Antibiotic resistance and the commensal flora: role of the commensal flora in the development and spread of antimicrobial resistance

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Antibiotic Resistance and the Commensal Flora

Role of the commensal flora in the development and spread of
antimicrobial resistance

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Antibiotic Resistance and the Commensal Flora

Role of the commensal flora in the development and spread of
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Ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
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in het openbaar te verdedigen op
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List of abbreviations	9
Chapter 1 General Introduction & Outline of the Thesis.	11
Chapter 2 Erythromycin resistance in the commensal throat flora of patients visiting the general practitioner: a reservoir for resistance genes for potential pathogenic bacteria. <i>International Journal of Antimicrobial Agents</i> (Available online 15 June 2005)	33
Chapter 3 Antibiotic resistance of faecal <i>E. coli</i> in healthy volunteers of nine developing countries. <i>Journal of Antimicrobial Chemotherapy</i> (2004) 54, 952-955	47
Chapter 4 Effect of hospitalization on the antibiotic resistance of fecal <i>Enterococcus faecalis</i> of surgical patients over time. <i>Microbial Drug Resistance</i> (2005) 11(2), 154-158	63
Chapter 5 Acute uncomplicated lower urinary tract infections in general practice: clinical and microbiological cure rates after three versus five day treatment with trimethoprim; a randomized-controlled trial. <i>Accepted for publication in European Journal of General Practice</i>	77
Chapter 6 Urinary tract infections in general practice patients: diagnostic tests versus bacteriological culture. <i>Submitted in Journal of Antimicrobial Chemotherapy</i>	87
Chapter 7 Acute uncomplicated urinary tract infections in community-dwelling elderly females: care as usual of the general practitioner and antimicrobial resistance. <i>Submitted in Clinical Microbiology and Infection</i>	103
Chapter 8 General Discussion & Summary.	119
Nederlandse Samenvatting*	129
Dankwoord	139
Curriculum vitae	140
List of Publications	141

ARI	acute respiratory tract infection
ATC	Anatomic Therapeutic Chemical
bp	base pair
cfu/ml	colony forming units per milliliter
CLSI	Clinical and Laboratory Standards Institute
DDD	defined daily doses
DNA	deoxyribonucleic acid
<i>erm</i>	erythromycin ribosome methylase
GI-tract	gastro-intestinal tract
GP	general practitioner
<i>mef</i>	macrolide efflux
MIC	minimal inhibitory concentration
MLS	macrolide, lincosamide, streptogramins
mRNA	messenger ribonucleic acid
NCCLS	National Committee of Clinical Laboratory Standards
NHG	Dutch College on General Practitioners
NIVEL	Netherlands Institute for Health Service Research
NPV	negative predictive value
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PPV	positive predictive value
RIVM	The national Institute of Public health and the Environment
RUTI	recurrent urinary tract infections
SWAB	The Dutch Foundation of the Working Party on Antibiotic policy
UTI	urinary tract infection
WHO	World Health Organization

Chapter 1

General Introduction & Outline of the Thesis

1. 2. 3.

The commensal flora

The human commensal flora comprises all the natural bacteria that live in and on a healthy person. This commensal flora is exceedingly complex and consists of more than 500-1000 different bacterial species.^{1,2} Little is known about the associations between humans and their normal flora, but they are thought to be dynamic interactions rather than associations of mutual indifference. Both host and bacteria are considered to derive benefit from each other, and the associations are mostly mutualistic. The host obtains from the normal flora certain nutritional benefits, stimulation of the immune system and colonization strategies that exclude potential pathogenic micro-organisms at the site, called colonization resistance.³⁻⁵ The bacteria on the other hand derive from their host a supply of nutrients, a stable environment, a constant temperature, and protection. For these reasons, different commensal bacteria are located at specific anatomical colonization sites. Within one host, e.g. humans, variation in the commensal micro-organisms has been observed related to factors as age,⁶ gender and dietary factors. However, within limits of these fluctuations, the bacterial flora of humans is sufficient constant to give a general description of the situation. The normal human flora comprises of about 10^4 bacteria/cm² on the skin, 10^{10} bacteria/ml inhabit the upper respiratory tract and 10^{14} cfu/ml colonize the gastro-intestinal tract (GI-tract). The latter two reservoirs which are those with the largest body surface area⁷ will be explained below in more detail.

The respiratory tract

The respiratory tract of a human being can be divided into two parts: the upper and the lower respiratory tract. The lower respiratory tract is essentially sterile. The upper respiratory tract on the other hand, is coated with a biofilm containing many different bacterial species. Among these are potential pathogens like *Streptococcus pyogenes* and *Streptococcus pneumoniae*, but also many harmless streptococci and other species. The upper respiratory tract forms a barrier preventing microbes from reaching and colonizing the lower respiratory tract. The resident commensals, like alpha-hemolytic throat streptococci have been shown to inhibit growth of potentially pathogenic bacteria present in the same area.⁸ Among these resident commensals inhabiting the nasopharyngeal area are the viridans group streptococci which colonize the mouth and throat area^{9,10} and are able to inhibit the adherence of other bacteria to the mucosal surfaces through the production of specific proteases.¹¹ *Streptococcus pneumoniae*, the infecting agent in for example community-acquired pneumoniae¹² and otitis media,¹³ is another common colonizer of the mouth and throat area. Pneumococcal disease however is caused when the host-bacterium balance is disturbed for example in patients with an impaired immune system due to illness. *Streptococcus pyogenes*, the etiological agent of acute streptococcal pharyngitis¹² and necrotizing fasciitis,¹⁴ is a natural inhabitant of the

human throat as well which can easily move to the site of infection and from there spread to other parts of the body.

The commensal bacteria are equally affected by antimicrobial treatment as pathogenic bacteria and the selective pressure for the development of antibiotic resistance will, thus be as great among the commensal flora as among pathogens. Therefore, the commensal throat flora is considered a large reservoir of resistance (genes) from where these genes might be transferred to (potentially) pathogenic bacteria.¹⁵ Monitoring the prevalence of antimicrobial resistance (genes) in the commensal throat streptococci is a good indicator for the resistance that might be present in (potentially) pathogenic micro-organisms of the respiratory tract.

The gastro-intestinal tract

The human and its gastro-intestinal micro-flora are inevitably linked. About 10^{14} bacteria per gram feces inhabit the human alimentary tract,⁵ which is about tenfold greater than the total number of host eukaryotic cells in the human body.⁷ The human GI-tract offers a relatively non-hostile environment with an abundance of nutrients for the residing bacteria. Along the tract, the luminal milieu varies per environmental niche resulting in the presence of aerobic, facultative anaerobic and/or anaerobic micro-organisms.¹⁶ Likewise, the human host benefits from products of the bacterial metabolism such as short-chain fatty acids and from the contributions that the bacterial flora makes to the immune and non-immune host defense.¹

More than 500 bacterial species colonize the GI-tract and all of these enter the tract via the mouth with swallowed food.¹⁶ Hardly any bacteria ($10^3/\text{ml}$, mostly lactobacilli) can be found in the gastric fluid due to low pH and the presence of proteolytic enzymes which kill most ingested micro-organisms.^{1,2} The proximal small intestine has a bacterial flora of 10^5 - 10^7 bacteria/ml, consisting mainly of lactobacilli and enterococci. The distal part of the small intestine on the other hand contains greater numbers of bacteria ($10^8/\text{ml}$) and additional species including coliforms and *Bacteroides* spp. The large intestine contains a high density of living bacteria, which achieve concentrations up to 10^{12} - 10^{14} cells/g of luminal content.¹ Anaerobic bacteria outnumber aerobic bacteria by a factor of 100-1000.⁵ Pre-dominant genera in the human intestinal flora are among others the *Bacteroides*, *Bifidobacterium*, and *Clostridium* species. Aerobes (facultative anaerobes) such as *Escherichia coli*, *Enterobacter* spp., *Enterococcus* spp., *Klebsiella* spp., *Lactobacillus* spp., *Proteus* spp. and others are among the sub-dominant flora.^{1,2}

Even though representing the sub-dominant species of the gut flora, these (facultative) aerobes and more specific the most common species among them, i.e. *E. coli* and enterococci,¹⁶ are considered good indicator bacteria to study the prevalence of antimicrobial resistance in the normal flora of healthy volunteers.¹⁷

Antibiotic use

Antibiotics are widely used in humans to treat and prevent infections. The principal goal of the antibiotic use in the treatment of infections is eradication of the pathogen as quickly as possible with minimal adverse effects on the host.¹⁸ Most antibiotic prescriptions (~80%) in the developed countries are from the community,¹⁹⁻²¹ especially from general practitioners (GPs). In most parts of the world beta-lactam agents play a major role, but sulphonamides (with or without trimethoprim), macrolides, lincosamides and streptogramins (the MLS-group), fluoroquinolones, tetracyclines, aminoglycosides and glycopeptides are also widely used, some in the community and some mainly in hospitals.^{18,22,23} In addition, it has been repeatedly demonstrated that 30-60% of the use of antimicrobial agents is inappropriate, with consequent waste of resources.²⁴

Antibiotic use in the community is important as it is a risk factor for the development of antibiotic resistance not only outside the hospital setting but also for the spread into the hospital. Nevertheless, international and world-wide studies on antibiotic prescriptions and sales as well as international comparisons of the antibiotic use have only been performed the last decade (Figure 1).^{22,23,25,26} To make such comparative studies possible the data should be classified according to the Anatomic Therapeutic Chemical classification system²⁷ and expressed in defined daily doses (DDD, a unit based on the average daily dose used for the main indication of the drug in adults).

In the studies of Cars et al.,²² Rønning et al.,²³ and Goossens et al.²⁶ who compared non-hospital antibiotic use data in 15, 18 and 26 European countries respectively, a large variation in the antibiotic use between the different countries was found as well as large percentages of inappropriate antibiotic use in many countries. However, these studies did find that the antibiotic prescription rate in general practice in The Netherlands is very low. The antibiotic use in The Netherlands will be discussed in more detail further in this paragraph.

To obtain comparable data on antibiotic use, antibiotic sales and the effectiveness of the antibiotic use in developing countries is even harder than in the industrialized countries. There are several reasons for this. In most developing countries antibiotics can be purchased without a prescription of a physician. Furthermore, prescriptions made by physician are not always appropriate due to a lack of well-educated health care workers.²⁸ Continuing medical education is time consuming and it involves great expenses. In addition, antibiotics are readily available from drugstores and roadside market stalls without any control.²⁹ These unofficial sources are generally more accessible than official ones (hospital pharmacies). Also, medicines can be bought in these drug outlets per tablet or capsule rather than in complete (5-, 7- or 10-day) courses because many drugs are expensive.³⁰ When the prescribed drugs are out of stock an (inappropriate) alternative drugs is sold by the unqualified drug sellers. Furthermore, when symptoms disappear treatment is discontinued by the patient and left-over capsules will be saved for self-medication when another infection is suspected. These

factors all make it very hard to perform studies in developing countries on the relation between antibiotic use and antibiotic resistance. Some of the limited data that are available on antibiotic use in some of the developing countries will be discussed at the end of this section.

Figure 1. The comparison of antibiotic use in the non-hospital setting of 15 European countries.

From Goossens H et al. 2005.²⁶

The Netherlands

The use of antimicrobial agents in human medicine can be divided in extramural antibiotic use, e.g. the antibiotic use in the community mainly by the GPs and intramural antibiotic use in hospitals and nursing homes. Of the total human consumption of antimicrobial agents, ~80% is prescribed in the community.¹⁹⁻²¹

In the annual report (NethMap 2004) of the Dutch Working Group on Antibiotic Policies (SWAB)²⁵ the consumption of antimicrobial agents in primary health care (1998-2002) as well as the hospitals use (1998-2001) has been described. The total antibiotic use in the community has remained almost constant during this period of time. Tetracyclines (mainly doxycycline) together with penicillin with extended spectrum (amoxicillin), combinations of penicillins with beta-lactam inhibitors (co-amoxiclav) and macrolides were the agents mostly prescribed in the community in 2002. In the hospitals on the other hand the total amount of antibiotics used increased from 47.8 DDD per 100 patient days in 1998 to 54.7 DDD/100 patient days in 2001. All penicillins combined represented more than half of all hospital antibiotic use in 2001 in The Netherlands.

Developing Countries

Approximately 4 billion people, or 80% of the total global population, live in developing areas of the world³¹ and these countries suffer enormously from the burden of infectious disease. However, the per capita drug expenditure is much lower because the per capita income in these countries is much lower²⁹ as compared to the industrialized countries. According to the WHO developing countries spend up to 40% of their total health care budget on drugs vs. 11% in the developed nations.³¹

A study on the availability of antibiotics³² and the antibiotic prescribing pattern³³ in Ghana showed that the antibiotics most commonly available (co-trimoxazole, metronidazole, tetracycline, penicillin V and amoxycillin) were also the least expensive ones. In a household and drugstore survey in Mexico³⁴ the most frequently purchased agents were amino-penicillins, neomycin, natural penicillins (penicillin G) and tetracyclines, the latter two most frequently without a prescription. Also in a study from Manila, the Philippines,³⁰ the majority of antibiotics (66%) were purchased without a prescription and again the agents most frequently sold were amino- and natural penicillins. Furthermore a Nigerian study showed that almost half of the samples tested ($n = 581$) did not contain the appropriate amount of active ingredient.³⁵ A recent surveillance in Kenya monitoring the overall antimicrobial use over a 5 year period (1997-2001) described that the mean yearly national antibiotic use in humans, obtained from import licenses and manufacturers' records, during this 5 year period was 20.21 DDD/1000 inhabitants/day.³⁶ As this number includes all drugs for both the use in the community and the hospitals in Kenya it can not be compared to the data in Europe as the latter studies express their hospital antibiotic use in DDD/100 patient-days. Penicillins, tetracyclines, and trimethoprim again were the agents mostly used.

Thus although there are (limited) data available on the antimicrobial use in the developing countries most of them report only the agents prescribed most, but hardly any data on the DDD/1000 inhabitants/day were found, excepted for one study in Kenya.³⁶ So, no trends in consumption or sales could be detected nor could a comparison be made with the data in the European countries.

Antibiotic resistance

Bacterial resistance to antimicrobial agents is an important obstacle for the successful treatment of bacterial infections, leading not only to treatment failure, but also to prolonged morbidity and even mortality.³⁷ Resistance is considered to be present if bacteria are not susceptible to a clinically relevant concentration of an antibiotic and/or the bacteria possess a mechanism or property which will render the antibiotic ineffective. Bacteria can be intrinsically resistant or unsusceptible which means that a whole species or genus of bacteria is not susceptible to a particular drug. A bacterium of

a normally susceptible species to a certain antimicrobial agent might become resistant to this agent by mutation³⁸ or acquisition of new exogenous genes which is called acquired resistance.³⁹⁻⁴² Antibiotic resistance due to chromosomal mutations could occur spontaneously in replicating bacteria due to the imperfect fidelity of DNA replication, and is therefore not influenced by the presence of antimicrobial agents. Resistance by the acquisition of new exogenous genes on the other hand, refers to the prior existence of a resistance gene in a reservoir that is acquired by another bacterium⁴³ through transformation, transduction or conjugation.⁴⁴

Once resistance has occurred in a few bacteria of a population they might be selected for when the conditions in the environment change in their benefit. Furthermore, once established in a population these resistant bacteria and their resistance genes might be spread throughout different populations. Mechanisms by which resistance emerges and the selection, dissemination, and transfer of resistance (genes) will be further described below. Resistance present in the commensal flora and surveillance of antibiotic resistance are enlightened as well.

Resistance mechanisms

Resistance genes, acquired exogenous genes or chromosomal mutations resulting in new resistance genes, in turn give rise to several biochemical mechanisms. These mechanisms include among others reduced permeability of the bacterial cell by alteration or elimination of the entry port (aminoglycosides), pumps that actively export the antimicrobial agent out of the bacterial cell (tetracyclines, fluoroquinolones), and alteration or inactivation of the drug target (erythromycin, tetracyclines, fluoroquinolones) or the drug itself (aminoglycosides, chloramphenicol, beta-lactams) by degrading or chemically modifying enzymes.^{37,41,45}

Selection by antibiotic use

At any point of time a minority of strains resistant to a certain antibiotic may be present in a population. Whether these resistant strains survive and replicate in this population depends on the selective pressure placed on them.⁴⁰ Mostly they disappear as spontaneously as they have emerged. Due to increasing antibiotic use susceptible bacteria will be eliminated and the resistant ones will proliferate at the expense of the susceptible strains and the majority of the population will become resistant.⁴⁶ However, susceptible bacteria might be reintroduced after disappearance of the selective pressure of the antibiotic use for two reasons: introduction of new susceptible strains from the environment and because resistant bacteria are less 'fit'.^{47,48} Small numbers of resistant strains will remain present, certainly after repeated exposure to antibiotics. This means that antibiotic use will cause a rise in the number of resistant bacteria in the endogenous flora of exposed individuals and populations.¹⁸ These (resistant) commensal bacteria in

an individual or a population form a large reservoir of resistance genes which consequently increases the risk of transfer of their resistance genes to (potentially) pathogenic micro-organisms.⁴⁹

Dissemination of antibiotic resistance

Next to the selection of antibiotic resistant bacteria, the dissemination of these resistant strains and their resistance genes is a very important factor in the spread of antimicrobial resistance. There are two different aspects of dissemination that may lead to colonization with drug-resistant bacteria or transfer of resistance genes to the commensal flora. First, dissemination can be caused by direct contact between individuals. For indirect dissemination a third party is involved in the process of the spread of resistance. For example among inpatients this spread might occur via health-care workers or medical equipment in hospitals.^{43,50-52} In turn, resistance acquired in the hospital might be spread into the community after discharge of the patient and vice versa, resistance obtained by a patient in the community might be introduced into the hospital after admission.²¹ In addition, increasing international travel provides great opportunities for both pathogenic and non-pathogenic antibiotic-resistant strains to be carried over long distances to other countries, thus further aiding the dissemination of resistant bacteria.⁵³⁻⁵⁵

Other factors influencing the spread of antibiotic resistant bacteria or resistance genes from one host to another are high population density (crowding in nursing homes, day care centers, hospitals, large cities),^{37,56,57} high antibiotic use (hospital settings), use of inappropriate antimicrobial agents and poor hygiene and sanitary facilities as well as drugs of substandard quality (developing countries)^{29,43,53,54,58,59} and antibiotic use in animals via the food chain.^{43,53}

Transfer of resistance

Resistance genes, located on the bacterial chromosome or on plasmids, can transfer by conjugation to other bacteria and even to other species. The resistance genes found on plasmids (R-plasmids) are embedded in small, mobile DNA units called transposons which can easily hop within one bacterium between the plasmid and its chromosome and even from bacterium to bacterium.^{38,42,45,49} The potential for the spread of resistance is related to the bacterial host ranges of these transposons, their efficiency of transfer, and the balance of their particular advantageous and disadvantageous properties.^{42,43} Transfer of resistance (genes) has been described between bacteria belonging to the same species and between micro-organisms of different species as well.⁵ Also, resistant bacteria from animal origin may transfer their resistance genes either to bacteria pathogenic for humans or to the commensal flora of humans through direct contact (for example with pet animals) or indirectly through meat products.⁴⁹

Many mobile antibiotic resistance elements encode multiple resistance determinants which provides selective advantages to bacteria in a broad range of clinical settings.⁴³

Antibiotic resistance in the commensal flora

In general, potentially pathogenic bacteria differ little in their basic cellular biology from commensal bacteria. These fundamental cellular processes, like transcription of the mRNA through ribosomes or the cell wall assemblage, are targets of classical antimicrobial agents.⁶⁰ This results in the elimination of both the benign commensal bacteria and the pathogens by antimicrobial therapy.⁶⁰ So, administration of antimicrobial agents prescribed for the treatment of infections can cause disturbances in the ecological balance between the host and its endogenous micro-organisms. The incomplete absorption of orally administered drugs for example or the secretion of an antimicrobial agent into the intestinal lumen maintain the presence of a low-dose antibiotic pressure which can influence the intestinal micro-flora at different habitats. As a consequence, antibiotic resistant bacteria in the normal flora may increase in numbers³ and constitute an enormous reservoir of resistance genes for (potentially) pathogenic bacteria,^{5,50} the latter being considered only the 'tip of the resistance iceberg'.⁴⁹ Moreover, the level of resistance in the normal flora is considered a good indicator for the selection pressure exerted by antibiotic use in that population.^{17,61}

Investigation of the prevalence of resistance of certain indicator bacteria like *E. coli* and enterococci in the intestinal tract and streptococci in the respiratory tract of humans makes it possible to compare the prevalence of antimicrobial resistance and resistance genes in different populations and over time. In this way, surveillance studies of antimicrobial resistance in the normal flora can serve as an early warning system for the antibiotic resistance problems to be expected in potentially pathogenic micro-organisms.

Surveillance of antimicrobial resistance

The aim of surveillance is to monitor the prevalence and degree of antimicrobial resistance and resistance genes being expressed in and transmitted through various populations of microbes.⁶² Surveillance of antimicrobial susceptibility will therefore improve empirical antimicrobial treatment in both the community and the hospital. Furthermore, surveillance studies will guide antimicrobial policy makers and all those involved in the use of antibiotics. In addition, these studies will monitor prospectively the activity and the usefulness of available agents, identify resistance problems and monitor changes in resistance patterns and track the spread of significant resistance patterns.⁴⁴ These surveillances can either be carried out to detect differences in the resistance prevalences of clinical isolates or in the prevalence of resistance in the endogenous flora of different populations. It is widely considered that most infections in a population result from the commensal flora carried within a population.⁶³ Moreover,

resistant bacteria of the normal flora not only constitute an enormous reservoir of resistance genes for (potentially) pathogenic bacteria, but also the level of resistance in the endogenous flora is considered to be a good indicator for the selection pressure exerted by antibiotic use on a population and for resistance problems to be expected in potential pathogens.^{17,61}

Urinary tract infections

An acute uncomplicated urinary tract infection (UTI) is one of the most common infections in women⁶⁴ and is characterized by the presence of dysuria, frequent and/or urgent urination and pyuria alone or in combination.⁶⁵ An acute uncomplicated UTI occurs primarily in healthy, pre-menopausal, non-pregnant women with an apparently normal urinary tract. It is estimated that 1 out of 3 women of 24 years of age has experienced at least one UTI episode and as many as 60% of women report having had a UTI in their lifetime.^{66,67} Approximately 44% of the patients will experience at least one recurrence in the 12-month follow-up after the index UTI.⁶⁸

Most uropathogens originate from the commensal gut flora⁶⁹ and enter the bladder in an ascending route via the urethra with an interim phase of per-urethral and distal urethral colonization.^{64,70} Vaginal colonization is a prerequisite to bladder infection and factors that increase the risk of UTI generally do so in part by facilitating vaginal colonization. In the majority of the acute uncomplicated community acquired UTIs *Escherichia coli* is the most likely candidate bacterium as uropathogen.⁷¹⁻⁷³ The remaining uropathogens include *Staphylococcus saprophyticus*, mainly in young, healthy female patients, *Enterococcus faecalis*, and *Proteus spp.*⁷⁴ *Klebsiella spp.*, *Pseudomonas spp.*, and *Enterobacter spp.* are less frequent causes of an acute uncomplicated UTI.

Management of urinary tract infections

An acute uncomplicated urinary tract infection is usually empirically treated by the GP based upon the complaints of the patient without urine culture or susceptibility testing.⁷¹ The GP can additionally perform a urinary dipstick test,⁶⁵ i.e. the nitrite and leukocyte-esterase tests, directly on the urine and the result will predict or rule out a UTI. This method has replaced microscopy because it is fast, cheap and very convenient for the GP. A positive nitrite test performed on fresh urine can almost certainly predict a UTI whereas a negative nitrite tests does not rule out a UTI. However, a negative nitrite test combined with a negative leukocyte-esterase tests does rule out the presence of a UTI in the majority of the cases.⁷⁵

In the Netherlands for the treatment of acute uncomplicated UTIs trimethoprim and nitrofurantoin are the drugs of choice according to the guidelines of the Dutch College on General Practitioners (NHG)⁷⁵ and preferably prescribed as a 3-day course of 3 DD

and 1 DD respectively for nitrofurantoin (100 mg) and trimethoprim (300 mg). In case of recurrent UTI another agent is chosen and therapy will be preceded by culture and susceptibility testing.

Recurrent urinary tract infections

Approximately 45% of the patients suffering from a UTI will experience one or more recurrent infections in the year following the index UTI.⁶⁸ A recurrent UTI (RUTI) is defined as 3 or more symptomatic UTIs in the past year or 2 such episodes in the past 6 months.⁷⁶ RUTIs form a substantial burden for the health-care system in terms of costs, sick leave, and morbidity.⁷⁷ In most cases, women suffering from RUTIs will receive low-dose prophylactic antimicrobial regimens for several months. This strategy consists of daily or thrice-weekly subtherapeutic doses of an antimicrobial agent effective for the treatment of an established UTI.^{76,77}

A study on host risk factors predictive of RUTIs in young women (18-30 years) found that both maternal history of UTIs and childhood onset of cystitis were associated with 2-4 fold increased risk for RUTIs. This suggest that (inherited) genetic factors may be important in women with recurrent infections.⁷⁶ Furthermore, sexual intercourse, intercourse frequency, and contraceptive exposure were also associated with RUTIs.

Urinary tract infections in the elderly

It has been shown that the frequency of bacteriuria increases with advancing age for both men and women.⁷⁸ Also, the prevalence of bacteriuria is very high in the more impaired elderly who require institutional care because of functional deficits and co-existing illnesses.⁷⁹ The contributing factors for bacteriuria in the non-institutionalized elderly women include loss of the estrogen effect on the genitourinary mucosa and the depletion of vaginal colonization of *Lactobacilli*,⁸⁰ increased residual urine, and genitourinary abnormalities such as cystocoeles, rectocoeles and bladder diverticula.

The etiology of UTIs in the elderly is in general distinct from that in younger women. *E. coli*, still the most prevalent uropathogen in these women, is proportionally less frequent detected,⁷⁹ but more *Proteus spp.* and *Klebsiella spp.* can be found.

Antibiotic resistance in uropathogens

As with many community-acquired infections, antimicrobial resistance among pathogens that cause community-acquired acute uncomplicated urinary tract infections is increasing.^{81,82} Many studies on the antimicrobial susceptibility of community acquired uropathogens however report data from selected isolates, i.e. isolates derived from urine specimens send to the laboratory by the general practitioner after prior treatment failure or after recurrent UTI episodes. As a consequence, the antibiotic

susceptibility percentages determined for those 'selected' uropathogens will be lower.^{83,84} However for the empiric treatment of acute uncomplicated UTIs it is important for the GP to have actual data on the susceptibility of 'unselected' uropathogens.

Trends for reduced antibiotic susceptibility of *E. coli* both world-wide as well as in the Netherlands have been described. For instance resistance for beta-lactams such as ampicillin and first generation cephalosporins increased in the past decade and now approaches 40% in the US.^{81,82} In Europe however, a recent study showed that the resistance percentages of *E. coli* for ampicillin ranged from ~20% in the North to ~50% in the Western and Southern countries.⁸⁵ Nitrofurantoin has retained *in vitro* activity against most uropathogenic *E. coli* isolates world-wide.^{81,82,85,86} Trimethoprim+/- sulfamethoxazole underwent the most substantial change over time and approached ~20% in the US in the late nineties.^{82,86,87} In Europe also for this agent an increasing trend was observed ranging from 5-10% in the North to 15-20% in the Southern countries.⁸⁵ Resistance to fluoroquinolones remained low in community-acquired isolates.^{82,86} However in 2003 in Spain it has reached 15% in the *E. coli* isolated from UTIs.⁸⁵

Aims of the study

The last decades an increase in antibiotic resistant bacteria has been observed worldwide. Most studies on antimicrobial resistance however, did focus on the increasing antibiotic resistance of clinical isolates, i.e. bacteria isolated as causative agents of an infection. Little is known on the prevalence of antimicrobial resistance of bacteria that belong to the human commensal flora.

Therefore, in this thesis the prevalence of antimicrobial resistance and the influence of a variable selective pressure on the prevalence of resistance of the commensal flora was determined. The studies focused on the respiratory tract and the gastro-intestinal tract as a reservoir of antibiotic resistant micro-organisms and the urinary tract as site of infections caused by (resistant) micro-organisms of the latter reservoir.

For the respiratory tract the prevalence of macrolide resistance among commensal throat streptococci was determined. In addition, the genes encoding for this resistance were characterized and the ability to transfer the resistance gene involved (*ermB*) was assessed. Transfer of resistance was studied in an *in vitro* model mimicking the *in vivo* situation of close contact.

The influence of the selective antibiotic pressure on the prevalence of antimicrobial resistant faecal *E. coli* was studied in the gastro-intestinal tract of healthy volunteers in several developing countries in Latin-America, the Sub-Saharan Africa and Asia. The resistance prevalence was compared between urban and non-urban populations for both the Eastern and Western hemisphere.

Furthermore, the influence of hospitalization, i.e. high antibiotic use and crowding of patients on the prevalence of antibiotic resistance in faecal *Enterococcus faecalis* isolates of patients after discharge was assessed in three university-affiliated hospitals in The Netherlands. Antibiotic resistance acquired by the patient during hospital stay might be disseminated into the community after discharge from the hospital. Therefore, this acquired resistance is a risk for an increase in the prevalence of resistance in the extramural situation, i.e. the commensal flora of people residing in the community.

The human gastro-intestinal tract serves as reservoir of micro-organisms causing urinary tract infections (UTIs) through the ascending route. The antimicrobial resistance present in these commensal faecal bacteria poses a risk for antibiotic resistant uropathogens. Therefore, the prevalence of antimicrobial resistance in unselected *E. coli* isolates from female patients with complaints of an acute uncomplicated UTI of all ages was determined. These data are important for the setup of national guidelines for the empiric treatment of urinary tract infections.

Outline of the Thesis

In this thesis several aspects of the role of the commensal flora in the development and spread of antimicrobial resistance have been enlightened. In **Chapter 1**, the General Introduction, a description of the human commensal flora and more specific the most prevalent micro-organisms of the normal flora of the respiratory and gastro-intestinal tract was given. Furthermore, surveillances of antibiotic use in European and developing countries were described. In addition, several features of antibiotic resistance were discussed. Finally, as urinary tract infections are caused by ascending bacteria from the gut the etiology and the management of UTIs together with some characteristics of recurrent UTIs and antimicrobial resistance in uropathogens were described.

As part of an ongoing national surveillance study of acute respiratory tract infections (the ARI-EL study) the prevalence of macrolide resistance was determined in the commensal throat flora for the period October 2000 till December 2002 (**Chapter 2**). In addition, the transfer of macrolide resistance genes from commensal streptococci to a potential pathogenic bacterium was studied *in vitro*.

Besides the respiratory tract, the gastro-intestinal tract forms a second large reservoir of commensal bacteria and very likely thus antimicrobial resistance (genes). To assess the magnitude of this reservoir in circumstances that provide a variable antibiotic selection pressure a study was conducted in nine different developing countries, i.e. Mexico, Peru, Venezuela, Curaçao, Ghana, Tanzania, Zimbabwe, Kenya and The Philippines (**Chapter 3**). The populations in these countries live close together, have poor hygienic and sanitary facilities and in most countries there is no defined antibiotic policy which all contributes to the selection and dissemination of antibiotic resistance within these populations. Therefore, the prevalence of antibiotic resistance of faecal *Escherichia coli* isolated from healthy volunteers living in urban and rural areas of these nine developing countries was determined.

The hospital environment is another setting with a high selective pressure for resistance, i.e. high antibiotic use and crowding of patients. Therefore the effect of hospitalization on the prevalence of antibiotic resistance in the gut flora of patients admitted to the surgical wards of three university-affiliated hospitals in The Netherlands was followed in time from admission to six month after discharge in order to measure the effect of a hospital stay after discharge, i.e. after disappearance of the selective pressure of the antibiotic use (**Chapter 4**).

The commensal (resistant) bacteria of the intestinal flora are an important source of micro-organisms able to cause an infection at otherwise sterile body-sites like the bladder. Through the ascending route these commensal gut bacteria, and especially *E. coli*, can reach the bladder and cause urinary tract infections. Urinary tract infections are very common in female patients and several antibiotic treatment regimens of different duration are suited for therapy. Recently some debate has been going on to increase the

length of treatment from 3 days to 5-7 days. Therefore, in **Chapter 5**, 'Acute uncomplicated urinary tract infections in general practice: self-reported and microbiological cure rates after three versus five day treatment with trimethoprim; a randomized-controlled trial' the differences in cure rate for respectively a three-day and a five-day course of trimethoprim were determined. Trimethoprim resistance in the isolated *E. coli* before therapy and 1 and 3 days after therapy was compared for both treatment regimens.

The NHG-guidelines for antimicrobial therapy for UTIs empirically prescribed by the general practitioner in The Netherlands are based on national susceptibility data. Most studies on antibiotic resistance in uropathogens however, are performed on selected isolates, i.e. isolates send in by the GP for susceptibility testing after prior therapy failure. The prevalence of antibiotic resistance among these selected isolates will probably be higher as compared to that of unselected uropathogens. Therefore, a national study on acute uncomplicated UTIs has been performed to determine the prevalence of resistance from unselected uropathogenic *E. coli* isolates from female patients of all ages. In female patients between 11-70 years of age the accuracy of the diagnostic tests, i.e. the nitrite and leukocyte-esterase test performed by the general practitioners when an acute uncomplicated UTI was expected, and the prescribing behavior of the GP were evaluated (**Chapter 6**) and compared with the bacteriological culture results. The antimicrobial susceptibility of the *E. coli* isolates was determined and compared between patients of different age groups. In **Chapter 7**, 'Acute uncomplicated urinary tract infections in community dwelling elderly female patients in The Netherlands' urinary tract infections in non-institutionalized elderly women over the age of 70 years were analyzed. The care as usual of the general practitioner, i.e. the performance of the nitrite and leukocyte-esterase tests and the prescription rate, were compared with the bacteriological culture results. Furthermore antimicrobial susceptibilities of the uropathogens most frequently isolated were determined for the different age groups.

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Chapter 2

Erythromycin resistance in the commensal throat flora of patients visiting the general practitioner: a reservoir for resistance genes for potential pathogenic bacteria.

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1. The first part of the proof is to show that f is continuous at a . Let $\epsilon > 0$ be given. Since f is bounded on $[a, b]$, there exists $M > 0$ such that $|f(x)| \leq M$ for all $x \in [a, b]$. Choose $\delta > 0$ such that $\delta < \epsilon/M$. If $|x - a| < \delta$, then $|f(x) - f(a)| \leq M|x - a| < M\delta < \epsilon$. Thus f is continuous at a .

2. The second part of the proof is to show that f is continuous at b . Let $\epsilon > 0$ be given. Since f is bounded on $[a, b]$, there exists $M > 0$ such that $|f(x)| \leq M$ for all $x \in [a, b]$. Choose $\delta > 0$ such that $\delta < \epsilon/M$. If $|x - b| < \delta$, then $|f(x) - f(b)| \leq M|x - b| < M\delta < \epsilon$. Thus f is continuous at b .

3. The third part of the proof is to show that f is continuous at c . Let $\epsilon > 0$ be given. Since f is bounded on $[a, b]$, there exists $M > 0$ such that $|f(x)| \leq M$ for all $x \in [a, b]$. Choose $\delta > 0$ such that $\delta < \epsilon/M$. If $|x - c| < \delta$, then $|f(x) - f(c)| \leq M|x - c| < M\delta < \epsilon$. Thus f is continuous at c .

Abstract

Antibiotic resistance in the commensal flora often precedes that in the pathogenic flora and is a reservoir for resistance genes. Unfortunately, little is known about the prevalence and mechanisms of resistance in the commensal throat flora in the Netherlands as well as the transfer of the resistance genes from the commensal bacteria to potential pathogenic strains.

From October 2000 until December 2002 the prevalence and mechanism of erythromycin resistance was determined in the commensal streptococcal throat flora of patients with an acute respiratory tract infection (ARI) and in control patients matched by age presenting to general practitioners participating in a GP network of the Continuous Morbidity Registration of the NIVEL. Their patient population accounts for about 1% of the Dutch population and is representative according to age, gender, regional distribution and degree of urbanization.

The prevalence of resistance to 1 and 16 mg/L erythromycin was 57% and 20% respectively. The percentage of the total commensal flora resistant within each patient ranged from 1-100% with a median of 1%. The *mefA* gene was predominantly found among isolates on the 1 mg/L plates; the *ermB* gene was present in 64% of the isolates on the 16 mg/L plates. Erythromycin resistance was transferred from a commensal streptococcus isolate to a clinical *Streptococcus pneumoniae* strain with a frequency of 1×10^{-9} .

Macrolide resistant streptococci were present in the commensal throat flora in the majority of GP patients in The Netherlands although in most patients in relatively low numbers of the total commensal flora. Judicious use of macrolide therapy will control the increase in number of patients with detectable macrolide resistant flora as well as the relative contribution of the macrolide resistant flora to the total commensal flora per patient. This is important to prevent transfer of resistance genes and to keep these antibiotics for therapy in the future.

Introduction

Respiratory tract infections have in most cases a viral etiology for which antimicrobial therapy is not beneficial. However, up to 60% of the patients with a common cold in general practice receive an antimicrobial treatment.¹ Consequently, the normal bacterial flora of the upper respiratory tract is often exposed to antibiotics.² Resistant commensal flora might be selected each time an antibiotic is administered irrespective of the infection it should cure.³ Through this selection, resistant commensal streptococci play a significant role as reservoir of antimicrobial resistance genes and might transfer different resistance traits to more pathogenic micro-organisms like *Streptococcus pneumoniae* and *Streptococcus pyogenes*.⁴ To date, only two studies in The Netherlands

have addressed the problem of resistance in commensal throat flora.^{5,6} These studies were directed at the problem of resistance in the commensal throat flora after clarithromycin and/or azithromycin therapy in placebo controlled double blind studies in specific populations.

For years, macrolides have been an effective alternative for the treatment of infections due to Gram-positive micro-organisms especially in case of allergy to beta-lactam antibiotics. However, the development of an increase in macrolide resistance has limited the use of these antimicrobial agents.⁷ Resistance to macrolides in streptococci is mainly due to two resistance mechanisms: modification of the ribosomal target of the antibiotic due to methylation or mutation and efflux of macrolides. Methylation is encoded by the *ermB* gene (MLS_B-phenotype) and efflux is mediated by the *mefA* gene (M-phenotype). *MefA* is presently used to refer to the genes originally named *mefE* and *mefA* by Suttcliffe et al.^{8,9} Strains with a MLS_B-phenotype usually show high-level resistance to macrolides, lincosamides and streptogramin B, whereas strains with a M-phenotype express low-level resistance to 14- and 15-membered macrolides only but no cross-resistance to other antimicrobial agents.^{10,11} So far, ribosomal methylation remains the most widespread mechanism of resistance to macrolides and lincosamides.⁷

The purpose of this study was to determine the prevalence of erythromycin resistance (genes) in the commensal streptococcal flora of patients with or without an acute respiratory tract infection (ARI) attending their general practitioner (GP) participating in a GP network of the Continuous Morbidity Registration of the NIVEL in The Netherlands. Furthermore, the mechanisms of erythromycin resistance, i.e. the presence of *mefA* and/or *ermB* genes was determined as well as the possibility to transfer erythromycin resistance (genes) from a commensal streptococcal isolate to *Streptococcus pneumoniae*.

Materials and Methods

Sample collection

As part of an ongoing national surveillance study of acute respiratory tract infections (the ARI-EL study) organized by the National Institute of Public Health and the Environment (RIVM) throat swabs were collected from GP patients with or without complaints of an acute respiratory tract infection from October 2000 till December 2002.¹² The samples were collected by 67 GPs from 45 practices of the GP network of the Continuous Morbidity Registration of the NIVEL. Their patient population accounts for about 1% of the Dutch population and is representative according to age, gender, regional distribution and degree of urbanization. Patients with a diagnosed ARI (case) were included if it was their first visit to the doctor in this episode of ARI, the complaints started not more than five days before the doctor visit and no antimicrobial

agents nor antivirals were used in the previous two weeks. Within seven days after a patient with ARI was included, a control patient without ARI in the last two weeks was asked to participate. Furthermore, the control patient had to be in the same age category of the case, may not belong to the same household as the case, and may not have used any antimicrobial agents nor antivirals in the previous two weeks.¹²

The specimens collected were analyzed at the RIVM for respiratory viruses and for respiratory bacteria at the Regional Laboratory of Public Health in Tilburg. Samples were then forwarded to the Microbiological Laboratory of The University Hospital Maastricht for further analysis of the commensal streptococcal throat flora. Upon arrival they were suspended in 3 ml of sterile saline (0,9% NaCl, w/v). Five-hundred microliter of this suspension was centrifuged at 14000 rpm for 10 minutes, the pellet was suspended in 1 ml of 0,7% peptone (w/v) with 30% glycerol (v/v) and stored at -20°C until further analysis.

Determination of the erythromycin resistance phenotype

The total indigenous oropharyngeal flora, i.e. alpha-hemolytic and non-hemolytic streptococci, was cultured to determine resistance to erythromycin. After thawing, the samples were centrifuged at 14000 rpm for 10 minutes and the pellet was suspended in 500 µl 0,9% NaCl (w/v). Then, 40 µl of tenfold dilutions were inoculated using a spiral plater (Eddy Jet, IUL Instruments, I.K.S., Leerdam, The Netherlands) onto Columbia agar (Oxoid, CM 331, Basingstoke, England) plates supplemented with 5% sheep blood and containing 0, 1 and 16 mg/L erythromycin. Of the samples showing growth after overnight incubation at 37°C colonies with the morphology of streptococci were counted. Per swab, only the colonies growing on the agar plate with the highest concentration of erythromycin (i.e. isolates of the 1 mg/L plates showing growth only on this plate or isolates of the 16 mg/L plates in case of growth on both antibiotic containing plates) were subcultured and stored at -70°C until further analysis of the resistance genes.

The prevalence of resistance for each concentration of erythromycin was calculated as the ratio between the number of samples showing growth at that specific concentration erythromycin and the total number of samples tested multiplied by 100%.

Detection of the erythromycin resistance genes

For the identification of the different erythromycin resistance genes (*ermB* and *mefA*), total DNA was extracted from bacteria grown on blood agar plates (Oxoid, Basingstoke, England). PCR was performed with in a Perkin-Elmer Cetus GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, Conn.) yielding PCR products of 425 bp and 348 bp respectively for *ermB* and *mefA* as described previously.^{8,13,14} Positive control strains for *ermB* JH2-2:Tn1545¹⁴ and *mefA/E* O2J1175¹³ were included in each run.

Conjugation experiment

The bacterial strain used as donor of the erythromycin resistance gene *ermB* was a rifampicin-susceptible isolate from this study. A clinical erythromycin-susceptible *S. pneumoniae* strain made rifampicin (25 mg/L) resistant was used as recipient. Transfer of the resistance gene was performed in suspension with both donor and recipient grown to the late exponential phase and then mixed at a donor/recipient ratio of 1:5. As a control, each of the donor and recipient strains were mixed with 0.9% NaCl in a ratio 1:5. After centrifugation of the mating mixtures, the pellet was suspended in 0.5 mL NaCl (0.9%, w/v) and plated on Columbia agar plates (CM331, Oxoid, Basingstoke, England) supplemented with 5% sheep blood, containing both erythromycin (16 mg/L) and rifampicin (25 mg/L). The inoculated plates were incubated at 5% CO₂ and 37°C. Putative transconjugants were characterized for the presence of the *ermB* gene by PCR and by pulsed-field gel electrophoresis (PFGE) to detect clonality with the recipient *S. pneumoniae* strain. The frequency of transfer was expressed as the number of transconjugants after mating per recipient colony forming units (cfu) in the mating mixture. The stability of the transconjugant was determined by five successive platings on blood agar without antibiotics.

Pulsed-field gel electrophoresis

In short, agarose plugs containing bacterial chromosomal DNA were digested with *Sma*I (Invitrogen, reference 15228-018, Breda, The Netherlands) for 6h at 37°C. Pulsed-field gel electrophoresis was carried out on a CHEF-DRII cell (Bio-Rad Laboratories, Richmond, CA) as described previously with minor modifications.¹⁵ The gel was stained with ethidium bromide for 15 min and photographed under UV irradiation. The restriction endonuclease digest patterns were visually compared and interpreted using the criteria of Tenover et al.¹⁶

Results

Sample collection

Of the 678 patients participating in the study, 60% were female. The age distribution of the participants was not significantly different from the distribution in the Dutch population, although the 0- to 4-year-olds were underrepresented and the 14- to 44-year-olds were overrepresented.¹²

Determination of the erythromycin resistance phenotype

Indigenous oropharyngeal flora, i.e. alpha-haemolytic and non-haemolytic streptococci, was present in all collected swabs (Table 1). The flora of 292 (43%) patients showed no resistance to either concentration erythromycin tested. On the 1 mg/L agar plates 57% of the samples showed growth and 20% were able to grow on the 16 mg/L agar plates. The proportion of erythromycin resistant streptococci in the total commensal throat flora of each patient ranged from 1-100% with a median of 1%. Furthermore, in 3% of the patients >50% of the total commensal streptococcal flora were erythromycin resistant (1 mg/L).

Table 1. Culture results of the oropharyngeal swabs and erythromycin resistance prevalence (%) of the streptococcal isolates.

	Total
Number of patients	678
Number of samples with growth on 1 mg/L plates	386
Number of samples with growth on 16 mg/L plates	138
Prevalence of resistance for 1 mg/L	57%
Prevalence of resistance for 16 mg/L	20%

Detection of the erythromycin resistance genes

The majority of the isolates showing growth only on the 1 mg/L erythromycin plates (78%) carried the *mefA* resistance gene. For the isolates on the highest erythromycin concentration plates the *ermB* gene was found in the majority of the samples (64%). Isolates of the higher erythromycin concentration also showed more often the combination of both *ermB* and *mefA* genes (9%) as compared to the isolates from the 1 mg/L agar plates (2%).

Conjugation experiment

Erythromycin resistance was successfully transferred from the randomly chosen commensal streptococcus isolate (*ermB* positive) to a clinical *S. pneumoniae* strain (ery^S, rif^R) with a frequency of $\pm 1 \times 10^{-9}$. The identification of the transconjugant was confirmed by API strep and was similar to that of the acceptor strain. Furthermore, the *ermB* gene was detected in the transconjugant by PCR and PFGE of both the recipient and transconjugant showed identical restriction patterns. No spontaneous mutations in the control experiments were detected. All the transconjugants were stable as

erythromycin resistance remained after 5 consecutive platings on antibiotic free agar plates.

Table 2. PCR results of the erythromycin resistant streptococcal isolates.

Concentration		Number (percentage) of isolates with the resistance gene			
erythromycin	n*	<i>ermB</i>	<i>mefA</i>	<i>ermB+mefA+</i>	<i>ermB-mefA-</i>
1 mg/L	294	29 (10)	230 (78)	5 (2)	30 (10)
16 mg/L	188	121 (64)	42 (22)	16 (9)	9 (5)

*number of isolates assayed by PCR

Discussion

The present study is to the best of our knowledge one of the few studies dealing with macrolide resistance in the commensal flora of general practice patients in The Netherlands. The prevalence of macrolide resistance in the majority of the patient population and the transferability of macrolide resistance by conjugation to potentially pathogenic micro-organisms with a frequency of 1×10^{-9} underscore the relevance of the commensal flora as source of antibiotic resistance genes. Furthermore, this transfer of resistance genes by conjugation is a process equal to the *in vivo* situation as bacteria of the commensal throat flora are also found in close proximity of one another.

The two published other studies in The Netherlands on commensal flora were primarily directed at the effect of clarithromycin and/or azithromycin on the appearance of resistance in the commensal flora in a double blind placebo-controlled fashion.^{5,6} In the study by Berg et al.⁵ 296 patients with documented coronary artery disease were randomized to receive either two weeks of slow release (SR) clarithromycin or placebo for two weeks. SR clarithromycin had a major effect on the appearance of resistance in the commensal throat flora, which persisted for at least 8 weeks after end of therapy. Matute et al.⁶ did not find any effect of a 3-day course of azithromycin or 7-day course of clarithromycin on the commensal throat flora in each of 6 healthy volunteers. Explanations for the lack of effect of the tested macrolides in the latter study are the small number of participants and the shorter duration of therapy.

The resistance percentage in our study was lower than the 71% macrolide-resistant viridans group streptococci (the predominant species of the oropharyngeal commensal flora) found in Belgian healthy volunteers in 2003¹⁷ and the rate reported in Spain in 2001.⁹ These results are in accordance to the lower antibiotic use in general¹⁸ and especially in macrolides¹⁹ in The Netherlands compared to both other countries. Also, in our study the resistance prevalence was determined in the total commensal streptococcal

throat flora as compared to only a selected sub-population of this flora in both studies which might have resulted in higher resistance percentages compared to our results.

Twenty percent of the patients in our study carried streptococci resistant to a high concentration of erythromycin (16 mg/L). As the majority of the streptococci growing on the 16 mg/L agar plates (64%) carried the *ermB* gene it seems that in the population studied resistance to a high erythromycin concentration is correlated with the MLS_B-phenotype that confers cross-resistance to macrolides, lincosamides and streptogramins. The same relation was also found by Pérez-Trallero et al.⁹ Furthermore, similar as in our study their study showed that all commensal streptococci with the M-phenotype had low MICs for macrolides, clindamycin and streptogramin B. In our isolates 80% of the isolates growing only on the 1 mg/L agar plates contained the *mefA* gene.

In 5% of the isolates growing on the higher concentration of erythromycin and 10% of the isolates on the lower concentration, none of the resistance genes studied were detected. The mechanism(s) of resistance in these isolates are therefore unknown. This finding might imply the existence of other resistance genes or mechanisms. For the lower erythromycin resistant strains a likely explanation might be an altered secretion pathway for the antimicrobial agent or a modified entry. For the higher erythromycin resistant strains the *ermA* gene, subclass *ermTR* might be responsible for the MLS_B-phenotype as this gene is known to code for the iMLS_B-phenotype in *S. pyogenes* isolates,²⁰ and in beta-hemolytic streptococci group B, C, G.^{4,21}

A limitation of the study was the identification of the isolates by morphology only. However, the aim of the study was to determine the prevalence of erythromycin resistance in the total commensal streptococcal flora. Thus the morphology only was sufficient to distinguish between streptococci and non-streptococci (mostly staphylococci).

The present results confirmed that the commensal throat flora forms a large reservoir of resistance genes able to transfer respectively their erythromycin resistance genes by horizontal gene transfer to a potential pathogenic *S. pneumoniae* which consequently becomes resistant to commonly used antimicrobials. How often this occurs depends on the number of genes involved in resistance, the ability of these genes to be transferred, the prevalence of pathogenic bacteria, the prevalence of resistant commensal throat flora, and the relative contribution of resistant flora to the total commensal flora. The latter two factors are influenced by the use of antibiotics. The transfer is relatively easy for macrolide resistance as this is often encoded by one gene only, often located on transposons^{22,23} and the transconjugants were resistant to the same erythromycin concentration as the donor strain. Thus potential pathogenic bacteria like *S. pneumoniae* might become resistant by horizontal gene transfer to commonly used antimicrobials (macrolides in case of penicillin allergy). How often this occurs depends on the number of genes involved in resistance, the ability of these genes to be transferred, the prevalence of pathogenic bacteria, the prevalence of resistant throat flora, and the relative contribution of resistant flora to the total commensal flora. The latter two

factors are influenced by the use of antibiotics. The transfer is relatively easy for macrolide resistance as this is often encoded by one gene only, which is often located on transposons.^{22, 23} As shown in a previous study²⁴ *mefE* could be transferred with a frequency of $\pm 1 \times 10^{-7}$ from viridans group streptococci to *S. pneumoniae* by transformation. Transformation however is considered an *in vitro* phenomenon with pre-competent *S. pneumoniae* R6 cells and total genomic donor DNA. Whether this situation occurs also *in vivo* is questionable. The conjugation experiment in this study was assayed with bacterial donor and recipient strains mixed in suspension to mimic the *in vivo* situation. Close contact between micro-organisms, not pre-treated to get competent cells is very likely a common situation *in vivo*.

Although in The Netherlands macrolide resistance in Gram-positive potential pathogenic bacteria is still relatively low, it is increasing.²⁵ Judicious use of macrolide antibiotics is essential to keep macrolides as alternatives for penicillins in the future especially in patients with severe forms of allergy to beta-lactam antibiotics.

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Chapter 3

Antibiotic resistance of faecal *Escherichia coli* in healthy volunteers from nine developing countries.

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Abstract

In many developing countries antibiotics are frequently misused and overused resulting in resistant bacteria. However there is paucity of accurate data on the prevalence of antibiotic resistance that would be useful in the formulation of appropriate policies. In this study the prevalence of antibiotic resistant *Escherichia coli* in healthy volunteers from urban (U) areas in Kenya, Mexico, Peru, Philippines and non-urban (NU) locations in Curaçao, Mexico, Venezuela, Tanzania, Ghana, Zimbabwe and Philippines was determined.

The collected 1474 faecal samples were analysed in one laboratory for the presence and antimicrobial resistance of *E. coli*. The susceptibility to 16 antibiotics was determined by the MIC-method. Genetic relatedness of the isolates was determined by PFGE of *Xba*I-digested genomic DNA.

Prevalence of resistance to oxytetracycline, ampicillin and trimethoprim was found in more than 50% of the samples. Ninety-six percent of the volunteers from NU Mexico carried *E. coli* resistant to oxytetracycline and trimethoprim. Ninety-five percent of the Peruvian participants had *E. coli* resistant to ampicillin; 82% of those from Ghana were resistant to chloramphenicol. Ciprofloxacin resistance ranged between 1-63% for the different countries. Significantly higher prevalences for ampicillin, oxytetracycline and trimethoprim were found for urban areas compared to non-urban ones. Ten percent of the *E. coli* were resistant to more than three antimicrobials. Genotyping of the multi-resistant strains resulted in 53 unrelated PFGE-patterns.

In these populations the prevalence of antibiotic resistant faecal *E. coli* from healthy volunteers was high for most antibiotics. Furthermore, these high resistance prevalences were not caused by the spread of clonally related strains within these populations.

Introduction

The development and introduction of antimicrobial agents into clinical practice is one of the most important achievements of human medicine in the twentieth century. Antibiotics have, until recently, been used successfully for the management of most bacterial infections. In the last few decades there has been a growing concern about the increase in antibiotic resistance, especially in the developing countries.¹ It is generally accepted that in a population antibiotic resistance and the frequency and amount of usage of a particular drug are related.^{2,3} In developing countries the availability of antibiotics over the counter without prescription, the use of subtherapeutic doses that are often of substandard quality,^{4,5} insufficient emphasis on patient education concerning the consequences of antibiotic use and misuse, insufficient adherence of the medical profession to an antibiotic policy if available, are among factors that contribute to the emergence and spread of resistant strains.^{6,7} Increasing international travel also provides

great opportunities for both pathogenic and non-pathogenic antibiotic resistant bacteria to be carried over long distances to other countries which might even be more important than the spread and dissemination of resistant bacteria within countries.^{8,9}

The faecal flora of the general healthy population represents a potentially large reservoir of antimicrobial resistant bacteria at sites where resistance genes can be transferred from the commensal flora to potentially pathogenic micro-organisms.^{8,9}

In this study *Escherichia coli*, the predominant Gram-negative, facultative aerobic organism of the faecal flora¹⁰ was used as an indicator organism to determine the prevalence of antibiotic resistance in faecal samples of healthy adult volunteers in 9 developing countries in Africa, Asia and South-America. In addition genotyping of these strains was performed to determine possible spread of clonal strains within each population studied.

Materials and Methods

Sample collection

Faecal samples were randomly collected by medical students of the University of Maastricht in towns and villages of nine developing countries between November 1998 and July 2002. Participating countries were: Ghana, Kenya, Tanzania, Zimbabwe, Peru, Mexico (urban and non-urban), Venezuela, Curaçao, the Philippines (urban and non-urban). Samples were obtained from four urban (U) areas and from seven non-urban (NU) areas. Classification as urban or non-urban was based on information about the population size of the respective town. The cut-off value for the urban population was $\geq 150,000$ inhabitants (Table 1). Healthy adult volunteers were asked to participate in the study by providing a fresh faecal sample (one sample per volunteer) and filling out a questionnaire on age, gender and antibiotic use. Criteria for exclusion were hospitalisation and presence of gastro-intestinal disease in the past month.

Because, as has been shown by mathematical modelling,¹¹ resistance rates increase exponentially, the emergence of resistance is more important from an epidemiological point of view than a small increase in resistance for example from 50% to 60%. This assumption was used to calculate the minimum sample size. To find such a difference in resistance prevalence at least 90 evaluable samples are needed in each group ($\alpha = 0.05$ and $\beta = 0.20$). Between 95 and 231 samples were therefore collected in each group.

Sample processing

On the day of collection the faecal samples were diluted 1:10 in sterile saline (0.9% (w/v) NaCl) with 20% (v/v) glycerol in the local laboratory and stored at -20°C until transported to the Medical Microbiology laboratory of the University Hospital

Maastricht, The Netherlands in dry ice using shipping services. Weekly Eosin Methylene Blue (EMB) agar (Oxoid, CM 69, Basingstoke, England) plates with and without antibiotics were prepared and stored at 4°C until further use within one week. Ampicillin and oxytetracycline containing agar plates were prepared daily. The antibiotic concentrations were based on the resistance breakpoints of the National Committee for Clinical Laboratory Standards (NCCLS)¹² guidelines and modified to make comparison with previous studies possible.^{3,13} The antibiotics tested and concentrations used were: ampicillin 25 mg/L, cefazolin 32 mg/L, ciprofloxacin 4 mg/L, chloramphenicol 25 mg/L, gentamicin 16 mg/L, oxytetracycline 25 mg/L and trimethoprim 8 mg/L. When using trimethoprim, 5% (v/v) lysed horse blood was added to the EMB-agar. Faecal samples were tenfold diluted and 40 µl from each dilution was inoculated on the EMB-agar plates with and without antibiotics, using a spiral plater (Eddy Jet, IUL Instruments, Leerdam, The Netherlands). After 18-24h of incubation at 37°C, *E. coli* appeared as purple colonies with a dark centre and a metallic green sheen. One colony of the dominant flora was randomly picked from each plate for identification using the indole and β-glucuronidase (β-glucuronidase discs, Rosco, Denmark) test. When both tests were positive the colony was considered *E. coli*, if there remained any doubt further biochemical tests were performed using API 20E strips (BioMérieux, Plainview, N.Y.).

The prevalence of resistance to a certain antibiotic was defined as the proportion of faecal samples showing growth of *E. coli* on agar plates containing that antibiotic compared to the total number of samples tested.

MIC Distributions

Minimum inhibitory concentrations (MICs) of *E. coli* were determined according to the NCCLS-criteria.¹² The microbroth dilution method was used with Mueller Hinton II broth cation adjusted (Becton, Dickinson and Company, Sparks, USA), an inoculum of 5×10^5 cfu/ml and an overnight incubation at 37°C. The MIC-plates with freeze-dried antibiotics were provided by MCS Diagnostics BV (NLDMCS1 and NLDMCS2, MCS Diagnostics BV, Swalmen, The Netherlands). The MIC-plates were delivered after quality testing by the manufacturers and a shelf life of approximately one year was guaranteed. The following antimicrobial agents (range in mg/L) were tested: amoxicillin (0.06-128), amoxiclav (0.06-128), trimethoprim (0.03-64), co-trimoxazole (0.03-64), gentamicin (0.03-64), norfloxacin (0.03-64), ciprofloxacin (0.008-16), nitrofurantoin (0.5-512), ceftazidime (0.06-128), cefuroxim (0.06-128), meropenem (0.03-64), piperacillin (0.25-512), piperacillin/tazobactam (0.25-512/4), doxycycline (0.06-128), amikacin (0.12-256), and imipenem (0.06-64). The reference strains *E. coli* ATCC 35218 and ATCC 25922 were used as control strains.

Table 1. General information about the eleven populations studied.

Country	Town or village	Population size*	Demographic category	Year of collection	Number of volunteers
The Americas					
Venezuela	Pueblo Llano	15000	Non-urban	1998	231
Netherland Antilles	Curacao	130627	Non-urban	2000	149
Mexico U	Mexico City	8605239	Urban	2001	99
Mexico NU	Puerto Angel	< 50000	Non-urban	2002	114
Peru	Lima	329023	Urban	2002	95
Sub-Saharan Africa					
Tanzania	Sengerema District	36938	Non-urban	1999	168
Zimbabwe	Musami	< 10000	Non-urban	1999	207
Ghana	Duayaw Nkwanta	< 20000	Non-urban	2001	100
Kenya	Nairobi	2143254	Urban	2001	100
Asia					
The Philippines U	Antipolo	470000	Urban	2000	105
The Philippines NU	Sapang Palay	< 50000	Non-urban	2000	106

U = urban, NU = non-urban. *Population size derived from www.citpopulation.de

Table 2. Characteristics of the 11 populations studied.

	Ven	Cur	Mex U	Mex NU	Per	Tan	Zim	Gha	Ken	Phi U	Phi NU
Number of volunteers	231	149	99	114	95	168	207	100	100	105	106
% male	30	N.A.	35	43	44	24	29	59	75	35	36
Age (year)	Mean	37	44	36	31	38	42	42	N.A.	N.A.	N.A.
	18-19	8	9	16	13	4	N.A.	1	0	0	33
	20-29	37	6	31	37	26	N.A.	17	87	49	14
	30-39	17	21	11	26	29	N.A.	27	6	34	27
	40-49	14	20	20	15	23	N.A.	28	4	16	12
	50-60	15	31	9	6	11	N.A.	17	3	1	3
	> 60	10	13	13	3	6	N.A.	11	0	0	1
Previous antibiotic use (%)	Yes	6	13	15	1	4	N.A.	10	11	13	N.A.
	No	91	86	85	99	96	N.A.	89	89	87	N.A.
	Unknown	3	1	0	0	0	N.A.	1	0	0	N.A.

U = urban, NU = non-urban. N.A. Not available. Venezuela (Ven), Curaçao (Cur), Mexico (Mex), Peru (Per), Tanzania (Tan), Zimbabwe (Zim), Ghana (Gha), Kenya (Ken), and the Philippines (Phi)

Pulsed-field gel electrophoresis

Sixty-two out of the 105 *E. coli* isolates resistant to at least amoxicillin, trimethoprim and doxycycline were at random chosen for genotyping by PFGE. For countries with 5 isolates meeting the resistance criteria all the isolates were genotyped, for countries with more multi-resistant isolates every other isolate was tested. Agarose plugs containing bacterial chromosomal DNA were digested with *Xba*I (BioLabs Inc., New England, USA) for 2h at 37°C. Pulsed-field gel electrophoresis was carried out on a CHEF-DRII cell (Bio-Rad Laboratories, Richmond, USA) as described previously with minor modifications.¹⁴ The gels were stained with ethidium bromide for 15 min and photographed under UV irradiation. The restriction endonuclease digest patterns were interpreted using the criteria of Tenover *et al.*¹⁵

Statistical analysis

Statistical differences in the prevalences of antibiotic resistance between the different populations were detected using The Mann-Whitney-U test.

Results

Study populations

Demographic information about the populations studied is shown in Table 1. In total 1474 healthy volunteers participated in the study, from Venezuela n=231, Curaçao n=149, Mexico U n=99, Mexico NU n=114, Peru n=95, Tanzania n=168, Zimbabwe n=207, Ghana n=100, Kenya n=100, the Philippines U n=105 and the Philippines NU n=106. The populations studied were derived from four urban (Mexico, Peru, Kenya and the Philippines) and seven non-urban regions.

The characteristics of the eleven populations studied are shown in Table 2. The majority of the volunteers were between 18 and 50 years old, mostly female (56%-76%), except in Ghana and Kenya (59% and 75% male respectively). One volunteer from Venezuela and 15 from the urban Mexico population did not deliver a faecal sample and were therefore excluded from further analysis.

The prevalence of resistance

The prevalences of resistance of the faecal *E. coli* for the antibiotics tested are shown in Table 3. Five percent (70/1458) of all volunteers showed no growth of faecal *E. coli*. The highest prevalence of resistance was found for the relatively inexpensive, broad spectrum, orally administered agents, particularly those that have been in use for several

Table 3. The prevalence of antibiotic resistant faecal *E. coli* (%) in healthy volunteers in the 11 populations studied.

Antibiotic	Conc mg/L	Venezuela ¹	Curaçao	Mexico U	Mexico NU ¹	Peru	Tanzania	Zimbabwe	Ghana	Kenya	The Philippines U	The Philippines NU
		n = 230	n = 149	n = 99	n = 99	n = 95	n = 168	n = 207	n = 100	N = 100	n = 105	n = 106
Ampicillin	25	43	48	78	94	95	55	49	89	89	87	75
Cefazolin	32	12	7	0	4	13	N.T.	0	4	2	25	18
Ciprofloxacin	4	1	1	15	51	36	2	1	8	1	63	35
Chloramphenicol	25	30	8	45	75	52	N.T.	19	82	45	68	60
Gentamicin	16	3	4	10	14	33	1	0.5	2	2	46	20
Oxytetracycline	25	52	56	86	97	93	69	59	90	92	94	85
Trimethoprim	33	33	32	76	96	93	67	64	89	88	93	86

U = urban, NU = non-urban; N.T. = not tested.

¹The number of samples tested was smaller than the number of volunteers that filled out the questionnaires.

decades. For instance over 50% of the samples from most populations were resistant to oxytetracycline, ampicillin and trimethoprim. The samples from volunteers of non-urban Mexico showed the highest prevalence of resistance to oxytetracycline (97%) and trimethoprim (96%), whereas participants from Peru and Ghana showed a high prevalence of resistance to ampicillin (95%) and chloramphenicol (82%), respectively. With the exception of Curaçao and Zimbabwe, more than 30% of the faecal samples from the other areas had *E. coli* that showed resistance to chloramphenicol.

Resistance to cefazolin, gentamicin and ciprofloxacin was less common, with the exception of the Philippines U. There was considerable variation in the prevalence of ciprofloxacin resistance. It ranged from 2% or lower in the African countries, Curaçao and Venezuela to over 30% in Mexico, Peru and the Philippines. Gentamicin resistance was the lowest in the African countries, Curaçao and Venezuela. Samples from the Philippines had the highest prevalence of resistance for three out of seven antibiotics tested.

Using the demographic information of each country, the prevalences of resistance for the urban and non-urban areas respectively were combined for the Eastern as well as the Western hemisphere respectively. Although the time bias was greater in the data from the Western hemisphere, the trends seen were the same in both hemispheres (Figure 1). The resistance percentages to ampicillin, oxytetracycline and trimethoprim were significantly higher in urban groups compared to non-urban groups ($p < 0.01$) in both hemispheres but no significant differences in antibiotic resistance could be detected between the two hemispheres.

Figure 1. The prevalence of antibiotic resistant faecal *E. coli* of 11 population of healthy volunteer in function of demographic differences

am = ampicillin, cef = cefazolin, chl = chloramphenicol, cip = ciprofloxacin, gen = gentamicin, oxy = oxytetracycline, and tri = trimethoprim.

* significantly higher resistance in urban groups compared to non-urban groups ($p < 0.01$) in both hemispheres.

Multi-drug resistance patterns

Ten percent (129/1388) of all the *E. coli* isolates tested were resistant to three or more antimicrobial agents. Of these strains, 77 were resistant to the combination of amoxicillin, trimethoprim, co-trimoxazole and doxycycline. Twenty-eight isolates showed an additional resistance to up to four other antibiotics including piperacillin, co-amoxiclav, gentamicin, norfloxacin, ciprofloxacin and nitrofurantoin. All isolates tested were sensitive to imipenem and only one urban Philippines isolate showed resistance to meropenem. This latter strain showed also resistance to cefuroxime and doxycycline.

Pulsed-field gel electrophoresis

In total 62 *E. coli* isolates (the Philippines NU, n=4, Peru, n=5, Curaçao, n=5, Ghana, n=5, Kenya, n=5, the Philippines U, n=5, Mexico U, n=6, Venezuela, n=6, Zimbabwe, n=10 and Tanzania, n=11) resistant to at least amoxicillin, trimethoprim and doxycycline were analysed using PFGE. Of these isolates, 44 were totally unrelated. The remaining 18 strains formed nine unrelated patterns.

Discussion

The prevalence of resistance of faecal *E. coli* isolated from eleven populations of healthy volunteers living in nine different developing countries was determined in one laboratory using the same method. A high prevalence of resistance to most agents was found in all populations, consistent with findings from previous developing-country studies.^{9,13,16,17} The highest prevalences of resistance were found for the orally administered, relatively inexpensive, broad-spectrum agents including ampicillin, trimethoprim and oxytetracycline. Furthermore, resistance to these antibiotics was the most common multi-resistance pattern seen in the *E. coli* isolates. This association has been found in previous studies with adults^{16,18} as well as children.⁶ The combination of resistance to these three antibiotics is very likely due to the fact that the genes encoding for resistance to these antimicrobials are located on the same plasmid.¹⁹ In order to rule out the possible spread of one or a few clonal strains in the populations studied we characterised multi-drug resistant *E. coli* isolates by PFGE and found that there were hardly any related strains in each population. The results indicate that the resistance prevalences found in this study were not derived from one common *E. coli* strain.

The prevalence of ciprofloxacin resistance ranged from one to more than sixty percent, in the Philippines U and NU, Mexico NU and Peru. A previous study by Rodriguez *et al.*²⁰ found lower resistance percentages for quinolones in Lima (Peru), i.e. 31-38%; in Caracas (Venezuela)²⁰ and in other studies^{3,6,21,22} resistance percentages ranged from 0-15%. An explanation for this high prevalence of resistance might be the use of

ciprofloxacin for treatment of cholera and typhoid in adults as alternative for the standard therapy when strains are resistant to commonly used antimicrobials.²³⁻²⁵

To our knowledge the present study was the first in which nine different developing countries on different continents were studied for the antibiotic resistance in healthy volunteers. Although time bias could not be avoided, the methods used for detecting faecal *E. coli* and determining the prevalence of antibiotic resistance were similar for the eleven populations studied. In their study van de Mortel *et al.*³ also found higher oxytetracycline ($p < 0.05$) and trimethoprim (not significant) resistances in the urban area of Mérida compared to the village of Grulla in Venezuela. In our study there were also significant differences in the prevalence of resistance between non-urban and urban areas for ampicillin, oxytetracycline and trimethoprim ($p < 0.01$). These differences in resistance between the rural and urban areas might be due to the availability of antibiotics²⁶ and the easy access of the drugs afforded by the greater accessibility of healthcare facilities and drug distribution points.²⁷ In cities a large variety of often inexpensive antibiotics are available in pharmacies, over the counter and in market stalls and most of them are inexpensive,⁷ whereas in rural areas only a few drugs are available and usually brought there by a visiting²⁸ or a resident physician.²⁹ Furthermore, crowding together with poor hygiene and poor sanitary facilities for sewage disposal in the cities might encourage the exchange of antibiotic-resistant bacteria in a population and the exchange of resistance genes among bacteria, thereby increasing the prevalence of antibiotic resistant strains.^{7,9,29}

Mathematical modelling suggests that the prevalence of resistance over time follows a sigmoidal curve and approaches equilibrium.¹¹ Depending on the antibiotic and the species involved it may take (some) time before the first resistant strain appears. Then, the resistance increases exponentially until equilibrium is reached. It may therefore be more effective to prevent the emergence of resistance than to reduce the prevalence of resistance once it has appeared, a factor that may account for the high levels of resistance in this study.³⁰

A limitation of this study was the time bias in sample collection which might have influenced the results. Although different medical students collected the samples each year, they were all instructed the same way with the same information and provided with the storage media they needed.

In conclusion, a high prevalence of resistance to most antibiotics tested among faecal *E. coli* from healthy individuals in developing countries was found. Moreover carriage of resistant *E. coli* was more common in urban than in rural areas. Unfortunately, we could not correlate these resistance prevalences to data on antibiotic use due to the lack of official data on the use of antibiotics and the over the counter sales. Also, the data provided by the volunteers on antibiotic use in the three months prior to the study were not consistent with percentages found in other studies.^{4,31,32}

More surveillance studies are certainly necessary in developing countries to monitor the antibiotic use and the antibiotic resistance over time. These data are important as a basis

for the implementation of an antibiotic policy. In addition, education of healthcare workers, patients and parents is also important in the control of antibiotic resistance over time.

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Chapter 4

**Effect of hospitalization on the antibiotic resistance of fecal
Enterococcus faecalis of surgical patients over time.**

$$\begin{aligned} \frac{1}{2} \left(\frac{1}{2} \right) &= \frac{1}{4} \\ \frac{1}{2} \left(\frac{1}{4} \right) &= \frac{1}{8} \end{aligned}$$

Abstract

The prevalence of antibiotic resistant *Enterococcus faecalis* was determined in fecal samples of 263 patients admitted to the surgical wards of three university-affiliated hospitals on admission, at discharge and 1 and 6 months after discharge.

A slight increase in the prevalence of antibiotic resistance of *E. faecalis* was found at discharge for the antibiotics tested compared to those on admission, vancomycin excepted. Six months after discharge the prevalence of resistance for amoxicillin (0%), ciprofloxacin (3%), erythromycin (47%) and oxytetracycline (60%) decreased to the level on admission (respectively 0%, 8%, 45% and 64%). Gentamicin resistance was the same at discharge (10%) as one month later (12%), but decreased as well six months after discharge (8%) to the level on admission (7%).

In conclusion, hospitalization resulted in the study population in a slight increase in the prevalence of resistant fecal *E. faecalis* isolates at discharge which decreased again (slowly) to the level on admission six months after discharge. Thus the influence of hospitalization on the prevalence of antibiotic resistance in the extramural situation disappears between one and six months after discharge in this population.

Introduction

Despite the tremendous benefits of antibiotics for the treatment of a wide range of pathogens, an increase in antibiotic resistant bacteria has recently been observed worldwide.¹⁻³ Of particular concern are the hospitals not only because of the antibiotic use both for therapy and prophylaxis⁴ but also because of the spread of resistant bacteria facilitated by a high patient population density and frequent contacts between health care workers and patients.^{3,5} Several studies addressed the relation between antibiotic use and antibiotic resistance⁶ in potential pathogenic micro-organisms as causative agents of nosocomial infections.⁷⁻⁹ Up till now little attention has been paid to the effect of hospitalization and antibiotic use on the prevalence of resistance in the commensal fecal flora like *E. coli* and enterococci. The resistance of the commensal flora acquired during hospitalization may disseminate into the community after discharge just as community acquired resistance may act as a reservoir of antibiotic resistance for hospital outbreaks. Only limited studies addressed the dissemination of resistance between both populations.¹⁰

Enterococci belong to the commensal flora of the gastro-intestinal tract of humans and animals.¹¹ They are an important cause of nosocomial infections.¹² Its success as a hospital pathogen is due to its intrinsic resistance to many antibiotics that are commonly used in hospitals such as cephalosporins and aminoglycosides¹ and the emergence of glycopeptide resistance in this genus by mutation or transfer of resistance genes on plasmids or transposons.^{2,3} The ban of avoparcin, a glycopeptide like vancomycin

widely used as a growth promoter in animal husbandry, resulted already in a decrease in glycopeptide resistant enterococci in healthy volunteers without a recent (three-month) history of hospitalization.¹³ In hospitals the use of cephalosporins (and glycopeptides) selects for glycopeptide resistant enterococci.^{14,15}

In this study the effect of hospitalization of patients admitted to surgical wards on the prevalence of antibiotic resistant *E. faecalis*, the dominant enterococcus species of the fecal flora, was studied in three university affiliated-hospitals in The Netherlands on admission, at discharge and 1 and 6 months thereafter. In addition, the resistance patterns of the *E. faecalis* isolated at different points of time were determined. The study focused on surgical patients rather than on patients of other wards or Intensive Care patients as surgical patients are considered being representative for the average hospitalized patient.

Materials and Methods

Patient population

From March 1999 until February 2002 patients admitted to the surgical wards of the University Hospitals Maastricht (azM) and Groningen (azG) and the Erasmus Medical Center Rotterdam (azR) were asked to participate in the study. Upon inclusion, patients were requested to deliver a fecal sample on admission, at discharge, and one and six months thereafter. Patients' characteristics including gender, age and antibiotic use during hospital stay were registered. One and six months after discharge the patients were reminded to provide a fresh fecal sample and to complete a questionnaire on antibiotic use and re-hospitalization in the previous period. The Medical Ethical Committees of the University Hospitals Maastricht and Groningen and the Erasmus Medical Center Rotterdam approved the study.

Exclusion criteria

Patients were excluded if they were: 1) less than 18 years of age, 2) unable to give informed consent, 3) not living in The Netherlands during the last 6 months, 4) referred from a nursing home (long-term care facility), 5) transferred from/to another ward of the same or another hospital, or 6) hospitalized for less than two days. Moreover, if one of the first three fecal samples, i.e. the one on admission, discharge or 1 month after discharge was missing the patient was excluded as well.

Analysis of the fecal samples

Fecal samples were diluted 1:10 in 0.9% NaCl (w/v) with 20% (v/v) glycerol and stored at -20°C at the microbiological laboratories of the respective hospitals. The samples of azR and azG were transported on dry ice to the microbiological laboratory of the azM for further analysis.

After thawing, 40 µl of the 10^{-1} and 10^{-3} dilutions of the samples in 0.9% NaCl (w/v) were inoculated onto KF-Streptococcus agar™ plates (Oxoid CM 701, Basingstoke, England) with and without antibiotics using a spiral plater (Eddy Jet, IUL Instruments, I.K.S., Leerdam, The Netherlands) as described previously.^{16,17} The antibiotic concentrations incorporated in the agar plates (Table 1) were based on the NCCLS breakpoints¹⁸ and adapted to make comparison with previous studies possible.^{16,17} After 48h incubation at 42°C typical red or pink enterococci colonies were counted on the plates with and without antibiotics. The minimum detection level of enterococci was approximately 300 colony forming units per g feces. One typical colony was randomly picked from each plate showing growth and identified to the *Enterococcus* genus using tolerance to bile, esculin hydrolysis, growth in 6.5% (w/v) NaCl, and a positive pyrrolidonyl-arylamidase reaction (pyrrolidonyl-arylamidase tablets, Rosco, Taastrup, Denmark). For the identification of *E. faecalis* tellurite reduction was determined using tellurite tablets (Rosco, Taastrup, Denmark) on blood agar plates (Oxoid, Basingstoke, UK) after overnight incubation at 37°C. In case of doubt, the identification was confirmed with the API 20Strep system™ (BioMérieux, Plainview, N.Y.). One *E. faecalis* isolate per fecal sample was stored at -70°C in 0.7% peptone (w/v) with 30% glycerol (v/v).

The prevalence of resistance was calculated as the number of samples with *E. faecalis* isolates resistant to a certain antibiotic divided by the total number of samples showing growth of *E. faecalis* multiplied by 100. These percentages were determined for each point of time.

MIC Distributions

Minimum inhibitory concentrations (MICs) of *E. faecalis* isolates of patients with this species in the first three samples (n=40) were determined according to the Standard of the Dutch working Group on Antibiotic Policy (www.swab.nl). The microbroth dilution method with Iso-Sensitest broth™ (Oxoid CM 473, Basingstoke, England), an inoculum of 5×10^5 cfu/ml and overnight incubation at 37°C was used. The following antimicrobial agents (range in mg/L) were tested: amoxicillin (0.06-128), vancomycin (0.12-256), teicoplanin (0.12-256), linezolid (0.03-64), ciprofloxacin (0.03-32), clarithromycin (0.06-128), tetracycline (0.06-128), and chloramphenicol (0.12-256). High level resistance against gentamicin (500 mg/L) and streptomycin (2000 mg/L) was determined by the agar dilution method using BHI-agar (Oxoid CM 375, Basingstoke,

England) according to NCCLS criteria.¹⁸ *E. faecalis* ATCC 29212 was used as reference strain.

Statistical analysis

Basic statistics were calculated using SPSS for Windows release 11.01 (SPSS inc., Chicago, Illinois). Confidence intervals for prevalences were calculated using the exact method.¹⁹ Calculations were performed using Microsoft Excel 2000 (Microsoft Corporation) and StaTable version 1.0.1 (Cytel Software Corporation) to obtain the needed F-values. If 95% confidence intervals (CI) did not overlap differences were considered statistical significant. MIC-values were converted to a logarithmic scale with base 2 before statistical analysis. The non-parametric Spearmans Rho correlation coefficient was calculated to determine relations between the MIC-values for the different antibiotics tested.

Results

Patient population

Of the 681 patients willing to participate in the study, 263 patients were included for the final analysis on admission, at discharge and one month thereafter. Six months after discharge 215 patients submitted a fecal sample. The main reasons for exclusion of patients were incompleteness of the samples (two or less), less than 2 days of hospital stay, and transfer to the ICU.

The number of patients on admission was comparable for the three hospitals as well as the mean age of the patients (58 ± 15 years). Furthermore, prophylactic antibiotics were used in 54% of the patients and 32% received antibiotics for therapy.

*Prevalence of antibiotic resistant *E. faecalis**

Enterococci were isolated in 76%-88% of the fecal samples from the patient population at the different points of time. The most commonly isolated species was *E. faecalis* (47%-61%) followed by *E. faecium* (26%-37%). Other species found in lower percentages were *E. gallinarum* (3%), *E. casseliflavus* (3%), *E. avium* (3%), *E. hirae* (7%) and *E. mundtii* (0,25%).

The prevalences of antibiotic resistance of the *E. faecalis* isolates at the different points of time are presented in Table 1. For all antibiotics tested vancomycin excepted, an increase in the prevalence of resistance was observed during hospitalization, i.e. between "on admission" and "at discharge". One month after discharge, the prevalence of resistance to ciprofloxacin and tetracycline decreased to a level being lower than that

Table 1. The resistance percentage of fecal *E. faecalis* isolates of surgical patients in time

Antimicrobial agent (mg/L)	Time point	Patients with <i>E. faecalis</i> (n)	Prevalence of resistant <i>E. faecalis</i> (%)	95% CI
Amoxicillin (25)	admission	97	0	
	discharge	136	5	2.7-12.6
	1 month	109	2	0.6-12.3
	6 months	104	0	
Ciprofloxacin (4)	admission	97	8	4.5-18.3
	discharge	136	9	5.3-16.8
	1 month	109	6	2.8-13.6
	6 months	104	3	1.2-12.8
Erythromycin (10)	admission	97	45	35.4-55.9
	discharge	136	52	42.7-60.2
	1 month	109	52	42.6-61.8
	6 months	104	47	37.3-57.2
Gentamicin HL (500)	admission	97	7	3.8-17.5
	discharge	136	10	5.9-17.4
	1 month	109	12	7.3-21.6
	6 months	104	8	4.2-17.2
Oxytetracycline (25)	admission	97	64	53.6-73.5
	discharge	136	67	58.4-74.8
	1 month	109	59	49.0-68.0
	6 months	104	60	49.5-69.1
Vancomycin (10)	admission	97	3	1.3-13.6
	discharge	136	2	0.5-9.8
	1 month	109	5	2.2-13.8
	6 months	104	2	0.7-12.8

HL= high level

on admission (5,5% compared to 8% on admission for ciprofloxacin and 58% compared to 64% for tetracycline). For amoxicillin, erythromycin and gentamicin the resistance prevalences of the "on admission" samples was reached six months after discharge. Resistance to vancomycin decreased during hospitalization, increased one month after discharge, but decreased again six months thereafter to the same level as at discharge. For erythromycin (45%, 52%, 52%, and 47% respectively) and oxytetracycline (64%, 67%, 59%, and 60% respectively) the highest prevalences of resistance were found for each point of time.

MIC-distribution

In total 120 *E. faecalis* isolates were tested (40 isolates on admission, at discharge and 1 month after discharge). All isolates were susceptible to amoxicillin, vancomycin, teicoplanin and linezolid. There were no differences in the number of multi-drug resistant *E. faecalis* isolates between the different points of time.

Analyzing the MIC-distribution patterns after conversion of the MIC-values to a logarithmic scale with base 2 a positive correlation was found between amoxicillin and respectively linezolid and ciprofloxacin. A positive correlation was also found between clarithromycin and respectively tetracycline and chloramphenicol.

Discussion

The rapid emergence of antibiotic resistant enterococci as important nosocomial pathogens has become a health care issue of great concern.^{2,3} The main reasons for resistance are selection by antibiotic use and dissemination of resistance genes and resistant bacteria, both factors which are important in a hospital setting.

The samples on admission, reflecting the extramural situation, did not show any significant differences in resistance prevalence between the three hospitals (data not shown). Similar results were obtained for the prevalences of resistance in the extramural situation for both *E. coli* and enterococci in the same three geographical regions.¹⁷ Based on these findings the total study population was analyzed as one group of patients. Furthermore only *E. faecalis*, the clinical most relevant species^{20,21} and the most isolated enterococcal species in this population, was analyzed.

The influence of hospitalization on the prevalence of antibiotic resistant fecal *E. faecalis* was determined in three university affiliated-hospitals in The Netherlands for patients admitted to the surgical wards on admission, at discharge and 1 and 6 months thereafter. The latter two samples were used to analyze the persistence of antibiotic resistance in the community over time.

Persistence of antibiotic resistance in the commensal flora of patients after discharge contributes to the reservoir of antibiotic resistant bacteria and resistance genes in the

community. In addition, as 80% of the antibiotic use in humans is prescribed outside the hospital, in primary health care, selection and dissemination of antibiotic resistance from the community into the hospital will contribute to the antibiotic resistance problems in hospitals.

In this study, the prevalence of resistance after discharge returned to the level before hospitalization within six months after discharge. Depending on the antibiotic tested, the time needed ranged from 1 month (ciprofloxacin and tetracycline) to six months (erythromycin and gentamicin). This difference might be explained by the difference in antimicrobials used in primary health care²² as well as differences in the properties of the antibiotics. In our patient population, unfortunately, no information was available on the individual use of antibiotics after discharge.

A limitation of the present study was that fecal samples were requested only one and six months after discharge. Therefore we do not know whether the level on admission was already reached shortly after one month. Monitoring the influence of hospitalization on the resistance prevalence after discharge collecting monthly fecal samples seems preferable but might negatively influence the compliance of the patients.

According to the power analysis ($\alpha = 0,05$ and $\beta = 0,2$) the minimum number of patients needed to detect a significant difference in the prevalence of resistance from 1-10% was ~100 for each point of time. To obtain this we included twice as many patients in each hospital. As the total number of patients in the final analysis was 263 and the number of patients with *E. faecalis* ranged from 97 to 136 sufficient patients were included at all time points. The large number of patients excluded was due to the lack of ≥ 2 fecal samples/patient as the study had a long follow-up in the home environment.

The antibiotic susceptibility as MIC was performed only for *E. faecalis* isolates, which was the dominant species in three consecutive samples of the same patient. No significant differences in MIC-distribution between the different points were observed, which might be due to the low number of isolates tested. The positive correlation between amoxicillin and fluoroquinolone resistance in enterococci was found previously;^{23,24} 91% of the ampicillin resistant enterococci in that study were ciprofloxacin resistant compared to 14% of the ampicillin susceptible strains. It is however not clear yet how this co-selection of ampicillin and ciprofloxacin resistance occurs. The relation between clarithromycin and tetracycline resistance could be explained by the co-location of the resistance genes on the same plasmid.^{25,26} The positive correlation between amoxicillin and linezolid could neither be explained by a similar mode of action²⁷ against bacteria nor by comparable mechanisms of resistance²⁷ against both agents. Since linezolid has only been recently available on the market, similar findings were not yet found in the literature and further investigation is needed to determine whether this finding is a coincidence or a real relation between both agents exists.

In conclusion, hospitalization caused in the population studied a slight increase in the prevalence of antibiotic resistance after discharge, which decreased again to the level on

admission within six months after discharge. In this population there is only a slight contribution of hospitalization to the persistence of antibiotic resistance in the commensal flora of patients after discharge. This might be due to the relatively short hospitalization period (mean $\pm$ 8 days/patient) and the low antibiotic use in this group of patients. This group was analyzed as being representative for the average hospitalized patient in The Netherlands. Further antibiotic resistance surveillance studies both in the hospital and the community, with different patient populations, are necessary including the dissemination from the hospital into the community and vice versa to analyze their mutual interaction.

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Chapter 5

Acute uncomplicated lower urinary tract infections in general practice: clinical and microbiological cure rates after three versus five day treatment with trimethoprim; a randomized-controlled trial

Accepted for publication in *The European Journal of General Practice*

$$\begin{aligned}
 T_1 &= \frac{1}{2} \int_0^1 (1-x)^2 dx = \frac{1}{2} \left[-\frac{1}{3}(1-x)^3 \right]_0^1 = \frac{1}{2} \left(-\frac{1}{3}(1-1)^3 + \frac{1}{3}(1-0)^3 \right) = \frac{1}{2} \left(0 + \frac{1}{3} \right) = \frac{1}{6} \\
 T_2 &= \frac{1}{2} \int_0^1 (1-x)^2 dx = \frac{1}{2} \left[-\frac{1}{3}(1-x)^3 \right]_0^1 = \frac{1}{2} \left(-\frac{1}{3}(1-1)^3 + \frac{1}{3}(1-0)^3 \right) = \frac{1}{2} \left(0 + \frac{1}{3} \right) = \frac{1}{6} \\
 T_3 &= \frac{1}{2} \int_0^1 (1-x)^2 dx = \frac{1}{2} \left[-\frac{1}{3}(1-x)^3 \right]_0^1 = \frac{1}{2} \left(-\frac{1}{3}(1-1)^3 + \frac{1}{3}(1-0)^3 \right) = \frac{1}{2} \left(0 + \frac{1}{3} \right) = \frac{1}{6}
 \end{aligned}$$

Abstract

Epidemiological studies indicate that acute uncomplicated urinary tract infections (UTIs) in women can be successfully treated with short treatment regimens. However, the findings from the literature do not match the daily practice experience.

In this study the microbiological and clinical (self-reported) cure rate in a randomized-controlled trial of a three-day vs. five-day treatment regimen with trimethoprim for UTIs in women were evaluated.

No statistically significant difference in bacteriological cure rate was found between the three-day and the five-day regimen. One day after the shorter regimen 44% of women considered themselves as "not-recovered" due to persistence of the symptoms compared to 35% after the five-day treatment ($p > 0.05$). Three days after therapy these percentages were 30% and 25% respectively.

The relatively high percentage of persistent complaints one day after the three-day regimen might be responsible for the experience of general practitioners believing that short regimens are not successful. It is therefore advisable to control urine samples at least three days after finishing treatment.

Introduction

Acute uncomplicated urinary tract infections (UTIs) are a frequent cause of consulting a general practitioner (GP): 40%-50% of all women suffer at least once in their life from a UTI.¹ Most lower urinary tract infections in women are caused by *Escherichia coli*, with *Proteus spp.*, *Staphylococcus saprophyticus* and *Klebsiella spp.* as less frequent pathogens.²

The optimal duration of antibiotic treatment remains a matter of debate. Single dose or short (three days) treatment improves compliance and reduces the chance of selection of antibiotic resistance³ of the causative as well as the commensal micro-organisms, and is therefore more advantageous for both patient and doctor. Several studies have suggested good efficacy of three-day treatment as compared to regimens of seven to ten days.⁴⁻⁷ However, experiences of GPs 'in the field' suggest that patients after a three-day treatment regimens return more often because of persisting complaints. Therefore, some GP's still prescribe five to seven days of therapy.

In the present study we evaluated the microbiological and clinical (self-reported) cure rate of a 3- versus 5-day treatment course with trimethoprim for uncomplicated UTIs in a blinded randomized-controlled trial in women in the general population of the city of Maastricht, The Netherlands. Furthermore, the prevalence of trimethoprim resistant *E. coli* was assessed before the start of therapy as well as 1 and 3 days after the end of therapy. In addition, the percentage persistent infections with a trimethoprim resistant *E. coli* was determined.

Materials and Methods

Patients

Non-pregnant female patients aged 13-77 year presenting to their GP with either dysuria, stranguria, urinary frequency or urgency in the past 24 hours were included in the study as shown in the flow chart (Figure 1). Exclusion criteria were known diabetes, known structural abnormalities of the urinary tract, indwelling catheters, use of immunosuppressive drugs and allergy to trimethoprim. Also patients with symptoms of acute pyelonephritis or with a known UTI within the last three months were excluded from the study. When the GP did not intend to treat the patient with an antibiotic the patient was not eligible for participation in the study. Informed consent was obtained from all participants before enrolment.

Upon inclusion, the patients received a prescription for either a three- or five-day regimen of trimethoprim in a closed envelope from their general practitioner. The treatment strategy was computer-aided randomized (www.randomizer.org) for each general practice, and blinded for the physician. Before treatment ($t=0$), a urinary sample was collected, as well as one and three days after completion of the therapy (respectively $t=1$ and $t=3$). On these days the patient filled out a questionnaire asking for (the relief of) symptoms (Figure 1).

In order to detect a higher efficacy of the 5-day regimen of 10% compared to the 3-day regimen with a cure rate of 90% for trimethoprim 142 patients per treatment regimen were needed ($\alpha = 0.05$ and $\beta = 0.2$). In total 324 patients were included by 9 general practitioners.

Sample Processing

Fresh urine samples were used for the preparation of a standard dipslide culture and subsequently sent to the department of Medical Microbiology of the University Hospital Maastricht, The Netherlands. For identification of the micro-organisms standard laboratory methods were used. The antimicrobial susceptibility was performed according to the SWAB (Stichting Werkgroep Antibiotica Beleid) standard (www.swab.nl).

Data Analysis

Women stating in the questionnaires (1 question on general well being and 7 specific questions on UTI symptoms) after treatment that they had no more complaints were considered as "recovered", those mentioning still urinary complaints were considered "not-recovered".

Figure 1. Flow chart of the study design

Only patients that completed both questionnaires and from whom all three urine samples were collected were included in the final analysis. Comparison of the treatment regimens, the prevalence of symptoms and side-effects were tested for statistical significance with the Pearson Chi-Square Test. A p-value < 0.05 was considered as statistically significant.

Results

A total of 324 patients were randomly assigned to either treatment group: 160 to the 3-day and 164 to the 5-day trimethoprim regimen (Figure 1). No baseline urine sample ($t=0$) was received from 68 and 70 patients of the 3- and 5-day treatment regimen respectively. Fifty-seven patients were excluded due to the lack of the follow-up urine samples ($t=1$ and $t=3$). In the final analysis for the three-day regimen 66 women (mean age: 43 ± 17 years) and for the five-day treatment group 63 women (mean age: 44 ± 16 years) were included.

E. coli was isolated in almost 65% of the urine samples before antibiotic treatment was started (Table 1). Both one and three days after the 3-day antibiotic therapy nine samples (14%) were still positive for *E. coli*. Trimethoprim resistant isolates were found in seven and eight of these follow-up samples respectively. One and three days after the five-day therapy course eleven out of twelve and nine out of ten samples respectively showed growth of trimethoprim resistant *E. coli*. Trimethoprim resistance observed before treatment persisted one and three days after both therapy regimens in all but two cases. In four patients trimethoprim resistance developed during therapy, two patients in each therapy regimen.

Table 1. Culture results of urine samples in numbers (percentages) respectively before, one day after and three days after treatment.

Micro-organism	Before treatment		1 day after treatment		3 days after treatment	
	3D	5D	3D	5D	3D	5D
Total <i>Escherichia coli</i>	42 (64)	38 (60)	9 (14)	12 (19)	9 (14)	10 (16)
Trimethoprim resistant <i>E. coli</i>	4	9	7	11	8	9
<i>Proteus mirabilis</i>	5 (8)	3 (5)	-	-	-	-
other	6 (9)	5 (8)	8 (12)	2 (3)	13 (19)	1 (2)
Negative ¹	13 (20)	17 (27)	49 (74)	49 (78)	44 (67)	52 (83)

3D: 3-day regimen (n=66); 5D: 5-day regimen (n=63)

¹Negative represents urine samples showing no growth or with $< 10^3$ colony forming units/ml

Table 2 depicts the results from the questionnaires combined with the number (percentage) of trimethoprim resistant *E. coli* isolated for both time points after the end of therapy. Twenty-two patients (35%) reported "not-recovered" one day after a five-day treatment regimen compared to twenty-nine patients (44%) after a three-day regimen ($p > 0.05$). Of those who considered themselves as "recovered" trimethoprim resistant *E. coli* were found in 10% to 24% of the patients. In the "not-recovered" patient group these percentages ranged from 24% to 36%.

Table 2. Recovery (rate) from urinary tract infections according to self-reported complaints in the questionnaires and the number (percentage) of trimethoprim resistant *E. coli*.

Self-reported		3-day regimen		5 day-regimen	
		Day after treatment		Day after treatment	
		day 1	day 3	day 1	day 3
recovered	patients	33 (50)	39 (59)	35 (56)	39 (62)
	Tri resistant	8 (24)	9 (23)	6 (17)	4 (10)
not-recovered	patients	29 (44)	20 (30)	22 (35)	16 (25)
	Tri resistant	7 (24)	6 (30)	8 (36)	5 (31)
unclear	patients	4 (6)	7 (11)	6 (10)	8 (13)
	Tri resistant	2 (50)	3 (43)	1 (17)	0

Tri = trimethoprim

Discussion

In the present study no significant differences in bacteriological cure rate were found between the three-day and five-day trimethoprim treatment regimen both one and three days after completion of the therapy. The percentage trimethoprim resistant *E. coli* was also similar in both groups. Results in this study support previous findings that short courses of 3 days of antibiotics are as effective as longer courses in the treatment of an uncomplicated UTI.^{8,9} In addition, a three-day treatment, with the advantages of lower costs, less side-effects and better patient compliance, is as effective as a longer treatment regimen. Similar findings were found for short treatment regimens with fluoroquinolones.³

However, relief of symptoms is also an important issue for the patient and for the GP. In the three-day treatment group twenty-nine patients (44%) considered themselves "not-recovered" one day after the end of the therapy. This means that these patients were not symptom-free when they delivered their control urine sample. A non-recovery rate of

44% is considerable and unsatisfactory for the GP. Although the recovery rate between the two treatment groups was not significantly different, we assume that the difference in self-reported cure rate, i.e. 56% versus 65%, is very likely the reason why experienced (and older) GPs consider a three-day regimen as less reliable especially as recommendations for shorter treatment strategies are relatively recent.

The relatively high percentage of trimethoprim resistant *E. coli* isolates before treatment (13/80, 16%) however, raises the question whether trimethoprim is still a good agent of first choice for (empiric) therapy of UTIs in The Netherlands. In the present study the isolates were derived from samples from the Maastricht area only, but recent susceptibility data of *E. coli* isolated from unselected urine samples of general practice patients all over The Netherlands showed similar resistance percentages.¹⁰ The relatively high percentages of trimethoprim resistant *E. coli* isolates in the group of patients who considered themselves as “recovered” underscores the natural course of an acute uncomplicated UTI.¹¹ Furthermore, patients who considered themselves as “recovered” more often showed a low count bacterial culture as those who considered themselves as “not-recovered”. It might be that due to this low number of bacteria the clinical symptoms are not as obvious and the patients considered themselves as recovered.

One limitation of the study is that the number of patients in the final analysis were not large enough to reach statistical significant differences between both treatment groups. It proved to be difficult to perform this kind of study in general practices, mostly because of perceived lack of time of the doctors and nurses. Despite this limitation and based on the data presented an advice for daily practice might be that GPs inform their patients that a three-day antibiotic therapy is effective but it takes several days for complete relief of the complaints. Comparing the recovery rate 6 days after the start of therapy, i.e. 3 days after the 3-day course and 1 day after the 5-day regimen similar percentages of patients reported themselves as recovered. This is probably due to the natural course of the disease. Therefore, if control urine samples are tested this has to be done at least three days or more after the end of the treatment. Christiaens *et al.*¹¹ used a seven-day period after treatment before samples were controlled. Control for clinical and bacteriological recovery directly after treatment will result in disappointment both for the patient and the GP.

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Chapter 6

Urinary tract infections in general practice patients: diagnostic tests versus bacteriological culture

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Abstract

Urinary tract infections (UTIs) are common bacterial infections encountered in general practice. For the optimal treatment of a UTI the general practitioner (GP) should rely on the results of diagnostic tests and recent antimicrobial susceptibility percentages of unselected uropathogens.

In a study on acute uncomplicated UTIs in 1993 female patients between 11-70 years old in 2003-2004 in The Netherlands the performance characteristics of the diagnostic test(s) used by the GP were determined and compared with the antibiotic prescription rate. For different age groups the choice and duration of the antibiotic treatment, the uropathogens and the antibiotic susceptibility of *Escherichia coli* were determined.

The positive predictive value (PPV, 96%) and the specificity (94%) of the nitrite test were high for all samples. A negative nitrite with a positive leukocyte-esterase test showed a high PPV (79%) and sensitivity (82%). When both nitrite and leukocyte-esterase tests were negative ~50% of the samples were culture positive. Antimicrobial therapy was prescribed in 96% of the patients with a positive nitrite and in 71% of those with a negative nitrite and positive leukocyte-esterase test. Nitrofurantoin and trimethoprim were the agents mostly prescribed. Nitrofurantoin prescription rate decreased and that of fluoroquinolones increased with increasing age ($p < 0.01$). Nitrofurantoin was prescribed with the same frequency for 3, 5 or 7 days in all patients. Trimethoprim was mostly prescribed for 3 days in patients 21-50 years and for 5 days in the other ones. *E. coli*, the uropathogen mostly isolated, decreased in frequency with increasing age. The frequency of *Proteus mirabilis* and *Staphylococcus saprophyticus* was age dependent, i.e. *P. mirabilis* was more found in the oldest patients and *S. saprophyticus* in the younger ones. The antimicrobial susceptibility of *E. coli* was not age related. The lowest percentages were found for amoxicillin (67%) and trimethoprim (77%). Fluoroquinolones resistance was emerging in the older patients.

For female patients with symptoms of an acute uncomplicated UTI a positive nitrite or a negative nitrite with a positive leukocyte-esterase test confirmed a UTI whereas a negative nitrite together with a negative leukocyte-esterase test did not rule out infection. For the empiric treatment of a UTI the GP should take into account the changing etiology with increasing age. Furthermore, prudent use of antimicrobial agents in general and more specific of fluoroquinolones remains recommended. As trimethoprim resistance reached 20% it is advisable to no longer use this agent as first choice in the treatment of acute uncomplicated UTIs in The Netherlands.

Introduction

An acute uncomplicated urinary tract infection (UTI) is one of the most common bacterial infections in women.¹⁻³ It is estimated that as many as 60% of all women report having had a UTI at least once in their lifetime.^{4,5} The majority of UTIs are caused by *Escherichia coli* followed by *Staphylococcus saprophyticus*, mainly in younger women. *Klebsiella*, *Proteus* and *Enterobacter* species are isolated less frequently.^{6,7}

For the diagnosis of a UTI the general practitioner (GP) can rely on, besides the symptoms of the patient, the results of the nitrite and leukocyte-esterase dipstick tests performed on the urine sample. In the first revision of the "Urinary Tract Guidelines" of the Dutch College of General Practitioners (NGH)⁸ a urine sample with a positive nitrite test is considered positive and a specimen with a negative nitrite together with a negative leukocyte-esterase test is considered negative. Furthermore, urine specimens of symptomatic female patients should be considered as culture positive at $\geq 10^2$ cfu/ml and are called low count bacteriuria.⁸

Several studies in The Netherlands on the antibiotic susceptibility patterns of uropathogens showed an increased resistance to widely used agents like trimethoprim and amoxicillin.⁹ However, most information concerning the isolated uropathogens and their antimicrobial susceptibility was derived from samples sent in by the general practitioners (GPs), after prior (once or twice) therapy failure has occurred.¹⁰ Therefore, these samples are likely to reflect a sample bias. Consequently an underestimation of the antibiotic susceptibility percentages of uropathogens from patients with an acute uncomplicated UTI is to be expected.¹¹⁻¹³

Recent data on the antimicrobial susceptibility of uropathogens isolated from patients with an acute uncomplicated UTI visiting their GP, i.e. the "unselected" uropathogens are needed for the setup of evidence-based guidelines such as those of the NGH.

Therefore a study on acute uncomplicated UTIs in non-pregnant female patients between 11 and 70 years of age attending 21 general practitioners from the Sentinel Station of The Netherlands Institute for Health Services Research (NIVEL) was performed. In these patients care as usual of the GP concerning diagnostic procedures (i.e. nitrite and leukocyte-esterase tests) and the treatment prescribed (choice and duration of the antimicrobial agent) were compared with the bacteriological culture results and the antimicrobial susceptibility of all the unselected *E. coli* isolates, the most important uropathogen, was determined.

Materials and Methods

Patients and urine sample processing

The patient population of the 21 general practices (GPs) from the Sentinel Station of The Netherlands Institute for Health Services Research (NIVEL) participating in the study accounts for about 1% of the Dutch population and is representative according to age, gender, regional distribution, and degree of urbanization.

From January 2003 until December 2004 the GPs recorded for non-pregnant female patients between 11 and 70 years old with symptoms of an acute uncomplicated UTI the care as usual, i.e. the results of the diagnostic tests (nitrite and leukocyte-esterase test) and the therapy prescribed (agent and duration). An acute uncomplicated UTI was defined as an infection with one or more of the following symptoms: dysuria, stranguria, urinary frequency, or urgency without the presence of fever $>38^{\circ}\text{C}$.

A fresh voided (midstream) urine sample was used for diagnostic purpose by the GP, i.e. nitrite dipstick and/or leukocyte-esterase test. Subsequently a dipslide (Uriline, 56508, BioMérieux, Plainview, N.Y) was prepared according to the manufacturer's instructions and sent to the laboratory of Medical Microbiology of the University Hospital Maastricht, The Netherlands for isolation and identification of the uropathogens and antibiotic susceptibility testing.

On the day of arrival, the dipslide was incubated overnight at 37°C (if necessary). Bacterial growth on the dipslides was recorded from no growth to 10^7 cfu/ml according to the manufacturer's instructions and considered positive at $\geq 10^3$ cfu/ml. Identification was performed using standard biochemical tests.¹⁴ If there remained any doubt about the identification API 20E, API20 NE, API 20 Strep or API Staph (BioMérieux, Plainview, NY) were used depending on the Gram-stain of the micro-organism. The isolated uropathogens were stored at -70°C until further analysis.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) of the *E. coli* isolates were determined according to the CLSI-criteria (formerly the NCCLS)¹⁵ using the microbroth dilution method with Mueller Hinton II broth cation adjusted (Becton, Dickinson and Company, Sparks, USA), an inoculum of 5×10^5 cfu/ml, and overnight incubation at 37°C . The MIC-plates with freeze-dried antibiotics were provided by MCS Diagnostics BV (NLDMCS1, MCS Diagnostics BV, Swalmen, The Netherlands) and delivered after quality testing by the manufacturers and a shelf life of approximately one year was guaranteed. The following antimicrobial agents (range in mg/l) were tested: amoxicillin (0.06-128), co-amoxiclav (0.06-128), trimethoprim (0.03-64), co-trimoxazole (0.03-64), norfloxacin (0.03-64), ciprofloxacin (0.008-16), and nitrofurantoin (0.5-512). The reference strains *E. coli* ATCC 35218 and ATCC 25922 were used as control strains.

The breakpoints for susceptibility were according to the CLSI-guidelines (formerly the NCCLS).¹⁵

Statistical analysis

The performance of the diagnostic tests, i.e. the positive predictive value (PPV), the negative predictive value (NPV), the sensitivity and the specificity of all samples was calculated for the nitrite test alone and for the leukocyte-esterase test in case of a negative nitrite test using culture $\geq 10^3$ cfu/ml as gold standard. Samples with a negative nitrite and an unknown leukocyte-esterase test result or both test results unknown were excluded for this analysis. For the analysis of the therapy prescribed (agent and duration), the causative uropathogens and the susceptibility of the *E. coli* isolates the patients were divided in three age categories: 11-20 year, 21-50 year and 51-70 year.¹⁶ For the analysis of the prescribed therapy norfloxacin, ciprofloxacin and ofloxacin therapy were totalized as the group of fluoroquinolones.

For the statistical analysis the program SPSS 11.0 for Windows was used. To detect significant differences between the age groups and the prescribed treatment, the bacteriological culture results and the antimicrobial susceptibility of the *E. coli* isolates the Mann-Whitney U test was performed. A p-value of $p < 0.05$ was considered significant.

Results

In total 1993 non-pregnant women with a mean age ($\pm$ SD) of 43 ± 17 year were enrolled in the study, half of which belonged to the age group 21-50 year.

The performance characteristics of the diagnostic tests performed by the GP for all patients are shown in Table 1 as there were no significant differences between the different age groups. The positive predictive value (PPV) of the nitrite test was 96% and the specificity 94%. The negative predictive value (NPV) and sensitivity were 30% and 44% respectively. In case of a negative nitrite test result, the PPV of the leukocyte-esterase was 79% and the sensitivity 82%.

The prescription rate of the GPs for all patients in function of the nitrite and leukocyte-esterase test results is shown in Table 2. Ninety-four percent of the patients with a nitrite positive urine sample received antimicrobial therapy. The majority of patients (71%) with urine samples negative for the nitrite test and positive for the leukocyte-esterase test did receive antimicrobial therapy. One fifth of the patients with urine samples showing both a negative nitrite and leukocyte-esterase tests were prescribed antibiotics.

Table 1. Performance characteristics of the diagnostic tests performed by the GP in all patients.*A. The performance of the nitrite test:*

Diagnostic test result ^a	Urine culture		PPV	NPV	Sens	Spec
	$\geq 10^3$ cfu/ml	$< 10^3$ cfu/ml				
Nitrite positive	662	25	96	30	44	94
Nitrite negative	845	360				

B. The performance of the leukocyte-esterase test in case of a negative nitrite test:

Diagnostic test result ^a	Urine culture		PPV	NPV	Sens	Spec
	$\geq 10^3$ cfu/ml	$< 10^3$ cfu/ml				
Leukocyte-esterase positive	693	186	79	53	82	48
Leukocyte-esterase negative	152	174				

PPV = positive predictive value, NPV = negative predictive value, Sens = sensitivity, Spec = specificity
^asamples with a negative nitrite and unknown leukocyte-esterase test result or both unknown test results were excluded.

Table 2. The number (rate) of prescriptions of the general practitioner compared with the diagnostic test results.

Diagnostic test result	Therapy prescribed		
	Yes	No	Unknown
Nitrite positive	648 (94)	28 (4)	11 (2)
Nitrite negative			
Leukocyte-esterase positive	623 (71)	233 (27)	23 (3)
Leukocyte-esterase negative	64 (20)	240 (74)	22 (7)
Unknown	63 (62)	16 (16)	22 (22)

Figure 1. The percentage antimicrobial agents prescribed as therapy in patients with acute uncomplicated UTI per age category.

Nit = nitrofurantoin, tri = trimethoprim, co-tri = co-trimoxazole, am = amoxicillin, co-am = co-amoxiclav, qui = fluoroquinolones

*significant decrease in the prescription rate between the youngest and the oldest patients ($p < 0.05$)

°significant higher prescription rate in the oldest patients compared to both younger groups ($p < 0.01$)

Figure 2. The duration of the antimicrobial therapy per age group for the three agents mostly prescribed.

Seventy percent of all the patients were prescribed antibiotic therapy. Trimethoprim and nitrofurantoin were the agents prescribed most frequently in all the patients (Figure 1). The latter agent was significantly less prescribed in the oldest age category as compared to the youngest one ($p < 0.05$). The prescription rate of the fluoroquinolones increased with increasing age ($p < 0.01$). The duration of the treatment regimen prescribed in this patient population ranged from 3 to 7 or more days. Nitrofurantoin therapy was in all age groups prescribed with the same frequency as a 3-, 5- or 7-day regimen (Figure 2). The majority of the patients 21-50 years old (80%) were prescribed a 3-day course of trimethoprim; a 5-day therapy was given to the other patients. Fluoroquinolones were mostly prescribed for 7 days in all the patients.

E. coli, the uropathogen isolated most frequently in all age groups, was proportionally less found in the oldest patients (Table 3). The frequency of isolation of *Proteus mirabilis* and *S. saprophyticus* was age dependent, i.e. the prevalence of *P. mirabilis* increased and that of *S. saprophyticus* decreased with increasing age. The highest frequency of isolation of *S. saprophyticus* was found for the youngest age group.

The antimicrobial susceptibility percentages of the *E. coli* isolates from patients of the three age groups are shown in Table 4. No significant differences in susceptibility percentages were found between the three age groups. The lowest susceptibility percentages were found for amoxicillin (67%) and trimethoprim (77%). Fluoroquinolone susceptibility was slightly lower in the patients over the age of 21 year.

Table 3. Percentage isolated uropathogens for each age group.

Micro-organism	Age category (years)			Total
	11-20 n=165	21-50 n=789	51-70 n=629	
<i>E. coli</i>	69	66	65	66
<i>P. mirabilis</i> *	1	4	6	5
<i>K. pneumoniae</i>	1	4	4	4
<i>Pseudomonas spp.</i>	2	2	2	2
<i>Acinetobacter spp.</i>	2	4	4	4
Other Gram-negatives	7	7	9	8
<i>S. saprophyticus</i> °	7	4	0.5	3
<i>E. faecalis</i>	4	3	3	3
Other Gram-positives	6	6	7	6

*significant difference between the youngest and oldest patient group (Mann-Whitney U test; $p < 0.02$)

°significant lower percentage in the oldest patients compared to both other groups (Mann-Whitney U test; $p = 0.000$)

Table 4. Susceptibility percentages of *E. coli* isolated per age category.

Antibiotic	Age category (years)			Total
	11-20 n=103	21-50 n=489	51-70 n=370	
Amoxicillin	75	65	68	67
Co-amoxiclav	98	98	98	98
Trimethoprim	81	77	78	77
Co-trimoxazole	83	80	80	80
Norfloxacin	100	97	97	97
Ciprofloxacin	100	97	97	97
Nitrofurantoin	99	99	100	99

Discussion

This study on acute uncomplicated urinary tract infections in non-pregnant female patients of 11 to 70 years of age among 21 general practices throughout The Netherlands was a nationwide study on acute uncomplicated UTIs dealing with the care as usual of the general practitioner, i.e. nitrite and leukocyte-esterase tests and the antibiotic therapy prescribed, bacteriological culture and the antimicrobial susceptibility of unselected *E. coli* isolates.

The nitrite dipstick test was very accurate in predicting a UTI (PPV = 96%) and GPs prescribed in > 90% of the patients with a positive nitrite test antimicrobial therapy as recommended by the NHG.⁸ A negative nitrite test combined with a positive leukocyte-esterase test showed a PPV of 79% whereas the NPV of a negative nitrite combined with a negative leukocyte-esterase test was moderate (53%). According to the NHG-guidelines the leukocyte-esterase test can be used to exclude a UTI in combination with a negative nitrite. However, our data showed that a positive leukocyte-esterase test with a negative nitrite test was predictive for a UTI in 79% of the patients and that in case of a negative leukocyte-esterase test (with a negative nitrite test) the chance on UTI was 53%. The daily practice data of this study showed that the GP used the leukocyte-esterase test often as diagnostic tool as 71% of the patients with a negative nitrite and positive leukocyte-esterase test were treated with antibiotic therapy. When both diagnostics test showed a negative result about 20% of the patients were treated with antibiotics whereas in almost 50% of these samples a positive culture ($\geq 10^3$ cfu/ml) was found. This discrepancy between the prescription rate (20%) and the percentage culture positive urine samples (50%) might be explained by the low cut-off value we used for

positive culture. In the latest recommendation of the NHG,⁸ however, it is stated that for symptomatic women a "low count" bacteriuria can be caused by 10^2 - 10^4 cfu/ml and these infections should be considered a UTI. Furthermore, as bacteria need over 4 hours to convert nitrate into nitrite at a level that is reliably detectable, the time span might have been too short before collection of these urine samples and false negative test results could be obtained.¹⁷ Also, the biochemical reaction detected by the nitrite test is associated with members of the *Enterobacteriaceae* family but not with other uropathogens like *S. saprophyticus*, *Pseudomonas spp.* or enterococci.¹⁷ Indeed, in the nitrite and leukocyte-esterase negative samples of this study significantly less frequent *E. coli*, but more often *Acinetobacter spp.*, other Gram-negatives and -positives and *E. faecalis* were found (Data not shown). In addition, by lowering the cut-off value for a positive urine sample to $\geq 10^3$ cfu/ml more low count urine samples are considered culture positive in which insufficient bacteria might be present to reduce nitrate in adequate amounts of nitrite for detection by the dipstick test. False positive nitrite test results were seen in 4% of the patients and might be due to anaerobic or fastidious micro-organisms not able to grow on the uricult dipslide.¹⁸ False positive leukocyte-esterase results when the nitrite test was negative were found in 186 (21%) urine samples and might be caused by the presence of bacteria of the vaginal fluid in the urine or the specimen might contain eosinophils which can act as a source of esterases.¹⁷

The antibiotic treatment prescribed most frequently in our patient population was according to the national guidelines.⁸ However the oldest patients were prescribed significantly less often nitrofurantoin and more frequently fluoroquinolones as compared to both younger groups. One reason for the GP to change his prescribing might be that nitrofurantoin is known to cause side-effects like nausea and vomiting, allergic, pulmonary, hepatic, neurological and hematological reactions, especially in older women.¹⁹ Another reason might be that older women more often have a history of UTI²⁰ and thus received more often therapy in the past. Fluoroquinolone therapy was also prescribed for longer durations in these older women probably to be sure of full eradication of the more resistant uropathogen. However, it is very likely that the increased prescription rate resulted in an increase in fluoroquinolone resistance in these patients.⁹

The uropathogens isolated in this patient population were similar to those in other studies.^{6,21-23} *E. coli*, still the most prevalent uropathogen was proportionally less isolated in the oldest patients. *P. mirabilis* was more frequently found with increasing age. In post-menopausal women due to the loss of estrogen and consequently the depletion of vaginal colonization with lactobacilli^{16,18} other Gram-negative bacteria than *E. coli* can enter more easily the urinary tract and cause an infection. The percentage *S. saprophyticus* in the study of Christiaens et al.²⁴ among patients between 15-50 years old (9%) was similar to the 7% found in our youngest age group (11-20 years) but higher than in our 21-50 years old (4%). This difference with our patients was probably caused by the differences in the age groups.

The antimicrobial susceptibility data were analyzed for the *E. coli* isolates only as this was the most prevalent uropathogen isolated in all age groups. The susceptibility percentages of the unselected *E. coli* isolates for the three age groups were similar for all agents tested (amoxicillin in the youngest age group excepted). However, a tendency to an increasing fluoroquinolone resistance was found in the patients who were more often prescribed these agents. The susceptibility percentages of isolates from public health laboratories were lower compared to our data.^{9,10} As in both studies most urine specimens analyzed were sent in by the GP after prior therapy failure or recurrent UTI, these "selected" isolates were more often exposed to antibiotics and thus more resistant. In conclusion, for symptomatic female patients between 11-70 years of age a urine sample with a positive nitrite test or with a negative nitrite test together with a positive leukocyte-esterase test should be considered having a UTI and treated accordingly. However, when both nitrite and leukocyte-esterase tests were negative, a UTI cannot be excluded and the samples should be further investigated by culture. Furthermore, the GP should take into account the age of the patient when prescribing antibiotic treatment as the etiology of a UTI is influenced by it. Prudent use of fluoroquinolones is strongly recommended as resistance is already emerging in the patients who use these agents more frequently. In addition, for an optimal empiric therapy the availability of actual data on antimicrobial resistance percentages of unselected uropathogens are required and these data should be made available to the GPs for implementation in their daily practice. Finally, as trimethoprim susceptibility in *E. coli* isolated in this study decreased to ~80% it might be advisable to limit its use as first agent in the treatment of an acute uncomplicated urinary tract infection in The Netherlands.

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Chapter 7

Acute uncomplicated urinary tract infections in community-dwelling elderly females: care as usual of the general practitioner and antimicrobial resistance.

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$$\begin{aligned} Y_1 &= \frac{1}{2} \left(\frac{1}{2} \right) = \frac{1}{4} \\ Y_2 &= \frac{1}{2} \left(\frac{1}{2} \right) = \frac{1}{4} \\ Y_3 &= \frac{1}{2} \left(\frac{1}{2} \right) = \frac{1}{4} \end{aligned}$$

Abstract

Urinary tract infections (UTIs), the most common bacterial infections in women, are more prevalent with increasing age. However, the care as usual of the general practitioner and the antimicrobial susceptibility of the causative uropathogens in acute uncomplicated UTIs in non-institutionalised elderly females have not been studied well. Therefore, a population-based study was conducted among women over the age 70 years with complaints of acute uncomplicated UTIs attending 21 GPs throughout The Netherlands. The performance of the nitrite and leukocyte-esterase tests, the treatment strategies used by the GP, the aetiology of infection and the antimicrobial susceptibility of the three most frequently isolated uropathogens were determined.

A positive nitrite test alone or a negative nitrite test in combination with a positive leukocyte-esterase test was very accurate in predicting a UTI (83%-99%) in these patients. The antimicrobial therapy most frequently prescribed in this population was nitrofurantoin, however its prescription rate decreased with increasing age. Fluoroquinolone and co-amoxiclav prescriptions increased (significantly) with increasing age. *Escherichia coli* was the uropathogen mostly isolated in all urine samples followed by *Proteus mirabilis* and *Klebsiella pneumoniae*. The etiology of infection was age depending: the former micro-organism decreased and the later increased in frequency with increasing age. For *E. coli*, *P. mirabilis*, and *K. pneumoniae* the lowest susceptibility percentages and the highest MIC₅₀ and MIC₉₀ values were found for amoxicillin, trimethoprim and co-trimoxazole. *K. pneumoniae* showed the lowest susceptibility for nitrofurantoin and the highest MIC₅₀ and MIC₉₀ values for both fluoroquinolones as well.

In conclusion, for the confirmation of a UTI in elderly females the GP can also rely on the results of both diagnostic tests. Furthermore, as trimethoprim susceptibility in these patients reached ~75%, it might be advisable to no longer use this agent as first choice in the treatment of acute uncomplicated UTIs.

Introduction

Urinary tract infections (UTIs) are among the most common infections in women and the prevalence increases substantially with increasing age,¹ i.e. from 5-10% for those until the age of 70 years to ~20% for those 80 years and older.^{2,3} Also, recurrent UTIs are more prevalent among postmenopausal women than among younger ones,⁴ and these infections are uncomfortable, costly and affect negatively the quality of life.⁵

The diagnosis of an acute uncomplicated UTI in older female patients is more complex than in younger women^{6,7} as older women often have an atypical clinical presentation and many common urinary symptoms (i.e. frequent urination and incontinence) are already present. Furthermore, obtaining a clean-catch (midstream) urine specimen is

more difficult⁶ and co-morbidities common to the elderly, such as neurologic, hepatic and cardiac conditions, can complicate the diagnosis and treatment of a UTI.⁷ Also, a positive culture, with or without the presence of pyuria, may be an asymptomatic bacteriuria, a condition similar to colonization which might not always benefit from antibiotic treatment.^{6,7} The nitrite and leukocyte-esterase dipstick test showed to be a reliable diagnostic tool to detect a UTI in young female general practice patients (< 70 years old).⁸⁻¹⁰ However, no data are available on the performance of both diagnostic tests in the elderly community dwelling female population.

The etiology of UTI among the elderly includes a broad range of Gram-negative and – positive micro-organisms. Consequently these infections require longer treatment duration with broad-spectrum antimicrobial agents for optimal treatment. Also, the uropathogens isolated in the elderly are more likely to be resistant to conventional antibiotic agents as trimethoprim probably by selection of more resistant strains by frequent antibiotic use in this population.^{7,11}

Most antibiotic surveillance studies on UTIs in the elderly female population however did focus on institutionalized patients or patients living in long-term care facilities.^{2,12} Acute uncomplicated UTIs in female patients of older age residing (independently) in the community have not been well studied. Therefore, this community-based study on female patients over 70 years of age attending 21 general practitioners from the Sentinel Station of the National Institute of Health Services Research in The Netherlands with complaints of a UTI was performed. In this surveillance study the antibiotic susceptibility of the most prevalent uropathogens was determined. Furthermore, the care as usual of the general practitioner, i.e. the nitrite and leukocyte-esterase test (if performed) and therapy prescribed, was evaluated.

Materials and Methods

Participating General Practitioners

Twenty-one General Practices from the Sentinel Station of The Netherlands Institute for Health Services Research (NIVEL) participating in the study were located all over The Netherlands. Their patient population accounts for approximately 1% of the Dutch population and is representative according to age, gender, regional distribution and degree of urbanization. Female patients over 70 years of age suffering from an acute uncomplicated UTI were included by the General Practitioners (GPs) from January 2003 until December 2004. The GPs were asked to include all patients with a UTI defined as the presence of one or more of the following complaints: acute signs of dysuria, stranguria, urinary frequency or urgency without the presence of fever >38°C. For each patient, age and the care as usual performed by the GP (i.e. nitrite and leukocyte-esterase test and the therapy prescribed) were recorded. All patients received

care as usual, i.e. diagnostic tests and empirical therapy according to the daily practice of the GP.

Urine Sampling and Processing

A fresh voided urine sample provided by the patients was used for nitrite dipstick and / or leukocyte-esterase by the GP (when part of the care as usual). Subsequently a dipslide (Uriline, 56508, BioMérieux, Plainview, N.Y) was prepared according to the manufacturer's instructions and send to the laboratory of Medical Microbiology of the University Hospital Maastricht, The Netherlands for identification and susceptibility testing of the uropathogens.

On the day of arrival, if necessary, the dipslide was incubated at 37°C for 16-20h. Thereafter the growth on the dipslides was compared with a chart provided by the manufacturer and recorded (from no growth to $\geq 10^7$ cfu/mL). Bacterial growth on the dipslides of $\geq 10^3$ cfu/mL was identified using standard biochemical tests.¹³ If there remained any doubt about the identification further biochemical tests were performed API 20E, API20 NE, API 20 Strep or API Staph (BioMérieux, Plainview, N.Y.). The bacteria isolated were stored at -70°C until further analysis.

Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of the three uropathogens most frequently isolated were determined according to the CLSI-criteria (formerly the NCCLS)¹⁴ using the microbroth dilution method with Mueller-Hinton II broth cation-adjusted (Becton, Dickinson and Company, Sparks, USA), an inoculum of 5×10^5 cfu/mL and an overnight incubation at 37°C. The MIC-plates with freeze-dried antibiotics (NLDMCS1, MCS Diagnostics BV, Swalmen, The Netherlands) were delivered after quality testing by the manufacturer and a shelf-life of approximately one year was guaranteed. The following antimicrobial agents (range in mg/L) were tested: amoxicillin (0.06-128), co-amoxiclav (0.06-128), trimethoprim (0.03-64), co-trimoxazole (0.03-64), norfloxacin (0.03-64), ciprofloxacin (0.008-16), and nitrofurantoin (0.5-512). The reference strains *E. coli* ATCC 35218 and ATCC 25922 were used as control strains. CLSI-guidelines (formerly the NCCLS)¹⁴ for resistance breakpoints were used.

Statistical Analysis

For the analysis of the culture results and the susceptibility percentages the patients were divided in three age groups: 71-80, 81-90 and >90 years.

The statistical program used was SPSS 11.0 for Windows. To detect significant differences in therapy prescribed, isolated uropathogens, and antibiotic susceptibility of the three uropathogens most frequently isolated the Mann-Whitney U test was performed. A p-value < 0.05 was considered significant.

Results

Care as usual of the General Practitioner

In total 917 female patients were included in the study with a mean age of 80.1 years (range 71-104 years). The performance characteristics of the diagnostic tests performed by the GP are shown in Table 1. The nitrite test alone showed a high positive predictive value (PPV) and a high specificity, respectively 99% and 97%. Furthermore, for urine samples with a negative nitrite test the leukocyte-esterase test showed a PPV of 83% and a sensitivity of 78%.

Table 1. Performance characteristics of the diagnostic tests performed by the GP in all patients.

A. The performance of the nitrite test:

Diagnostic test result ^a	Urine culture		PPV	NPV	Sens	Spec
	≥10 ³ cfu/ml	<10 ³ cfu/ml				
Nitrite positive	409	3	99	22	52	97
Nitrite negative	373	103				

B. The performance of the leukocyte-esterase test in case of a negative nitrite test:

Diagnostic test result ^a	Urine culture		PPV	NPV	Sens	Spec
	≥10 ³ cfu/ml	<10 ³ cfu/ml				
Leukocyte-esterase positive	285	59	83	34	78	42
Leukocyte-esterase negative	82	42				

PPV = positive predictive value, NPV = negative predictive value, Sens = sensitivity, Spec = specificity
^asamples with a negative nitrite and unknown leukocyte-esterase test result or both unknown test results were excluded.

Ninety percent of the patients with a nitrite positive urine sample were prescribed antibiotics by their GP (Table 2). Two third of the patients whose urine samples showed a negative nitrite test and a positive leukocyte-esterase test received an antibiotic prescription as well. Of the patients with a urine sample with both the nitrite and the leukocyte-esterase test negative 27% received antibiotic therapy.

Table 2. The number (rate) of prescriptions of the general practitioner compared with the diagnostic test results.

Diagnostic test result	Therapy prescribed		
	Yes	No	Unknown
Nitrite positive	372 (90)	30 (7)	10 (2)
Nitrite negative			
Leukocyte-esterase positive	221 (64)	113 (33)	10 (3)
Leukocyte-esterase negative	33 (27)	84 (68)	7 (6)
Unknown	9 (56)	2 (13)	5 (31)

Figure 1. The percentage antimicrobial agents prescribed as therapy in patients with acute uncomplicated UTI per age category.

Nit = nitrofurantoin; Tri = trimethoprim; Co-tri = co-trimoxazole; Co-am = co-amoxiclav; Am = amoxicillin; Qui = quinolones.

*significant difference between the youngest and both other age groups (Mann Whitney U test; $p < 0.05$)

The antimicrobial agent most frequently prescribed in patients of all age groups was nitrofurantoin (Figure 1). Its prescription rate ranged from 41% in the oldest to 51% in the youngest patients. One fourth of the patients received fluoroquinolones and trimethoprim was prescribed in 10%-21% of all patients. The prescription rate of co-amoxiclav increased significantly from 2% to 8% with increasing age.

Nitrofurantoin therapy was equally prescribed as a 3-, 5- and 7-day regimen (Figure 2). A seven-day course of fluoroquinolones was most frequently prescribed whereas trimethoprim was most frequently prescribed for 5 days in the patients of all age groups.

Figure 2. The duration of the antimicrobial therapy per age group for the three agents mostly prescribed.

Nit = nitrofurantoin; Tri = trimethoprim; Qui = fluoroquinolones.

Culture Results

In total 88% (808/917) of the urine samples yielded a positive culture of $\geq 10^3$ cfu/mL (Table 3). *Escherichia coli* was the most frequently isolated uropathogen in all age groups (57%-62%). *Proteus mirabilis* was more frequently isolated in the younger patients (10%). *Klebsiella pneumoniae* was significantly more found with increasing age, i.e. from 3% in the patients 71-80 years of age to 16% in the patients > 90 years old. No *Staphylococcus saprophyticus* was isolated in this population.

Susceptibility Data

Susceptibility percentages of the three uropathogens most frequently isolated per age group are shown in Table 4. The lowest susceptibility percentages for the three micro-organisms were found for amoxicillin (*K. pneumoniae* is intrinsic resistant to this

agent), trimethoprim and co-trimoxazole. *K. pneumoniae* susceptibility for nitrofurantoin (53%-78%) was the lowest (*P. mirabilis* excluded) and the MIC₅₀ and MIC₉₀ of this micro-organism for this agent were higher compared to those of the *E. coli* isolates. Furthermore, *K. pneumoniae* showed the highest MIC₉₀ for both fluoroquinolones (respectively 16 and 0.5 mg/L). On the other hand, for trimethoprim and co-trimoxazole this uropathogen showed the lowest MIC₉₀, respectively 16 and 4 mg/L

Table 3. Percentage isolated uropathogens for each age group.

Micro-organism	Age category (years)			Total
	71-80 n=460	81-90 n=292	>90 n=56	
<i>E. coli</i>	62	60	57	61
<i>P. mirabilis</i>	10	6	4	8
<i>K. pneumoniae</i> *	3	8	16	6
<i>Pseudomonas spp.</i>	5	4	7	5
<i>Acinetobacter spp.</i>	4	2	2	3
Other Gram-negatives	12	13	11	12
<i>E. faecalis</i>	3	3	2	3
Other Gram-positives	3	3	2	3

*significant difference between the youngest and both other age groups (Mann-Whitney U test; $p < 0.001$)

Discussion

This study is to the best of our knowledge the first community-based study on acute uncomplicated UTIs in more than 900 non-institutionalized elderly females over the age of 70 years in The Netherlands.

The diagnosis of a UTI in elderly female patients is not always very straightforward. In younger female patients it is known that the nitrite and leukocyte-esterase have a high positive predictive value for infection.⁸⁻¹⁰ The performance characteristics of the nitrite and leukocyte-esterase dipstick tests showed that in this elderly female population a positive nitrite test result predicted an infection with a very high specificity. Furthermore, when the nitrite test was negative, a positive leukocyte-esterase test still predicted a UTI in more than 80% of the urine samples positive in culture. The sensitivity of this test was also high (78%). Therefore, both tests have proven to also accurately predict a UTI in the elderly non-institutionalized female population.

Table 4. Susceptibility percentages, the MIC₅₀ and the MIC₉₀ of the three uropathogens most frequently isolated.

Uropathogen	Antibiotic	Age category (years)			MIC ₅₀	MIC ₉₀
		71-80	81-90	>90		
<i>E. coli</i>		n=254	n=137	n=25	n=416	n=416
	Amoxicillin	69	74	80	4	>128
	Co-amoxiclav	95	96	96	4	16
	Trimethoprim	76	80	84	0.5	>64
	Co-trimoxazole	79	83	84	0.06	>64
	Norfloxacin	93	98	96	0.06	0.25
	Ciprofloxacin	93	98	96	0.015	0.06
	Nitrofurantoin	98	97	100	16	32
<i>P. mirabilis</i>		n=30	n=7	n=1	n=38	n=38
	Amoxicillin	70	86	100	1	128
	Co-amoxiclav	93	100	100	1	8
	Trimethoprim	73	86	100	4	>64
	Co-trimoxazole	77	86	100	0.12	>64
	Norfloxacin	100	100	100	0.06	0.12
	Ciprofloxacin	100	100	100	0.03	0.06
	Nitrofurantoin	17	14	0	128	256
<i>K. pneumoniae</i>		n=8	n=15	n=9	n=32	n=32
	Amoxicillin	13	20	22	64	>128
	Co-amoxiclav	100	100	100	2	8
	Trimethoprim	75	80	100	1	16
	Co-trimoxazole	88	80	100	0.12	4
	Norfloxacin	100	80	100	0.12	16
	Ciprofloxacin	100	80	100	0.03	0.5
	Nitrofurantoin	75	53	78	64	512

However, the combination of both diagnostic tests resulted in 17% of the urine samples in a false negative test result which might be explained by contaminating micro-organisms as obtaining a clean-catch urine specimen in the elderly is difficult.⁷ Furthermore, for the production of nitrite from nitrate at a reliable level bacteria need ≥ 4 hours, so the nitrite dipstick test requires preferably first morning urine.¹⁰ If the time span was too short for this conversion false negative test results will be obtained. Furthermore, the biochemical reaction detected by the nitrite test is associated with members of the family of *Enterobacteriaceae*, but not with other uropathogens like *Pseudomonas spp.* or enterococci.¹⁰ As the etiology of UTIs in this elderly population shifted toward more *Pseudomonas spp.* and other Gram-negatives as compared with a younger patient population (Unpublished data) false negative test results could be obtained.

For the prescription of antimicrobial therapy the general practitioner did rely on the outcome of the diagnostic tests as 90% of the patients with a nitrite positive urine sample received antimicrobial therapy. For patients with a negative nitrite and positive leukocyte-esterase urine sample the prescription rate of the GP dropped to 68% and went even further down to 56% in patients for whom both tests were not performed. As shown from the results of this study the GP can rely even more on the diagnostics tests available for the detection of a UTI in the elderly community dwelling female population and subsequently the prescription of antimicrobial therapy.

Even though nitrofurantoin was mostly prescribed in the three age groups, there was an inverse relation between the prescription rate of this agent and the increasing age of the patients. In contrast, the number of patients prescribed co-amoxiclav and fluoroquinolones increased (significantly) with increasing age. Similar results were described by Goettsch et al.¹⁵ The oldest patients in that study received more often a prescription of fluoroquinolones compared to the younger ones. There might be several reasons for the GP to choose in this older patient population another first line agent than those recommended by the NHG,¹⁶ i.e. nitrofurantoin and trimethoprim. First, nitrofurantoin treatment regimens might have several adverse effects, especially in older women, including nausea and vomiting, allergic, pulmonary, hepatic, neurological and hematological reactions.¹⁷ In addition, elderly females are more likely to have experienced several UTIs in their life-time and therefore they might have received more often antimicrobial therapy in the past.²⁻⁴ Therefore it is to be expected that the GP considered the standard agents as no longer effective and preferred to use co-amoxiclav or fluoroquinolones. These agents are also prescribed for longer durations to be sure of full eradication of the causative uropathogen.

The etiology of UTIs among the non-institutionalized elderly women differed from that of younger female adults (Unpublished data), i.e. *E. coli*, still the most common isolated uropathogen, was proportionally less detected but more *P. mirabilis*, *K. pneumoniae*, and other Gram-negatives were isolated. Due to the depletion of hormones in these

elderly patients the vaginal flora is no longer dominated by the presence of lactobacilli⁵ and it might be easier for uropathogens to enter the urinary tract.

The lowest susceptibility percentages for the three most frequently isolated uropathogens were found for amoxicillin and trimethoprim, agents very often used in general practice. Resistance for fluoroquinolones was emerging in these three uropathogens as well. However, *E. coli* isolated in this patient population showed higher susceptibility percentages for amoxicillin, co-trimoxazole, norfloxacin and nitrofurantoin as compared to uropathogenic *E. coli* isolated from residents of 2 nursing homes in the South of The Netherlands.¹² The differences in antibiotic susceptibility between the isolates from GP patients and nursing home residents is very likely due to the presence of additional risk factors for the development of antibiotic resistance in the latter population including antibiotic use, crowding, underlying pathology and the presence of invasive devices.^{12,18}

In conclusion, as the elderly community dwelling females mostly present to their GP with vague clinical symptoms of a UTI, it is important for the GP to perform the nitrite and leukocyte-esterase dipstick tests to confirm their perception of a UTI. Furthermore for the treatment of UTIs in these elderly females actual data on the susceptibility of pathogens isolated from this group of patients is essential for making an empiric choice. Therefore, as trimethoprim and co-trimoxazole susceptibility percentages for the three most frequently isolated uropathogens in this population ranged from 73%-86% it might be advisable to no longer use this antibiotic as an agent of first choice for the treatment of acute uncomplicated urinary tract infections in The Netherlands. Furthermore, national surveillance studies generating data on the resistance percentages of the agents currently used for the treatment of UTIs in patients of different age groups should be continued in the future and should be made available to the GPs for implementation into their daily practice.

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Chapter 8

General Discussion & Summary

The role of the commensal flora of healthy volunteers and patients attending their general practitioner in the epidemiology of antimicrobial resistance, in particular the development and spread of resistance was studied. The commensal flora at different body-sites constitutes a large reservoir of antimicrobial resistance (genes) and some aspects which might be of influence on the increase of antimicrobial resistance in the commensal bacteria were analyzed. Furthermore, the consequences of this reservoir of antibiotic resistance (genes) for potentially pathogenic bacteria was enlightened as well.

In a case-controlled study the prevalence of erythromycin resistant streptococci, the predominant species of the oropharyngeal commensal flora, was studied (**Chapter 2**). Almost 60% of the patients attending their general practitioner (GP) for an acute respiratory tract infection (cases) and controls carried erythromycin resistant streptococci in their commensal throat flora. One fifth of all patients studied carried commensal streptococci that were highly resistant not only for erythromycin and other macrolides but also for lincosamides and streptogramin antibiotics due to cross-resistance conferred by the *ermB* gene. The 60% erythromycin resistance percentage in the GP patients was lower compared to the 71% macrolide-resistant viridans group streptococci found in Belgian healthy volunteers in 2003¹ and the rate reported in Spain in 2001.² The lower resistance percentage in the Dutch population might be due to the lower antibiotic use in general³ and especially of macrolides⁴ in The Netherlands compared to both other countries. In addition to demonstrating this reservoir of erythromycin resistance (genes) in the commensal throat flora, transfer of erythromycin resistance by conjugation to a *Streptococcus pneumoniae* with a frequency of 1×10^{-9} was demonstrated. The conjugation experiment in this study was performed with bacterial donor and recipient strains mixed in a small volume to mimic the *in vivo* situation of close contact. Although transfer of macrolide resistance by transformation has been previously described⁵ it is to be expected that conjugation is a more common phenomenon *in vivo* than transformation. This transfer of resistance genes emphasizes the risk of antibiotic resistance in the commensal flora as reservoir for resistance for potentially pathogenic bacteria.

Besides the respiratory tract, the gastro-intestinal tract, with the second largest body surface area,⁶ comprises another enormous reservoir of commensal bacteria (10^{12} - 10^{14} cfu/g)⁷ and consequently resistance (genes). To assess the magnitude of this reservoir of resistance in populations with different selective pressure for resistance, i.e. populations with a variable antibiotic use the prevalence of antimicrobial resistance in faecal *Escherichia coli* isolates from healthy volunteers in nine developing countries was determined (**Chapter 3**). A high prevalence of resistance to most agents tested was found in all populations, consistent with findings from other studies.⁸⁻¹⁰ The highest prevalences of resistance were found for the orally administered, relatively inexpensive, broad-spectrum agents including ampicillin, trimethoprim and oxytetracycline.

Resistance to these antibiotics was the most common multi-drug resistance pattern in the *E. coli* isolates and has been found previously both in adults^{8,11} and children.¹² The combination of resistance to these three antibiotics is very likely due to the localization of the resistance genes on the same plasmid.¹³ In addition, resistance to fluoroquinolones was already emerging in some populations of Asia and Latin-America where ciprofloxacin is used for treatment of cholera and typhoid fever in adults as alternative in case of resistance.¹⁴⁻¹⁶ Furthermore, there were significant differences in the prevalence of resistance between non-urban and urban areas for ampicillin, oxytetracycline and trimethoprim. This might be due to crowding together with poor hygiene and poor sanitary facilities for sewage disposal,^{10,17,18} the easy availability¹⁹ and over the counter sales¹⁷ of antibiotics in the cities whereas in rural areas only a few drugs are available and usually brought there by a visiting²⁰ or a resident physician.¹⁸

Another population experiencing a selective pressure for resistance, i.e. high antibiotic use and crowding are patients admitted to the hospital. In patients admitted to the surgical wards of the University Hospital Groningen, Maastricht and Rotterdam an increase in the prevalence of resistance in the faecal *Enterococcus faecalis* isolates was found during hospitalization compared to the situation on admission (**Chapter 4**). However, when the patients returned to the community and the selective pressure of the hospital environment disappeared the prevalence of resistance in their commensal flora decreased over time. Depending on the antibiotic tested, the time needed to return to the pre-hospitalization level ranged from 1 month (ciprofloxacin and tetracycline) to six months (erythromycin and gentamicin). This difference might be explained by the difference in antimicrobials used in primary health care²¹ as well as differences in the properties of the antibiotics.

As commensal bacteria from the gastro-intestinal tract are able to cause urinary tract infections (UTIs) via the ascending route²²⁻²⁴ the reservoir of resistance (genes) in the faecal flora poses a risk for antibiotic resistant uropathogens. Antibiotic therapy administered for UTIs might select for resistant commensal bacteria in the gut therefore the optimal duration of therapy for one of the drugs of first choice according to the guidelines of the NHG²⁵ was studied. The results of a three- versus five-day treatment regimen of trimethoprim on the bacteriological and clinical cure rate have been described in **Chapter 5**. No significant differences in bacteriological cure rate were found between the two treatment regimens both one and three days after treatment. These findings support a shorter treatment with the advantages of lower cost, less side-effects and better patient compliance. However, relief of symptoms is an equally important issue for both the patient and the GP. In the 3-day regimen group 29 patients (44%) considered themselves “not-recovered” one day after treatment and were thus not symptom-free when they delivered their control urine sample. Although the recovery rate between the two regimens was not significant different, we assume that the

difference in self-reported cure is very likely the reason why experienced GPs consider a three-day regimen as less reliable. One day after the end of both treatment regimens a trimethoprim resistant *E. coli* was more often isolated from patients who considered themselves as “not-recovered” compared to the “recovered” patients. The relatively high percentages of trimethoprim resistant *E. coli* isolates in the latter patients underscores the natural course of an acute UTI.²⁶ Due to the relatively low numbers of patients included in the final analysis no firm conclusions could be drawn.

For the treatment of patients with symptoms of a UTI the choice of the empiric therapy is mainly based on the recommendations of the NHG.²⁵ For these recommendations the data on the susceptibility of unselected uropathogens have to be taken into account. Until now most studies performed determined the susceptibility of selected isolates as samples were send in after prior therapy failure.^{27,28} Therefore a national study was conducted on female patients visiting their general practitioner with acute complaints of UTIs. The studies described in **Chapter 6** and **Chapter 7** described the results of the antimicrobial susceptibility of unselected uropathogens in different age groups. In addition the care as usual of the general practitioner, i.e. the accuracy of the nitrite and leukocyte-esterase test and the antimicrobial prescription rate in relation with bacteriological culture results, were determined.

For symptomatic women between 11 and 70 years (**Chapter 6**) a urine sample with a positive nitrite test (PPV = 96%) or a negative nitrite test together with a positive leukocyte-esterase test (PPV = 79%) should be considered as having a UTI and the patient should be treated accordingly. However when both nitrite and leukocyte-esterase tests are negative (NPV = 53%), a UTI cannot be excluded and urine samples should be further investigated by culture. Furthermore, the GP should take into account the age of the patient when prescribing antibiotic treatment as the causative uropathogen is age related. Prudent use of fluoroquinolones is recommended as resistance is already emerging in these patients. As trimethoprim susceptibility in *E. coli* isolates of this study decreased to ~80% it might be advisable to limit its use as agent of first choice in the treatment of an acute uncomplicated UTI in The Netherlands.

As elderly community dwelling females mostly present to their general practitioner with vague clinical symptoms of a UTI it is important that the GP performs diagnostic tests like the nitrite and leukocyte-esterase test (**Chapter 7**). Both test have proven to accurately predict a UTI in this elderly population. However, when both tests were negative, a UTI could not be excluded and bacteriological culture of the urine specimen is required. To prevent false negative test results the use of urine samples in which the micro-organism(s) present have sufficient time to convert nitrate in to nitrite (such as morning urine samples) is strongly recommended. The antimicrobial therapy prescribed by the GP was age related as less nitrofurantoin and more fluoroquinolones and co-amoxiclav were prescribed in these elderly women. These changes in the prescribing behavior of the GP might be due to the fact that elderly females have more often suffered from a UTI in the past and probably more often received antibiotic therapy.

Both agents were also prescribed for longer durations to be sure of full eradication of the probably more resistant uropathogen. The lowest susceptibility percentages for the three uropathogens most frequently isolated in these women (>70 years) were found for trimethoprim and amoxicillin. Resistance for fluoroquinolones was emerging in this age group. As trimethoprim susceptibility decreased to ~70% it is questionable whether this it should be further used as agent of first choice in the treatment of acute uncomplicated UTIs in The Netherlands. Nitrofurantoin susceptibility remained high in patients of all ages and can be further used as agent of first choice in the treatment of acute uncomplicated UTIs in The Netherlands.

In conclusion, the commensal flora of different sites of the human body constitutes a large population of micro-organisms regularly exposed to antimicrobial agents prescribed for the treatment of bacterial infections. Due to this repetitive exposure the commensal bacteria might become resistant to commonly used antibiotics and form a large reservoir of resistance genes. The resistance present in potentially pathogenic micro-organisms is only the tip of the 'resistance' iceberg as compared to the resistance in the normal flora. Therefore the availability of actual susceptibility percentages of the most prevalent commensal bacteria as well as potentially pathogenic bacteria causing common infections is important to be able to tune in with empiric treatment on the rapidly changing and emerging resistances.

Optimal antibiotic treatment for bacterial infections will directly lead to a reduction in the antibiotic resistance. However, other approaches which will also contribute to control the problem of antibiotic resistance are optimization of the diagnosis of bacterial infections and the use of alternative agents instead of antibiotics for the prevention and / or treatment of bacterial infections.

Optimization of the diagnosis of a bacterial infection is especially a problem in case of respiratory tract infections (RTIs) as these infections have in most cases a viral etiology for which the prescription of antimicrobial therapy is not beneficial. However, more than half of the patients with a common cold presenting to their general practitioner receive antimicrobial therapy.²⁹ Clinical differentiation between viral and bacterial RTIs will contribute substantially to reduce the unnecessary use of antibiotics. The detection of C-reactive protein (CRP) in a blood sample may be predictive for bacterial infections.³⁰ However, the cost-effectiveness of implementation of this test in general practice is necessary. It should be determined whether application of this test results in a reduction of the antibiotic prescriptions and thus in antibiotic resistance.

Another field of interest for optimization of the diagnostic tests currently used are the urinary tract infections (UTIs). In case of an acute uncomplicated urinary tract infection most general practitioners decide whether to prescribe antibiotic therapy after evaluation of the patients complaints. Also, they can rely on diagnostic tests like the nitrite and leukocyte-esterase dipstick tests, for this decision. The efficacy of most tests

available has been separately evaluated. However, there is great need to study how the test results of the different diagnostic tests relate to each other, what is their additional value to the medical history of the patient and how accurate are they in predicting an infection as compared to urine culture.

Next to the accurate diagnosis of bacterial infections, the prevention of infectious diseases is a major issue of interest in order to control antibiotic use and antibiotic resistance. As urinary tract infections are the most common bacterial infection seen in women in general practice and women often suffer from recurrent UTIs (≥ 3 episodes within one year) this is a large population that would benefit from effective (non-antibiotic) prophylaxis. In post-menopausal women, recurrent UTIs can also occur due to the loss of estrogen and consequently the depletion of vaginal colonization with lactobacilli. Currently, the prophylaxis available for women suffering from recurrent UTIs is a low dose of antibiotics, also used to treat actual infections, for 6 months or longer. However, this treatment poses a risk for the development of antibiotic resistance. Therefore, the use of alternative agents like probiotics (lactobacilli) or cranberry extract for the prevention of UTIs are good alternatives. The former prophylaxis is based on the concept that "good" bacteria will prevent vaginal colonization with potentially pathogenic micro-organisms and consequently infection by these micro-organisms. The latter one prevents adhesion of bacteria to epithelial cells of the urogenital tract. The efficacy of both non-antibiotic prophylaxis treatments in the prevention of recurrent urinary tract infections as well as in the reduction of the development of antimicrobial resistance should therefore be studied.

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Nederlandse Samenvatting

De rol van de commensale flora van gezonde vrijwilligers en huisartspatiënten in de epidemiologie van antibioticumresistentie, en in het bijzonder de ontwikkeling en verspreiding van de resistentie, werd onderzocht in dit proefschrift. De commensale flora aanwezig in / op verschillende delen van het lichaam vormt een groot reservoir van antibioticumresistentie genen en antibioticumresistente bacteriën. Een aantal aspecten van belang voor de stijging van de antibioticumresistentie in de commensale flora werden geanalyseerd. Bovendien werden enkele gevolgen van dit "commensaal reservoir" van antibioticumresistentie (genen) voor potentieel pathogene micro-organismen toegelicht.

De prevalentie van erythromycine resistente streptokokken, de meest voorkomende species van de oropharyngeale commensale flora, werd bestudeerd in een case-control studie uitgevoerd onder huisartspatiënten in Nederland. De huisartsen (van het NIVEL peilstation project) includeerden patiënten met klachten van een acute luchtweginfectie en controle patiënten van dezelfde leeftijdscategorie. Bijna 60% van de geïnccludeerde patiënten (cases en controles) waren drager van erythromycine resistente streptokokken. Bij een vijfde van alle patiënten waren de streptokokken niet alleen resistent voor erythromycine en andere macroliden maar ook voor lincosamiden en streptogramin antibiotica. Deze kruisresistentie werd veroorzaakt door de aanwezigheid van het *ermB* gen in de streptokokken. Het resistentie percentage van erythromycine bij de huisartsenpatiënten was lager in vergelijking met dat van gezonde vrijwilligers in België (71%) en het resistentie percentage in Spanje in 2001. Verder werd overdracht van het erythromycine resistentie gen *ermB* van een commensale streptokok naar een *Streptococcus pneumoniae* isolaat door middel van conjugatie met een frequentie van 1×10^{-9} aangetoond. Deze transfer van resistentiegenen benadrukt de rol van de commensale flora als reservoir van antibioticumresistentie genen voor potentieel pathogene micro-organismen.

Het gastro-intestinale stelsel is naast het luchtwegstelsel een tweede groot reservoir van commensale bacteriën (10^{12} - 10^{14} kolonie vormende eenheden per gram darminhoud) en dus ook van antibioticumresistentie genen en antibioticumresistente bacteriën. Om een indruk te krijgen van de omvang van het antibioticumresistentie reservoir in de darmflora in populaties met een variabele selectiedruk voor resistentie werd de prevalentie van antibioticaresistente fecale *Escherichia coli* van gezonde vrijwilligers uit negen ontwikkelingslanden bepaald. In de onderzochte populaties werd voor de meerderheid van de geteste antibiotica een hoge resistentie prevalentie gevonden. Voor de orale, relatief goedkope, breed spectrum middelen zoals ampicilline, trimethoprim en tetracycline werden de hoogste resistentie percentages gevonden. Verder werden significante verschillen gevonden in de prevalentie van resistentie voor deze drie middelen tussen stedelijke en landelijke populaties. Opmerkelijk was de resistentie tegen fluoroquinolonen in verschillende populaties in Azië en Latijns-Amerika

(Filippijnen, Mexico en Peru). Mogelijk dat het gebruik van ciprofloxacin als alternatief voor de behandeling van cholera en tyfus aan deze resistentie heeft bijgedragen.

Ziekenhuizen vormen door het antibioticagebruik en door de "concentratie" van relatief veel patiënten met verminderde weerstand in een beperkte ruimte een belangrijke bron voor antibioticaresistentie. In patiënten opgenomen op de afdeling chirurgie van het Academisch Ziekenhuis Maastricht, het Universitair Medisch Centrum Groningen en het Erasmus Medisch Centrum in Rotterdam werd een stijging gevonden in de prevalentie van antibioticaresistentie in fecale *Enterococcus faecalis* isolaten tijdens het ziekenhuisverblijf in vergelijking met de situatie voor opname. Na ontslag uit het ziekenhuis en dus na het verdwijnen van de selectie druk van het ziekenhuis verminderde de prevalentie van de resistentie in de commensale darmflora in de tijd. Afhankelijk van het geteste antibioticum verschilde de tijd nodig voor de resistentie om terug te keren naar het niveau voor opname tussen 1 maand (ciprofloxacin en tetracycline) en 6 maanden (erythromycine en gentamicin).

Aangezien de commensale bacteriën van het gastro-intestinaal stelsel in staat zijn om via de opstijgende route urineweginfecties te veroorzaken is de fecale flora het belangrijkste reservoir voor antibioticaresistente uropathogenen. In de laatste drie hoofdstukken werd voor acute ongecompliceerde urineweginfecties de optimale therapieduur en de prevalentie van antibioticaresistente uropathogenen in relatie tot de leeftijd onderzocht. In de studie naar de optimale therapieduur werd zowel op dag 1 als op dag 3 na het einde van een 3- en een 5-daagse kuur met trimethoprim geen significant verschil gevonden in het bacteriologisch genezingspercentage. Deze bevindingen ondersteunen een korte behandelingsduur met de voordelen van lagere kosten, minder nevenverschijnselen en betere compliance van de patiënt. Echter, voor de patiënt en de huisarts is een negatieve bacteriële kweek (afwezigheid van bacteriën in de urine) even belangrijk als het verdwijnen van de symptomen / klachten van de patiënt. Bij de patiënten in de 3-daagse kuur beschouwde 44% zichzelf als "niet-genezen" 1 dag na het beëindigen van de therapie. Alhoewel het percentage herstelde patiënten niet significant verschillend was tussen beide therapiegroepen (50% vs. 58% 1 dag na het stoppen van de kuur) is het aannemelijk dat het verschil in zelf gerapporteerde genezing de oorzaak is waarom huisartsen een drie daagse kuur als minder betrouwbaar ervaren. Opvallend is dat het percentage trimethoprim resistente *E. coli* in de controle urines van patiënten die zich wel en niet-genezen beschouwen gelijk was. Verder werd een aanzienlijk percentage trimethoprim resistente *E. coli* isolaten gevonden in de urine van zowel de patiënten die zichzelf als niet-genezen beschouwden als ook bij diegene die zichzelf opgeknapt vonden. Deze resultaten ondersteunen het natuurlijk verloop van een urineweginfectie. Helaas konden geen harde conclusies getrokken worden door het verlies van patiënten in de follow-up periode waardoor het aantal patiënten dat uiteindelijk geanalyseerd kon worden relatief klein werd.

Voor de empirische behandeling van patiënten met een UWI in de huisartsenpraktijk zijn richtlijnen gebaseerd op de gevoeligheidspercentages van ongeselecteerde uropathogenen nodig. Aangezien deze gegevens in Nederland slechts beperkt beschikbaar zijn werd een nationale studie uitgevoerd bij vrouwelijke patiënten die hun huisarts bezochten met klachten van een ongecompliceerde UWI. De antibioticagevoeligheid van de ongeselecteerde uropathogenen (*E. coli*) geïsoleerd bij patiënten van verschillende leeftijdscategorieën werd bepaald en vergeleken met de door de huisarts voorgeschreven therapie. Tevens werd de door de huisarts uitgevoerde diagnostiek (nitriet en leukocyten-esterase test) vergeleken met de bacteriële kweekuitslag, het voorschrijven van antibiotica door de huisartsen en de bacteriële kweekuitslag.

Bij symptomatische vrouwen tussen de 11 en 70 jaar oud wordt een positieve nitriettest (PPV = 96%) of een negatieve nitriettest in combinatie met een positieve leukocyten-esterase test (PPV = 79%) als een urineweginfectie beschouwd en zou als dusdanig behandeld moeten worden. Echter, indien beide testen een negatief resultaat opleveren (NPV = 53%) is verder bacteriologisch onderzoek van de urine vereist. Voor het voorschrijven van de empirische therapie zal de huisarts rekening moeten houden met de leeftijd van de patiënt aangezien deze gerelateerd is met de etiologie van de infectie. Vanwege de toename van resistentie tegen norfloxacin en ciprofloxacine in deze patiënten categorie lijkt het beter het gebruik van fluoroquinolonen te beperken. Aangezien het gevoeligheidspercentage van *E. coli* voor trimethoprim gedaald is tot 80% is te overwegen dit middel niet langer als middel van eerste keuze te gebruiken in deze patiëntenpopulatie in Nederland.

Aangezien oudere patiënten (>70 jaar) in de huisartsenpraktijk meestal vage klachten / symptomen van UWIs vertonen, is het verrichten van de diagnostiek met behulp van de nitriet en / of leukocyten-esterase test ook in deze populatie belangrijk. Gebruik van ochtendurine (bacteriën hebben immers minimaal 4u nodig om nitraat om te zetten in een detecteerbare hoeveelheid nitriet) ter voorkoming van een vals negatieve testuitslag is daarom essentieel. Alhoewel nitrofurantoïne nog steeds de therapie van eerste keuze was in deze groep, daalde het percentage voorschriften met een stijgende leeftijd en was er een stijging in het voorschrijfpercentage co-amoxiclav en fluoroquinolonen met een stijgende leeftijd. Deze verandering in voorschrijfgedrag bij de oudere vrouwen kan veroorzaakt zijn doordat deze vaker in het verleden een UWI gehad hebben en dus waarschijnlijk ook vaker antibiotische therapie kregen. Daarom wordt in deze populatie ook vaker een antibioticakuur van ≥ 5 dagen voorgeschreven. Deze veranderingen in voorschrijfgedrag zijn verwant met de verschuivingen in de etiologie van een UWI in de oudere populatie; er werd proportioneel minder vaak een *E. coli*, maar vaker een *P. mirabilis* en een *K. pneumoniae* geïsoleerd. De laagste gevoeligheidspercentages werden voor de drie uropathogenen het meest geïsoleerd bij deze oudere patiënten

gevonden voor amoxicilline en trimethoprim. Resistentie voor fluoroquinolonen werd in deze drie micro-organismen ook reeds gevonden. Aangezien trimethoprim resistentie ook in de meest frequent voorkomende uropathogenen van de 70 plussers 30% benaderde is het de vraag of dit antibioticum een middel van eerste keuze moet blijven voor de behandeling van acute ongecompliceerde UWI in Nederland.

Samengevat vormt de commensale flora van de luchtwegen en het maagdarm kanaal een grote populatie aan micro-organismen die vaak wordt blootgesteld aan antimicrobiële agentia tijdens de behandeling van bacteriële infecties. Door deze herhaaldelijke blootstelling kunnen de commensale bacteriën resistent worden tegen vaak gebruikte antibiotica en een groot reservoir aan resistentiegenen en resistente bacteriën vormen. De resistentie aanwezig in potentieel pathogene micro-organismen is dus slechts de tip van de "resistentie" ijsberg in vergelijking met de resistentie aanwezig in de normale flora. Daarom is de beschikbaarheid van actuele gevoeligheidspercentages van de meest voorkomende commensale bacteriën als ook van potentieel pathogene micro-organismen van groot belang om de empirische therapie snel te kunnen aanpassen aan de veranderende en beginnende antibioticumresistenties (een "early-warning" systeem).

Dankwoord, Curriculum vitae & List of Publications

Dankwoord

Ik heb lange tijd nagedacht over wat ik hier zou gaan schrijven. Het is misschien niet meteen geworden wat jullie verwacht hadden, maar het is dan ook mijn dankwoord!

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Als we het rijtje verder aflopen komen we bij het lab: Bac-Research. Alhoewel het lab gedurende de 3,5 jaar dat ik hier ben veranderd is van een 90% vrouwengroep naar een fifty-fifty verdeling qua geslacht is er 1 regel die nog steeds geldt: "Women are the best"! Allemaal bedankt voor de leuke werksfeer, het bijspringen in de werkzaamheden indien nodig en natuurlijk de onvergetelijke labuitjes... Lucy, heel erg bedankt om mij in te wijden in het praktisch reilen en zeilen van de Microbiologie, al het werk dat je in de verschillende studies hebt gestopt en in het bijzonder voor de leuke tijden die we samen hebben doorgebracht. Verder natuurlijk Ruud en Christel, mijn paranimfen, jullie weten wat jullie voor mij betekenen! Heel erg bedankt ook voor alle hulp ivm de promotie.

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Curriculum vitae

Sita Nys werd geboren op 21 juni 1977 te Genk, België. Ze behaalde het diploma van het secundair onderwijs (Wetenschappen-Wiskunde) in 1995 aan het Koninklijk Atheneum I te Hasselt. Na een verblijf van 11 maanden in Chagrin Falls Ohio (US) als uitwisselingsstudent behaalde ze het diploma van 'High School'. In 1996 begon ze met de opleiding Biologie aan het Limburgs Universitair Centrum te Diepenbeek. Na het behalen van het diploma Kandidaat in de Biologie zette ze haar studie in 1999 verder aan de Universiteit Antwerpen waar ze in 2001 de graad Licentiaat in de Algemene Biochemie behaalde. In januari 2002 trad ze in dienst bij de afdeling Medische Microbiologie van het Academisch Ziekenhuis Maastricht als onderzoeker in opleiding. Daar was zij werkzaam in het gebied van de antibioticumresistentie in de commensale flora van de mens. De resultaten van die onderzoeksperiode staan beschreven in dit proefschrift. Sinds april 2005 is haar onderzoek gericht op de preventie van wederkerende urineweginfecties met behulp van niet-antibiotische profylaxe.

List of Publications

Papers

Bruinsma N, Filius PM, van den Bogaard AE, Nys S, Degener J, Endtz HP, Stobberingh EE. (2003) Hospitalization, a risk factor for antibiotic-resistant *Escherichia coli* in the community? *Journal of Antimicrobial Chemotherapy* **51**(4):1029-3

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Abstracts

S. Nys, N. Bruinsma, M. Filius, J. Degener, A.E. van den Bogaard and E.E. Stobberingh (2003) Effect of hospitalization on the antibiotic resistance of faecal Enterococci after discharge. NvMM voorjaarsvergadering, Arnhem, Netherlands.

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Nys S., Tjhie J.H.T., Spee K., Bartelds A.I.M., Heijnen M.L.A., Peeters M.F., Stobberingh E.E. (2004) The potential risk of erythromycin as empiric therapy for therapy failure in acute respiratory tract infections. World Conference on magic bullets, Neurenberg, Germany.

Nys S., Tjhie J.H.T., Spee K., Bartelds A.I.M., Heijnen M.L.A., Peeters M.F., Stobberingh E.E. (2004) The potential risk of erythromycin as empiric therapy for therapy failure in acute respiratory tract infections. Joint Scientific Meeting on Infectious Diseases and Microbiology, Amsterdam, Netherlands.