Urinary tract infections and antimicrobial agents : a study into factors influencing the efficacy of antimicrobial agents

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URINARY TRACT INFECTIONS AND ANTIMICROBIAL AGENTS

A study into factors influencing the efficacy of antimicrobial agents.

URINARY TRACT INFECTIONS AND ANTIMICROBIAL AGENTS

A study into factors influencing the efficacy of antimicrobial agents

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
aan de Rijksuniversiteit Limburg te Maastricht,
op gezag van de Rector Magnificus, prof.dr. H. Philipsen,
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen op
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door

THEODORA ANTOINETTA MARIA TRIENEKENS

geboren te Tegelen

"ALL IN VERHAGEN BV" - KATWIJK

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In herinnering aan mijn ouders.

Aan Quir.

De uitgave van dit proefschrift werd mede mogelijk gemaakt door een bijdrage van Merck, Sharp and Dohme, Haarlem.

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CHAPTER I

INTRODUCTION **AIMS OF THE STUDY.**

INTRODUCTION

One of the most common diseases encountered in general practice are urinary tract infections (UTI)^{1,2}. This disease accounts for a high morbidity rate in otherwise healthy people. A standardized general practice will have two visits a week from patients with complaints of UTI. Antimicrobial agents will be prescribed to patients with complaints of UTI many times.

The first known description of UTI was presented by ar-Rhazi or Rha-zes in the ninth century (AD). He was a physician in Bagdad and described in one of his more than 150 publications a patient undoubtedly suffering from pyelonephritis. He also mentioned an effective therapy for this patient. The patient was given diuretics to clean his bladder until the urine became free of pus (leucocytes) and the fever disappeared. In older literature more descriptions of UTI and its therapy were presented, but they did not reach the therapeutic level of the Arabian approach³.

In 1863 Pasteur observed that urine was a good culture medium for bacteria, but it was not until 1881 that Roberts described the relation between the presence of bacteria in urine and complaints of cystitis. In 1894 Escherich isolated from the urine of children with cystitis bacteria which were later named after him: *Escherichia coli*. This micro-organism is still the most frequent causative agent of UTI in general practice.

A renewed interest into UTI occurred in the thirties of this century⁴. The relations between chronic pyelonephritis and hypertension, premature delivery and end-stage renal failure was investigated. As a result of bacteriuria, arteriosclerotic arteries developed in the kidneys without the presence of overall arteriosclerosis. This renal arteriosclerosis was believed to be the cause of the hypertension and the end-stage renal disease in these patients. In pregnant women with asymptomatic bacteriuria, an increased chance of pyelonephritis existed, because of the altered anatomy, which might lead to premature birth.

Bacteriuria was the key in this and Kass investigated what number of bacteria caused a significant bacteriuria in asymptomatic women⁵. This criterion ($\geq 10^5$ cfu/ml) could then be used to search for patients at risk. At that time treatment had improved. In the beginning urinary acidification with mandelic acid was used but the simultaneous discovery of the sulphonamides in about 1935 was more promising. A great search for other and more active antimicrobial agents was undertaken⁶. By now an enormous number of antibacterial agents have been developed and synthesized, some of which have been proved to be of use in UTI in general practice.

EPIDEMIOLOGY

UTI is a very common disease. Incidence ranges from 15 - 65 per 1000 patients per year depending on the population studied^{6,7}. Until the age of one year 0.7-3.5% boys and 0.3-1.0% girls experiences a UTI⁸. After the age of one the majority of patients is seen in girls and women. From one to ten years of age 1% of the boys and 3% of the girls will experience a period of UTI⁹. The prevalence increases with age to about 10%-15% in women aged 60 years and older. For the Netherlands the following data are available from a study of Lamberts et al. of the department of general practice of the university of Amsterdam in 1989 among 1005 patients complaining of UTI².

Table I DIFFERENCES BETWEEN MEN AND WOMEN AND AGE IN GENERAL PRACTICE ².

AGE (YEARS)	MEN		WOMEN		TOTAL	
	NUMBER	(%)	NUMBER	(%)	NUMBER	(%)
0-4	2	1.0	13	1.6	15	1.5
5-14	6	2.9	24	3.0	30	3.0
15-24	11	5.3	110	13.8	121	12.0
25-44	30	14.4	206	25.9	236	23.5
45-64	46	22.0	135	17.0	181	18.0
65-74	62	29.7	150	18.8	212	21.1
75+	52	24.9	158	19.8	210	20.9
total	209	20.8	796	19.2	1005	100.0

UTI can be divided into asymptomatic bacteriuria (ABU), cystitis (CYS) and pyelonephritis (PYE)¹¹. The most prevalent symptoms in CYS and PYE are dysuria, stranguria, frequency and urgency, whereas PYE also shows symptoms of fever ($> 38^{\circ}\text{C}$), chills and flankpain. All three have in common their significant bacteriuria ¹². With respect to the management of UTI a division can be made between complicated and uncomplicated UTI¹³. In uncomplicated infections no factors other than the bacterial infection of the urinary tract play a role, while in complicated infections factors such as urinary tract anomalies, stones and urine reflux play a role.

Infections can also be divided into upper and lower UTI, where upper UTI are located above or ascending from the bladder into the kidneys¹⁴. All divisions but also combinations of them are used in literature to describe infections of the urinary tract, depending on the situation.

The bacteria causing UTI are usually facultative aerobic Gram negative rods⁸. In uncomplicated infections *Escherichia coli* is the most frequent causative agent¹⁵. In more complicated infections other Gram negative bacteria such as *Proteus* spp, *Klebsiella* spp and *Enterobacter* spp together with *Pseudomonas* spp, enterococci and staphylococci become more and more important¹³. *Chlamydia* spp and other fastidious microorganisms might play a role as well in complicated UTI¹². Anaerobic pathogens rarely cause urinary tract infections^{16,17}. If the UTI is treated cure or failure might occur. A failure means recurrence of the bacteria into the bladder. A relapse is a post-treatment recurrence of the same organism as was originally isolated. Reinfection means recurrence of bacteriuria after treatment due to another organism¹⁸.

HOST DEFENCES AND PATHOGENESIS

There are two ways by which bacteria can spread into the urogenital tract; the ascending and descending pathway³. In the latter pathway the bacteria originate from an infection elsewhere in the body and infect the urinary tract via the bloodstream. However this rarely occurs in humans. More important is the spread via the ascending route in which bacteria originate from the bowel flora and ascend into the urinary tract.

The first and most important step in the pathogenesis of UTI is bacterial adhesion to the epithelium, followed by colonization and infection¹⁹.

Before adherence takes place, the microorganisms first have to overcome a number of defence mechanisms of the host. Although urine is considered to be a good culture medium, it does possess antibacterial activity especially for anaerobic and other fastidious organisms of the urethral flora. A high urea concentration and a low pH are growth inhibiting. The natural flushing mechanism to clear the bladder of bacteria, and the presence of a bladder surface mucopolysaccharide, glycoaminoglycan, seem responsible for a natural resistance to bacterial adherence. Once infection has occurred, other defence mechanisms of the bladder uroepithelium such as mobilization of leucocytes, phagocytosis and bacterial destruction are used to combat the bacteria³.

The way in which bacteria colonize the urinary tract has led to two theories.

The first one is the so-called "host-susceptibility theory"; i.e. the colonization of the vaginal introitus and periurethral region by microorganisms

always precedes an UTI. Host factors rather than specific pathogenicity of the microorganisms are the important determinants of colonization.

The second is the "chance colonization theory"; i.e. not the host but the bacterial characteristics determine whether or not an infection will take place²⁰⁻²⁴. In this concept the periurethral colonization with a specific microorganism precedes a significant bacteriuria with the same organism.

However, the organisms must not only bind to the epithelium but also multiply and bind to newer epithelial cells, because older cells attached with bacteria will be swept away by fluid flow. This means that adhesion is an important factor in pathogenicity and a prerequisite for local bacterial proliferation. However, bacteria do not cause disease by adhesion only, other virulence factors are important as well²⁵.

VIRULENCE FACTORS

The research performed with *Escherichia coli* on the specific virulence factors that enable bacteria to overcome host defences have shown that in addition to fimbriae that mediate adherence, also the presence of the aerobactin system, the production of hemolysin and the presence of certain O and K antigens are important virulence factors²⁶⁻³⁰. In table II the association of these virulence factors with different types of UTI are shown as described by Johnson³¹.

Table II PREVALENCE OF VIRULENCE FACTORS ³¹

URINARY TRACT INFECTION	VIRULENCE FACTORS		
	P FIMBRIAE	AEROBACTIN	HEMOLYSIN
pyelonephritis	70	73	49
cystitis	36	49	40
asymptomatic bacteriuria	24	38	20
faeces	19	41	-

ADHERENCE AND FIMBRIAE

As already stated, adherence is considered to be the first step in colonization and an essential step prior to infection³². It is generally accepted that adhesion is mediated by adhesins called fimbriae. Fimbriae have the property to agglutinate certain erythrocytes, a reaction by which they can be characte-

rized. They are roughly divided into mannose resistant hemagglutinating (MRHA) and mannose sensitive hemagglutinating (MSHA) fimbriae²⁷. Evidence for the importance of fimbrial adhesion was, among others, given by Svanborg-Eden³³, who demonstrated that fimbriae which mediated MRHA were significantly more prevalent in bacterial strains isolated from patients with symptomatic UTI than in asymptomatic bacteriuria. In addition, it was shown that adherent bacteria cause a decrease in urethral motility and were able to ascend to the kidney³⁴.

Morphologically, fimbriae are thin fiberlike appendages with a length of approximate 0.2-20 μm and a diameter of 2-7 nm. Chemically fimbriae are protein polymers composed of approximate 1,000 identical subunits per fimbriae. The molecular mass ranges from 15,000 to 30,000²⁵.

The relevant receptors for most fimbriae are glycoconjugates, which act as lectins using carbohydrates as their receptor sites. The fimbriae can be distinguished on basis of their receptor specificity. P fimbriae are able to bind to the α -D-Gal-(1-4)- β -D-Gal part of glycolipid structures. P₁, P₂, P^k₁ and P^k₂, but not the p blood group³⁵ antigens are oligosaccharides containing a Gal(α 1-4)Gal β part. Therefore P blood group erythrocytes can be used for detecting P fimbriae using an HA assay. Other fimbriae bind to α -Sialyl-(2-3)- β -Gal (S fimbriae)³⁶ or a N-acetyl-glucosamine (GlcNAc) containing receptor (G fimbriae)³⁷. Fimbriae, whose specific adhering property is not yet identified, are called X fimbriae. Type I fimbriae are MSHA fimbriae binding to α -D-mannose. Also non-HA such as type 1C fimbriae³⁸ and M fimbriae³⁹ exist, along with nonfimbrial or afimbrial adhesins (afa)^{40,41}.

Apart from their binding capacity, fimbriae can be serologically discriminated: type I fimbriae form group F1A, whereas P fimbriae form group F7 to F14⁴². Monoclonal antibodies against the several serotypes of the P fimbriae used to immunize patients in an attempt to prevent UTI do not cross-react. It appears that anti-P-fimbrial immunity protects against renal infection with homologous P fimbriae, but not with heterologous P fimbriated strains. Therefore, it is not very likely that prevention of all UTI with anti-P-fimbrial antibodies will be successful^{3,43,44}.

The most important fimbriae in UTI are P fimbriae. The actual genes involved in the production of P fimbriae are located on the chromosome. The P fimbrial proteins PapA to I are encoded by a gene cluster called Pap⁴⁵. PapA is necessary for the formation of fimbriae and is responsible for the long chain of identical polymerized subunits forming the fimbriae⁴⁶. PapG is the actual adhesin molecule⁴⁷, which is linked to the fimbriae by PapE and F (called minor pilins)^{48,49}. PapC forms the assembly platform for fimbrial growth. PapD is a periplasmic protein which forms complexes with the fim-

bril subunits before assembly. PapH affects the length of the pilin and PapB and I play a role in the regulation of the gene cluster⁵⁰. The physical map of the P fimbrial gene cluster is similar among the various F types⁵¹.

The distribution of strains expressing P fimbriae is shown in table II³¹. In addition a number of studies suggested that the fecal carriage of P fimbriated strains appears to be higher in patients with pyelonephritis than in normal controls^{35,52}.

AEROBACTIN

Iron is needed in essential biochemical reactions and in oxygen transport⁵³. Therefore organisms have developed efficient mechanisms for the acquisition, storage and transport of iron⁵⁴. In most uropathogenic *Escherichia coli* strains the siderophore aerobactin is present. Aerobactin competes with transferrin and lactoferrin for bound iron and transports the iron²⁸.

Aerobactin is a hydroxamate derivative of two lysine molecules coupled to one citric acid and after binding, transports the iron to the bacterial membrane receptor protein (74 kDa). The aerobactin system is encoded by a five gene operon. Four genes are encoding for the aerobactin synthesis termed iuc (iron uptake chelate), the fifth encodes the outer membrane receptor protein and is termed iut (iron uptake transport). In short, the aerobactin production is as follows: lysine is oxydized by an oxygenase (product of iudD), then catalyzed by the product of iucB. The synthetase (products of iucA and iucC) occurs in the next step (two discrete steps). Gene iutA encodes the outer membrane receptor^{55,56,57}.

Aerobactin production is commonly detected using a bioassay⁵⁸. A bioassay to determine the sensitivity to cloacin can also be used, because the same receptor is responsible for both aerobactin and cloacin uptake. Nowadays DNA hybridization assays are performed. A probe either encoding the biosynthesis genes or for the receptor is used. The bioassay and the probes give the same results⁵⁹.

Aerobactin was identified as a virulence factor in pioneering studies. These investigators used isogenic strains, which only differ in their ability to produce aerobactin and they clearly demonstrated the contribution of aerobactin to the virulence of the bacteria^{60,61,62}.

HEMOLYSIN PRODUCTION

Escherichia coli strains commonly secrete the protein α -hemolysin, which is active against erythrocytes of various animals and humans⁶³. In addition to lysing erythrocytes, hemolysin is cytotoxic to a variety of cells such as polymorphonuclear neutrophils (PMNs), monocytes and granulocytes, whereas lymphocytes are relatively resistant.

Hemolysin production is encoded by a four-gene operon termed *hly A-D*, which is located on the chromosome of *Escherichia coli* strains⁶⁴. HlyA encodes for the structural hemolysin protein. To bind the erythrocytes HlyA must be activated by HlyC prior to its secretion. The first step in hemolysin secretion is an energy dependent process involving HlyB, whereas release from the outer membrane is passive and requires HlyD.

The distribution of bacteria from different types of UTI expressing hemolysin production is shown in table II. Although most hemolytic uropathogens are closely associated with P fimbriae a number of studies clearly demonstrated the importance of hemolysin production as a virulence factor.

O AND K ANTIGENS.

In the same way the association between virulence factor and UTI the pathogenicity for certain O and K serotypes was documented. Only a limited number of O and K serotypes were associated with UTI e.g. O1, O2, O4, O6, O7, O18 and O75 and K1, K2, K3, K12, K13⁶⁵⁻⁷⁰. However, the relation between serologic typing and other virulence factors has not been elucidated and also the role of O and K antigens in pathogenicity is not yet clear.

TREATMENT OF UTI

EFFICACY AND RESISTANCE

A considerable amount of antimicrobial agents is used in general practice for the empirical treatment of UTI. Treatment of UTI in a symptomatic patient is generally started before the result of the urine culture is known, on the basis of history, physical examination and microscopic examination of the urine sediment³ with a general accepted duration of 7 to 10 days.

Efficacy of antimicrobial therapy depends among others on the most likely causative microorganism and its susceptibility to the antimicrobial agent used. It is also obvious that a recent knowledge of the percentages of resistant bacteria causing acute cystitis in general practice will be of help to the general practitioner to prescribe an adequate therapy. He should have knowledge of the frequencies of susceptibility, because differences between regions, type of infections and patients have been described. In this regard the most important factor, in addition to safety, tolerance, risk of bacterial resistance, costs and way of administration are the susceptibilities of *Escherichia coli*. Recent data of these susceptibility rates for the mostly used antimicrobial agents are described below.

Sulphonamides have been widely used in the treatment of UTI. They are still recommended by the Dutch General Practitioners Association (NHG)⁷¹ as one of the three first-choice agents, together with trimethoprim and nitrofurantoin.

toin. However, 22% of *Escherichia coli* isolated in general practice in the south of the Netherlands are resistant to sulphonamides⁷². Even higher percentages were found in other parts in the Netherlands and in other countries^{73, 74, 75}. Although sulphonamides are attractive antimicrobial agents for treatment with respect to costs, the relative high frequency of resistance decreases its efficacy.

Trimethoprim is a recommended antimicrobial agent for UTI in the Netherlands. Resistance occurs in about 10% of *Escherichia coli*⁷⁶. However, in *Escherichia coli* strains isolated in Finland a resistance percentage of 40% is observed^{77, 78} and therefore it is not recommended for treatment of UTI in this country. In countries where the susceptibility is still high, it is still suitable.

The combination of sulphamethoxazole and trimethoprim is effective against the majority of common urinary pathogens. The resistance percentage recorded was found to be 10-20% in *Escherichia coli*^{74, 76, 79}. The effect of the combination is probably due to the combination. Therefore trimethoprim alone should be preferred instead of the combination⁴.

Amoxicillin has been widely used in the treatment of UTI. Several studies have shown that 30% to 40% of *Escherichia coli* in general practice are resistant in the Netherlands^{73, 76}. Since the resistance is caused by a β -lactamase it is apparent that the combination of amoxicillin and a β -lactamase inhibitor, clavulanic acid, is active against almost all general practice isolates^{73, 76}.

Another effective agent in the treatment of general practice derived UTI is nitrofurantoin. Although its efficacy is slightly limited by the intrinsic resistance of *Proteus* spp, it is still a suitable agent, because in general practice *Escherichia coli* is the most frequent cause of uncomplicated UTI in female patients. Therefore this agent is still recommended to Dutch general practitioners as a first choice agent for acute cystitis treatment⁷¹.

Recently a new group of antimicrobial agents, the fluoroquinolones became available for treatment of infections. Two components, norfloxacin and ciprofloxacin, are very attractive for treating UTI and they have a wide spectrum of activity. In addition to the usually causative pathogens they are in general active against less frequently isolated pathogens in GP such as *Pseudomonas aeruginosa* and group D streptococci^{80, 81}. Although they have a very low frequency of resistance, development of resistance to these agents can be expected and has been described. Therefore it is questionable whether these agents must be used in general practice.

THREE DAYS TREATMENT

As mentioned before, the generally accepted course for UTI is 7 to 10 days. A number of considerations including the high spontaneous cure rate, the limited compliance of patients to treatment and the observation that bacteria are eradicated from the urine within 24 to 48 hours with adequate antimicrobial agents have raised the question if a 7 to 10 days' course is optimal. In addition the exposure of bowel flora to antimicrobial agents became an increasing concern, because the fecal reservoir is believed to be the place where resistance development occurs. The shorter the treatment duration the lesser the chance of resistance.

Both these areas of concern have led to a large number of studies into efficacy of short term treatment courses⁸²⁻⁹¹. Reviewing recent studies on a single-dose and a short course treatment of antimicrobial agents for acute uncomplicated UTI in non-pregnant female patients, the most striking fact was that although for a long period of time short-term treatment had received a lot of attention, no well designed and performed randomized clinical trials were available. As pointed out by Fihn and Stamm⁹², and also by Philbrick⁹³ in their reviews on single dose treatments for uncomplicated UTI, the majority of studies do not fulfill the basic principles of randomized controlled trial design. One of the major objections is that the number of patients is too low to draw valid conclusions (a type II error).

Recently Norrby⁹⁴ evaluated 28 published trials, which fulfilled the minimum criteria of randomized controlled trials. He concluded that a three days' course of antimicrobial agents was the most optimal treatment duration. The results of these studies evaluated by Norrby, although many trials were not double-blinded, have resulted in a change with respect to the treatment of UTI. A short course is now generally advocated as an acceptable approach. This is also indicated by recent recommendations of the NHG, the National Organization for Quality Insurance in Hospital (CBO) and the Drug Bulletin (Geneesmiddelenbulletin)^{95,96}. However, as Norrby pointed out, it must be kept in mind, that the results cannot be extrapolated to other antimicrobial agents, therefore adequate and additional studies are necessary.

SUBINHIBITORY CONCENTRATIONS

From the earliest days on, numerous reports were published on the effects of subinhibitory concentrations of antimicrobial agents on bacteria⁹⁷⁻¹⁰³. Gram negative bacteria, elongated and filamentous, and Gram positive bacteria enlarged with incomplete fission were observed in the presence of subinhibitory concentration of antimicrobial agents.

The effect of subinhibitory concentrations of antimicrobial agents on bacteria is of interest, in that the disturbance of bacterial metabolism, although

not always resulting in growth inhibition might influence production and expression of virulence factors¹⁰⁴. With respect to the pathogenicity of uropathogenic *Escherichia coli* subinhibitory concentrations of antimicrobial agents might influence the capacity for adhesion and possibly the production of hemolysin and aerobactin production. In clinical practice this might be of importance to explain the efficacy of low dose treatment for the prevention of UTI^{105,106}. In his study on subinhibitory concentrations Chopra¹⁰⁷ explained the effect on bacteria in four different ways; alteration of the overall shape of the bacteria, promotion of the release of adhesins from the bacterial cell surface, promotion of the formation of functionally deficient adhesins (fimbriae) or suppression of the synthesis or secretion of surface located adhesins.

Zhanel¹⁰⁸ reviewed more specifically nine reports on the effects of subinhibitory concentrations on bacterial adherence of *Escherichia coli* to uroepithelial cells. The *Escherichia coli* strains in these studies were chosen either because of their known and well characterized fimbriae or because of their adhering capacity. Except for two studies^{108,109} most of the investigators isolated uroepithelial cells from the sediment of fresh urine from healthy women. A variety of antibiotics in subinhibitory concentrations were tested. The results of the study are summarized in table III;

In general and as pointed out by Zhanel it is obvious that a decreased adherence is observed, although many factors apparently do influence the results. Zhanel suggested that the elongated bacteria, because of clumsy movement, had a lower chance of finding epithelial cells. All nine studies were performed with well adhering bacteria. However, these might not be representative for the usually causative microorganisms. But also within this group of well adhering bacteria with respect to adherence to bladder uroepithelial cells considerable strain to strain variation with the same antibiotic was observed. This might be due to differences in types and amounts of fimbriae, and the receptor sites of the epithelial cells.

With regard to the effects of subinhibitory concentrations of antimicrobial agents on hemolysin production the scant data available do not yet allow conclusions¹¹⁰.

The studies on the influence of subinhibitory concentrations of antibiotics on siderophore production indicate that siderophore production is inhibited¹¹¹⁻¹¹⁴.

The results of the studies reviewed above suggest effects of subinhibitory concentrations of antimicrobial agents on expression and production of virulence factors. It is apparent that the effect of adherence to uroepithelial cells is influenced by antimicrobial agents. It is difficult to predict and it cannot always be explained how interactions between bacteria and antimicrobial

agents might lead to decreased or increased expression of virulence factors of the antibiotic exposed bacteria.

Table III EFFECT OF SUBMICS OF SEVERAL ANTIMICROBIAL AGENTS¹⁰⁸

ANTIBIOTICS	MIC	EFFECT
penicillin	1/2, 1/4	↓
nitrofurantoin	1/2	↑
chloramfenicol	1/4	↑
tetracycline	1/2, 1/4 1/8, 1/32	↓ ↑
doxycycline	1/2, 1/4	↔
sulfonamides	1/2 to 1/32	↓
trimethoprim	1/2 to 1/32	↓
aminoglycosides	1/2	↓
quinolones	1/4 1/8, 1/16	↓ ↑

↑: both effects observed
 ↔: no effects observed
 ↓: diminished effect
 ↑: increased effect

AIMS OF THE STUDY

With respect to the literature as discussed in this chapter several questions are still unanswered. The present study is designed to answer the following problems;

I the prevalence of resistance to antimicrobial agents of bacteria isolated from patients with uncomplicated urinary tract infections from general practice.

II The determination of the optimal duration of antimicrobial treatment for urinary tract infection.

III The effect of subinhibitory concentrations of antimicrobial agents on virulence factors; fimbriae, aerobactin and hemolysin production.

OUTLINE OF THE THESIS.

In chapter I a review of the literature is given. Factors influencing the efficacy of antimicrobial therapy, with an emphasis on bacterial resistance to short term treatment regimens and the effect of subinhibitory concentrations of antimicrobial agents are reviewed.

Chapter II, entitled 'antibiotic susceptibility patterns of uropathogens isolated from general practice patients from the south of the Netherlands' describes the antibiotic susceptibilities of uropathogens (n=1300) isolated from patients from general practice and the mechanism of resistance to amoxicillin and to trimethoprim are analyzed. The mechanism of resistance to amoxicillin is investigated by β -lactamase detection and characterization. The mechanism of high level trimethoprim resistance is characterized with probe specifying type I and type V mediated resistance.

In chapter III and IV the results of a double-blind randomized trial are described, in which a three days course of an antimicrobial agent is compared with a seven days course in patients with acute uncomplicated cystitis presenting to the general practitioner. Special attention has been paid to the number of patients needed to draw valid conclusions. Chapter III studies co-trimoxazole and the paper is entitled: Different lengths of treatment with co-trimoxazole for acute uncomplicated urinary tract infections in women. Chapter IV studies norfloxacin and is entitled: A double-blind randomized study of three days versus seven days norfloxacin for acute uncomplicated urinary tract infections in general practice.

In chapter V entitled 'influence of fluoroquinolones on aerobactin and virulence associated factors of *Escherichia coli*', the presence of aerobactin, hemolysin, hemagglutination and O and K antigens in two groups of uropathogens is described. The first group included strains isolated from female patients seeing their general practitioner with complaints of an uncomplicated UTI, the second group comprised *Escherichia coli* isolated from the urine of pregnant women with an asymptomatic bacteriuria. A control group consisted of bacteria isolated from patients with an urosepsis. The influence of quinolones (norfloxacin, ofloxacin and ciprofloxacin) on the hemolysin production was investigated for all strains. The influence of subinhibitory concentrations of antimicrobial agents on the aerobactin encoded RNA was investigated with a specific DNA probe in six randomly chosen strains.

Chapter VI, entitled 'influence of quinolones on the adherence and production of P fimbriae of *Escherichia coli*', presents the results of the study of the influence of quinolones on the adherence to uroepithelial cells and especially on P fimbriae. The same bacteria as described in chapter V were

used. The influence of subinhibitory concentrations of ciprofloxacin, ofloxacin and norfloxacin on bacteria to the adherence of a T24 urothelial cell line was investigated. The obvious influence of subinhibitory concentrations of norfloxacin on the bacteria was further investigated. The P fimbriated bacteria were investigated with a specific DNA probe against P fimbrial RNA and with monoclonal antibodies against the fimbriae.

Chapter VII is the summary and general discussion. In this chapter the results of the studies presented are summarized and discussed.

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CHAPTER II

ANTIBIOTIC SUSCEPTIBILITY PATTERNS OF UROPATHOGENS ISOLATED FROM GENERAL PRACTICE PATIENTS FROM THE SOUTH OF THE NETHERLANDS.

11.

$$\frac{1}{2} \left(\frac{1}{2} + \frac{1}{2} \right) = \frac{1}{2}$$

SUMMARY

In the present study the antibiotic susceptibilities of nearly 1200 uropathogens isolated from general practice patients from the south of the Netherlands were determined. The lowest susceptibility rates of *Escherichia coli* were observed to amoxycillin and sulfamethoxazole (60 and 78 % respectively), the highest for norfloxacin and fleroxacin (100 %).

All but one amoxycillin resistant strain were susceptible to the combination of amoxycillin + clavulanic acid. Also nitrofurantoin susceptibility was rather high (93 %) in *Escherichia coli*. Most of the trimethoprim resistant strains (79 %) (MIC > 8 µg/ml) were high level resistant (MIC ≥ 512 µg/ml). Colony blot hybridization revealed the presence of DHFR type I and type V in 92 % and 8 % of those strains respectively.

The decrease in antibiotic susceptibilities over time, as demonstrated in an additional analysis of research reports over the period 1971-1991, and the seasonal variations in susceptibility warrant the participation in surveillance systems to monitor the antibiotic resistance of in- and outpatient isolates.

INTRODUCTION

Urinary tract infection (UTI) is a very common disease, especially in women. The incidence of UTI increases strikingly in young women during late adolescence^{1,2}. Between 16 and 35 years one out of five women has at least one episode of acute cystitis each year, making UTI the most frequent infection in this age group. Of all oral antibiotics prescribed by general practitioners 11 % are for the treatment of UTI and this represents therefore, an important group of infections treated with antimicrobial agents³.

For the treatment of UTI the antimicrobial agent should be active to the likely uropathogens and minimally disrupt the normal gut and perineal flora⁴. In general practice patients, antibiotic therapy is mainly empiric. Although high in-vitro and in-vivo activity of several antimicrobial agents has been described by several authors, also increased resistance of uropathogens was observed⁵. In addition, differences in antibiotic susceptibility of pathogens from in- and outpatients as well as differences between regions in the same country have been reported^{3,5,6}. To provide the general practitioner with antimicrobial susceptibility data one has to be careful to extrapolate data from the nearby hospital or from other institutions or regions to the outpatient populations of the general practitioner. Adequate epidemiological data on antibiotic susceptibility of uropathogens of outpatients will support the empiric therapy of general practice. In the Netherlands, however, only

limited data are available on antibiotic susceptibility on general practice isolates. In 1988 407 *Escherichia coli* strains isolated from general practice patients were investigated in the south of the Netherlands⁸ and in 1990 149 uropathogens were tested at the National Institute of Public Health and Environmental Hygiene⁷.

In the present study the antibiotic susceptibility of uropathogens isolated from patients in the south of the Netherlands attending their general practitioner was determined. In addition, the mechanism of resistance to amoxycillin and trimethoprim in *Escherichia coli* isolates was analyzed.

MATERIALS AND METHODS

BACTERIAL STRAINS

The strains (n=1181) were isolated from consecutive patients consulting the general practitioner with complaints of UTI. A urine sample was considered to be positive if $\geq 10^5$ colony forming units (CFU) per ml of one species were found using the streak plate method. The microorganisms isolated were identified by means of standard bacteriological methods, including the Analytic Profile Index (API20E) (API Monierieux, France) system for Enterobacteriaceae.

SUSCEPTIBILITY ASSAY

The antibiotic susceptibility tests were performed as a microbroth dilution method using an inoculum of 10^5 cfu/ml in ISO sensitest broth (Oxoid CM 473) prepared by dilution of a fresh overnight broth culture. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the antimicrobial agent at which no visible growth was apparent after 18-24 hr of incubation at 37°C. *Escherichia coli* ATCC 25922 was used as the reference strain.

ANTIMICROBIAL AGENTS

The antimicrobial agents used were kindly provided by their respective manufacturers. The breakpoints for determining resistance were according to the guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing: amoxycillin > 32 µg/ml, amoxycillin + clavulanic acid (2:1) > 32 µg/ml, sulfamethoxazole > 128 µg/ml, trimethoprim > 8 µg/ml, co-trimoxazole > 8 µg/ml, nitrofurantoin > 32 µg/ml, norfloxacin > 8 µg/ml, and fleroxacin > 8 µg/ml.

β-LACTAMASE DETERMINATION.

The characterization of the type of β-lactamase produced was performed as described earlier⁹. In short, an overnight culture was diluted 1:10 in Nutrient broth (Oxoid CM67) and incubated, while shaking at 37°C. After 4

h of incubation the cells were collected by centrifugation, washed once with 0.01M phosphate buffer (pH 7.0), suspended in the same buffer and ultrasonicated. The broken cells were centrifuged at 10,000g for 30 min at 4°C and the supernatant was used as the crude enzyme extract and stored at -20°C until use.

The concentration of protein was estimated by the method of Biorad with bovine serum albumin as the standard ¹⁰.

For the β -lactamase characterization, analytical isoelectric focusing was performed in polyacrylamide gels containing Pharmalyte in the pH range 3-10 using nitrocefin as indicator according to the method of Matthew⁹.

CHARACTERIZATION OF TRIMETHOPRIM RESISTANT STRAINS (MIC $\geq$ 512 mg/l)

Plasmid DNA was isolated by the method of Sambrook¹¹. Two probes encoding trimethoprim resistance were used: one was a 0.49 kb KpnI/BamHI fragment of pLKO627 encoding for the dhfrI gene and the other was a 0.49 kb HpaI fragment of pLKO22 encoding for the dhfrV gene^{12,13}. (The probes used were kindly provided by L.Sündström). The probes were labeled with digoxigenin according to the manufacturers instructions (Boeringer Mannheim, Mannheim, Germany).

Colony blot hybridization on nylon membranes (Nytran) was performed according to the manufacturers instructions (Nytran, Schleicher & Schuell, Den Bosch, the Netherlands). Prehybridization and hybridization were done under stringent conditions at 42°C in formamide 50%, 5*SSC (0.75 M sodiumchloride, 0.075 M sodiumcitrate), sarcosyl (n-laurylsarcosine) 0.1%, SDS (sodium dodecylbisulfate) 0.02%, salmon sperm 0.5% and blocking reagent 2% (Dig kit, Boeringer Mannheim, Mannheim, Germany) with washes in 2*SSC and 0.1% SDS at 68°C and in 1*SSC and 0.1%SDS at room temperature.

Detection of digoxigenin labeled nucleic acids, using positive (strains pLKO267 and pLKO22) and negative (strain HB101) controls, was performed by chemoluminescence on radiographic films (Kodak X omat AR).

RESULTS

Of the 1181 uropathogens isolated, 79% were *Escherichia coli* (n=934), followed by 7.5% coagulase negative staphylococci (n=89) and 6.8% *Proteus* spp (n=81). Other Enterobacteriaceae were isolated with a frequency of 5.1% (n=58). The rest 1.6% (n=19) were other bacteria.

The antibiotic susceptibility of the strains tested is shown in table 1. The positive effect of clavulanic acid on the susceptibility to amoxycillin of

Table 1 ANTIBIOTIC SUSCEPTIBILITIES OF UROPATHOGENS ISOLATED FROM GENERAL PRACTICE PATIENTS

BACTERIAL SPECIES AND ANTIBIOTIC (NUMBER OF ISOLATES)	MIC (MG/L)	SUSCEPTIBILITIES			
	RANGE	MIC ₅₀	MIC ₉₀	GEOM MEAN	%
<i>all uri isolates (1181)</i>					
amoxycillin	≤0.062- > 256	8	256	12.3	62
amoxycillin + clavulanic acid	≤0.062-256	2	8	2.4	99
sulphamethoxazole	≤0.062- > 256	16	256	19.0	80
trimethoprim	≤0.062- > 256	0.25	64	0.7	87
co-trimoxazole	≤0.062- > 256	0.5	256	1.0	86
nitrofurantoin* (1100)	≤0.25- > 1024	4	128	6.1	88
norfloxacin	≤0.062- > 8	0.062	0.125	0.2	100
floxacin	≤0.031-8	0.031	0.5	0.1	100
<i>Escherichia coli (938)</i>					
amoxycillin	≤0.062- > 256	8	256	14.9	60
amoxycillin + clavulanic acid	≤0.062-256	4	8	3.2	100
sulphamethoxazole	≤0.062- > 256	16	256	21.6	78
trimethoprim	≤0.062- > 256	0.5	128	0.6	88
co-trimoxazole	≤0.062- > 256	0.5	64	1.0	88
nitrofurantoin	≤0.25- > 1024	4	32	5.8	93
norfloxacin	≤0.062-8	0.062	0.125	0.1	100
floxacin	≤0.031-8	0.062	0.25	0.1	100
<i>Proteus spp. (81)</i>					
amoxycillin	≤0.062- > 256	2	256	4.6	75
amoxycillin + clavulanic acid	≤0.062-64	1	4	0.9	100
sulphamethoxazole	≤0.062-256	8	256	11.6	80
trimethoprim	≤0.062- > 256	1	8	1.6	91
co-trimoxazole	≤0.062- > 256	0.5	4	0.9	91
norfloxacin	≤0.062-8	0.062	0.125	0.1	100
floxacin	≤0.031-8	0.062	0.25	0.1	100
<i>Staphylococci spp. (89)</i>					
amoxycillin	≤0.062- > 256	1	256	2.2	78
amoxycillin + clavulanic acid	≤0.062-256	0.25	2	0.3	100
sulphamethoxazole	≤0.062- > 256	8	128	9.8	90
trimethoprim	≤0.062- > 256	0.5	32	1.0	87
co-trimoxazole	≤0.062- > 256	0.5	8	0.9	91
nitrofurantoin	≤0.25- > 1024	4	16	5.2	90
norfloxacin	≤0.062-4	0.125	1	0.2	100
floxacin	≤0.031-8	1	4	0.8	100

* *Proteus spp.* are not included

Figure I MICs OF ALL UROPATHOGENS AND *ESCHERICHIA COLI* ISOLATES TESTED TO EIGHT ANTIMICROBIAL AGENTS. (UPPER ALL UROPATHOGENS, LOWER *ESCHERICHIA COLI*)

strains was quite obvious. The geometric mean of amoxycillin alone was 12.3 and of the combination 2.4 $\mu\text{g/ml}$. The same pattern applied to the antibacterial activity of the combination of sulfamethoxazole and trimethoprim (cotrimoxazole) compared to the activity of sulfamethoxazole alone. Both quinolones, norfloxacin and fleroxacin, were highly active to all strains tested: 90% of the strains were inhibited by 0.5 $\mu\text{g/ml}$ or less although fleroxacin tended to be slightly less active than norfloxacin (figure 1). The resistance of *Escherichia coli* isolates to amoxycillin (40%) was distinctly

higher than to sulfamethoxazole (22%).

From all bacteria isolated (n=1181), resistance to three or more antimicrobial agents was observed in 144 (12.2%) strains, 487 strains (41.2%) were susceptible to all agents tested. From the *Escherichia coli* isolated (n=938) 86 (9.2%) were resistant to three or more antimicrobial agents, 438 (46.7%) were fully susceptible. (Table 2)

Table II MULTIPLE RESISTANCE TO ALL BACTERIA ISOLATED AND TO ALL *ESCHERICHIA COLI* ISOLATED.

NUMBER OF ANTIMICROBIAL AGENTS	ALL ISOLATES (1181)		ESCHERICHIA COLI (938)	
	FREQUENCY (%)	CUMULATIVE (%)	FREQUENCY (%)	CUMULATIVE (%)
0	41.2	41.2	46.7	46.7
1	31.4	72.6	29.8	76.5
2	15.2	87.8	14.3	90.8
3	4.1	91.1	3.3	84.1
4	6.6	98.5	4.7	98.8
5	1.5	100.0	1.2	100.0

All but one amoxycillin resistant *Escherichia coli* (n=933) were susceptible to amoxycillin in combination with clavulanic acid and were found to have a TEM-1 plasmid-mediated resistance. The only *Escherichia coli* resistant to the combination of amoxycillin + clavulanic acid produced two β -lactamases, a TEM-1 (pI= 5.2) and a second enzyme with a pI of 9.0. The relatively low basal enzyme activity increased more than tenfold after induction. This and the high pI strongly suggests that the second enzyme is a chromosomal encoded β -lactamase.

High level trimethoprim resistance (MIC $\geq$ 512 μ g/ml) was observed in 79% (n=75) of all trimethoprim resistant *Escherichia coli*. Further characterization using the same probes as were described previously revealed that 69 (92%) strains harbored a type I mediated resistance and one strains a type V (1%) mediated resistance.

DISCUSSION

In the present study the antibiotic susceptibility of nearly 1200 uropathogens was determined. The susceptibility to those compounds especially used for the treatment of UTI (trimethoprim, nitrofurantoin, co-trimoxazole

and norfloxacin) was rather high (88%-100%).

The susceptibility to sulfamethoxazole and amoxycillin was quite remarkable. A study performed in the Netherlands in the same region with almost the same general practitioners revealed a susceptibility to sulfamethoxazole and amoxycillin of 71% and 84% compared to 78% and 60% respectively in the present study⁷. Thus no decrease in susceptibility to sulfamethoxazole (rather a slight increase), however a distinct decrease in amoxycillin susceptibility was observed in a two year period.

Table III ANTIBIOTIC SUSCEPTIBILITIES OF ISOLATES OF GENERAL PRACTICE.

SURVEY	NUMBER OF ISOLATES	YEAR	COUNTRY	ANTIMICROBIAL AGENTS						
				AMP	AUG	SUL	TRI	COT	NIT	NOR
ESCHERICHIA COLI										
Gruneberg ⁵	340	1971	GB	91	-	77	99	99	98	-
Abbas ¹⁴	960	1979	GB	71	89	-	-	87	-	-
Beale ¹⁵	34734	1984	GB	67	93	63	86	83	93	-
Gruneberg ⁵	1543	1989	GB	66	-	67	82	83	97	-
Baselier ²	121	1983	NETH	87	-	35	-	-	89	-
Stobberingh ⁸	407	1988	NETH	84	96	71	90	91	98	110
de Neeling ⁷	149 ³	1990	NETH	66	95	55	76	76	97	99
Trienekens (this study)	938	1991	NETH	60	97	78	88	88	93	100
ALL UROPATHOGENS ISOLATED										
Gruneberg ⁵	433	1971	GB	88	-	76	94	97	86	-
	2129	1989		62	-	67	83	84	88	-
Naber ¹⁶	349	1990	GER	90	90	-	94	95	-	-
Trienekens (this study)	1181	1991	NETH	62	62	80	87	86	88	100

¹GB = Great Britain

NETH = the Netherlands

GER = Germany

²AMP = amoxycillin

AUG = amoxycillin + clavulanic acid

SUL = sulphamethoxazole

TRI = trimethoprim

COT = co-trimoxazole

NIT = nitrofurantoin

NOR = norfloxacin

³12 bacteria were not derived from urine samples

Comparing the results of different studies, in Great Britain a decrease in susceptibility to both compounds is observed (table 3)^{2,5,7,8,14,15,16}. The antimicrobial activity of amoxycillin in combination with clavulanic acid was very high, almost all amoxycillin resistant strains were susceptible to this combination. Over time no decrease in susceptibility is observed. Also the susceptibility of nitrofurantoin remained stable over time. Neither in Great Britain nor in the Netherlands a decrease was observed, despite the use of this antimicrobial agent in for at least 35 years.

Trimethoprim susceptibility is still at a fairly stable level (88%). However, it was remarkable that the combination of trimethoprim with sulfamethoxazole had the same susceptibility (88%). Thus no synergistic effect of trimethoprim with sulfamethoxazole was observed in this study. Therefore the use of trimethoprim as a single agent for the treatment of UTI is highly recommended. Sulfamethoxazole only adds to the side effects but does not increase the antibacterial activity if used in combination with trimethoprim¹⁷.

Comparing the trimethoprim resistance in the present study with that of the study of Heikkilä¹⁸ some differences could be observed. The overall resistance was 12% and 40% respectively. High level resistance in these strains was 79% and 97% respectively and the percentage of type I DHFR 92% and 79% respectively of these high level resistant strains.

The higher percentages of trimethoprim resistance in Finland might be due to the higher use of trimethoprim compared to that in the Netherlands¹⁸. The source of the isolates, outpatients or isolates from geriatric units or hospital strains might play a role as well. In another study in 1985 comprising 19 *Escherichia coli* strains a type I DHFR could be demonstrated in 15 (79%) of the high level resistant isolates¹⁹.

Vorland²⁰ described a seasonal dependence in incidence and prevalence of antimicrobial resistance. In the present study no seasonal variation was observed. The mean number of monthly isolates was 43.3 (sd 19.2). In addition the monthly prevalence of resistance to sulfamethoxazole, amoxycillin and nitrofurantoin was rather stable during the study period (figure II).

Not only differences over time, differences between regions within one country are important as well. Also in the Netherlands differences over time were as important as the differences in susceptibility between different regions. Susceptibility percentages to sulfamethoxazole in general practice uropathogens isolated in three regions (east, middle and south) were 35%, 55%, and 78% respectively. The lowest percentage of susceptibility were found in a study in the east of the Netherlands², the highest susceptibility

rates were found in the present one (1991).

Figure II PERCENTAGE OF MONTHLY ISOLATED *ESCHERICHIA COLI* STRAINS RESISTANT TO SULPHAMETHOXAZOLE.

It might be possible that the differences in susceptibility are due to differences in antibiotic prescription in the different regions. Unfortunately, no data on the use of antibiotics by general practitioners are available.

In conclusion the recommendations of the Infectious Disease Society of America as to the need for surveillance of out- and in-patient isolates to provide the medical doctor with up to date data on antibiotic susceptibility are underscored with our data²¹.

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CHAPTER III

DIFFERENT LENGTHS OF TREATMENT WITH CO-TRIMOXAZOLE FOR ACUTE UNCOMPLICATED URINARY TRACT INFECTIONS IN WOMEN.

ABSTRACT

Study objective - To compare a 3 days and 7 days course with co-trimoxazole in women with acute dysuria, stranguria, urinary frequency or urgency.

Design - Randomized, double blind placebo controlled trial.

Setting - General practices in the south east of The Netherlands.

Patients - 327 Non-pregnant female patients aged 12 to 65.

Intervention - 161 Women were allocated to 3 days treatment (co-trimoxazole 960 mg twice a day) 166 women were allocated to 7 days treatment (cotrimoxazole 960 mg twice a day).

Main outcome measure - The rates for resolution of symptoms were not significantly different between the two groups. Cumulative rates of recurrence after 3 days and 7 days treatment were 31/139 (22%) and 23/151 (15%) respectively six weeks after entry ($p=0.16$). Adverse effects occurred in a quarter of women given 3 days treatment compared with a third of women receiving 7 days treatment ($p=0.29$). In only 2 patients did adverse effects necessitate stopping treatment.

Conclusions - 3 days of co-trimoxazole seems to be as effective as a 7 days course for treating acute urinary tract infection in non-pregnant women.

INTRODUCTION

The prevalence of acute uncomplicated urinary tract infection in women is fairly high at about 50/1000/year^{1,2}. The optimal duration of antibiotic treatment, however, remains a point of discussion. Single dose therapy offers several advantages over the conventional duration, including improved compliance, reduced adverse effects, decreased risk on disturbing bacterial flora and selecting resistant microorganisms and lower costs^{3,4}. In the past 20 years several studies have suggested that single dose therapy is as effective as the conventional regimens of 10 or 14 days⁵⁻⁸. No study, however, has included enough patients to prevent the type II error⁹. Freiman et al.¹⁰ and Fihn et al.¹¹, reviewing 71 and 62 clinical studies respectively, concluded that most trials studied too few patients to detect a meaningful difference between the therapeutic regimens. In a recent study with adequate statistical power no symptomatic difference was observed between a single dose and 10 days course of co-trimoxazole at 3 and 13 days and 6 weeks after the start of the treatment¹². Adverse effects, however, were twice as common in the group allocated to 10 days of treatment compared with the patients

receiving single dose treatment. the authors therefore suggested that an intermediate duration of treatment might be optimal.

We carried out a randomised double blind placebo controlled clinical trial of 3 days versus 7 days of co-trimoxazole for treating acute uncomplicated urinary tract infections in women.

PATIENTS AND METHODS

Non pregnant female patients aged 12-65 presenting to their general practitioner with either dysuria, stranguria, urinary frequency or urgency in the past 24 hours were included in the study. Patients were excluded if they had signs and symptoms of acute pyelonephritis, were known diabetics, had known structural abnormalities of the urinary tract, had indwelling catheters, had recently received immunosuppressive drugs, were allergic to trimethoprim, sulfamethoxazole or co-trimoxazole, or received antimicrobial treatment within the previous 4 weeks. Patients with a known urinary tract infection within the past 3 months were also excluded. Informed consent was obtained from all participants before enrolment.

Patients were assigned in a random and double blind way to receive either 3 days of co-trimoxazole 960 mg twice a days followed by 4 days of placebo twice a days (3 days regimen) or 7 days co-trimoxazole 960 mg twice a days (7 days regimen). Blinding was achieved with the double dummy technique by using placebo tablets that looked identical to the active tablets (Bactrimel Forte[®]).

Clean voided urine specimens were obtained from all patients for standard quantitative and dip-slide culture (Orion Diagnostics, Espoo, Finland) and antimicrobial susceptibility tests. For isolating microorganisms standard laboratory methods were used. Bacterial counts were performed by the quantitative surface streak technique using a 0.03 ml standard drop on to 7% sheep blood agar and MacConkey agar plates. The isolated microorganisms were identified by standard bacteriological methods that included the analytic profile index (API Montalieu-Vercieu, France) for Enterobacteriaceae. Coagulase negative staphylococci resistant to novobiocin were identified as *Staphylococcus saprophyticus*. *Escherichia coli* serotyping (for O and K antigens) was performed in cooperation with the National Institute for Public Health and Environmental Hygiene, Bilthoven (dr. Guinée) on the initial and subsequent isolates from most patients whose follow up cultures yielded positive results for *Escherichia coli*. The O and K antigens were identified by bacterial agglutination. Urine samples containing more than two species of bacteria were considered to be contaminated and not

included in the analysis. The presence of growth inhibiting factors was detected by using a *Bacillus subtilis* as an indicator strain.

All patients were asked to return to their general practitioner one, two and six weeks after enrolment. On return visits they filled in a questionnaire about relieve of symptoms and possible antimicrobial side effects such as headaches, nausea, vomiting or diarrhoea. In addition, clean voided urine specimens were obtained at each follow up visit and processed in the same way as the initial specimen. Severe side effects were defined as those that needed treatment or that would preclude treatment with co-trimoxazole in the future. From some patients a urine sample was obtained without the questionnaire so the total number of patients for whom data on symptoms and bacteriological response were available were different.

Patients were diagnosed as having an urinary tract infection if they presented with acute dysuria, stranguria or urinary frequency or urgency, and a bacterial colony count $\geq 10^5$ cfu/ml. The effectiveness of therapy was based on the short and long term effect of cure, persistence, relapse and reinfection¹¹. Persistence was defined as presence of the causative organism in the urine after treatment had stopped and relapse as the absence of the organism at or after the end of treatment but reappearance of the same organism in the 2 weeks (early relapse) or 6 weeks (late relapse) after enrolment. Reinfection was defined as appearance of another infecting organism in the 1 or 2 weeks (early reinfection) or 6 weeks (late reinfection) after enrolment, whereas cure was defined as absence of the causative organism at and during follow-up. Failure of treatment was defined as all cases of persistence, relapse and reinfection as well as those cases of persistence of symptoms or occurrence of adverse drug reactions for which the doctor had to prescribe another drug. Unassessable cases were those for which evaluating bacteriological response was not possible, because no follow up cultures were obtained¹³.

Based on data from our pilot study¹⁴ bacteriuria will be eradicated in 90% of women treated with co-trimoxazole 960 mg twice a day. We took a 10% increase in effectiveness of 7 days'treatment over 3 days' to be the minimum; less would not be clinically meaningful because of the lower costs, greater compliance of patients, and fewer adverse effects associated with shorter treatment. Our null hypothesis was that both treatment with 3 days'and 7 days' co-trimoxazole would be successful in 90% of the patients, and assuming a significance of $p \leq 0.05$ and a power of 80% we calculated that we required 142 patients for each treatment group^{15,16}. The comparisons of treatment regimens and differences in the prevalence of adverse reactions were determined by χ^2 analysis.

RESULTS

Nineteen general practitioners from the south east of The Netherlands participated in the study. From January 1988 till April 1989, 327 women were enrolled in the study: 161 received a 3 days' treatment and 166 a 7 days' treatment. The mean (SD) ages of both the two groups were 35.0 (4.6) and 38.4 (5.4) years respectively. Follow-up cultures at 1, 2 and 6 weeks were obtained from 145 (90%), 132 (82%) and 139 (86%) patients receiving the short treatment course 152 (92%), 136(82%) and 151(91%) patients receiving the long term treatment respectively.

Table I NUMBERS (PERCENTAGES) OF PATIENTS WITH URINARY PATHOGENS BEFORE TREATMENT WITH CO-TRIMOXAZOLE.

URINARY PATHOGENS	LENGTH OF TREATMENT	
	3 DAYS (N=161)	7 DAYS (N=166)
<i>Escherichia coli</i>	90 (56)	83 (50)
coagulase negative staphylococci	9 (6)	12 (7)
<i>Proteus mirabilis</i>	6 (4)	10 (6)
other Enterobacteriaceae	6 (4)	6 (4)
<i>Staphylococcus aureus</i>		2 (1)
negative	48 (30)	53 (32)

Table II RESPONSE OF SYMPTOMS IN PATIENTS WITH URINARY TRACT INFECTION TREATED WITH CO-TRIMOXAZOLE. FIGURES ARE NUMBERS (PERCENTAGES) OF PATIENTS.

	SYMPTOMS PRESENT		SYMPTOMS PRESENT AND BACTERIOLOGICALLY PROVED	
	3 DAYS' TREATMENT	7 DAYS' TREATMENT	3 DAYS' TREATMENT	7 DAYS' TREATMENT
symptoms absent or improved one week after entry	131/142 (92)	129/145 (89)	88/97 (91)	89/97 (92)
symptoms absent or improved two weeks after entry	110/121 (91)	108/121 (89)	77/83 (87)	74/81 (91)
symptoms absent six weeks after entry	97/116 (84)	106/123 (86)	74/85 (87)	72/80 (90)

Table I lists the urinary pathogens isolated. In both treatment groups *Escherichia coli* was the most commonly isolated pathogen followed by coagulase negative Staphylococci and *Proteus mirabilis*. Other Enterobacteriaceae isolated were klebsiella, citrobacter and enterobacter. No significant differences were found in the rate of isolation of the uropathogens between the different treatment groups. Table II shows that the proportions of women with acute urinary symptoms whose symptoms were relieved after 1, 2, and 6 weeks were similar in both groups.

Table III CUMULATIVE RATES OF RECURRENT INFECTIONS IN PATIENTS WITH URINARY TRACT INFECTIONS TREATED WITH CO-TIMOXAZOLE. FIGURES ARE NUMBERS (PERCENTAGES) OF PATIENTS.

TIME AFTER ENROLMENT (WEEKS)	LENGTH OF TREATMENT		P VALUE
	3 DAYS	7 DAYS	
1	14/145 (10)	4/152 (3)	0.02
2	19/132 (14)	10/136 (7)	0.10
6	31/139 (22)	23/151 (15)	0.16

Table IV NUMBERS OF PATIENTS WITH RECURRENT INFECTION IN PATIENTS WITH URINARY TRACT INFECTION TREATED WITH CO-TRIMOXAZOLE.

TYPE OF FAILURE	LENGTH OF TREATMENT	
	3 DAYS	7 DAYS
persistence	6	4
early relapse	3	4
early reinfection	10	2
late relapse	5	4
late reinfection	7	9

The cumulative rates of failures were similar in the 2 treatment groups, with a slightly higher but non-significant rate in the patients allocated to the 3 days' treatment (Table III). Table IV gives the classification of the failure of treatment in both groups. In the group allocated to the 3 days' treatment 12 of the 14 *Escherichia coli* isolates from follow up cultures in cases of persistence and relapse were similar in serotype and antibiotic susceptibility or both to the original infecting microorganism; 2 could not be precisely characterized. The reinfections were caused by *Escherichia coli* (6), coagulase negative staphylococci (5), *Proteus mirabilis* (4) and pseudomonas

(2). The isolated microorganisms differed in species, serotype or antibiogram from the originally infecting strain. *Escherichia coli* (10), *Klebsiella* spp (1) and coagulase negative staphylococci (1) were isolated from samples from cases of treatment failure in patients allocated to (7) days' treatment. Eight of the 10 *Escherichia coli* strains were similar in serotype or antibiotic susceptibility or both to the originally infecting strain. The remaining 2 strains could not be precisely characterized. The *klebsiella* and coagulase negative staphylococci were both similar in species and antibiogram. *Escherichia coli* and, coagulase negative staphylococci were isolated 5 times and *Streptococcus faecalis* only once; they were different in terms of serotype or antibiogram, or both, from the original isolates. Failure of treatment because of resistance to co-trimoxazole occurred in 5 patients allocated to 3 days' treatment and in 4 allocated to 7 days' treatment.

The compliance of the patients was evaluated by using a questionnaire and by testing the urine specimen for the presence of growth inhibiting factors one week after enrolment. Growth inhibiting factors were found in 10 out of the 91 specimens in the group allocated to 3 days' treatment and in 74 of the 101 specimens in the group allocated to 7 days' treatment. Unfortunately, first control specimens taken 1 week after enrolment were not available from all patients.

Table V ADVERSE EFFECT OF TREATMENT WITH CO-TRIMOXAZOLE. FIGURES ARE NUMBERS (PERCENTAGES) OF PATIENTS.

EFFECT	LENGTH OF TREATMENT	
	3 DAYS (N = 161)	7 DAYS (N = 166)
nausea	12 (7)	19 (11)
other gastrointestinal complaints	10 (6)	15 (9)
vaginal discharge	2 (1)	1 (1)
rash	3 (2)	6 (4)
headache	5 (3)	
others	8 (5)	10 (6)
totals	40 (25)	51 (31)

Subjective adverse effects occurred in 40 patients (25%) receiving 3 days' treatment compared with 51 (31%) receiving 7 days' treatment ($p=0.29$). These included gastro-intestinal upset in 22 (14%) and 34 (20%) patients ($p = 0.137$) and allergic reactions in 3 (2%) and 6 (4%) patients (Table V). Severe side effects occurred in only two patients (one developed

rash, one developed gastrointestinal complaints), both of whom had been allocated to 7 days' treatment.

DISCUSSION

Our study protocol fulfilled the 12 methodological criteria mentioned by Philbrick et al.⁹ and the recommendations of Fihn and Stamm¹¹ to be used in clinical trials of treating uncomplicated urinary tract infections in women. Our results showed no difference in symptomatic response up to 6 weeks after enrolment between the different treatment regimens either in the group of patients with a bacteriologically proved urinary tract infection or in the group with acute urinary tract symptoms. A similar clinical response was observed in the study of Gossius and Vorland¹⁸.

The difference in the rate of bacteriological response 1 week after enrolment might have been due to the presence of growth inhibiting factors in the group allocated to 7 days' treatment, enabling early reinfection to occur. Growth inhibiting compounds were found in 74 of the 101 urine samples from the patients allocated to 7 days' treatment. Early reinfection was observed in 10 patients receiving a 3 days' treatment and in only 2 patients receiving 7 days' treatment. The compliance of the patients allocated to 7 days' treatment was 74% as indicated by the presence of growth inhibiting factors in the urine samples. This is similar to the 75% compliance with a treatment regimen of twice a day for about 14 weeks found in the study of Cramer et al.¹⁹.

Six weeks after enrolment the cure rates in the groups allocated to 3 and 7 days' treatment were 76% and 83% respectively. These proportions were in general lower than those found in other studies^{6,8,17,18}. The difference might have been due to the fairly short follow up period and the different definitions of cure rate used in other studies. We defined cure rate as relief of initial symptoms plus a sterile urine culture throughout the follow up period. Some investigators used the same definitions but included those patients who were reinfected with a different organism, because these patients were cured of the original infection (in our study the so-called re-infections)^{4,6,8,18}. If we used that definition the cure rates were 90% and 92% respectively 6 weeks after enrolment. These rates are slightly higher than found in the study of Fihn et al.¹² but lower than those described by Gossius and Vorland¹⁸.

An important issue for the patient is relieve of symptoms. Most patients showed relieve after 1 week. After 6 weeks most patients were still free of symptoms in both treatment groups. In 9 out of 31 bacteriological

failures in the group allocated to 3 days' treatment and 5 out of 23 bacteriological failures in the group allocated to 7 days' treatment, however, the infection was also symptomatic. In the other patients the infection was asymptomatic. In the bacteriologically cured group 2 patients allocated to 3 days' treatment and 3 in the group allocated to 7 days' treatment still had complaints of urinary tract infection. The cause of these complaints might have been due to the urethral syndrome caused by organisms such as *Chlamydia trachomatis*¹². No significant differences in side effects were observed between treatment regimens.

Major adverse effects -that is, enough to stop treatment- occurred only twice in the patients allocated to 7 days' treatment. These occurred less often than they did in other studies^{6,12}.

Based on our results- that is, the similar symptomatic and bacteriological response 6 weeks after enrolment and the similar occurrence of adverse effects in the 2 treatment groups in a study that included an adequate follow up period and a large enough sample to allow statistical analysis- we conclude that 3 days' treatment with co-trimoxazole is as effective as 7 days for the treatment of acute urinary tract infection in non-pregnant women. This study, however, was carried out in patients with more or less similar socioeconomic backgrounds and easy accessibility to well organised medical care. Whether these data could be generalized to other populations from lower socioeconomic backgrounds or with other forms of medical care remains to be elucidated.

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CHAPTER IV

TREATING ACUTE URINARY INFECTIONS. AN RCT OF 3-DAY VERSUS 7-DAY NORFLOXACIN.

ABSTRACT

A randomized double blind placebo controlled trial was carried out to compare two courses of treatment in women with complaints of acute urinary tract infection in general practice. The 3-day course of treatment was found to be as effective as, and cheaper than, the 7-day therapy for acute cystitis in general practice.

INTRODUCTION

Uncomplicated urinary tract infection (UTI) is a common disease in women, especially in general practice^{1,2}. The length of treatment of uncomplicated UTI, i.e. 3 days versus 7 to 10 days has been a hot topic during the past 10 years³⁻¹⁰.

Both treatment schedules have their benefits. Advantages of short-term treatment are better patient compliance, lower cost, and less frequent adverse effects¹¹. An advantage of long-term treatment is the reduced possibility of recurrent infections¹². Although some studies promote 3-day treatment, most have not had a sufficient number of patients to support their conclusions^{8,10,11,13,14}.

Recently, a new group of antimicrobial agents has been developed, of which norfloxacin is often prescribed for the treatment of UTI^{15,16}. Many papers have been published on the clinical efficacy of different lengths of treatment with norfloxacin for UTI¹⁷⁻¹⁹. Although a 3-day course of trimethoprim-sulphamethoxazole is now accepted as adequate for the treatment of UTI, no extrapolations from one antibiotic to another should be made^{8,20}.

In this study, a 3-day course of norfloxacin was compared with a 7-day course of norfloxacin for the treatment of women visiting their family physicians in the southern part of The Netherlands with complaints of acute uncomplicated UTI. End points of evaluation were the clinical and bacteriological outcome, the number of adverse effects and cost.

METHODS

Included in the survey were women who were non-pregnant aged 18 to 65 years consulting their general practitioners with complaints of at least one of the following symptoms during the last 24 hours: dysuria, strangury, and frequent or urgent urination. Excluded from the survey were patients who had signs and symptoms of pyelonephritis, known abnormalities of the renal

tract or diabetes. Also excluded were patients who had received either chemotherapy or antibiotics during the past month, patients who were known to be hypersensitive to nalidixic acid derivatives and patients who had had a UTI within the last 3 months. General practitioners (n = 12) from the south of the Netherlands were asked to participate in this study.

The protocol was approved by the Medical Ethics Committee of Maastricht University Hospital. All patients received oral and written information and all gave informed consent. They were allocated to one or the other course of treatment in a double-blind, randomized way by means of the double dummy technique. The 2 strategies were: 400 mg of norfloxacin twice daily for 7 days or 400 mg of norfloxacin twice daily for 3 days followed by 4 days of placebo twice daily.

A fresh urine sample was requested from all patients before the start of the medication, at the end of the medication (7 days later) and at least 6 weeks later. Patients were also asked to complete a questionnaire on compliance, relief of symptoms, and side effects. Patients were told to return to their general practitioners if symptoms did not disappear or if side effects occurred. Uropathogens were isolated and identified by standard microbiologic methods using API 20E (API Montalieu, Vercieu, France) for Enterobacteriaceae.

Cultures of $\geq 10^5$ colony forming units (cfu)/ml were considered to be positive. Cultures with 2 or more pathogens were regarded as mixed cultures. To evaluate patient compliance 1 week after starting medication, the presence of growth-inhibiting factors was tested. Clinical outcome was evaluated 1 and 6 weeks after starting medication through the questionnaire and data from the general practitioners. If patients returned to their physician with persistent complaints of UTI, the therapy was considered a failure.

Bacteriological outcome was scored as the elimination, persistence, or recurrence of the same or a different microorganism¹¹. No distinction was made between relapse and reinfection because from the standpoint of the patient (and the physician), this distinction is unimportant⁸.

The short term cure rate was defined as the number of patients from whom the initial pathogen was eliminated compared with the number of evaluable patients. The accumulated cure rate was defined as the number of patients in whom cure was noted at the last control visit, compared with the number of evaluable patients¹⁴.

Adverse effects were also evaluated on the basis of the data of the questionnaire and information supplied by the physicians. If medication was stopped because of side-effects, the therapy was considered a failure.

Statistical significance was calculated by means of chi-square analysis.

For an evaluation of the cost, it was assumed that a 3-day course was cheaper unless there was a statistically significant increase in the number of recurrences or a worse clinical outcome in this group. On the other hand, a shorter period of medication could be expected to produce fewer side effects in the 3-day treatment group. Therefore, the number of failures and the number of patients with additional visits to their general practitioners or to the urologist were compared in both groups.

Assuming a short term cure rate of 95% with 7 days of treatment, a type I error (α) of 0.05, a type II error (β) of 0.20, and a clinically important difference between treatments (δ) of 10%, 140 patients in each group are required^{8,11,13,21}.

The randomization was done by Merck Sharp & Dohme (Haarlem, the Netherlands), which also supplied the medication. The code was not known to the investigators, the physicians or the patients. It was kept at the laboratory in a sealed envelope and was broken 6 weeks after the last patient was included.

RESULTS

From April 1989 to October 1990, 395 patients were randomized to 1 of the 2 groups. One hundred ninety-nine were allocated to the 3-day and 196 were allocated to the 7-day treatment. Eleven patients (6 in the short-term and 5 in the long-term group) did not return at all, so 384 patients (193 and 191 respectively) were evaluable. A bacteriologically positive urine sample was obtained from 144 and 141 patients, respectively. *Escherichia coli* was the most frequently isolated organism (104 and 111 respectively), followed by *Staphylococcus* spp. and *Proteus* spp. No differences between the 2 treatment groups were observed (table I).

Table I MICROORGANISMS ISOLATED IN TREATMENT GROUPS.

MICROORGANISMS	TREATMENT GROUP N (%)*	
	3 DAYS	7 DAYS
<i>Escherichia coli</i>	104 (73)	111 (79)
<i>Proteus</i> spp	16 (11)	6 (4)
<i>Staphylococcus</i> spp	15 (10)	16 (11)
other Enterobacteriaceae	9 (6)	5 (4)
<i>Streptococcus</i> spp		3 (2)

* Percentage of positive urine samples

Patient compliance was recorded by using the answers provided by the patients on the questionnaire assessing the presence of growth-inhibiting factors in the urine 1 week after starting medication. The patients reported a 96% compliance; however, in only 76% of the urine samples of the 7-day treatment group was the presence of a growth-inhibiting factor demonstrated.

For the clinical outcome 1 week after starting medication, 348 patients were evaluable, 175 in the short-term treatment group and 173 in the long-term treatment group. No significant differences in clinical outcome were observed between the groups (Table II). Twenty patients (10 in each group) still had complaints of dysuria; 15 of these (7 and 8 patients in the short-term and long-term groups, respectively) returned to their family doctors. Another antibiotic was prescribed to 4 out of the 7 in the first group and to 7 out of the 8 in the second group. Trimethoprim-sulphamethoxazole for 5 to 7 days was then the drug of choice. After the first week without any treatment, 3 patients in the 3-day treatment group and 8 in the 7-day group recovered. Some patients had symptoms only after the first week (but not at the first control visit).

Table II RELIEF OF SYMPTOMS 1 WEEK AND 6 WEEKS AFTER INITIATION.

OUTCOME	TREATMENT GROUP N (%) *		P VALUE
	3 DAYS	7 DAYS	
1 WEEK AFTER INITIATION	175	173	0.13
relief of symptoms	166 (95)	155 (90)	
same or worse complaints	9 (5)	18 (10)	
6 WEEKS AFTER INITIATION	141	137	0.8
relief of symptoms	131 (93)	127 (93)	
same or worse complaints	10 (7)	10 (70)	

* percentage of total number of patients

The bacteriological outcome was assessed as the short-term cure rate and the accumulated cure rate 1 week and 6 weeks after therapy, respectively. The short-term cure rates were 92% and 95% for the 3-day and 7-day groups, respectively ($p=0.30$). The accumulated cure rates were 82% for the 3-day group and 88% for the 7-day group ($p = 0.30$) (table III). Adverse effects (gastrointestinal complaints, followed by headache and fatigue) occurred in 26 (13%) and in 29 (15%) patients respectively ($p=0.79$).

Table III SHORT-TERM AND ACCUMULATED CURE RATES. ASSESSMENT 1 WEEK AND 6 WEEKS AFTER THERAPY.

TIME AFTER START OF THERAPY	3 DAYS	TREATMENT GROUP		3 DAYS	7 DAYS	P VALUE
		TOTAL	N (%)	TOTAL	N (%)	
one week	169	155 (92)		169	161 (95)	0.30
six weeks	152	124 (82)		144	126 (88)	0.30

The 3-day course of treatment was cheaper. Seven patients from the 3-day and 8 patients from the 7-day group returned to their general practitioner. No difference was seen in the follow-up of these patients; most received an antimicrobial agent and none were sent to a hospital for further examination. Attention was also paid to the period of sick leave granted. No sick leave was recorded for women without a paid job. No significant differences were found between the 2 treatment groups. In total, 28 patients had a period of sick leave. The mean number of days of absence from work was 2.8 days in the 3-day group 2.6 days in the 7-day group. The mean number of days of sick-leave per subject (patients without sick-leave included) was 0.4 days.

DISCUSSION

This study observed the recommendations made by Fihn and Stamm¹¹ and the criteria suggested by the Working Party of the British Society of Antimicrobial Chemotherapy²². Even after 6 weeks, the number of patients with clinical symptoms of UTI and with a bacteriologically proven UTI was sufficient to avoid a type II error.

The difference between compliance levels reported by the patients on the questionnaires and those revealed by urinalyses demonstrated that the patients' answers were not fully reliable. This was also mentioned by Urquhart and colleagues²³, who tried to overcome this problem by developing a computerized pillbox to optimize the measurement of patient compliance.

Clinical outcome was considered the most important parameter for the patient. No difference in clinical failure rate was observed between the 2 treatment groups. Similar failure rates have been observed in other studies. Remarkably enough, when the patients (7 and 8 in the short-term and long-term groups respectively) returned to their general practitioner, a different medication was prescribed in 4 and 7 cases, respectively, irrespective of the

presence of significant bacteriuria. A positive urine culture was present in only 2 and 5 patients, respectively.

In the economic evaluation no significant differences between both treatment regimens were observed, either in terms of return to the general practitioner or in terms of sick-leave. The only difference, then was the initial cost of the medication, which was, of course, lower in the shorter treatment course. It was significant that 76% of the patients in the 7-day treatment group (using data on growth inhibiting factors in urine) complied. This means that 24% of the patients do not complete their therapy. It is not known whether they stopped after 1 day or after 5 days, but the fact that so many patients do not complete their antibacterial course suggests that general practitioners could be misled about the efficacy of 7-day treatment programs for UTI.

CONCLUSIONS

The results of this randomized, double-blind study conducted with a sufficient number of patients and adequate follow up, indicate a similar clinical and bacteriological cure rate 1 week and 6 weeks after the start of the medication, the same number of adverse effects, and lower costs for the 3-day treatment. We can conclude from this that a 3-day course of norfloxacin is as effective as, and cheaper than, a 7-day course in the treatment of acute uncomplicated urinary tract infections in women who are not pregnant.

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CHAPTER V

AEROBACTIN AND OTHER VIRULENCE ASSOCIATED FACTORS OF ESCHERICHIA COLI AND INFLUENCE OF SUBMINIMAL CONCENTRATIONS OF FLUOROQUINOLONES.

 

SUMMARY

In the present study the influence of subinhibitory concentrations of three quinolones (norfloxacin, ofloxacin and ciprofloxacin) on virulence associated factors in *Escherichia coli* isolated from asymptomatic bacteriuria (n=70) and cystitis strains (n=45) was described. The virulence associated factors studied were presence of the aerobactin genes, serotype, hemagglutination and the production of hemolysin. No significant differences between the 2 groups of strains with respect to the virulence factors were observed.

A different approach to study the influence of the quinolones on the aerobactin genes was made: aerobactin encoded RNA was detected with a specific DNA probe.

No influence of subminimal concentrations of the 3 quinolones on the aerobactin encoded RNA was observed either on the hemagglutination or on the hemolysin production .

INTRODUCTION

Escherichia coli have to produce one or more virulence factors (VF) to overcome the normal host-defences and to cause disease in hosts¹. In the past VFs studied were adherence², fimbriae³, hemolysin⁴ and O and K serology^{5,6}. In the last few years renewed interest has been shown to another virulence factor, the production of aerobactin⁷. Aerobactin is a specific bacterial siderophore, which extracts iron from the environment. Iron is needed by all living cells⁸ and is therefore a very important factor for the survival of bacteria in the host, including the urinary tract. Sublethal concentrations of antibiotics produced a reduction in siderophore production^{9,10,11}. However, the influence of these concentrations on the aerobactin production have not been studied quite extensively.

Aerobactin can be quantified by the so-called Csàky method¹², which is labourious and in which toxic chemicals are used¹³. A bioassay has also been described, but this method is susceptible to trivial variations in bacterial physiology¹³. Because up till now little is known about the mechanism of the influence of quinolones on the aerobactin production, a specific aerobactin probe¹³ was used to detect aerobactin expressing RNA. Two groups of uropathogenic *Escherichia coli* were studied: i.e. strains isolated from uropregnant women with asymptomatic bacteriuria (ABU) and strains from non-pregnant women with an acute cystitis (CYS). Also the prevalence of 3 other

VF e.g. serology (SER), hemagglutination (HA) and hemolysin production (HLY) were studied, as well as the influence of 3 quinolones on the last 2 VF.

MATERIALS AND METHODS

BACTERIA

Escherichia coli strains isolated from patients with CYS and from pregnant women with ABU were studied. CYS strains (n=45) were defined as follows: bacteria isolated from the urine in a number of $\geq 10^5$ cfu/ml of non-pregnant women, presenting with complaints of dysuria, stranguria, urinary frequency or urgency in the past 24 hours. ABU strains (n=70) were isolated from pregnant women, who visited the antenatal clinic of the University Hospital in Maastricht for a routine screening at a gestational age between 8 and 13 weeks. If $\geq 10^5$ cfu/ml were present in 2 consecutive urine samples the strains were included in the present study.

HEMAGGLUTINATION ASSAY

The hemagglutination assay was performed in microtiter plates as previously described¹⁴. The titers were expressed as the highest dilutions of bacteria showing hemagglutination. Strains were tested for MSHA (mannose sensitive hemagglutination i.e. type I fimbriae) and MRHA (mannose resistant hemagglutination) with guinea pig and human erythrocytes respectively in the presence and absence of mannose. The presence of P fimbriae in MRHA strains was determined by performing a commercially available latex agglutination test (Orion Diagnostica, Finland). MRHA strains not agglutinating were considered to have X fimbriae.

SEROTYPING OF *ESCHERICHIA COLI*

The somatic (O) and capsular (K) antigens were determined using an agglutination assay at the National Institute for Public Health and Environmental Hygiene, Bilthoven, the Netherlands.

HEMOLYSIN PRODUCTION

Hemolysin production was assessed qualitatively in nutrient agar (Oxoid CM3) with 5% sheep erythrocytes. A haemolytic zone larger than the overlying colony was considered as positive.

PURIFICATION OF AEROBACTIN PROBE AND RNA ISOLATION

Plasmid DNA was isolated by the method of Sambrook¹⁵. The aerobactin probe consisted of a 2.2 kb *Ava*I fragment of pABN5¹³ encoding a part of the biosynthesis genes. (The probe was kindly provided by

K.G.Wooldridge). The probe was labeled with digoxigenine according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

RNA was isolated by the guanidine-thiocyanate method (15). In short, bacteria were lysed with lysozyme (2.5 mg/ml) and the RNA was extracted with a solution containing guanidine-thiocyanate (4M), sodium citrate (20mM), β -mercapto-ethanol (0.5%) and sarcosyl (0.5%), purified with phenol-chloroform (49:1) extractions and precipitated with pure ethanol. RNA was then transferred to nylon membranes (Nytran. Schleicher & Schuell, Den Bosch, The Netherlands).

Prehybridization and hybridization were done under stringent conditions at 42°C in formamide 50%, 5*SSC, sarcosyl 0.1%, SDS 0.02%, salmon sperm 0.5% and blocking reagent (Dig kit, Boehringer Mannheim, Mannheim, Germany) with washes in 2*SSC and 0.1% SDS at 68°C.

Detection of digoxigenine labeled nucleic acids was performed by chemoluminescence on radiographic films (Kodak X Omat AR).

ANTIMICROBIAL AGENTS

The antibiotics used were norfloxacin, (Merck, Sharp and Dohme, Rahway, New Jersey, USA) ofloxacin, (Hoechst AG Frankfurt, Germany) and ciprofloxacin (Bayer AG Leverkusen, Germany). The compounds were kindly provided by their respective manufacturers.

INFLUENCE OF ANTIBIOTICS ON VIRULENCE ASSOCIATED PROPERTIES.

To study the effect of subinhibitory concentrations ($\frac{1}{2}$ and $\frac{1}{4}$ MIC) of the quinolones on HA activity an overnight culture of the microorganisms was washed and diluted to a concentration of 10^5 cfu/ml in fresh broth. After 2 hours of incubation at 37°C with shaking the antibiotic was added and the culture was further incubated for 2 hours. Then the bacteria were washed to remove the drug and were adjusted to the concentration of bacteria needed. Subsequently the hemagglutination assay was performed as described above. The influence of the quinolones on the hemolysin production was assessed in blood agar plates containing $\frac{1}{2}$ and $\frac{1}{4}$ MIC of the antimicrobials studied.

To study the influence of the quinolones on the expression of the aerobactin production, 3 P fimbriated CYS and 3 P fimbriated ABU strains were randomly selected. The microorganisms were cultured overnight in the presence of $\frac{1}{2}$ and $\frac{1}{4}$ MIC of the quinolones in iron deficient minimal medium containing 25mM 2,2'dipyridyl (Sigma). Subsequently the RNA isolation was performed as described before.

STATISTICS

Differences in prevalence of VF between the 2 groups were compared by chi-square test. $P < 0.05$ was considered to be significant.

RESULTS

According to the HA pattern, the *Escherichia coli* strains could be classified as P, type I, X or non-haemagglutinating (Table I). The prevalence of type I strains was quite similar among CYS and ABU isolates (i.e. 57% in both groups). In the CYS group more P, X and non-HA strains were observed compared to the ABU group, but the differences were not significant (ns). The mean HA titer of the strains with P adhesins was 1:16 and 1:32 for the CYS and for the ABU group respectively. (Titers are not shown). The mean HA titer of the type I fimbriated strains (1:16 or 1:32) was quite similar for both ABU and CYS strains. For the P fimbriated strains the HA titer in both the CYS and ABU strains (1:8 or 1:16) tended to be lower than in the X fimbriated strains (1:16 or 1:128).

Table I VIRULENCE PROPERTIES OF *ESCHERICHIA COLI*.

VIRULENCE FACTORS		ESCHERICHIA COLI				
		ABU N=70 (%)		CYS N=44 (%)		P VALUE ¹
HEMAGGLUTINATION	mannose sensitive (type I)	40	(57)	26	(59)	NS
	mannose resistant (P fimbriae)	8	(11)	7	(16)	NS
	mannose resistant (X fimbriae)	1	(1)	4	(9)	NS
	no hemagglutination	21	(30)	7	(16)	NS
		14	(20)	13	(30)	NS
HEMOLYSIN PRODUCTION		23	(33)	24	(53)	P<0.05
SEROLOGY (O2,4,6,7,8,16,18,25,75)		25	(36)	20	(45)	NS
AEROBACTIN PRODUCTION						

¹significance by means of χ^2 analysis

NS: non significant

The uropathogenic associated O serotypes (O1, 2, 4, 6, 8, 9, 25, and 75) occurred slightly more in the CYS than in the ABU group (Table I). The O2 serotype was the most prevalent type among CYS strains (20%), 4 strains belonged to the O2K1 serotype. The O6 type was found in 7 out of 70 (10%) ABU strains.

Also the differences in Hly production of ABU strains and CYS strains were not statistically different (19% and 29% respectively) (Table I).

AER positive strains were found among ABU and CYS strains with a prevalence of 36% and 45% respectively. Although more AER positive strains belonged to the CYS group, the difference was not statistically significant ($p > 0.05$) (Table I)

INFLUENCE OF FLUOROQUINOLONES ON VIRULENCE ASSOCIATED FACTORS

The HA titer was not affected by the presence of $\frac{1}{2}$ or $\frac{1}{4}$ MIC of the three quinolones studied. In contrast, subMIC of the compounds used did influence the HLY production. Two CYS and 3 ABU strains, which showed no HLY production in the absence of the quinolones, did so in the presence of each of the 3 agents. In addition, 1 CYS and 4 ABU strains became HLY positive but only in the presence of norfloxacin. Culturing of the ABU ($n=3$) and CYS ($n=3$) strains in the absence or the presence of $\frac{1}{2}$ or $\frac{1}{4}$ MIC of the quinolones studied did not influence the amount of RNA encoding for the expression of aerobactin. (Figure I)

Figure I AEROBACTIN ENCODED RNA INCUBATED WITH AND WITHOUT SUBINHIBITORY CONCENTRATIONS ($\frac{1}{2}$ AND $\frac{1}{4}$ MIC) OF THREE QUINOLONES: NORFLOXACIN (NOR), OFLOXACIN (OFLO), AND CIPROFLOXACIN (CIP) DETECTED WITH A DIGOXIGENIN LABELED DNA PROBE. A POSITIVE (+) (pABN5) AND NEGATIVE (-) (HB101) CONTROL STRAIN WERE INCLUDED IN THE ANALYSIS.

	P40	P89	P116	P331	P577	P714
1/2 MIC NOR	++	++	++	++	++	++
1/2 MIC OFLO	++	++	++	++	++	++
1/2 MIC CIP	++	++	++	++	++	++
1/4 MIC NOR	++	++	++	++	++	++
1/4 MIC OFLO	++	++	++	++	++	++
1/4 MIC CIP	++	++	++	++	++	++
WITHOUT	++	++	++	++	++	++
CONTROL	++ POS	- NEG	++ POS	- NEG	++ POS	- NEG

DISCUSSION

In the present study the prevalence of virulence associated factors studied among CYS strains were lower than those mentioned by other investigators. MRHA were present among 35% and 31% of the strains studied by Hagberg¹⁶ and Sandberg¹⁷ respectively; Dalet¹⁸ and Stenqvist¹⁹ mentioned 20% and 19% respectively, compared to 14% in this study. Hemolysin production was observed in 48% and 49% of the strains studied by Orskov²⁰ and Sandberg¹⁷ respectively. In the present study 29% was found. Also the prevalence of aerobactin producing strains was in the studies of Orskov²⁰ and Carbonetti²¹ slightly higher than in the present study i.e. 56%, 60% and 45% respectively.

It is not very likely that the differences in prevalence observed might be due to differences in methods used. All studies mentioned above including the present one used the same methods for aerobactin production, MRHA, as well as hemolysin production. The variations in data observed might be due to differences in populations studied. In the present paper only outpatient women with complaints of cystitis were studied. Sandberg¹⁷ included patients who were referred to a hospital, Orskov²⁰, Carbonetti²¹ and Hagberg¹⁶ included children in the population studied. Dalet¹⁸ and Stenqvist¹⁹ included acute uncomplicated cystitis strains of adults and pregnant women respectively. The last two studies were similar to the present one, not only in terms of the populations studied, but also in terms of the percentages observed. The data suggest that strains from hospitalized patients and from children are more virulent than from outpatient women. Jacobson²² already described that pyelonephritogenic strains from children were more virulent than the pyelonephritogenic strains from adults. He also found a difference between cystitis strains from children and adults²³. Therefore it might be possible that differences between lower UTI strains isolated from children and from adults do exist.

Similar to the study of Kovarik et al.²⁴ in the present study no effect of subMIC quinolones on the hemagglutination titer was observed. In both studies a correction was made for the number of bacteria used. The effect on the hemagglutination titer found in other studies might be due to a reduction in the number of bacteria used in the HA assay. Most investigators did not correct for the number of bacteria^{25,26,27}.

Although there was a tendency that subMIC norfloxacin might increase the hemolysin production, there was in general no significant influence of norfloxacin, ofloxacin and ciprofloxacin on the hemagglutination, the hemolysin and the expression of the aerobactin genes noticeable.

In the present study no influence of each of the 3 quinolones on the expression of aerobactin encoded RNA in *Escherichia coli* was found. Similar data were obtained by Morris¹¹ with ciprofloxacin and *Pseudomonas aeruginosa*. However, in the same study the use of subMIC of aminoglycosides did reduce the aerobactin production. Similar results were obtained by Courcol⁹ in *Staphylococcus aureus*.

In this study the aerobactin genes and amount of aerobactin encoded RNA was detected with a specific DNA probe instead of using a bioassay (28). A disadvantage of the bioassay is that it does not always work satisfactorily (29). A pitfall might be that many strains could produce colicins - other than aerobactin - to which the test strain is susceptible (instead of aerobactin-resistant). A positive bioassay might be overlooked in these circumstances. An advantage of gene detection is that insight in molecular processes can be obtained. The amounts of aerobactin encoded RNA (and thus the amount of the aerobactin molecule itself produced) can be compared. A comparison between strains incubated with and without quinolones can be made. However, the influence of the quinolones on the amount of aerobactin-encoded RNA detected was not different between the strains incubated with and without quinolones.

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CHAPTER VI

INFLUENCE OF QUINOLONES ON THE ADHERENCE AND PRODUCTION OF P FIMBRIAE OF *ESCHERICHIA COLI*

SUMMARY

The influence of subinhibitory concentrations of norfloxacin on the production and expression of P fimbriae of uropathogenic *Escherichia coli*. The effect of a range of subminimal concentrations of norfloxacin (1/2 to 1/32 MIC) on P fimbriae production and on expression of P fimbrial RNA - was measured with an inhibition ELISA using monoclonal antibodies against the different serotypes of P fimbriae and a P fimbrial DNA probe respectively. Trimethoprim in the same subinhibitory concentrations was used as the control agent. Electron microscopy was used to visualize the presence and the amount of P fimbriae.

Norfloxacin resulted in the ELISA as well as the RNA assay in a decrease of P fimbrial production and expression. Electron microscopy revealed no significant decrease in amount of P fimbriae of the strains tested in the presence trimethoprim or norfloxacin.

Moreover adhesion of *Escherichia coli* strains (n=6) to a T24 uroma celline after exposure to norfloxacin, ofloxacin and ciprofloxacin was tested. Adhesion diminished significantly in five out of six strains in the presence of 1/2 MIC of norfloxacin. Exposure to ofloxacin resulted in a decrease in 1 and in an increase in 2 strains, ciprofloxacin only resulted in a decrease in 1 strain.

In conclusion, rather no influence of ciprofloxacin and ofloxacin on adhesion of *Escherichia coli* to a T24 celline was observed, whereas norfloxacin showed a decrease in adhesion (adherence assay) and in adhesive capacity (ELISA assay and DNA probe) but not in the number of fimbriae (EM). These data suggest, that not the total fimbriae production, but rather the adhesive structure itself is affected by subinhibitory concentrations of norfloxacin.

INTRODUCTION

P fimbriae are the most important group of fimbriae of uropathogenic *Escherichia coli* involved in the pathogenesis of urinary tract infections (UTI)^{1,10,14,17}. The role of these fimbriae in bacterial adherence to uroepithelial cells as well as in facilitating bacterial colonization and invasion into uroepithelial cells is widely accepted now^{6,10,11,12,26}. However, bacterial adherence can be inhibited by subinhibitory concentrations (subMICs) of some antimicrobial agents and thus prevent bacterial colonization and infections².

Most studies on the effect of subMICs on bacterial adhesion have been performed with uropathogenic *Escherichia coli*, human urinary bladder epithelial cells and antimicrobial agents used for the treatment of UTI. In general a decreased adherence in the presence of subMICs was observed although the reversed was found as well^{13,20,22,23,27,28,30}. As to the mechanism of the altered adherence mediated by subMICs, only trimethoprim is known to alter fimbrial production⁴.

Recently, a new group of antimicrobial agents i.e. the fluoroquinolones has been introduced, which can be used for the treatment of UTI^{7,29}. Only few data on the effect of subMICs of these agents on the bacterial adherence of uropathogenic *Escherichia coli* are available¹³.

In this study, the influence of subMICs of norfloxacin (1/2 to 1/32) on the adherence of 14 clinical isolates of *Escherichia coli* to T24 human urinary bladder celline was assessed using P fimbrial monoclonal antibodies. In addition the effect on the P fimbrial production and expression was assessed. Moreover, the effect of subMICs of ciprofloxacin, norfloxacin and ciprofloxacin on the adherence of six of these fourteen strains to the T24 celline were assessed by light microscopy.

MATERIALS AND METHODS

BACTERIAL STRAINS

Escherichia coli strains were isolated from patients suffering from cystitis (CYS) (n=7) or from pregnant women with an asymptomatic bacteriuria (ABU) (n=7). Strains were isolated if $\geq 10^5$ colony forming units (cfu)/ml were present in 1 or 2 consecutive urine samples respectively. Bacteria were isolated and identified as *Escherichia coli* using standard microbiological methods including API20E (APIlab, Montereux, France). The presence of P fimbriae was determined using a commercially available latex agglutination test according to the manufacturers instructions (PF test, Orion Diagnostica, Finland). Strains were cultured on colonization factor antigen (CFA) agar to promote P fimbriae expression.

Seven ABU and 7 CYS strains and 4 reference P fimbriated strains were used in the ELISA assay. Six of these strains (3 ABU and 3 CYS) were also used in the adherence and probe assay (table I).

ANTIMICROBIAL AGENTS

The antibiotics used were kindly provided by their respective manufacturers. The minimum inhibitory concentration (MIC) was determined using a microbroth dilution method in ISO sensitest broth (Oxoid CM 473). Serial twofold dilutions of the antibiotic in ISO sensitest broth were

inoculated with 10^5 cfu/ml bacteria and incubated without shaking at 37°C for 18 hr. The lowest concentration of antibiotic agent that inhibited visible growth was defined as the MIC.

Table I *ESCHERICHIA COLI* STRAINS USED IN THIS STUDY.

STRAIN	REFERENCE	FIMBRIAE	SOURCE	MONOCLONAL ANTIBODIES
C1018	J M de Ree	F9	pyelonephritis	M4-7
C1976		F11	pyelonephritis	M7-7
C1979		F12	pyelonephritis	M14-5
C1212		F7 ₁ , F7 ₂	pyelonephritis	M6-3, M7-5, M2-11
HB101		none		
P40 *		F11	asymptomatic bacteriuria	M7-7
P89 *		--	asymptomatic bacteriuria	--
P116 *		F8	asymptomatic bacteriuria	M17-9S
134		F8	asymptomatic bacteriuria	M17-9S
3030		F11	asymptomatic bacteriuria	M7-7
24		F11	asymptomatic bacteriuria	M7-7
53		F7 ₂	asymptomatic bacteriuria	M2-11
134		F8	asymptomatic bacteriuria	M17-9S
P714 *		--	cystitis	--
P331 *		F11	cystitis	M7-7
P577 *		F7 ₁	cystitis	M6-3
493		F8	cystitis	M17-9S
441		F11	cystitis	M7-7
605		F9	cystitis	M4-7
600		--	cystitis	--

--no fimbriae could be detected with the MAbs available.

* strains used in the adherence and DNA probe assay.

ANTIBIOTIC TREATMENT

To study the effect of subinhibitory concentrations ($\frac{1}{2}$ MIC to $\frac{1}{32}$ MIC) of the antibiotic agent tested an overnight culture of bacteria was washed with PBS and diluted into fresh broth to a concentration of 10^5 cfu/ml. After 2 hours of gentle shaking the antibiotic agent was added and incubated another two hours at 37°C . Then the bacteria were washed again and a suspension containing 10^7 bacteria/ml in fresh broth was used in the adherence assay. For the ELISA and DNA probe assay an overnight culture

without shaking was used, grown in the presence of the appropriate subinhibitory concentration of norfloxacin or trimethoprim if necessary.

ADHERENCE ASSAY.

The method of Daifuku and Stamm³ was performed with slight modifications using the T24 human urinary bladder carcinoma cell line (ATCC HTB-4). In short, nonconfluent monolayers of T24 cells (approximately 10^4 cells) in 6 wells dishes (Costar) were obtained by culturing in MEM (GIBCO) containing 10% fetal bovine serum (GIBCO) in an atmosphere of 5% CO₂ overnight. A suspension containing *Escherichia coli* (diluted from an overnight culture to 10^7 cfu/ml) was added to the T24 cells. After 1 hr incubation at 37°C under gentle shaking the suspension was removed and the cells were rigourously washed at least 5 times with 0.9% NaCl solution, dried, fixed with ice-cold methanol and stained with Giemsa 30% (v/v). The number of bacteria adhered to 25 cells in 1 well were counted by light microscopy. Each experiment was performed in triplicate. So for each bacteria/antibiotic combination 75 cells were counted and the adherence was expressed as the median number of bacteria adhered per cell.

Morphology after 4hr or overnight incubation of treated and non-treated bacteria was assessed by light microscopy.

To control for the number of bacteria used in the assays the Miles and Misra method¹⁵ was used both from a 4 hr and an overnight culture in the presence and absence of the antimicrobial agent.

MONOCLONAL ANTIBODIES.

Monoclonal antibodies (MAbs) against F7₁, F7₂, F8, F9, F11 and F12 fimbriae were produced and described by de Ree et al.¹⁶

WHOLE BACTERIA ELISA.

An ELISA with whole bacteria as antigen was carried out as described by de Ree et al.¹⁶. Briefly, bacteria were coated to microtiter plates and preincubated with 1% w/v BSA followed by incubation of MAbs. Antifimbriae activity was detected colorimetrically after incubation with goat-anti-mouse antiserum, conjugated with peroxidase and addition of the substrate o-phenylenediamine. Absorbance at 495 nm was determined with a Titertek multiscan ELISA plate reader. Cross reactions of the MAbs against other fimbriae were checked as described by de Ree¹⁶. Strain HB101 was used as a negative control strain. Results of the ELISA assay were given as the optical density (OD) minus the control value of MAbs incubated without bacteria.

PURIFICATION OF P FIMBRIAE PROBE AND RNA ISOLATION.

Plasmid DNA was isolated by the method of Maniatis¹⁹. The P fimbrial probe consisted of a 7.3 kb ClaI fragment of AM1727 pPil 288-10¹⁶ encoding part of the fimbrial genes. (The strain was kindly provided by J.de Ree). The

physical map of the gene cluster encoding F9 fimbriae of pPil 288-10 is shown in Figure I. The subunit encoding the adhesin, called minor fimbrellins, are included. The major pilin subunit is not required for binding and was excluded from the probe fragment used in this assay. The probe was labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany).

Figure I RESTRICTION MAP OF pPIL 288-10 (24 KB). THE SOLID BLACK BAR REPRESENTS THE VECTOR pJB8, THE STRIPED BAR THE PART ENCODING THE MAJOR FIMBRILLIN, THE DOTTED BAR THE REST OF THE GENE CLUSTER ENCODING THE FIMBRIAE AND THE PART OF pPIL 288-10 THAT WAS USED AS A PROBE IN THIS ASSAY. THE BLANK BAR REPRESENTS THE PARTS OF pPIL 288-10 NOT USED.

RNA was isolated by the guanidine-thiocyanate method¹⁹. In short, bacteria were lysed with lysozyme (2.5 mg/ml) and the RNA was extracted with a solution containing guanidine-thiocyanate (4M), sodium citrate (20mM), β -mercaptoethanol (0.5%) and sarcosyl (0.5%), purified with phenol-chloroform (49:1) extractions and with pure ethanol. RNA was then transferred to nylon membranes (Nytran, Schleicher & Schuell, Den Bosch, The Netherlands). Purity of RNA was checked by gelelectrophoresis and OD 280:260 ratios.

Prehybridization and hybridization were done under stringent conditions at 42°C in formamide 50%, 5*SSC, sarcosyl 0.1%, SDS 0.02%, salmon sperm 0.5% and blocking reagent (Dig kit, Boehringer Mannheim, Mannheim, Germany) with washes in 2*SSC and 0.1% SDS at 68°C. Detection of digoxigenin labeled nucleic acids was performed by chemoluminescence on radiographic films (Kodak X Omat AR).

AM1727 pPil288-10 (16) and HB101 were used as the positive and negative controls respectively. The amount of RNA was assessed semiquantitatively.

ELECTRON MICROSCOPY

Escherichia coli P 577 was streaked on CFA agar plates with and without norfloxacin (1/2 to 1/32 MIC) and trimethoprim (1/4 and 1/8 MIC). After overnight incubation colonies were suspended in Ammoniumacetate (0.1 M, PH 6.8) and one drop of this suspension was placed on carbon-plated

ERRATUM

Op pagina 79 is de restriction map van pPIL288-10 weggevalen.

copper grids and allowed to stand for a few minutes at room temperature; the excess suspension was drained off, and the adherent bacteria were negatively stained with methylamine tungstate (Biorad) for 5 seconds. Grids were examined by electron microscopy (EM) (Philips CM10) at 80 kV.

STATISTICS.

Difference between proportions were tested with the chi-square test for independent proportions, using a two-sided confidence level of 5%.

For the adherence assay the median instead of the mean was used because of a non-Normal distribution¹⁸. The nonparametric Mann-Whitney-U test²² was applied to the results of the adherence assay.

RESULTS

The MIC of the *Escherichia coli* strains used ranged from 0.063 to 0.125 $\mu\text{g/ml}$ for norfloxacin, for ofloxacin the MIC was for all but 1 strain (P139) 0.063 $\mu\text{g/ml}$, for ciprofloxacin the MIC ranged from 0.004 to 0.016 $\mu\text{g/ml}$, and for trimethoprim the MIC ranged from 0.25 $\mu\text{g/ml}$ to 64 $\mu\text{g/ml}$.

After incubation with different concentrations of the 3 quinolones during 2 and 24 hours rather no morphological changes were visible by light microscopy. Only incubation with $\frac{1}{2}$ MIC norfloxacin during 2 and 24 hours revealed bacterial elongation in all 6 strains.

Viable counts revealed no differences between antibiotic exposed strains as compared with the control one.

Figure II INFLUENCE OF SUBMICS OF NORFLOXACIN IN AN ELISA USING MONOCLONAL ANTIBODIES AGAINST P FIMBRIATED BACTERIA. MONOCLONAL ANTIBODIES USED ARE LISTED IN TABLE I.

Figure III INFLUENCE OF SUBMICS OF TRIMETHOPRIM IN AN ELISA USING MONOCLONAL ANTIBODIES AGAINST P FIMBRIATED BACTERIA. MONOCLONAL ANTIBODIES USED ARE LISTED IN TABLE I.

The results of the whole bacteria ELISA of the strains exposed to norfloxacin and trimethoprim are presented in figures II and III respectively. Concentrations from 1/32 to 1/2 MIC of norfloxacin and trimethoprim resulted in almost all strains in a decrease in OD₄₉₅. If an effect was observed with trimethoprim a similar decrease was observed with norfloxacin. In most cases the amount of P fimbrial RNA decreased correspondingly (Table II). The effect was most pronounced with 1/4 MIC and 1/8 MIC of norfloxacin and 1/4 MIC of trimethoprim.

No differences in total amount of fimbriae of strain P577 was observed using electron microscopy in the presence of (1/2 to 1/32 MIC) of trimethoprim or norfloxacin compared to the control without antibiotic.

All 6 *Escherichia coli* strains tested in the adherence assay adhered quite well to T24 uroepithelial cells (Table II). Differences between the strains, with respect to the median number of bacteria adhering to the T24 uroepithelial cells, did occur. The median number of bacteria ranged from 36 to 191 bacteria per uroepithelial cell for CYS strains and from 22 to 45 bacteria per cell for ABU isolates.

In all strains, except one ABU strain, adherence decreased significantly in the presence of 1/2 MIC of norfloxacin. Ciprofloxacin gave a significant decrease in only one P fimbriated strain. The presence of ofloxacin resulted

in one CYS strain in a decrease of adherence, an increase in adherence was observed in two ABU isolates (Table III).

Table II INFLUENCE OF TRIMETHOPRIM AND NORFLOXACIN ON THE RELATIVE AMOUNT OF RNA DETECTED WITH A SPECIFIC P FIMBRIAL DNA PROBE.

MIC ($\mu\text{g/ml}$)	STRAINS					
	40	89	116	331	577	714
	SEROTYPE					
	F11	--	F8	F11	F7 ¹	--
0	++	++	++	++	++	++
1/32 norfloxacin	++	+	++	+	+	+
1/16 norfloxacin	-	+/-	+/-	+/-	-	+/-
1/8 norfloxacin	-	+/-	-	-	-	-
1/4 norfloxacin	-	-	-	-	-	-
1/2 norfloxacin	+/-	+	-	++	+/-	+/-
1/4 trimethoprim	+/-	+/-	+/-	+/-	+	+
	CONTROL STRAINS					
	HB101	CI018				
0	-	+++				
1/4 trimethoprim		+				

+++, ++, +, +/-, - are the relative amounts of RNA detected.

Table III THE MEDIAN OF THE NUMBER OF BACTERIA ADHERED TO 75 UROMA T24 CELLS, IN THE ABSENCE AND IN THE PRESENCE OF THE INVESTIGATED QUINOLONES.

STRAINS	WITHOUT 1/2 MIC NORFLOXACIN	1/2 MIC CIPROFLOXACIN	1/2 MIC OFLOXACIN
1281 P577	57	6 ¹	42
139 P331	36	2 ¹	35
889 P714	191	62 ¹	60 ¹
590 P89	22	16 ¹	19
4620 P116	45	26 ¹	38
4313 P40	35	26	22
			82 ²

¹ p < 0.05 DEcreased adherence in the presence of 1/2 MIC of the investigated quinolone.

² p < 0.05 INcreased adherence in the presence of 1/2 MIC of ofloxacin

DISCUSSION

In the present study a decrease in adherence of CYS and ABU *Escherichia coli* strains to uroepithelial cells was observed in the presence of norfloxacin.

An important issue is the way the results were analyzed. Rosenstein¹⁸ reported in a meta-analysis of adherence assays and observed that bacteria adhered to epithelial cells in a non-normal way. It meant that bacteria adhered to some epithelial cells with large numbers, while other epithelial cells had only small numbers of adhering bacteria. Therefore in the analysis of the results a non-parametric analysis applied to medians has to be used. Also in the present study the adherence of the strains showed a non-normal distribution, so the results were analyzed applying the Mann-Whitney-U-test²² because a parametric test was not the correct choice¹⁸.

With respect to the results of the ELISA an obvious decrease of the OD in the presence of subMICs of quinolones was observed. The monoclonal antibodies used in this assay are directed against the adhesin protein of the fimbriae. The probe used for the detection of the P fimbrial RNA is directed against all genes involving fimbrial production except the gene encoding for the major fimbrillin part. In both assays a concentration dependent influence of subMICs of norfloxacin was observed. Both assays can detect the influence on the adhesin part, therefore the results of the present study suggest an influence of norfloxacin on the adhesin of the fimbriae, instead of an influence on the presence of the fimbriae. Absence of fimbriae in the presence of subMICs are not confirmed with our data of EM, because EM did not show any difference between the amount of fimbriae of bacteria cultured in the presence and absence of subMICs of antimicrobial agents. However if the adhesin itself was influenced, as suggested by the results of the ELISA and RNA assay, this could not have been detected with EM. Further analysis to the possible mechanism of its decreased adherence in the presence of subMICs of trimethoprim or norfloxacin suggests furthermore a strain dependent effect. The effect of the subMICs was not in all strains equal. A possibility is that the serotype might influence the effect, although from the strains tested no such effects were observed.

With respect to the results of the other quinolones in the adherence assay as assessed with light microscopy rather no influence with ofloxacin and ciprofloxacin was observed. Although the 3 quinolones are structurally and chemically related, there are differences lipophilicity and in antibacterial

activity. It might be possible that these differences play a role in the differences in effect observed in the adherence assay.

In conclusion the results of this study indicate that subMICs of norfloxacin decreases the adhesion of *Escherichia coli* strains to uroepithelial cells. The data of the ELISA and RNA assay and the electron microscopic studies suggest that this influence is directed against the adhesive part itself of the P fimbriae. However a strain dependent influence was observed.

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CHAPTER VII

GENERAL DISCUSSION

INTRODUCTION

This thesis describes factors influencing the efficacy of antimicrobial agents in urinary tract infections (UTI): the bacterial susceptibility, the optimal treatment duration and the effect of subinhibitory concentrations of antimicrobial agents on bacterial virulence factors.

BACTERIAL RESISTANCE

The results of the antibiotic susceptibility testing of 1200 uropathogens isolated in the south of the Netherlands in the period from 1989 until the beginning of 1991 showed that the susceptibility rates of *Escherichia coli* isolates to sulfamethoxazole and amoxycillin were 78% and 60% respectively, and to trimethoprim, co-trimoxazole, nitrofurantoin and norfloxacin 88%, 88%, 93% and 100% respectively. These data illustrate the general trend that the longer an antimicrobial agent has been used the lower the susceptibility rates of the bacteria are. One example is norfloxacin, which has been introduced recently. Although a decrease in susceptibility rates of *Escherichia coli* to 95.6% has occurred in the period from 1988 to 1991 in Spain¹, where norfloxacin has been used more extensively than in the Netherlands, the susceptibility rates in the present study are still about 100%. Sulfamethoxazole and amoxycillin have both been prescribed for a longer period and consequently the susceptibility of *Escherichia coli* to those agents has substantially declined. The still high percentage of bacteria susceptible to nitrofurantoin is an exception to this rule². This might be explained by pharmacokinetic properties: two third is rapidly metabolized, the other one third is excreted unchanged into the urine, and as a consequence the bowel flora is not exposed³. Since the general concept is that only in the bowel the sufficient numbers of bacteria are present that are required for the development of resistance, resistance might hardly develop⁴.

It is obvious that the results of antibiotic susceptibility surveys are relevant to general practitioners. Of importance are the observed differences between regions. A study from the National Institute of Public Health (RIVM), performed in the middle of the Netherlands in 1989, showed antibiotic susceptibility rates for amoxycillin, sulphamethoxazole, trimethoprim, co-trimoxazole and nitrofurantoin of 66%, 55%, 76%, 76% and 97% respectively⁵. These percentages differ from the present results, in that the susceptibilities of *Escherichia coli* isolates from UTI are lower than the susceptibilities to sulphamethoxazole, trimethoprim and co-trimoxazole found in the present study. However, a comparison with the study by Stobbe-

ringh⁶, a study performed in the south of the Netherlands from 1987 to 1988, shows an overall similar pattern of the susceptibilities as observed in the present study. This indicates a certain stability of susceptibilities within one region, although minor differences in susceptibilities do occur. Gruneberg⁷ observed in his studies on resistance development important trends over a period of 20 years. However between two consecutive years only minor differences were observed, suggesting that changes develop over a period of years. The present study and the studies mentioned above illustrates that to provide the general practitioner with relevant data on susceptibility patterns, prospective methodological studies should be performed. The surveillance should include representative collections of strains, performed regularly and, because regional differences do occur, set up on a regional basis. In addition it is important to study in depth the types and clonality of bacteria and the mechanisms of resistance by application of molecular biological techniques. The data from these studies, especially when related to the use of antimicrobial agents, might provide means for early detection of resistance development⁸⁻¹⁴.

On the basis of the susceptibilities of the uropathogens as shown in the present study, amoxycillin and sulphamethoxazole can not be recommended as first choice agents. Instead trimethoprim, co-trimoxazole and nitrofurantoin should be preferred, despite the fact that more adverse effects might be expected with the use of nitrofurantoin and co-trimoxazole. With respect to the use of trimethoprim, however, the reports in the literature strongly indicate that the sulpha-component accounts for the majority of adverse effects, whereas it adds little to the antibacterial activity¹⁵. It might therefore be preferable to prescribe trimethoprim instead of co-trimoxazole for UTI. In the present situation norfloxacin should not be used, but reserved for treatment failures or in case of documented resistance. With this approach resistance development against norfloxacin might be delayed.

THREE DAYS' TREATMENT

As discussed in chapter I valid conclusions could not be drawn from the majority of studies on therapeutic efficacy because they did not fulfill the recommendations on clinical evaluation of antimicrobial agents in randomized controlled trials as made by the Working Party of the British Society of Antimicrobial Chemotherapy and Philbrick et al.^{16,17}. The most frequent requirement not fulfilled was the inclusion of a sufficient number of patients: if the number of patients was too small a true difference could not be detected¹⁸.

Statistical calculations indicated that 280 patients were sufficient to reach the required power in our study¹⁸. The number of patients included in the present study was based on the intention to treat method. The patients were included in the trial by the general practitioner on the basis of clinical signs of UTI and received medication and all patients had to be evaluated at the end of the trial. It was anticipated that a number of included patients would be lost during the follow-up period. Therefore the number of patients included was higher than the 280 that were required as shown by the calculations. The number of patients finally included in the two studies was 327 and 384 respectively, and 280 and 290 patients were finally left for evaluation.

Another question to discuss is that not in all patients infection could be verified by a positive urine culture and it might therefore be expected that these patients did not have an UTI. Moreover at least two considerations demand that they remain included in the study group. First, from a statistical point of view all manipulations of the treatment groups after randomization have the danger to invalidate the data²². Second, the inclusion criteria of the patients are complaints of an acute UTI and in this respect the evaluation was done. For studies in general practice it might be emphasised, that this is the only relevant approach. Moreover, routine urine cultures may be false negative and therefore a clearly symptomatic patient is a candidate for treatment even in case of a negative culture.

Fihn et al.²⁰ compared a single dose versus a 10 days' course of co-trimoxazole, in a trial with a sufficient number of patients and concluded that a single dose co-trimoxazole was less effective than a 10 days' course, and suggested that a trial with a three days' course should be conducted. Also Norrby²¹ concluded, that single-dose treatment was less effective than a seven to ten days' course of antimicrobial therapy for the treatment of acute uncomplicated urinary tract infections. His recommendations included that a three days' course of co-trimoxazole and the fluoroquinolones should be studied. Therefore the present studies tested co-trimoxazole as well as norfloxacin, comparing a three versus a seven days' course. The results obtained in both studies indicate that a three days' course of antimicrobial treatment is as effective as a seven days' course in the treatment of uncomplicated urinary tract infection in non-pregnant female patients in general practice. Based on the literature and on our results a three days course of antimicrobial agents can therefore be recommended in uncomplicated UTI, although extrapolations to other than the investigated antimicrobial agents should be made carefully²¹⁻²³.

A three days' course with co-trimoxazole, trimethoprim and nitrofurantoin as first choice agents, is also recommended by the Dutch General Practitioners Association (NHG)²⁴. However the National Organization for Quality Insurance in Hospital (CBO) and the Drug Bulletin (Geneesmiddelenbulletin)^{25,26} favor a single dose treatment whereas in the latter especially amoxycillin and fosfomycin trometamol are recommended. For amoxycillin, reviewed by Philbrick¹⁷, sufficient data are available to support this approach. However, resistance percentages to this antimicrobial agent in the present study of 30-40% in uropathogens derived from uncomplicated urinary tract infections in general practice, indicate that this approach is not applicable in all situations. Fosfomycin trometamol might be an adequate medication as a single dose agent, but data from a randomized controlled trial with a sufficient number of patients in general practice to prove its efficacy, are not yet available.

A shorter treatment course reduces costs, the exposure time to the antimicrobial agent and the number of adverse effects and warrant a better patient compliance²⁷. A considerable advantage is also the shorter exposure time of the bacteria in the bowel flora to the antimicrobial agents, reducing the likelihood on resistance development.

In conclusion the two present studies indicate and further substantiate the more and more accepted approach, as mentioned before, that a three days' course of antimicrobial treatment is probably an optimal treatment for acute UTI in general practice.

SUBINHIBITORY CONCENTRATIONS

It has been observed that in general subinhibitory concentrations of antimicrobial agents do effect bacteria, among others their virulence factors. In UTI it has been shown that important virulence factors are adherence, P fimbriae, hemolysin and aerobactin production.

Inhibition or suppression of hemolysin and aerobactin production due to the subinhibitory concentrations of antimicrobial agents tested could not be demonstrated in this study.

The published results indicate that trimethoprim influences the adherence and the production and expression of P fimbriae^{29,30}. Since little information was available on the effect of subinhibitory concentrations of fluoroquinolones on adherence, three of the newer quinolones, norfloxacin, ofloxacin and ciprofloxacin were studied in comparison with trimethoprim.

With respect to the effect of these quinolones on adhesion the results indicated that norfloxacin diminished the adherence of *Escherichia coli*. A

similar inhibitory effect of ciprofloxacin and ofloxacin could however not be demonstrated, however the number of strains investigated was limited and data in literature indicate that strains do vary in their susceptibility to subinhibitory effects. Apparently the similarity in structure and mechanism of action of the three quinolones does not exclude differences in antimicrobial effects³⁰.

Further analysis of the influence of norfloxacin on adherence by means of a P fimbrial probe indicated that the amount of P fimbrial RNA isolated from bacteria treated with subinhibitory concentrations of norfloxacin was reduced. Using monoclonal antibodies against P fimbriae in an ELISA also indicated that P fimbrial adhesion was diminished as a result of subinhibitory concentrations of norfloxacin. Electron microscopic studies showed that the density and the number of fimbriae in comparison to untreated bacteria was not reduced³¹.

The clinical significance of the use of subinhibitory concentrations of antimicrobial agents is the prevention and prophylaxis of UTI. In this respect a number of studies are of interest. In a study by Ben Redjeb³², patients with acute uncomplicated UTI were treated with subinhibitory concentrations of amoxycillin and a cure rate of 80% was observed. The value of this observation is, however, limited because of the relatively small number of patients. In addition, other studies on prophylaxis of UTI showed that UTI could be prevented with subinhibitory concentrations of several antimicrobial agents³³⁻³⁶. In the light of the present findings with norfloxacin it seems therefore of interest to explore further the efficacy of norfloxacin in low doses for the prevention and prophylaxis of UTI.

GENERAL CONCLUSIONS AND RECOMMENDATIONS

With respect to the factors influencing the efficacy of antimicrobial agents in urinary tract infections described in this thesis the following conclusions and recommendations can be made.

The most important recommendation concerns the choice of the antimicrobial agent made in the light of the antimicrobial susceptibilities of the uropathogens. A three days' treatment with trimethoprim or cotrimoxazole as first, nitrofurantoin as second and norfloxacin as third are the preferred choices. Sufficient data are available to support this, and it seems worthwhile to study a short-term course with nitrofurantoin. A single-dose treatment with amoxycillin could also be supported, but care must be taken because of the high percentage of resistant uropathogens.

With respect to the prevention of UTI sufficient data from the literature and the present study support the use of trimethoprim, co-trimoxazole, norfloxacin and to a lesser degree of amoxycillin in low doses for the prophylaxis of UTI. With respect to norfloxacin it should not be recommended as long as trimethoprim and nitrofurantoin are still effective for therapy.

As previously indicated it is strongly recommended to establish surveillance stations, which at regular intervals collect a sufficient and representative number of strains to study bacterial susceptibilities. Recommendations for initial therapy to general practitioners should be made on the basis of these susceptibility rates. In addition studies in depth with a limited number of selected strains should be performed to elucidate the epidemiology of the strains and the mechanisms of resistance. These data might provide a more anticipatory approach of the use of antimicrobial agents in UTI.

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SUMMARY

Urinary tract infections (UTI) are very common, especially in women. The infections are especially encountered in general practice, and remain a source of medical research. In this dissertation attention is paid to a number of factors that are important to the treatment of UTI with antibiotics. As a great deal has already been written on UTI, only the subjects relevant to this study are introduced in chapter I, namely the epidemiology of UTI, the defence mechanisms of the host, the virulence factors of *Escherichia coli* and the treatment of UTI.

In the majority of the cases the microorganism that causes UTI in individual patients in general practice is unknown. This means that the antimicrobial treatment is prescribed blindly, so without the general practitioner knowing the susceptibilities of the bacteria to antibiotics. Therefore epidemiological knowledge of the anticipated microorganisms with their susceptibilities to antibiotics is of the outmost importance to a successful treatment and, as such, also to curing the patient.

In chapter II we describe the research into the susceptibilities of the bacteria isolated from urine samples of women who saw their general practitioner with acute UTI complaints in the Maastricht area. The most common causative agent of UTI in general practice is *Escherichia coli* (80%). The susceptibilities to the antibiotic amoxycillin and sulphamethoxazole is so low, 60% and 78% respectively, that starting treatment blindly is too likely to fail. The susceptibilities of the bacteria to trimethoprim, co-trimoxazole, nitrofurantoin and norfloxacin is better, varying from 88 to 100%.

Besides similar percentages, also differences in susceptibilities are found in literature. An important cause of the differences in the literature found is the decreasing susceptibility in time as a result of acquiring resistance in the bacteria investigated. Also there are differences between countries and even between areas in one country. Therefore it is advisable to monitor the prevalence of resistance to antibiotics at regular intervals with the help at surveillance stations in the various areas in a country. Besides, research should be done into the underlying mechanism of resistance with the help of molecular biological techniques.

With respect to the treatment-advice for general practice this means that on the basis of our susceptibilities trimethoprim, co-trimoxazole, nitrofurantoin and norfloxacin are a good choice to start with blindly. However, as a part of the prevention of resistance to antibiotics it is advisable to restrict the use of norfloxacin to those cases where other antibiotics fail.

The length of the treatment also plays a role in the development of resistant bacteria. Bacteria acquire their resistance to antibiotics through mutation. In the large intestine sufficient bacteria ($>10^9$) are present for mutation to take place. The longer the treatment lasts, the longer concentrations of antibiotics are present in the large intestine and the greater a chance of a resistant mutant. So, if a shorter treatment can be given, this is also desirable with respect to the prevention of resistance.

In literature there is a lack of well-designed studies on short-term treatment for the various antibiotics carried out with a sufficient number of patients. In chapter III and VI the double-blind randomised study into the effectiveness of a three days' treatment is described, with the antibiotics co-trimoxazole and norfloxacin, research that was carried out with a sufficient number of patients. It appeared that a three days' course, with co-trimoxazole as well as with norfloxacin was as effective as a seven days' course to cure patients with acute uncomplicated UTI.

Not only the development of resistance, but also the compliance, side-effects and costs are thus affected favourably.

Not only shortening of the treatment period but also the dosage of the antibiotic is worth investigating. Since the time treatment with antibiotics was started it has been known that antibiotics in subinhibitory concentrations can affect bacterial growth without really killing them. It is possible that virulence factors of the bacteria, which are relevant to the adherence are affected by subinhibitory concentrations in such a way that the bacteria are not able to cause UTI any more.

In chapter V and VI the research into the influence of subinhibitory concentrations of three quinolones, namely norfloxacin, ofloxacin and ciprofloxacin, on some virulence factors of *Escherichia coli* is described. The virulence factors studied are: the hemolytic activity, the adherence, P fimbriae and the production of aerobactin which all play an important role in the development of UTI.

The aerobactin production and the hemolytic activity were not affected by subinhibitory concentrations of these antibiotics. The quinolones did cause a decreased adherence of bacteria to uroepithelial cells. Also the adherence of P fimbriae was reduced by norfloxacin. Electron microscopic pictures showed no reduction or absence of fimbriae, however. Probably norfloxacin affects the receptors at the ends of the P fimbriae without preventing all production of fimbriae.

So the most important step in the development of UTI, namely the adherence of bacteria to the epithelium, is decreased by the presence of subinhibitory concentrations of quinolones. Considering this, prophylaxis of

UTI by giving subinhibitory concentrations of quinolones could be envisaged. However further research is necessary here.

In the concluding chapter the results as presented in the chapters II to VI are examined and discussed and recommendations are made as mentioned in this summary.

SAMENVATTING

Urineweginfecties (UWI) zijn een zeer vaak voorkomende kwaal, vooral bij vrouwen. Deze worden met name gezien in de huisartsenpraktijk, en blijven een bron van onderzoek voor de medische wereld. In dit proefschrift wordt aandacht besteed aan een aantal factoren die van belang zijn bij de behandeling van UWI met antibiotica. Omdat er al zeer veel geschreven is over UWI wordt in het eerste hoofdstuk alleen die onderwerpen ingeleid, die van belang zijn voor dit onderzoek, te weten, de epidemiologie van UWI, de verdedigingsmechanismen van de gastheer, de virulentiefactoren van *Escherichia coli* en de behandeling van UWI.

In de meerderheid van de gevallen is het microorganisme, dat de UWI bij individuele patiënten in de huisartsenpraktijk veroorzaakt niet bekend. Dat betekent, dat de antimicrobiële therapie blind wordt voorgeschreven, dus zonder dat de huisarts de antibioticagevoeligheden van de bacterie weet. Epidemiologische kennis van de te verwachten microorganismen met hun antibiotica-gevoeligheden is dan ook van het grootste belang voor het slagen van de therapie en als zodanig ook voor de genezing van de patient.

In hoofdstuk 2 wordt het onderzoek beschreven dat werd verricht naar de gevoeligheden van de bacteriën, geïsoleerd uit urines van vrouwen die met klachten van acute UWI bij hun huisarts in de regio Maastricht kwamen. De meest voorkomende verwekker van UWI in de huisartsenpraktijk is de *Escherichia coli* (80%). De gevoeligheden van deze bacteriën voor de antibiotica amoxycilline en sulfamethoxazole is zodanig laag, 60% en 78% respectievelijk, dat blind starten met therapie een te grote kans op falen geeft. Voor trimethoprim, co-trimoxazole, nitrofurantoin en norfloxacin is de gevoeligheid van de bacteriën beter, variërend van 88% tot 100%.

In de literatuur worden naast overeenkomstige, ook verschillen in percentages gevoeligheden gevonden. Een belangrijke oorzaak van de gevonden verschillen is de afnemende gevoeligheid in de tijd ten gevolge van het verwerven van resistentie in de onderzochte populatie. Ook is er verschil tussen landen en zelfs tussen regio's onderling. Daarom is het goed om de prevalentie van antibioticaresistentie met geregelde tussenpozen te monitoren met behulp van peilstations in de verschillende regio's binnen een land. Bovendien zou met behulp van moleculair biologische technieken onderzoek gedaan moeten worden naar de onderliggende mechanismen van resistentie.

Met betrekking tot het therapieadvies voor de algemene praktijk betekent dit dat trimethoprim, co-trimoxazole, nitrofurantoin en norfloxacin op basis van deze gegevens goede keuzes zijn om blind mee te starten. Echter in het kader van het voorkómen van antibioticaresistentie is het raadzaam het gebruik van norfloxacin te reserveren voor die gevallen waarin andere antibiotica falen.

Ook de duur van de therapie speelt een rol bij het ontstaan van resistente bacteriën. Bacteriën verwerven hun resistentie tegen antibiotica via mutaties. In de dikke darm zijn voldoende bacteriën ($>10^9$) aanwezig, om mutaties plaats te laten vinden. Des te langer de therapie duurt, des te langer antibioticaconcentraties in de dikke darm aanwezig zijn, en des te groter dus de kans op een resistente mutant. Als dus een kortere kuur gegeven kan worden, is dit dan ook wenselijk met betrekking tot het voorkómen van resistentie. De literatuur over kortere kuren vertoont echter nog een gebrek aan goed opgezette studies voor de verschillende antibiotica uitgevoerd met een voldoende aantal patiënten.

In hoofdstuk 3 en 4 wordt het dubbelblind gerandomiseerd onderzoek beschreven, met de antibiotica co-trimoxazole en norfloxacin, dat werd verricht met een voldoende aantal patiënten naar de effectiviteit van een driedaagse kuur. Hieruit bleek dat een driedaagse kuur, zowel met co-trimoxazole alsook met norfloxacin, even effectief was als een zevendaagse kuur om patiënten met een acute ongecompliceerde UWI te genezen.

Niet alleen de resistentieontwikkeling, maar ook de therapietrouw, bijwerkingen en de kosten worden zo in gunstige zin beïnvloed.

Net zoals verkorting van de therapieduur is ook de dosering van het antibioticum onderzoek waard. Al sinds de tijd dat antibiotica gebruikt worden is het bekend, dat antibiotica in subinhibitoire concentraties bacteriën in hun groei kunnen beïnvloeden, zonder dat ze daardoor werkelijk gedood worden. Het is mogelijk, dat virulentiefactoren van de bacteriën, die van belang zijn bij de aanhechting, door de subinhibitoire concentraties zodanig beïnvloed worden, dat de bacteriën niet meer in staat zijn om een UWI te veroorzaken.

In hoofdstuk 5 en 6 wordt dan ook het onderzoek beschreven naar de invloed van subinhibitoire concentraties van drie quinolonen, te weten norfloxacin, ofloxacin en ciprofloxacin, op enkele virulentiefactoren van *Escherichia coli*. De onderzochte virulentiefactoren zijn, de hemolytische activiteit, de aanhechting, P fimbriae en de aerobactine productie, welke allen een belangrijke rol spelen bij het ontstaan van UWI.

De aerobactine productie en de hemolytische activiteit werden niet beïnvloed door subinhibitoire concentraties van deze antibiotica. De quinolonen veroorzaakten echter wel een verminderde aanhechting van bacteriën aan uroepitheelcellen. Ook de P fimbriae werden in hun aanhechting verminderd door norfloxacin. Electronenmicroscopische opnames lieten echter geen vermindering of afwezigheid van fimbriae zien. Waarschijnlijk heeft norfloxacin dus een invloed op de receptoren, die aan het uiteinde van de P fimbriae zitten, zonder de gehele fimbriaeproductie te verhinderen.

De belangrijkste stap in het ontstaan van UWI, namelijk de aanhechting van bacteriën aan het epitheel, wordt dus verminderd door de aanwezigheid van subinhibitoire concentraties quinolonen. In dit kader zou de profylaxe van UWI door het geven van subinhibitoire concentraties quinolonen overwogen kunnen worden. Hiervoor moet echter nog nader onderzoek gedaan worden. Ter afsluiting worden in het laatste hoofdstuk de resultaten, zoals die beschreven zijn in de hoofdstukken 2 tot en met 6, besproken en bediscussieerd en worden aanbevelingen gedaan zoals die ook in deze samenvatting vermeld staan.

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En toen was het af. Echter dit onderzoek is zeker niet alleen door mij tot stand gekomen. Vele mensen hebben aan dit onderzoek een bijdrage geleverd. Enkele hiervan wil ik met name noemen en hiervoor bedanken.

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Nancy, die eerst als stagiaire met plasjes, later zelf als AIO met poepjes werkzaam, altijd voor een goede sfeer op het lab zorgde, zeker bij het gezamenlijk formulieren invoeren, en die met haar eerlijkheid soms onverwachte reacties opriep.

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CURRICULUM VITAE

De schrijfster van dit proefschrift is op 9 januari 1961 in Tegelen geboren. In 1979 behaalde ze het Gymnasium- β diploma aan het Collegium Marianum te Venlo.

Na haar eindexamen, uitgeloot voor geneeskunde, studeerde ze een jaar Pedagogiek aan de Rijks Universiteit Leiden, waarna ze overstapte naar de HBO-A analistenopleiding aan de Analistenschool te Venlo.

In 1981 werd ze ingeloot voor geneeskunde aan de Rijksuniversiteit Limburg in Maastricht. In 1988 werd het artsexamen behaald en kreeg ze een baan bij de vakgroep Medische Microbiologie in Maastricht. Eerst als part-time onderzoeker, vanaf april 1989 als assistent in opleiding, werkte ze aan het onderzoek, dat in dit proefschrift beschreven is.

Vanaf april 1993 is ze in opleiding tot medisch microbioloog bij professor C.P.A. van Boven in het Academisch Ziekenhuis Leiden.