patients, nursing home residents, intensive care unit patients and urology services patients. These populations have expected differences in prevalence of resistance probably caused by differences in the amount and choice of antibiotics. The implications of the differences in antibiotic resistance on empiric antibiotic treatment are also discussed.

The micro-organisms of interest were *S. aureus*, as the representative of the Gram positive commensal microbiota and, *Eschericha coli* and *Klebsiella pneumoniae* representing the Gram negative commensal microbiota. The commensal microbiota was chosen as target micro-organisms as most infections are caused by the patient' own commensal microbiota[6-9]. Information on the prevalence of resistance of the commensal microbiota is, therefore relevant to make an optimal empiric choice for the treatment of infections. Another reason to choose representatives of the commensal microbiota is that the commensals are considered the main reservoir for antibiotic resistance genes and resistant microorganisms[10, 11]. Resistance traits can be transferred from the resistant commensals to the potential pathogens via mobile genetic elements (horizontal gene transfer)[10]. Therefore, the commensal microbiota is considered the main reservoir for antibiotic resistant microorganisms; the resistance in the infecting microorganisms represents only the tip of the antibiotic resistance 'iceberg'.

Nursing home residents

Nursing home residents are a frail and vulnerable, mostly elderly population who are living relatively close together and often need skilled nursing care due to co-morbidities such as chronic wound infections and incontinence for feces and or urine. The prevalence of infections among nursing home residents is relatively high[12], which has led to a high use of antibiotics.

In the Province of Limburg in the Netherlands the antibiotic resistance of *E. coli* isolated from nursing home residents was high, e.g. amoxicillin-clavulanic acid 23% and trimethoprim-sulfamethoxazole 19% (chapter 2). Among the *E. coli* isolates especially the high resistance to the fluoroquinolones (16%) is a point of concern. This might be due to the high use of these agents (14.8 DDD/1000 residents/day). Fluoroquinolones are easy to administer, have a broad antibacterial spectrum, favorable pharmacodynamics and minor side effects[13], but due to the high resistance rates the empirical use of these agents is not advisable. Resistance to nitrofurantoin was almost absent and, therefore, appropriate for empiric treatment. However, the poor tissue penetration[14] makes it unsuitable for complicated UTIs. We also demonstrated a difference in resistance between the participating nursing homes. This could be caused by differences in antibiotic use and characteristics of the nursing homes, such as size and ward dynamics. Furthermore, we demonstrated multi drug resistant *E. coli* isolates with the same PFGE profile in different nursing homes suggesting spread of these bacteria between nursing home residents.

The prevalence of resistance among *S. aureus* was investigated for nursing home residents in the Province of Limburg in the Netherlands and the regions of Euskirchen and Daun in Germany (chapter 5). The prevalence of MRSA was relatively high (3.5%) in the German nursing homes compared with the Dutch nursing homes (<1%). The German MRSA isolates were also resistant to the macrolides and ciprofloxacin, limiting the treatment options in case of an infection. The German MRSA isolates (n=20) belonged to two globally prevalent clones[15] and were found in six out of the ten nursing homes. In four nursing homes at least two residents were colonized with MRSA isolates with the same PFGE profile. These results strongly suggested the spread of resistant bacteria within the nursing homes and probably between nursing homes and hospitals and vice versa, a revolving door.

Among the Dutch nursing home residents the prevalence of antibiotic resistant *S. aureus* was low, e.g. erythromycin 2%, clindamycin 0%, as was the prevalence of MRSA (<1%). For empiric treatment of a *S. aureus* infection many antibiotic options remain among both the German and Dutch nursing home residents. Only the use of clindamycin should be discouraged among the German nursing residents, since the prevalence of resistance was 15%, exceeding the 10% resistance cut-off for appropriate empiric treatment[4]. Moreover, based on the spread of MRSAs among the German nursing homes, adherence to a nursing home specific infection control policy is warranted.

Hospital patients

In this thesis two groups of hospital patients were included: the ICU patients, a group of patients with a high use and variety of broad spectrum antibiotics, and the urology patients, a patient group with a high use of a limited group of antibiotics e.g. fluoroquinolones. The urology isolates were collected from 9 services in the Euregion Meuse-Rhine.

Probably, due to the frequent use of antibiotics, many *E. coli* isolates were resistant to the agents of first choice for a complicated UTI, e.g. amoxicillin-clavulanic acid, fluoroquinolones and trimethoprim-sulfamethoxazole (chapter 2). However, the prevalence of resistance varied significantly in the three different regions isolates were collected from. In particular, resistance among the Belgian *E. coli* isolates was high: e.g. amoxicillin-clavulanic acid 39%, ciprofloxacin 37% and trimethoprim-sulfamethoxazole 36%. Subsequently, alternatives options for oral empiric treatment are few. Only piperacillin-tazobactam, the 3rd and 4th generation cephalosporines, nitrofurantoin and aminoglycosides of which the prevalence of resistance was 10% or lower might be suitable for empiric treatment. However, they are unsuitable based on other factors such as oral unavailability and limited tissue penetration.

This chapter together with the prevalence of resistance among *K. pneumoniae* isolates from ICU and urology patients (chapter 7) also underlined that the prevalence of resistance differs between the different hospital wards. An antibiotic, which has a prevalence of resistance of less than 10% for ICU patients and is, therefore, appropriate for empiric treatment, might have a prevalence of more than 10% and unsuitable for urology patients, or vice versa. For example the prevalence of resistance among *K. pneumoniae* collected from ICU patients was 26% for amoxicillin-clavulanic acid, 19% for ciprofloxacin and 23% for trimethoprim-sulfamethoxazole. These percentages were 15%, 9% and 28% among the urology isolates, respectively. This is probably related to the differences in antibiotics most used at the different wards. Therefore, current antibiotic surveillance data of the target population at a ward specific level are needed to help physicians make an optimal choice for empiric antibiotic treatment.

Nevertheless, just as for the nursing home residents, further research into alternative treatment options is warranted. This could be the development of new agents (not necessarily the classic chemical antibiotic substances) or the use of already existing agents with different indications. An example is fosfomycin. Until now this agent is mostly used and registered only for the treatment of uncomplicated UTIs. However, limited research has demonstrated its potential use for more severe infections and infections caused by different bacteria, such as *S. aureus*[16-18], and further studies are warranted.

A high prevalence of resistance is also a point of concern among *K. pneumoniae* among ICU and urology patients in the Netherlands. *K. pneumoniae* is mostly an opportunistic pathogen often causing infections in those already ill[19]. Therefore, the appropriateness of empiric treatment is very important since the impact of inappropriate therapy is expected to be high among immunocompromised patients. However, over time also the prevalence of resistance among these bacteria increased especially for

ceftazidime, ciprofloxacin and trimethoprim-sulfamethoxazole among the ICU isolates and cefuroxime and trimethoprim-sulfamethoxazole among the urology isolates, decreasing the antibiotic alternatives for empiric treatment (chapter 7).

Spread of resistance

Not only the emergence or increase of resistance is a point of concern but also the spread of the resistant strains is a problem. Bacteria do not recognize political borders, could spread to another country and pose a problem when causing an infection, which requires empiric antibiotic treatment.

Over the years there have been many reports of globally emerging resistant strains[20, 21], which pose a threat to human health, such as the multi drug resistant ST131 *E. coli*[22, 23]. In this thesis the presence of this strain was demonstrated in the entire Euregion among the general practice patients, nursing home residents, ICU and urology patients. Also the ST393 and ST88 *E. coli* isolates were found in the entire Euregion (chapter 4). PFGE analysis among the ST131, ST393 and ST88 isolates showed comparable and similar profiles in different parts and populations of the Euregion (chapter 2, 3 and 4). This demonstrates the presence and spread of ST131 in the Euregion. However, it is difficult to prove actual transfer from one healthcare facility in the Euregion to another. Previous research has demonstrated that resistant strains predominantly spread in a health care network[24] and although there is cooperation between the health care institutes in the Euregion it might not be enough (in terms of patient transfer) to cause a spread of resistant strains. Follow up of the *E. coli* population is required to determine patterns of spread in the Euregion. Also, the results from the Euregion should be compared with results from the three individual countries separately to detect any differences in genetic background and to aid in the detection of spread of particular patterns of resistance.

Overall, the prevalence of antibiotic resistance is lower in the Netherlands compared with Germany and Belgium[1]. If resistant strains would spread from Belgium and Germany to the Netherlands it would affect the prevalence of resistance in the province of Limburg compared with the remaining provinces in the Netherlands. However, the differences in antibiotic resistance and population structure of *S. aureus* isolates collected from nursing home residents and general practice patients in the province of Limburg and the remaining provinces of the Netherlands were not significant. Therefore, the inhabitants of Limburg are probably not more or not yet at risk for an infection with a resistant *S. aureus* strain than the other Dutch inhabitants.

However, one might argue that there still could be an increased risk but that the threat of international travel to countries with a high prevalence of multi drug resistant bacteria might play an even more important role than the regional cross border movements. This is a threat for all Dutch residents not only those in Limburg. Nowadays people travel around the globe also to countries with a high prevalence of multi drug resistant bacteria. These international movements facilitates the spread of resistant strains[25].

Future recommendations

Due to the globally increasing prevalence of antibiotic resistance among many clinically relevant potentially pathogenic micro-organisms it is essential to make the choice for empiric treatment with consideration. Since antibiotic use is the leading driving force for increasing antibiotic resistance[2, 26-28], it is of importance to use antibiotics only when necessary. To accomplish this many antibiotic stewardship programs have been developed. Antibiotic stewardship programs are an ongoing effort to optimize antimicrobial use in order to improve patient outcomes, ensure cost effective therapy and reduce adverse sequelae of antimicrobial use[29]. These programs have three goals[30]: 1) treatment with appropriate antibiotics according to the 4Ds as described by Joseph and Rodvold[31] (right drug, right dose, right duration and de-escalation to pathogen directed therapy), 2) prevent antibiotic overuse, misuse or abuse, 3) minimize the development of antibiotic resistance.
These programs have to be initiated and maintained in cooperation with all health care practitioners, not only in the hospitals but also in nursing homes and in general practice. We have to find the balance between what is best for the individual patient and what is best for the entire population. To determine the appropriateness of empiric antibiotic treatment regarding antibiotic resistance, accurate data on antibiotic resistance and the prevalence of multi drug resistance is essential[32, 33]. Also, proper infection control is crucial as is optimal information on the clusters of resistant bacteria among specific patient populations.

For the optimal empiric choice surveillance of antimicrobial resistance is essential and should be continued both at a local, a (Eu)regional and a(n) (inter)national level to ensure a favorable patient outcome. These survey results should be used to adapt stewardship programs, treatment protocols and infection control policies, preferably to a local level.
An effort is required from all those working in healthcare, on associated research projects and also the patients themselves to prevent further increase and spread of antimicrobial resistance.

- Working together for a healthier tomorrow -

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Summary

Chapter 1: The commensal microbiota is an indispensable part of the human body. However, it also contains many potential pathogenic microorganisms such as *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae*, which can cause many different infections, mild but also severe.
In the previous century antibiotic agents, such as penicillin, were introduced and were a cure for many infectious diseases, which would have been lethal without these antibiotics. During the second half of the 20th century many antibiotics have been developed, all with their own mechanism of action and, therefore, all with their own antibacterial spectrum. However, parallel to the development and introduction of new antibiotics, the bacteria developed ways to survive the antibiotic action and be resistant. Well known examples are the methicillin resistant *S. aureus* (MRSA) and the extended spectrum beta-lactamase (ESBL) producing Gram negative bacteria. The MRSA is resistant due to an alteration of the drug target side and ESBL producers due to production of an enzyme that breaks down beta-lactam antibiotics. Resistance mechanisms to all antibiotics have been described. The main reason for the development and increase of resistance is the use of antibiotics. Other risk factors include the presence of invasive devices or foreign materials, co-morbidities, poor functional status and wounds.
Another major concern is the spread of the resistant bacteria. In our globalized world everything is becoming more interconnected, which also affects the spread of resistant bacteria. Previous research has shown that several clones of MRSA isolates and types of ESBLs and ESBL producers have emerged all around the globe but with differences between countries. Since bacteria do not stop at our borders, they could easily spread from one country to another, which hampers patient mobility, especially in a region with intensive cross border traffic, such as the Euregion Meuse-Rhine. Therefore, the prevalence of resistance should be monitored and action should be taken to control this increasing prevalence. Close cooperation in the Euregion regarding prevalence of resistance, antibiotic policies and infection control policies is required.

Chapter 2 describes the prevalence of resistance among *E. coli* isolates collected from urine samples from urology service patients in the Euregion Meuse-Rhine. There were significant differences in prevalence of resistance between the three subregions of the Euregion, which was highest among the Belgian isolates. The prevalence of ESBL producing isolates, of which most produced a CTX-M 15, was highest among the German isolates. For several tested antibiotics the prevalence of resistance exceeded 10% or even 20% and these agents are, therefore, not appropriate as empiric antibiotic treatment. These include amoxicillin-clavulanic acid, the fluoroquinolones and the folate pathway inhibitors. However, resistance to nitrofurantoin was low. The prevalence of the multi drug resistant *E. coli* isolates ranged from 11% in the Dutch region to 27% in the Belgian region ($p<=0.001$). MLST ST131 was the most prevalent type and several of those ST131 isolates, which were collected in the three regions of the Euregion, had a comparable or similar PFGE profile. This indicates the spread of ST131 *E. coli* among patients from the urology services in the Euregion.

The prevalence of resistance among *E. coli* isolates collected from residents in five nursing homes in the province of Limburg in the Netherlands and the antibiotic use among those residents are presented in **Chapter 3**. The prevalence of resistance was higher compared with that among general practice patients, especially for the fluoroquinolones. Since the prevalence of resistance exceeded 10% for many antibiotics including the fluoroquinolones, those agents were not suitable for empiric antibiotic treatment. Nevertheless, the prevalence of resistance varied between the different nursing homes.
Multi drug resistant *E. coli* was observed among 13% of the isolates. Many of those belonged to ST131 and had a similar PFGE pattern. This suggested that spread within and between nursing homes spread of multi drug resistant isolates did occur.
Variations in the use of antibiotics between the different nursing homes were found but overall the use of amoxicillin-clavulanic acid and the fluoroquinolones were highest. Often high use could be linked to high prevalence of resistance but also other risk factors for resistance and differences in need for care might play a role.

Chapter 4 presents the prevalence of resistance, genetic background and spread of (multi drug resistant) *E. coli* isolates collected from general practice patients, nursing home residents, urology and ICU patients in the Euregion Meuse-Rhine. The prevalence of resistance varied significantly between the four populations. Amoxicillin-clavulanic acid reistance was highest among the ICU isolates, and ciprofloxacin resistance among the URO isolates. Approximately 11% of the *E. coli* isolates was multi drug resistant and/or an ESBL producer. The most prevalent ESBL type was CTX-M 15. In total, 47 differents STs were observed of which ST131 was the most prevalent one. PFGE analysis of the ST131 isolates suggested the spread of isolates belonging to the same PFGE group in the entire Euregion. The emergence of resistant strains, such as ST131, might be a precursor for an increasing prevalence of resistance in the near future.

In **Chapter 5** the prevalence of resistance among MSSA isolates and the prevalence of MRSA isolates collected from nursing home residents in the province of Limburg in the Netherlands and the regions of Euskirchen and Daun in Germany are described. The MRSA prevalence was higher among the German nursing home isolates compared with the Dutch ones. The German MRSA isolates consisted of two globally prevalent clones and the isolates belonging to each clone had a very comparable PFGE profile. This demonstrated the spread of MRSA within and between the German nursing homes.
The prevalence of resistance among the MSSA isolates was highest among the Germany nursing home isolates, especially for the macrolides and clindamycin. Overall, the prevalence of resistance to the fluoroquinolones was high. The population structure determined with *spa* typing and BURP analysis also showed differences between the Dutch and German MSSA isolates disputing the spread between nursing homes cross the border.

The influence of cross border traffic from Belgium and Germany to the Province of Limburg on the prevalence of resistance and population structure among *S. aureus* collected from general practice patients and nursing home residents in the province of

Limburg compared with isolates from the remaining provinces in the Netherlands is discussed in **Chapter 6**. No differences in prevalence of resistance and population structure for the two groups of general practice isolates were observed and only a lower resistance for trimethoprim-sulfamethoxazole among the nursing home isolates in Limburg province compared with the remaining provinces. Among the nursing home isolates in Limburg the prevalence of *spa*-CC 084 was higher and that of *spa*-CC 002 was lower compared with isolates from the nursing homes in the remaining provinces. Despite great differences in resistance in the intramural setting in the Euregion Meuse-Rhine, intensive cross border traffic does not result in a higher prevalence of resistance and population structure in Limburg compared with the remaining provinces in the Netherlands in the extramural setting and in the nursing homes.

The prevalence of resistance of *K. pneumoniae* collected from ICU and urology patients in the Netherlands over a period of 12 years is described in **Chapter 7**. An increase in prevalence of resistance was observed, especially for ceftazidime, ciprofloxacin and trimethoprim-sulfamethoxazole among the ICU isolates, and cefuroxime and trimethoprim-sulfamethoxazole among the urology isolates. Overall, the prevalence of resistance was highest among the ICU isolates except for the carbapenems and trimethoprim-sulfamethoxazole where the prevalence of resistance was comparable. The high prevalence of resistance may render several antibiotics, especially among the ICU isolates, unsuitable for empiric treatment.

Chapter 8: The high prevalence of resistance, the prevalence of ESBLs and the emergence and endurance of specific resistant clones, such as *E. coli* ST131 and ST225-MRSA-II, in Euregion Meuse-Rhine and in the Netherlands, emphasize the need for continuation of antibiotic surveillance studies. Also further epidemiological studies investigating the genetic background of the resistant bacteria are warranted. These data are essential for development and adaptation of empiric antibiotic treatment protocols.
This thesis demonstrated differences in prevalence of resistance between countries and between different patient populations, but also within countries and within different patient populations. This underlines the need for surveillance at a local level, development and adaptation of empiric treatment protocols at a local level and the need for cooperation between health care institutes also in a cross border region.
Efforts from the entire health care sector are required to control the further increase of the prevalence of resistance. This includes the set up of local infection control policies. However, antibiotic stewardship programs including an empiric antibiotic treatment protocol should be implemented to decrease the overall use of antibiotics and the missuse of these agents. Due to the increasing prevalence of resistance the number of antibiotic agents suitable for empiric treatment is declining. Further studies to new or alternative agents are warranted.

Samenvatting

Hoofdstuk 1 beschrijft de rol van de commensale flora, het gebruik van antibiotica, het voorkomen en verspreiden van antibiotica resistentie en risicofactoren voor de toename van antibiotica resistentie. De commensale flora, bestaande uit vele soorten micro-organismen, is een onmisbaar onderdeel van het menselijk lichaam. Deze flora bestaat deels uit potentieel ziekmakende micro-organismen zoals *Staphylococcus aureus*, *Escherichia coli* en *Klebsiella pneumoniae*. Deze bacteriën kunnen verschillende infecties veroorzaken variërend van onschuldig tot zeer ernstig.
In de vorige eeuw zijn verschillende antibiotica, zoals penicilline, geïntroduceerd. Deze antibiotica waren en zijn nog steeds essentieel voor de behandeling van vele infectieziekten, die anders dodelijk zouden zijn geweest. Echter, gelijktijdig met de ontwikkeling van antibiotica hebben de bacteriën een manier ontwikkeld om de werking van deze middelen te overleven. Ze werden resistent. Bekende voorbeelden zijn de methicilline resistente *S. aureus* (MRSA ook bekend als de zogenaamde ziekenhuisbacterie) en de extended spectrum beta-lactamase (ESBL) producerende Gram negatieve bacteriën. De voornaamste oorzaak voor de ontwikkeling en toename van resistentie is het gebruik van antibiotica. Een hoog gebruik leidt tot meer resistentie. Ook patiënt specifieke factoren zoals de aanwezigheid van kathethers of andere lichaamsvreemde materialen, onderliggend lijden, slechte lichamelijke conditie en/of aanwezigheid van wonden spelen een rol bij de toename van resistentie.
Behalve antibiotica gebruik leidt ook verspreiding van antibiotica resistente bacteriën tot een toename van het resistentie probleem. Bacteriën worden niet tegengehouden door landsgrenzen. Gebleken is dat bepaalde types van MRSA en ESBL producerende bacteriën zich over de hele wereld hebben verspreid, echter er zijn wel verschillen tussen landen.
De verspreiding van bacteriën over de landsgrenzen heeft negatieve gevolgen voor de zogenaamde “cross border mobiliteit” van patiënten zoals in de Euregio Maas-Rijn: de grensregio bestaande uit de provincie Limburg in Nederland, de provincie Limburg en Luik en de Duitssprekende gemeenschap in België en Regio Aken in Duitsland.

Hoofdstuk 2 beschrijft het voorkomen van resistentie bij *E. coli* isolaten afkomstig van urine monsters van urologie patiënten uit 9 ziekenhuizen in de Euregio Maas-Rijn. Er waren significante verschillen in het voorkomen van resistentie in de drie delen van deze Euregio. Resistentie was over het algemeen het hoogst bij de Belgische isolaten. Het voorkomen van ESBL producerende bacteriën, voornamelijk type CTX-M 15, was het hoogst bij de Duitse isolaten (8%). Voor verschillende geteste antibiotica, zoals amoxicilline-clavulaanzuur, de fluoroquinolen en de foliumzuursyntheseremmers, was de resistentie hoger dan 10% of zelfs 20%. Deze antibiotica zijn derhalve niet geschikt voor empirische antibiotische behandeling, omdat de kans op falen van de therapie dan groot is. Echter, de resistentie voor nitrofurantoïne was laag in alle landen. Het voorkomen van multi resistente (resistentie voor 3 of meer klassen van antibiotica) E. coli isolaten varieerde van 11% in de Nederlandse tot 27% in de Belgische regio ($p \leq 0.001$). *E. coli* type ST131 was het meest voorkomende type en verschillende van

deze ST131 isolaten, uit de gehele Euregio, hadden een overeenkomend PFGE profiel, wat wijst op genetische verwantschap en verspreiding van dit ST131 type onder urologie patiënten in de Euregio. Deze bevinding is overeenkomstig de literatuur: *E. coli* ST131 is wereldwijd een veelvoorkomend vaak multi resistent type.

Het voorkomen van resistentie bij *E. coli* isolaten van bewoners van vijf verpleeghuizen in de provincie Limburg in Nederland en het antibiotica gebruik in deze huizen wordt beschreven in **Hoofdstuk 3**. Het voorkomen van resistentie was hoog vooral voor de fluoroquinolonen (16%). De resistentie was voor meerdere antibiotica, waaronder de fluoroquinolonen, hoger dan 10%. Deze middelen zijn daarom niet geschikt voor empirisch antibiotische behandeling. Tussen de verpleeghuizen werden wel verschillen in prevalentie van resistentie waargenomen, evenals verschillen in antibiotica gebruik. Over het algemeen was het gebruik van amoxicilline-clavulaanzuur en de fluoroquinolonen het hoogst. Vaak bleek een hoge resistentie in een bepaald verpleeghuis gerelateerd te kunnen worden aan een hoog antibiotica gebruik, maar ook andere risicofactoren en verschillen in zorgzwaarte spelen een rol.
Multi resistentie werd vastgesteld bij 13% van de isolaten. De meeste van deze isolaten waren van het ST131 type en hadden een vergelijkbaar PFGE profiel. Dit suggereert een verspreiding van multi resistente bacteriën in en tussen verpleeghuizen. Dit werd ook gezien in ziekenhuis isolaten (Hoofdstuk 2).

Hoofdstuk 4 beschrijft het voorkomen van antibiotica resistentie, de genetische achtergrond en verspreiding van (multi) resistente *E. coli* isolaten van vier verschillenden patiënten populaties: huisarts patiënten, verpleeghuisbewoners, urologie- en IC patiënten in de Euregio Maas-Rijn. Het voorkomen van resistentie verschilde significant tussen de vier populaties, verschillen tussen de landen waren relatief klein. De resistentie voor amoxicilline-clavulaanzuur was het hoogst bij de IC isolaten en de ciprofloxacine resistentie het hoogst bij de urologie isolaten. Ongeveer 11% (n=182) van de *E. coli* isolaten (n=1651) was multi resistent en/of produceerde een ESBL. Het meest voorkomende ESBL type was CTX-M 15. In totaal werden er 47 verschillende E. coli types (ST types) aangetoond, waarvan het ST131 type het meeste voorkwam. Resultaten van de PFGE analyse van deze ST131 isolaten suggereerde een verspreiding van deze isolaten met een vergelijkbaar PFGE profiel in de hele Euregio. Het opkomen van bepaalde resistente types, zoals ST131, kan een voorbode zijn voor een toename van antibiotica resistentie in de nabije toekomst.

In **Hoofdstuk 5** wordt het voorkomen van de antibiotica resistentie bij methicilline gevoelige *S. aureus* (MSSA) en methicilline resistentie S. aureus (MRSA) isolaten bij verpleeghuisbewoners in de provincie Limburg in Nederland en de regio's Euskirchen en Daun in Duitsland beschreven. Het voorkomen van MRSA was hoger bij de Duitse verpleeghuisisolaten (3,5%, n=20) vergeleken met de Nederlandse (<1%, n=2). De Duitse MRSA isolaten behoorden tot twee wereldwijd voorkomende types (ST225-MRSA-II en ST22-MRSA-IV) en hadden een vergelijkbaar PFGE profiel. Dit suggereert de verspreiding van deze MRSA types in en tussen Duitse verpleeghuizen. Het voorkomen van resistentie bij de MSSA isolaten was ook het hoogste bij de Duitse

verpleeghuisisolaten, met name de resistentie voor de macroliden en clindamycine (beiden 15%). Bij zowel de Nederlandse als Duitse isolaten was de resistentie voor de fluoroquinolonen relatief hoog (25% en 34% respectievelijk). De populatie structuur van de MSSA isolaten, bepaald met *spa* typering en BURP analyse, liet verschillen zien tussen de Nederlandse en Duitse isolaten. Dit spreekt eventuele verspreiding tussen verpleeghuizen tussen Nederland en Duitsland tegen.

De invloed van grensverkeer van België en Duitsland naar de provincie Limburg in Nederland op het voorkomen van resistentie en de populatie structuur van *S. aureus* bij huisarts patiënten en verpleeghuisbewoners in de province Limburg vergeleken met de andere provincies in Nederland wordt bediscussieerd in **Hoofdstuk 6**. Er waren geen verschillen in het voorkomen van resistentie en de populatie structuur van *S. aureus* tussen de twee groepen huisartsisolaten. Bij de verpleeghuisisolaten werd alleen een lagere resistentie voor trimethoprim-sulfamethoxazole gevonden in de provincie Limburg vergeleken met de andere provincies. Ook werd een verschil in populatie structuur gevonden: het voorkomen van *spa*-CC 084 was hoger en van *spa*-CC 002 was lager in de provincie Limburg vergeleken met de andere provinces. Ondanks verschillen in het voorkomen van antibiotica resistentie in de ziekenhuizen in de Euregio Maas-Rijn, leidt (intensief) grensverkeer blijkbaar niet tot een hogere prevalentie van resistentie en een andere populatie structuur bij huisartspatiënten en verpleeghuisbewoners in de provincie Limburg vergeleken met de andere provincies.

Het voorkomen van resistentie bij *K. pneumoniae* (n=1578) bij IC en urologie patiënten van 14 ziekenhuizen in Nederland in een periode van 12 jaar wordt beschreven in **Hoofdstuk 7**. Een toename van het voorkomen van resistentie werd met name gezien voor ceftazidime (tot 11%), ciprofloxacine (tot 19%) en trimethoprim-sulfamethoxazole (tot 23%) bij de IC isolaten en, cefuroxime (tot 8%) en trimethoprim-sulfamethoxazole (tot 28%) bij de urologie isolaten. Over het algemeen was de resistentie het hoogste bij de IC isolaten, maar niet voor de carbapenems en trimethoprim-sulfamethoxazole. Voor deze antibiotica was de resistentie in beide populaties vergelijkbaar. Door de hoge prevalentie van resistentie zijn vele van deze antibiotica, zoals amoxiciline-clavulaanzuur (26%), piperacilline-tazobactam (15%) en ciprofloxacine bij de IC patiënten en amoxicilline-clavulaanzuur (15%) en trimethoprim-sulfamethoxazole bij de urologie patiënten, niet geschikt voor empirische antibiotische behandeling.

Hoofdstuk 8: De relatief hoge prevalentie van antibiotica resistente isolaten, het voorkomen van ESBLs en bepaalde antibiotica resistente types, zoals *E. coli* ST131 en MRSA ST225-II, in de Euregio Maas-Rijn en in Nederland, benadrukt de noodzaak van (Euregionale) antibiotica surveillance studies en epidemiologische studies, die het voorkomen en de genetische achtergrond van resistente bacteriën onderzoeken. Deze data zijn essentieel voor het opstellen en aanpassen van empirische antibiotische behandelprotocollen.
Dit proefschrift toont aan dat er verschillen zijn in het voorkomen van resistentie tussen landen en tussen verschillende patiënten populaties, maar ook binnen landen en binnen patiënten populaties. Dit onderstreept de noodzaak van surveillance, het

opstellen en aanpassen van protocollen op lokaal niveau en de noodzaak van samenwerking tussen zorginstanties, ook of met name in een grensregio.
Om verdere toename in resistentie te beheersen is de inzet van de gehele zorg sector vereist. Dit houdt o.a. in het opstellen van lokale infectie preventie protocollen. Ook een antibiotica stewardship programma zou geïmplementeerd moeten worden om het inadequaat gebruik van antibiotica terug te dringen.
Door het stijgen van de antibiotica resistentie is het aantal antibiotica dat geschikt is voor empirische behandeling afgenomen. Verder onderzoek naar nieuwe middelen of nieuwe toepassing van oude middelen is noodzakelijk.

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Curriculum vitae

Christel van der Donk werd geboren op 19 november 1984 in ’s-Hertogenbosch en zij groeide op in het kleine plaatsje Middelrode. Van 1997 tot 2003 heeft zij het middelbaar onderwijs gevolgd aan het Gymnasium Bernrode te Heeswijk. In 2003 werd de grote overstap gemaakt naar het Limburgse Maastricht waar zij startte met de opleiding Geneeskunde aan de Universiteit van Maastricht. Tijdens deze opleiding heeft zij haar horizon verder uitbreid met verschillende stages in Birmingham, Mount Isa en Dublin.
Het laatste jaar van haar opleiding heeft zij haar klinische en wetenschappelijke stage gevolgd aan de afdeling medische microbiologie in het academisch ziekenhuis Maastricht, waarna zij haar opleiding afrondde in 2009. Na de geneeskunde opleiding kreeg zij de mogelijkheid om promotieonderzoek te starten bij dezelfde afdeling onder begeleiding van dr. Ellen Stobberingh en prof. dr. Cathrien Bruggeman. De resultaten van haar promotieonderzoek zijn te lezen in dit proefschrift. Tijdens de stage in het laatste jaar van de geneeskunde opleiding en gedurende haar promotieonderzoek is ze helemaal in de ban geraakt van de medische microbiologie. Sinds januari 2013 is zij in opleiding tot arts-microbioloog in het Erasmuc MC. Ook is zij na haar Limburgse avontuur weer teruggekeerd in het Brabantse, waar zij samen met haar vriend Giel Gaajetaan in Eindhoven woont.

List of publications

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WLJ Hansen, **C van der Donk**, CA Bruggeman, EE Stobberingh, PFG Wolffs. A real-time PCR-based semi-quantitative breakpoint to aid in molecular identification of urinary tract infections. *Accepted in PLoS ONE*.

CFM van der Donk, JMGA Schols, V Schneiders, K-H Grimm, EE Stobberingh. Resistance and population structure of *S. aureus* in nursing homes in the border region of the Netherlands and Germany. *submitted*.

CFM van der Donk, JHB van de Bovenkamp, H Bamelis, CC Driessen, K-H. Feldhoff, WA Kalka-Moll, K Magerman, EE Stobberingh. Prevalence and spread of multi drug resistant *E. coli* including ST131 in different patient populations in the Euregion Meuse-Rhine. *submitted*

From now on, you shall be known as: Sharkbait
Gill (Finding Nemo)