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Antibiotic resistance in the non-hospital environment

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ANTIBIOTIC RESISTANCE
IN THE
NON-HOSPITAL ENVIRONMENT

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NON-HOSPITAL ENVIRONMENT

PROEFSCHRIFT

Ter verkrijging van de graad van doctor
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INTRODUCTION

INTRODUCTION

I. General background

Since the introduction of antimicrobial agents an increase in the prevalence of antibiotic-resistant microorganisms has been observed. In the pre-antibiotic era pathogenic bacteria of medical importance generally showed a lower prevalence of antibiotic resistance than after the introduction and (extensive) use of antibiotics. In 1940, for example, nearly all *Staphylococcus aureus* isolates were susceptible to penicillin, at present more than 80% of hospital as well as extramural isolates are penicillin-resistant.

To describe the extent of the problem of antibiotic resistance it is convenient to consider the hospital and the community as two separate ecosystems, different among aspects in populations, selective pressures and reservoirs and each with a characteristic pattern of emergence, prevalence and transmission of antibiotic-resistant microorganisms. However, the ecosystems are not completely isolated from each other as an exchange of antibiotic resistance genes and antibiotic-resistant microorganisms does take place. Both ecosystems have in common an increasing problem of antibiotic resistance among microorganisms, which may have serious consequences for public health.

The impact of the emerging antimicrobial resistance on health care can be summarized as follows: First, treatment failure in infections caused by resistant microorganisms treated with what is considered an appropriate empirical therapy. This leads to an increased morbidity and mortality and an increase in health care costs. Second, antibiotic resistance is sometimes encoded on plasmids that also encode for virulence factors. This phenomenon of "indirect selection" will therefore result in more severe infections with a higher morbidity and mortality as compared to those infections caused by susceptible microorganisms. Third, treatment of an infected person with an antibiotic to which the causative microorganism is resistant will provide a selective advantage for this resistant microorganism since in many cases the susceptible bacteria of the patient's own microflora will be eradicated. This results in a longer period of persistence of the resistant microorganisms in the host.

Finally, an essential part of the infection-prevention strategy for communicable diseases is effective eradication of the source. Persons who have infections and who are

inadequately treated because of antibiotic resistance will therefore remain a potential source for a longer period of time.

It is generally accepted that antimicrobial usage relates to the emergence, persistence and dissemination of antibiotic-resistant microorganisms. However, a direct causal relationship between antibiotic usage and emergence of resistance is sometimes difficult to prove. Some authors have described this phenomenon in a single hospital with a single specific antibiotic. Other cofactors that contribute to or exert an influence on the selection of the bacteria are the presence of an environmental or human reservoir, the socio-economic circumstances and the characteristics of the microorganisms.

The presence in nature of dense bacterial populations, e.g. the human gut flora, can be considered as ecological niches in which a large reservoir of genetic material, including resistance genes, is maintained, continuously introduced, and exchanged. Among the socio-economic circumstances that contribute to the resistance problem are the increase in numbers of elderly and immunocompromised patients in the population, resulting in an increased risk of infection and the usage of antimicrobial agents. This, in turn, increases the chance for the spread of resistant microorganisms. Also crowding and malnutrition are well known factors that contribute to the persistance and transmission of infectious diseases. The characteristics of the microorganisms concerned include their intrinsic resistance, their ability to survive adverse environmental conditions, their capacity to colonize and infect susceptible hosts and their capacity to exchange genetic material.

Several strategies have been developed in order to reduce the risk of emergence, persistence and spread of antibiotic-resistant microorganisms. Among these, the effort to reduce the inappropriate use of antibiotics in humans and in animals is by far the most important one. Relevant and up to date information on antibiotic resistance patterns of medically important bacterial species is an important tool for guiding the empirical choice of antibiotics and for improving appropriate therapy. Active population-based surveillance on a national and an international level is necessary to provide these data.

In this context, the present study was performed to look into several aspects of antibiotic resistance in the non-hospital environment and the results are described in this thesis.

II. Outline of the thesis

In chapter I relevant literature is reviewed concerning the prevalence of antibiotic resistance in several human populations that differ in their exposure to risk factors for the emergence of resistance and selection of resistant strains. Especially, the available data in several groups of healthy humans, i.e. pig farmers, abattoir workers, (sub)urban residents, and general practice patients, the mechanisms of bacterial resistance and the effect of antimicrobial therapy on antibiotic resistance are emphazised.

Chapter II, entitled 'Resistance in faecal *Escherichia coli* isolated from pigfarmers and abattoir workers' describes the prevalence and degree of antibiotic resistance of faecal *Escherichia coli* isolated from pig farmers and abattoir workers as compared to a control group of (sub)urban residents.

The variation in prevalence and degree of antibiotic resistance and the reproducibility of sampling over time in a certain group of healthy people was the subject of the study presented in chapter III, entitled 'Antibiotic resistance of faecal Enterobacteriaceae isolated from healthy volunteers, a 15-week follow-up study'.

Chapter IV, entitled 'Carriage of antibiotic-resistant *Escherichia coli* by healthy volunteers during a 15-week period' presents the results of the study of the antibiotic susceptibilities of *Escherichia coli* strains isolated from healthy volunteers with special emphasis on the variation in antibiotic resistance patterns over a 15-week period. In addition, the mechanism of high level trimethoprim resistance (i.e. MIC $\geq$ 1024 mg/l) is characterized with probes specifying type I and type V mediated resistance.

The effect of amoxycillin or doxycycline therapy on the antibiotic resistance of faecal *Escherichia coli* isolated from general practice patients treated for a respiratory tract infection is presented in chapter V, entitled 'Effect of antibiotic therapy on the antibiotic resistance of faecal *Escherichia coli* in patients attending general practitioners'.

Chapter VI, entitled 'Comparison of virulence factors of urinary and faecal *Escherichia coli* isolated from the same patient' presents the results of the study in which faecal and urinary *Escherichia coli* isolated from patients with an uncomplicated urinary tract infection from general practice were compared with respect to virulence factors, serotype and antibiotic susceptibility in order to determine the role of the faecal flora as a reservoir for uropathogens.

Chapter VII, entitled 'A piperacillin-tazobactam resistant *Escherichia coli* strain isolated from a faecal sample of a healthy volunteer', describes the mechanism of a TEM-1 "like" producing *Escherichia coli* strain resistant to the combination of piperacillin and the ß-lactamase inhibitor tazobactam.

In chapter VIII the results of the studies presented are reviewed, discussed and conclusions and recommendations are formulated.

CHAPTER I

REVIEW OF THE LITERATURE
&
AIMS OF THE STUDY

Antibiotic resistance

Nowadays bacterial resistance to antimicrobial agents is an important obstacle for the successful treatment of bacterial infections, leading not only to treatment failures but consequently to prolonged morbidity and even mortality [40,58,59,60]. Since the introduction of antimicrobial agents into clinical practice, microorganisms have been labelled sensitive or resistant. In clinical practice "resistance" means that an infection caused by an antibiotic-resistant microorganism of a normally sensitive species cannot be treated successfully with that particular agent using standard dosages. For microbiologists, resistance means that an antimicrobial agent, even used in higher doses, has little or no effect on a particular microorganism or that an isolate has become less susceptible to an antimicrobial agent while other bacteria of the same species still remain susceptible [5,26].

In considering the problems caused by antibiotic-resistant bacteria it is usually the emergence of resistance in previously susceptible bacteria that is of concern and not the intrinsically resistant microorganisms. Lack of susceptibility (also called natural or intrinsic resistance) is species-restricted; this means that an antimicrobial agent cannot reach its target or binding site or because these sites are absent in the species involved. For example Enterobacteriaceae are intrinsically resistant to benzylpenicillin, because penicillin cannot penetrate the outer membrane of these Gram-negative bacteria. Therefore this compound cannot be used for the treatment of *Escherichia coli* infections [26,70,73].

There are three ways for a microorganism to become resistant to an antimicrobial agent. First, selection. Even before the introduction of an agent a few strains are already found to be resistant. The classic example of this type of resistance is *Staphylococcus aureus* and penicillin [17]. Second, resistance due to chromosomal mutations. The frequency of these spontaneous mutations is not influenced by exposure to antimicrobial agents unless these agents are mutagens. Such mutations usually involve the genes encoding the target site of the agent, or cell structures affecting access of the agent to the target site. This kind of resistance is not transferable to other species, but only vertically transferred to the next generation of the microorganism involved. If this spontaneous mutation frequency is high enough, use of the antimicrobial agent will select for resistant

strains which will then replace the sensitive bacterial population. However, in the absence of that agent these resistant strains have no particular survival advantage and mostly disappear sooner or later [22,40,58,69,70,73]. The third kind of antibiotic resistance is transferable resistance. The genes responsible for this kind of resistance, the so-called resistance factors (R-factors) are mainly located on extrachromosomal circular elements of DNA: plasmids. R-plasmids can transfer themselves from one bacterial cell to another by conjugation, i.e. conjugative plasmids. Transfer of this type of resistance occurs not only between bacteria belonging to the same species but also between bacteria of different species. In addition to conjugative plasmids, bacteria may possess transposons, the so-called "jumping genes", which are usually small pieces of DNA. Unlike plasmids, transposons do not rely on a particular host cell or any particular host DNA in order to exist or multiply. Transposons can "jump ship" on to the host chromosome or on to a resident plasmid already stably present in the bacterial cell and while the new host may lose the plasmid and even its copy, the transposon will remain with the new cell since it has found a stable place in the new host. Other possible mechanisms for transferring resistance genes include transformation and transduction. Transformation involves the pickup by a recipient bacterial cell of pieces of naked DNA released after death by the donor bacteria. Once taken up, the DNA can become part of the DNA of the recipient cell if donor and recipient belong to the same species. Transduction is the process by which genes , both from plasmid(s) or from their chromosome, are transferred to a new host via bacterial viruses called bacteriophages. Phages infect only those bacterial hosts that have a particular membrane site to which they can attach. Once there, the phage injects its DNA into the bacterial cell and gene exchange can take place as soon as the phage and its DNA piece integrate into a second host's chromosome of a bacterial cell of the same species.

The most common way of transferring resistance *in vivo* in Gram-positive as well as in Gram-negative bacteria, however, is by plasmid-mediated conjugation [32]. In contrast to chromosomally-mediated antibiotic resistance, plasmid-mediated resistance to one or several antibiotics, once acquired, may remain in the environment for prolonged periods, even in the absence of selection by exposure to that antibiotic. This is because the resistance factors are often linked to plasmids encoding for other virulence factors and/or to resistance to other antibiotics, so-called multiple antibiotic resistance [41,70]. Multiple resistance occurs when genes conferring resistance to several unrelated antimi-

crobial agents are linked together into one plasmid and are transferred from one bacterial cell to another. Use of one agent will not only select for an increase of resistance to that agent but to the other agents as well. In 1959, Japanese workers showed that multiple resistance , in this case resistance to chloramphenicol, tetracycline, streptomycin and sulphonamides, was transmissible from shigellae to *Escherichia coli* [56]. In a nosocomial outbreak among children in Mexico City, the *Shigella dysenteriae* involved was not only resistant to chloramphenicol, tetracycline, streptomycin and sulphonamides but the microorganism had also acquired resistance to ampicillin [56]. Since that time many additional outbreaks due to multiply resistant pathogens have been recognized and documented all over the world.

Mechanisms of antibiotic resistance

Microorganisms can become resistant to antibiotics in a number of ways. The most common mechanism is inactivation of the antimicrobial agent. An example of this is the hydrolysis of ß-lactam antibiotics by ß-lactamases [74]. By far the most common of the plasmid-mediated ß-lactamases is TEM-1 [75]. To overcome the action of these enzymes, ß-lactamase inhibitors such as clavulanic acid and tazobactam have been developed [76]. Other mechanisms of resistance include alteration of the antibiotic target or binding site [2], reduction in bacterial accumulation of the agent involving active efflux of the agent out of the bacterial cell or reduced penetration of the agent into the cell due to alterations in outer membrane proteins (OMPs) [10,12], development of a separate metabolic pathway that allows bypassing of the target metabolic process of the antibiotic [8], alteration of a molecule so that it does not effectively bind to its receptor site [18], elimination or reduction of the requirement for a metabolite [37], or elimination of enzymes whose action stimulated by the substance resulted in destruction of the bacteria [30].

Antibiotic use and antibiotic resistance

It is generally accepted that antibiotic resistance is related to the frequency and amount of usage of that particular agent in a certain population [50,65]. Several studies in

humans dealing with the relation between antibiotic consumption and antibiotic resistance have been performed in hospitals and describe a positive correlation between usage and resistance [11,33,55]. In contrast, other studies have either failed to observe any relationship between usage and resistance [31,34] or have shown a decrease in resistance despite an increase in usage of a particular agent [55].

Apart from antibiotic use in humans, both in general practice and in hospitals, veterinary and agricultural usage of antibiotics may also be partly responsible for the increase in resistant bacteria, but it is still not clear whether antibiotic use in humans or in animals has contributed most to the environmental pool of resistant microorganisms [44]. Just as is the case with humans, animals receive antibiotics prescribed by veterinary surgeons therapeutically and prophylactically. However, quantitatively much more antibiotic is used in agriculture for crop protection and given to animals in small subtherapeutic levels as growth promotors [5,41].

Concern for the public health implications of indiscriminate antibiotic use in animals resulted in the Swann Report (1969) in Great Britain and an Interim Report of a Food and Drug Administration Task Force (1972) in the USA. Both reports recommended that antimicrobials agents be excluded from animal feed as growth promoter if these antibiotics were also used as therapeutic agents in human or animal medicine (i.e., penicillins or tetracyclines) or if they were associated with the development of cross-resistance to drugs that were registered for use in humans [23]. However, although the recommendations of these reports have been implemented in the member states of the European Union, they have not yet been implemented in the USA.

In human medicine, resistance to antimicrobial agents is mainly a problem in hospitals, in which large quantities of antibiotics are frequently used for treating infections. As in the hospital environment new microorganisms are constantly being introduced from new patients and personnel, this setting most probably represents the greatest reservoir of significant resistant bacteria [32]. The heavy use of antibiotics exerts a selective pressure on these resistant microorganisms which may consequently accumulate in the hospital and may be spread to the open population as soon as the patients are discharged back to the community [14,70].

The prevalence of antibiotic-resistant microorganisms in general practice patients and the spread of these resistant strains in that population are less well documented

[25,72,77,78]. General practitioners prescribe most antimicrobial agents for the treatment of urinary tract infections or respiratory tract infections [24]. The choice of antibiotic is usually made and treatment started on an educated guess before laboratory data on the susceptibility of the causative organism are available. For an educated guess and an optimal antibiotic therapy knowledge of the probable infecting organism and its likely susceptibility is essential [71].

The prevalence of antibiotic resistance in clinical isolates might be considered as the top of the iceberg as the usage of antibiotics also selects for antibiotic-resistant strains in the human gut flora. Several studies have analysed the effect of antimicrobial therapy on selection of antibiotic-resistant microorganisms in the faecal flora [1,64]. Therapeutic oral courses of ampicillin or a sulphonamide had only slight effect in selection of faecal *Escherichia coli* resistant to that particular agent [15]. Similar observations were made after use of trimethoprim or co-trimoxazole [36], but tetracycline, a broad spectrum antibiotic, exerted strong selection, not only for tetracycline resistance but also for resistance to ampicillin, streptomycin, chloramphenicol and sulphonamides [15].

Another aspect of the problem of antibiotic resistance is the contamination and possible colonization of the human gut with resistant microorganisms from an exogenous source. Colonization or even transit of the human gut with exogenous resistant microorganisms seems to be an important factor for the introduction of new resistant genes into commensal microorganisms and into (potentially) pathogenic microorganisms [5,70]. Some studies described the colonization of the human gut with antibiotic-resistant animal isolates [29,41]. However, other studies suggest that under certain circumstances *Escherichia coli* strains carrying R-factors may not be such good colonizers as those that are drug-sensitive [4] and that it is very difficult to prove that the *Escherichia coli* strains colonizing the human gut are indeed the same as those found in animals [48].

If R-factors of colonizing resistant *Escherichia coli* are transferred to pathogens such as *Salmonella* spp. or *Yersinia* spp. the chance of infection after eating a product contaminated with these pathogens is higher in people taking antibiotics to which the microorganism is resistant. In the USA *Salmonella newport* caused serious disease in 18 persons, of whom 12 had taken antibiotics recently for other reasons. All of them had

prepared and eaten minced beef (hamburger) in the week before illness. It was found that the meat used for the hamburgers was obtained from beef cattle which had been fed subtherapeutic doses of chlortetracycline. It seemed very likely that the resistant strain had a selective survival advantage in the antibiotic-treated people [29]. The importance of person-to-person spread [9] and food-borne illness resulting from contamination of foods with *Salmonella* spp. transmitted by human carriers must be considered along with animal sources [23].

Reservoirs of antibiotic-resistant *Escherichia coli*

The intestinal flora is considered the main reservoir of antibiotic-resistant *Escherichia coli* and antibiotic resistance genes in the population as well as in hospitals [20,28,35,38,43,51,66].

The bacterial flora of hospitalized patients consists of bacterial strains which they brought with them to the hospital, i.e. the flora of the community, plus the microorganisms acquired in the hospital, i.e. the nosocomial flora [62]. In the early years of the antibiotic era, Gram-positive cocci (*Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*) were the most common causes of hospital infections. More recently they have been replaced by facultatively anaerobic Gram-negative bacilli and multi-resistant *Pseudomonas aeruginosa* strains as the most important cause of nosocomial infections [52]. Nowadays, however, the importance of Gram-positive cocci is again increasing. When patients leave the hospital, the resistant microorganisms can be transferred to people in the population either directly, e.g. by close contact, or indirectly, e.g. by food handling [13,16].

To reduce the chance of emergence of resistance often combinations of antimicrobial agents are used, such as a cephalosporin or broad-spectrum penicillin plus an aminoglycoside [53]. The improved hygienic practices in the hospital to avoid the spread of multi-resistant strains from patient to patient seem to be effective. Also an understanding of resistance mechanisms will assist the clinician in the rational and appropriate use of antibiotics [73].

Antibiotic resistance in animals: an additional reservoir

As the environments of animals and humans are not separate, exchange of bacteria and bacterial genes occurs between both groups. The indirect effects caused by the use of antibiotics in animals on the acquisition of antibiotic-resistant bacteria affecting humans include the possible transfer of resistant bacterial strains and R-factors from animal to man. The transfer of bacterial strains can take place after direct contact with the animal, after contact with animal faeces or contaminated animal carcasses as is the case by farmers, abattoir workers or butchers or following the preparation and ingestion of contaminated meat. Levy *et al.* demonstrated cross infection of *Escherichia coli* from chickens to the humans in contact with the birds. The *Escherichia coli* strain involved was marked by a temperature-sensitive chloramphenicol-resistance [39]. Transfer of R-factors between man and domestic pets can also occur and is likely to take place since the contact between owner and pet is often very close [19]. It is to be expected that people having close contacts with pigs or pig carcasses have a greater risk of becoming contaminated with animal intestinal bacteria which might harbour R-plasmids than people without or with less contact. Abattoir workers are in close contact with pig carcasses contaminated with gut contents following slaughter [46] and pig farmers also have close and regular contact with their animals and waste [63,79]. Direct contact with antibiotics used for treatment of pigs and with their antibiotic-enriched feed is another possible risk factor for selection of resistant strains in the intestinal flora of pig farmers [61,63]. As the number of people having direct contact with food animals (cattle, pigs, poultry) is relatively small, it is very likely that the effect on the population is of minor importance. Consequently this route of cross-infection is only important for individuals [48]. The main route by which resistant *Escherichia coli* and resistant genes pass to the human population from outside is via the food chain: slaughter, packaging, distribution, handling raw meat, cooking, and eating [47,48,49,68].

Prevalence of antibiotic-resistant *Escherichia coli* in developing countries

The prevalence of antimicrobial resistance in the healthy human population is often higher in developing countries than in developed ones. In developing countries antibiotics

are available over the counter, without prescription. Even the antimicrobial agents which are easy and inexpensive to obtain, but which are no longer effective because of common resistance, are still continuously used. Since the bacteria involved are often multiresistant, use of one antibiotic will select for the same multi-resistant strain and perhaps coupled with poor hygiene and daily ingestion of high numbers of faecal bacteria in contaminated water supplies, the high prevalence percentages of resistance will be maintained in those countries [3,38,44,51,66,67]. The extensive reservoirs of resistance genes in commensal bacteria from developing countries may also serve to introduce resistance genes into the bacterial populations of developed countries [3]. In a study of travellers to Mexico from the USA, 57% of those who had no trimethoprim-resistant *Escherichia coli* in their commensal faecal flora on entering the country had nevertheless acquired them on return [57]. The major problem of antibiotic resistance in developed countries is caused by indiscriminate and excessive consumption of antibiotics.

Prevalence of antibiotic-resistant *Escherichia coli* in developed countries

Relatively little is known about the prevalence of antibiotic-resistant bacteria in the healthy human population not subjected to antimicrobial chemotherapy or prophylaxis. In Dublin, faecal *Escherichia coli* strains from healthy infants were examined for antibiotic resistance [54]. In 81 out of a total of 100 faecal samples antibiotic-resistant strains were isolated; resistance to ampicillin was found in 62%, and to streptomycin and tetracycline resistance in 63% and 67%, respectively. Eighty-four percent of these 81 strains were shown to possess R-factors. Only a low correlation between previous antibacterial therapy and antibiotic-resistant enterobacteria was found, i.e. 7 (39%) of 18 infants who had received either penicillin or ampicillin recently had a predominantly ampicillin-resistant bacterial flora [54].

Similarly, Datta (1969) examined faecal samples collected from patients before admission to hospital for elective surgery and found that 52% contained antibiotic-resistant *Escherichia coli*. Resistance to sulphamethoxazole and tetracycline occurred most frequently, i.e. 38% and 34%, respectively. Resistance to streptomycin and ampicillin was found in 27% and 17% of the patients, respectively [14].

A significant difference in the prevalence of antibiotic-resistant strains was found

between children and adults, in both rural and urban groups in the study of Linton *et al.* (1972). For the children in the urban group the prevalence of resistance was 64%, for the rural group even higher(73%) although not significantly. For the adults the figures were 42% and 49%, respectively. In addition, in the rural group 63% of faeces from adults of farming families contained resistant coliform bacilli compared with a significantly lower proportion (29%) of adults in non-farming rural families. Overall 61% of the antibiotic resistance was transferable; ampicillin, tetracycline, streptomycin and sulphamethoxazole resistance alone or in various combinations was transferred most frequently [45].

Levy *et al.* (1988) found a significantly higher prevalence of resistance to ampicillin for hospitalized patients than for healthy individuals; all of the latter were known not to be taking antibiotics. This group consisted of laboratory workers, as well as urban and rural dwellers. No significant differences in prevalence of resistance to any of the antibiotics tested were found among the subpopulations of the ambulatory group, although the general trend was towards lower numbers of resistant bacteria in the rural samples. Eight to nine years later, in 1987, the urban group was again studied and yielded similar results for the prevalences of resistance; also the high degrees of resistance ($\geq$ 50% of the faecal flora resistant) were of the same order of magnitude as in the previous study: 11.3% for ampicillin, 17.5% for streptomycin, 21.3% for tetracycline, and 7.5% for kanamycin [43].

Prevalence of antibiotic-resistant *Escherichia coli* in the Netherlands

In the Netherlands only limited data are available on the prevalence of antibiotic resistance in healthy people [6,7,20,21,27].

In the study of Guinee *et al.* (1970) the prevalence of *Escherichia coli* carrying R-factors was examined in meat-consuming individuals (military kitchen personnel and office employees) and in those not consuming meat (vegetarians and babies below the age of 6 months). Of the office employees, 38% yielded tetracycline-resistant *Escherichia coli*; 22% yielded one or more *Escherichia coli* strains with transferable resistance. For the adult vegetarians the respective figures were 52% and 27%, and for the babies 49% and 31%. It was assumed that these babies had acquired the resistant organisms from

their mothers. The percentages found for the vegetarians were higher than those found for the non vegetarians; so the most important mechanism with regard to the incidence of resistant *Escherichia coli* in man in this study was not likely to have been due to the human consumption of meat containing antibiotic-resistant *Escherichia coli* [27]. Raw vegetables and salads often carry large numbers of resistant bacteria due to the use of antibiotics in agriculture [42].

Degener *et al.* (1978-1980 and 1987) determined the faecal carriage rates for antibiotic-resistant *Escherichia coli* in an urban population in Zoetermeer, a new town in the West of the Netherlands. In the period 1978-1980 the prevalence of resistance to ampicillin was 25%, for tetracycline 42% and sulphamethoxazole 45%. In 1987 these percentages were 27%, 20% and 46%, respectively. The high degree of resistance, i.e. $\geq$50% of the faecal *Escherichia coli* flora in one individual being resistant, increased from 5% to 11% for ampicillin, decreased from 12% to 6% for tetracycline and remained almost the same for sulphamethoxazole,i.e. 19% and 21% [20,21].

In a more recently performed study of Bonten *et al.* (1990) the antibiotic resistance level of *Escherichia coli* isolated from faecal samples from 172 students in Maastricht, a city in the South of the Netherlands, was determined. In 165 out of 172 samples resistance to either ampicillin, tetracycline, sulphamethoxazole, trimethoprim or nitrofurantoin was observed. The highest percentages were found for sulphamethoxazole (86%), ampicillin (76%) and tetracycline (47%). For ampicillin the high degree of resistance was 8%, for tetracycline and sulphamethoxazole 11% and 37% were found [6].

Comparison of the data of these studies on the healthy population not using any antibiotics is difficult because of differences in methods and in selective antibiotic concentrations used as well as in the populations studied. Furthermore, except for the study of Levy *et al.* (1988), who analysed two faecal samples from the same individual and found that 90% of all individuals showed a gain (47.6%) and/or loss (65.7%) of one or more detectable resistances [43], data from other studies were based upon only one faecal sample per individual. There are thus no data on the reproducibility of sampling, or on the intra-(or within) individual variation regarding the presence of antibiotic resistance in faecal isolates and possible changes in resistance over time.

AIMS OF THE STUDY

With respect to the literature as discussed in this chapter several questions concerning the prevalence of antibiotic resistance in non-hospitalized patients still remain unanswered. The present study has been designed to answer the following questions;

I The prevalence of resistance to antimicrobial agents and the antibiotic resistance patterns of faecal *Escherichia coli* isolated from healthy volunteers, i.e. pig farmers, abattoir workers and (sub)urban residents. In the last group special attention has been paid to the reproducibility of sampling and the intra-individual variation over time.

II The effect of antibiotic therapy on antibiotic resistance of faecal *Escherichia coli* in general practice patients with respiratory tract infections.

III To provide evidence for the reservoir function of the faecal *Escherichia coli* flora as uropathogens.

IV To determine the mechanism of resistance to the combination of a ß-lactam antibiotic and a new ß-lactamase inhibitor in an isolate from a healthy individual who had not undergone any antibiotic therapy in the previous three months.

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CHAPTER II

RESISTANCE IN FAECAL *ESCHERICHIA COLI* ISOLATED FROM PIG FARMERS AND ABATTOIR WORKERS.

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SUMMARY

Faecal samples collected from three populations of healthy adult volunteers (290 pig farmers, 316 abattoir workers, 160 (sub)urban residents) living in the south of The Netherlands were analysed for the prevalence and degree of antibiotic resistance of *Escherichia coli*. Significant differences in prevalence of resistance to amoxycillin, neomycin, oxytetracycline, sulphamethoxazole and trimethoprim were observed. The pig farmers showed the highest percentages of resistance and the (sub)urban residents the lowest. In contrast no significant differences in high degrees of resistance were observed, except for neomycin. Although both pig farmers and abattoir workers have regular contact with pigs differences in prevalences of resistance were observed. However, because abattoir workers with intensive and less intensive pig(carcass) contact did not show significant differences, this is probably not the only important source of resistant *Escherichia coli* in pig farmers.

The high antibiotic use by pig farmers (5%) and abattoir workers (8%) than by (sub)urban residents (0%) did not result in significantly different resistance percentages.

INTRODUCTION

Since their introduction antimicrobial agents have been successfully used for treatment and prophylaxis of bacterial diseases in man and other species. The availability of antibiotics means that many previously severe infections can now be treated. In addition, antibiotics are used for growth promotion in animal husbandry and in agriculture for crop protection. As antibiotics are not only very effective, but also remarkably safe drugs this safety may have provoked liberal, even lavish, use in man and other animals. The use of antibiotics, however, leads inevitably to emergence of resistance in the endogenous bacterial flora of treated persons and animals alike, against the antibiotics used or to other drugs [4,18]. These enteric microorganisms may colonize other persons and animals and may spread resistance further by transfer of resistance plasmids to their faecal flora. Consequently the environmental bacterial population may be contaminated after faeces excretion. Lester *et al.*[9] showed that persons with a few resistant bacteria in their intestinal flora will have more chance of developing an infection with resistant bacteria

after antibiotic therapy than persons with no resistant strains at all.

Many studies have examined the resistance of enteric bacteria in humans after antibiotic therapy [1,6,8,17], but there is much less information available on the prevalence of antibiotic resistance in the faecal flora of healthy adults who have not used antibiotics recently [2,3,5,13,14]. However, such subjects are potential recipients of antimicrobial agents.

Farmworkers can directly become colonized by resistant bacteria due to close contact with animals and their faeces [10,11,21], but are also directly exposed to antibiotics used for treatment or prevention of diseases in animals [12]. Abattoir workers have daily contact with contaminated carcasses and gut contents [7,15,16,26]. A common risk factor for colonization with resistant microorganisms in all three groups is personal use of antibiotics.

To elucidate the importance of spread of resistance from food-animals to man we studied in the same region the antibiotic resistance in three populations with different risks of exposure to faecal bacteria from pigs i.e. pig farmers, abattoir workers and as a control group, (sub)urban residents.

As the faecal flora is considered the most important reservoir of resistant microorganisms and the antibiotic resistance of this flora is an indicator for the resistance of potentially pathogenic bacteria in a population [8,9,13,28], faecal samples of these three populations were analyzed for the prevalence and degree of antibiotic resistance of *Escherichia coli*.

MATERIALS AND METHODS

Collection of the faecal samples.

Faecal samples, one from each person, were received from adult pig farmers (290), pig-abattoir workers (316, of which 73 were meat inspectors) and (sub)urban residents (160) all living in the same area. After receipt, the samples were diluted (10^{-1}) in physiological saline, containing 20% (v/v) glycerol and stored at -20°C until examined. All participants were asked to answer a questionnaire concerning antibiotic use in the previous three months. Additional information about recent hospital stay and previous antibiotic use by family members was obtained from the pig farmers and abattoir

workers. The abattoir workers were also asked to give some information about keeping domestic animals or pigs and their daily duties at the slaughterhouse.

Bacteriological analysis of the faecal samples.

The methods used to determine the prevalence and degree of resistance were as described before [20]. In brief, after thawing the samples (10^{-1}), tenfold dilutions (10^{-2}-10^{-5}) in physiological saline were made. Thirty-seven μl of these dilutions were inoculated with a spiral plater on Levine-agar plates (BBL 11221, [27]), a selective medium for *Escherichia coli*, with and without antibiotics. The antibiotic concentrations (Table 1) were based on NCCLS guidelines and modified where appropiate so that the data were comparable with those of previous studies [2,3]. Only colonies with the appearance of *Escherichia coli* (i.e. purple with a black center and a metallic green shine) were counted. The total number and the number of resistant *Escherichia coli* were determined. The minimum detection level of bacterial growth was 10^3 colony forming units (CFU) /g faeces. From each agar plate without antibiotics one colony with the appearance of *Escherichia coli* was picked and tested for growth at 42°C overnight in tryptone water (Oxoid L42) and for the indole reaction. If these tests were positive the microorganism was considered to be *Escherichia coli*. For the first 50 isolates this identification was confirmed with Api-20E test (BioMerieux, Den Bosch, The Netherlands).

Prevalence of antibiotic resistance:

The prevalence of antibiotic resistance was defined as the percentage of faecal samples showing any growth of *Escherichia coli* on antibiotic-containing plates.

Degree of antibiotic resistance:

The degree of resistance of each sample was calculated as the percentage of the total number of colonies that was resistant.

Two degrees of antibiotic resistance to a particular antimicrobial agent were distinguished [3,13], namely the proportion of faecal samples with a ratio **< 50%** was defined as **low degree of resistance**, and the proportion of faecal samples with a ratio **≥ 50%** was defined as **high degree of resistance** (thus the majority of the strains showed resistance to

that agent).

The antimicrobial agents used in this study were selected because these or closely related compounds are regularly used for the treatment of humans and pigs in The Netherlands, except apramycin which is only registered for use in animals but is not extensively used in The Netherlands.

Statistical analysis.

In the analysis of the differences in prevalence and degree of antibiotic resistance of the faecal samples of the three populations a Chi Square test with continuity correction was performed. A Fisher Exact test was used if the expected frequency in at least one cell was five or less. A two-sided significance level of ≤ 0.05 was used.

Multiple logistic regression was used to analyse the contribution of the origin of the three study populations (independent variable) to the prevalence of resistance (dependent variable) to a particular antibiotic. The antibiotics other than the dependent variable were considered to be independent variables simultaneously, a two-sided significance level of ≤ 0.05 was used.

The error of the method by using the spiral plater and by making tenfold dilutions, calculated from the standard error of the mean, was 0.5 $\log_{10}$.

RESULTS

Ninety-five percent of the pigfarmer colonies, 94% of the abattoir workers colonies and 92% of the (sub)urban residents colonies that grew on Levine-agar showing the morphology typical of *Escherichia coli* were identified as *Escherichia coli*. The other colonies tested were also Enterobacteriaceae: *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp. Finally, 278 samples of pig farmers, 289 of abattoir workers and 150 of (sub)urban residents were included in the analysis. The other samples failed to grow on the agar plates without antibiotics.

Antibiotic use was recorded by 15 pig farmers and 17 of their family members. Two farmers had been hospitalized recently. Twenty-five abattoir workers and 25 family members recorded antibiotic use, 5 abattoir workers had been hospitalized recently. By the (sub)urban residents no antibiotic use in the three previous months was recorded.

Intensive contact with pigs or pig carcasses was recorded by 182 abattoir workers, whereas 104 workers had other duties as well or no direct contact. No information about contact with pigs/carcasses was obtained from the remaining abattoir workers (n=30). Fifty-two percent of the abattoir workers kept at least one domestic animal, whereas only three persons kept pigs.

Prevalence of antibiotic resistance:

The prevalence and high degree of resistance are shown in Table 1 and Figure 1. The most significant differences were noticed between pig farmers and (sub)urban residents. The highest prevalence percentages were found for the pig farmers and the lowest for the (sub)urban residents. The highest percentages (i.e. 47%) in the abattoir workers and in the (sub)urban residents group were found for oxytetracycline and amoxycillin, respectively, and in the pigfarmer group for sulphamethoxazole (84%).

Further analysis as to the patterns of prevalence of resistance to amoxycillin, neomycin, oxytetracycline and trimethoprim of *Escherichia coli* isolated from the three populations studied showed that the highest percentage of fully susceptible strains (34%) as well as the lowest percentage of completely resistant isolates (4%) were found in the (sub)urban residents. The converse was observed for the pig farmers.

Logistic regression analysis was performed to estimate the relative risk of prognostic and risk factors (i.e. antibiotics used and population groups) with regard to the (sub)urban residents. The odds ratio (OR), with the 95% confidence interval (CI), for resistance to a particular antibiotic under consistent circumstances was calculated. Both pig farmers (OR 0.4, CI 0.2-0.6) and abattoir workers (OR 0.5, CI 0.3-0.9) showed a low odds ratio for amoxycillin resistance. The pig farmers showed a high odds ratio for neomycin (OR 3.6, CI 2.5-5.4), sulphamethoxazole (OR 6.5, CI 4.0-10.6) and trimethoprim (OR 2.1, CI 1.4-2.9). Resistance to oxytetracycline appeared to be independent of the population tested. For the other antibiotics tested no significant prognostic and risk factors were found.

Table 1: Prevalence and high degree of antibiotic-resistant *Escherichia coli* (%).

Antibiotic[a]	Prevalence			High degree		
(mg/L)	PF[b]	AW	UR	PF	AW	UR
	n=278	n=289	n=150	n=278	n=289	n=150
AMX (25)	62	42*	47/ **	7	9	13
AP (32)[c]	3	1	nt[d]	0	0	nt
CIP (4)	1	0	0	0	0	0
NA (32)	5	3	1	1	0	0
NE (8)	66	36*	25/ **	7	2*	2/ **
FT (50)	8	4	3	0	0	0
OT (25)	79	47*	36/ ‖ **	10	15	8
SMX (100)	84	45*	40/ **	17	13	10
TMP (8)	53	23*	15/ **	4	4	3

[a]AMX=amoxycillin, AP=apramycin, CIP=ciprofloxacin, NA=nalidixic acid, NE=neomycin, FT=nitrofurantoin, OT=oxytetracycline, SMX=sulphamethoxazole, TMP=trimethoprim

[b]PF=pig farmers, AW=abattoir workers, UR=(sub)urban residents

[c]Apramycin was only tested for the abattoir workers and the last 116 pig farmers faecal samples.

[d]nt= not tested

Significantly different ($P \leq 0.05$): PF and AW: *, PF and UR: /, AW and UR: ‖; PF and AW and UR **

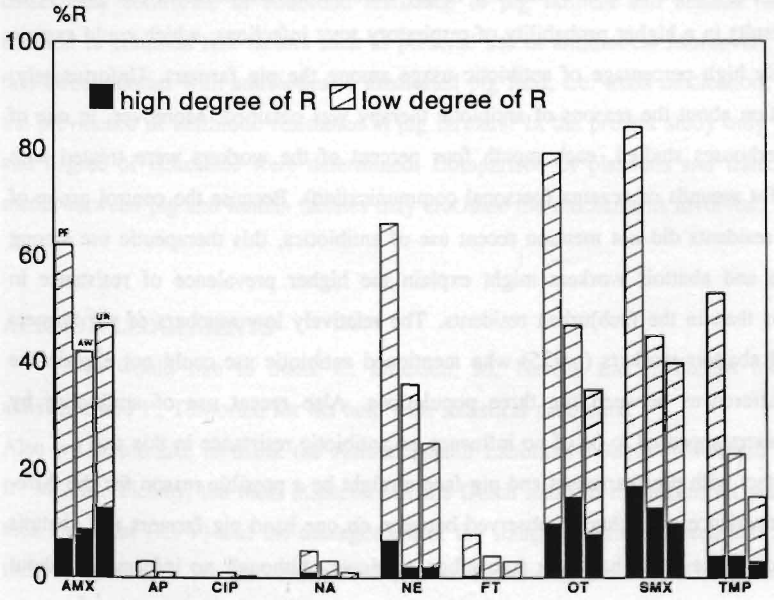
Degree of antibiotic resistance:

As presented in Figure 1 all three populations showed, except for neomycin, similar percentages for high degree of resistance, but distinct variations in low degree of resistance. The prevalence and degree of resistance of the meat inspector samples were not significantly different from those of the abattoir workers. In addition, no differences in prevalence and degree of resistance were observed between abattoir workers with and without domestic animals. Because only three abattoir workers kept pigs no conclusions about the influence of regular contact with pigs could be drawn.

No significant differences could be observed between abattoir workers with intensive and those without or with less intensive contact with pig faecal contents or pig carcasses.

No significantly different prevalence or degree of resistance rates were observed for those people who had recently used antibiotics compared with those who had not used antibiotics recently (pig farmers 5%, abattoir workers 8%). Nor were differences observed for those recording recent hospital stay (pig farmers 1%, abattoir workers 2%) or antibiotic use by family members (pig farmers 6%, abattoir workers 8%) when compared with those who did not record these factors.

Figure 1. Prevalence of antibiotic resistance (%) of *Escherichia coli* from pig farmers (PF, first bar per antibiotic), abattoir workers (AW, second bar) and (sub)urban residents (UR, third bar). Also shown are the proportions (%) of low degree (<50%) and high degree (≥50%) of resistance. AMX=amoxycillin; AP=apramycin; CIP=ciprofloxacin; NA=nalidixic acid; NE=neomycin; FT=nitrofurantoin; OT=oxytetracycline; SMX=sulphamethoxazole; TMP=trimethoprim.



DISCUSSION

The present study showed significant differences in prevalence of resistance between pig farmers and (sub)urban residents for antibiotics extensively used in human and veterinary medicine in The Netherlands [19]. In contrast the prevalence of a high degree of resistance was, except for neomycin, not significantly different.

Several investigators have also observed differences in resistance of the faecal flora of pig farmers/abattoir workers and urban residents [14,22,24] suggesting that contact with live- stock was one route by which antibiotic resistance entered the human gut flora. In contrast, Levy *et al.* [13] found no significant difference between rural and urban residents. The general trend in their study was for lower numbers of resistant bacteria to be found in rural samples.

Remarkably, in the present study 15 (5%) pig farmers and 25 (8%) abattoir workers used antibiotics during the three months previous to faecal sampling, whereas none of the (sub)urban residents mentioned recent antibiotic use. This might be an indication that people in contact with pigs or pig carcasses have a greater risk of bacterial infections. A recent study about occupational risk factors for pig farmers showed that pig farmers often suffer from chronic non-specific respiratory tract afflictions, due to regular exposure in pig-stables to dust containing fungi, endotoxins, disinfectants etc. [23]. This exposure results in a higher probability of respiratory tract infections, which could explain the relatively high percentage of antibiotic usage among the pig farmers. Unfortunately, no information about the reasons of antibiotic therapy was obtained. Moreover, in one of the slaughterhouses studied, each month four percent of the workers were treated with antibiotics for wounds or eczema (personal communication). Because the control group of (sub)urban residents did not mention recent use of antibiotics, this therapeutic use among pig farmers and abattoir workers might explain the higher prevalence of resistance in these groups than in the (sub)urban residents. The relatively low numbers of pig farmers (n=15) and abattoir workers (n=25) who mentioned antibiotic use could not explain the observed differences between the three populations. Also recent use of antibiotics by family members appeared to be of no influence on antibiotic resistance in this study.

Contact with pigs/carcasses and pig faeces might be a possible reason for the differences in prevalence of resistance observed between on one hand pig farmers and abattoir workers and on the other hand the (sub)urban residents. Although no information about

the professions of the last group was obtained, it is to be expected that they do not have regular direct contact with pigs. However, no significant differences were observed between the abattoir workers with intensive and those with less intensive pig contact. Therefore, other factors such as more intensive faecal contact, less personal hygiene and protection taken by farmers as compared to abattoir workers might have contributed to these differences. Moreover, it is very likely that direct contact with antibiotics used for treatment of pigs is an additional risk factor for emergence of resistance and selection of resistant strains in the intestinal flora of pig farmers. The results of the logistic regression analysis underscore these suggestions.

Remarkably, significantly different prevalences and high degrees of resistance to neomycin were observed for the pig farmers. Because neomycin is seldom used orally and never parenterally in human medicine but frequently in pigs, the suggestion seems likely that it is not human but mainly veterinary use of neomycin that is responsible for a higher prevalence and high degree of resistance in pig farmers.

This study showed significant differences in the prevalence of antibiotic resistance in the faecal flora of the three populations tested. Direct contact with pigs and pig carcasses may contribute to antibiotic resistance in pig farmers and abattoir workers, in addition to common risk factors such as personal use of antibiotics. Moreover, it is likely that direct contact with antibiotics in medicated pig feed, i.e. mass medication, influences the prevalence of antibiotic resistance in pig farmers. In the present study only prevalence and degree of resistance were determined. Comparison of plasmids and transfer experiments between pig and human isolates may elucidate the mechanisms involved.

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CHAPTER III

ANTIBIOTIC RESISTANCE OF FAECAL ENTEROBACTERIACEAE ISOLATED FROM HEALTHY VOLUNTEERS, A 15-WEEK FOLLOW-UP STUDY.

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SUMMARY

From 25 March to 1 July 1991 faecal samples from healthy volunteers of two cities and their rural surroundings, Weert (n=91) and Roermond (n=96) were collected weekly and analysed for the presence of Enterobacteriaceae, resistant to ampicillin, ciprofloxacin, nalidixic acid, neomycin, nitrofurantoin, oxytetracycline, sulphamethoxazole or trimethoprim. In total, 682 and 690 samples from Weert and Roermond, respectively were analysed for the prevalence and the degree of resistance to each antimicrobial agent. The mean prevalence of resistance of the samples from Weert varied from 28% ± 12% (mean ± S.D.) for ampicillin to 0.1% ± 1% (range 0% - 2%) for ciprofloxacin. For Roermond the prevalence of resistance varied from 41% ± 7% for sulphamethoxazole to 0% for ciprofloxacin.

The high degree of resistance (i.e. >50% of the faecal flora of one particular individual) varied from 8% ± 4% for sulphamethoxazole to 2% ± 2% for trimethoprim for Weert. For Roermond the figures varied from 14% ± 4% for sulphamethoxazole to 0.3% ± 1% (range 0% - 2%) for nalidixic acid. High degrees of resistance were not found for ciprofloxacin and nitrofurantoin in either city.

In Weert ampicillin-resistant Enterobacteriaceae were isolated from 69/91 individuals.

INTRODUCTION

Emergence of antibiotic-resistant microorganisms continues to be an important problem for the treatment of bacterial infections [3,14,15,20]. There is general agreement that antibiotic use in general practice and in veterinary medicine is responsible in part for the increase in the numbers of resistant bacteria. However, it is still not clear how the use in humans (hospital and/or general practice) or that in animals has contributed to the environmental pool of resistant microorganisms [1,8,17,25,27]. In hospitalized patients relatively large amounts of antibiotics are used both for therapy and prophylaxis, resulting in a high prevalence of antibiotic-resistant pathogens [21,23,24]. In veterinary medicine antimicrobial agents are used in both these indications and as growth promoters at subtherapeutic concentrations [2,10,16]. In addition, multiple-resistant bacteria are

increasingly being isolated, and it is evident that these microorganisms can be spread from animals to the human population as well as from person to person [5,10,15,17,19,26]. Furthermore, resistant bacteria, e.g. *Salmonella* spp. and *Campylobacter jejuni*, can colonize the human intestinal tract after ingestion of contaminated food [6,13,16].

The intestinal tract of the human open population is considered to be an important reservoir of antibiotic-resistant bacteria [12], and several reports emphasize the importance of the availability of information of the prevalence of antibiotic-resistant isolates from faecal flora [7,18,22]. Relatively little is known about the numbers of resistant bacteria in the healthy human population not receiving antimicrobials for therapy or prophylaxis [7,18,22]. In addition, hardly any data are available on the reproducibility of sampling, the intra-(or within) individual variation regarding the presence of antibiotic resistance in faecal isolates and possible changes in resistance over time.

In this study, the prevalence and the degree of resistance for each antimicrobial agent were calculated for all faecal samples each week and for the entire 15-week period for each individual in the study.

MATERIALS AND METHODS

Sample collection

From 25 March to 1 July 1991 faecal samples were collected once weekly for a period of 15 weeks in two medium sized cities, Weert and Roermond, (41×10^4 and 43×10^4 inhabitants, respectively) 30 km apart in the south of the Netherlands. Volunteers were randomly selected at the registry office. Of the first 100 people in Weert and Roermond who consented to participate in the study, 91 and 96 subjects finally entered the study. Of these individuals 61/91 (67%) and 63/96 (66%), respectively, could be followed up for at least ten weeks. Only one individual reported having taken antibiotics (tetracycline) in the previous 15 weeks.

Sample processing

One gram of fresh faeces was suspended in 9 mL of 0.9%(w/v) NaCl, supplemented with 20%(w/v) glycerol and stored at -20°C until assayed. After thawing

this suspension, serial ten-fold dilutions were made up to a final dilution of $1:10^5$ and 0.037 mL of these dilutions were inoculated on to Levine agar plates (Oxoid CM69) with and without antibiotics using a spiral plater (Lameris Laboratorium B.V., Breukelen, the Netherlands). On Levine agar plates *Escherichia coli* has a purple appearance with a black centre and a green metallic sheen. Sometimes the green metallic sheen disappears if antibiotics are added to the Levine agar. Other bacteria that grow on these agar plates are: *Pseudomonas* spp, *Klebsiella* spp, *Enterobacter* spp, *Citrobacter* spp and *Proteus* spp. The following antibiotic concentrations were used based on NCCLS guidelines and modified where appropriate so that the data were comparable with that of previous studies [7]: ampicillin 25 mg/L, ciprofloxacin 4 mg/L, nalidixic acid 32 mg/L, neomycin 8 mg/L, nitrofurantoin 50 mg/L, oxytetracycline 25 mg/L, sulphamethoxazole 100 mg/L and trimethoprim 8 mg/L. If trimethoprim was used 5%(v/v) lysed horse-blood was added to the agar. After incubation at 37°C for 18-24 hours the total number and the number of resistant Enterobacteriaceae were determined. The lowest number of Enterobacteriaceae detectable was 10^3 cfu/g faeces, the highest concentration approximately 10^9 cfu/g faeces. One colony with the colonial morphology of *Escherichia coli* was randomly picked out from each agar plate ± antibiotics and tested for indole reaction and growth at 42°C. Isolates yielding positive results for both tests were considered to be *Escherichia coli* and stored at -70°C in small plastic tubes containing beads (Microbank, PRO-LAB Diagnostics, the Netherlands).

The prevalence of antibiotic-resistance was defined as the number of faecal samples with resistant Enterobacteriaceae divided by the total number of samples tested multiplied by 100%. The degree of resistance of each sample was defined as the ratio (in %) between the number of colonies grown on the agar plates ± antibiotics. If the ratio was ≥50 % or <50% the degree of resistance was defined as high or low. The prevalence and the degree of resistance were calculated over time for all the samples tested (n=682 and n=690) for Weert and Roermond, respectively, and for each individual participant (n=91 and n=96 for Weert and Roermond, respectively).

For each week, the prevalence and the degree of resistance to each antibiotic were calculated on the basis of all the samples with a positive growth on the agar plate without antibiotics. The mean values for the entire 15 week period were also calculated. In

addition, for each individual the mean values for the entire 15 week period were calculated.

Statistics

The error in the method by making serial ten-fold dilutions and by using a spiral plater was 0.5 ^{10}log (i.e. $\pm$ 5x10^{x}). If the number of colonies found on the agar plates without antibiotics from the faecal samples of one particular individual was not significantly different from the mean value, the corresponding number of colonies on the agar plates with antibiotics was included in the analysis of prevalence and degree of resistance.

The F-test, Students *t*-test and chi-square test were used to analyse statistical differences ($P<0.05$, two-tailed) between the results for the cities of Weert and Roermond. Students *t*-test was also used to predict the number of individuals with a high degree of resistance to any of the antibiotics used.

The intra-(or within) individual variation was determined by analysis of variance. Variation of the prevalence and the degree of resistance within one person was due to measurement error as well as biological variation.

RESULTS

Prevalence of antibiotic resistant Enterobacteriaceae in faecal samples

The total number of faecal samples analysed was 943 and 956 for Weert and Roermond, respectively. After incubation at 37°C for 18-24 hours no growth was visible on the agar plates without antibiotics in 249 and 255 samples from Weert and Roermond, respectively. Only those samples with a colony count on the agar plates without antibiotics, 682 and 690 samples for Weert and Roermond, respectively, were analysed. All further calculations were based upon those samples.

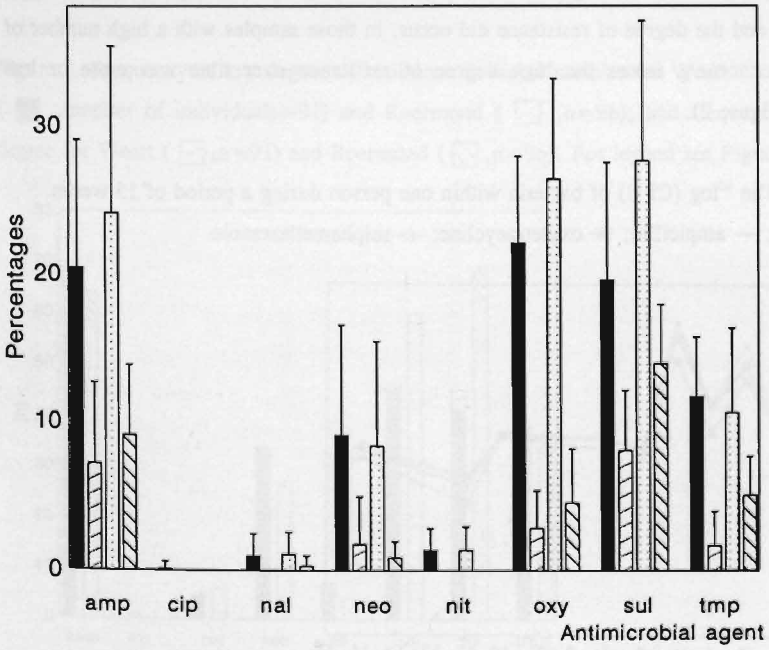
The mean total number, i.e. ^{10}log(CFU), of Enterobacteriaceae in the samples tested was for Weert 6.02$\pm$1.25 per gram faeces with a range between 3.43 and 8.43. For Roermond 6.30$\pm$1.00 was found, with a range between 3.43 and 8.43.

Of the randomly picked colonies 93% (3007/3226) were considered to be *Escherichia coli*. No resistant Enterobacteriaceae were isolated from 10/91 and 9/96

individuals, for Weert and Roermond, respectively.

The mean prevalence of antibiotic-resistant Enterobacteriaceae varied in the samples from Weert (n=682) from 28% for ampicillin and sulphamethoxazole to 0.1% for ciprofloxacin (Figure 1, only one colony of *Klebsiella* spp. was found on the agar plate containing ciprofloxacin). For the samples from Roermond (n=690) the percentages varied from 41% for sulphamethoxazole to 0% for ciprofloxacin (Figure 1). The mean prevalence of resistance to oxytetracycline and sulphamethoxazole in Roermond was significantly higher than in Weert ($P<0.05$, two-tailed).

Figure 1. Frequency of low degree of resistance for Weert (■ ,n=682) and Roermond (□ ,n=690) and frequency of high degree of resistance for Weert (▨ ,n=682) and Roermond (▧ ,n=690).
Amp=ampicillin; cip=ciprofloxacin; nal=nalidixic acid; neo=neomycin; nit=nitrofurantoin; oxy=oxytetracycline; sul=sulphamethoxazole; tmp=trimethoprim.

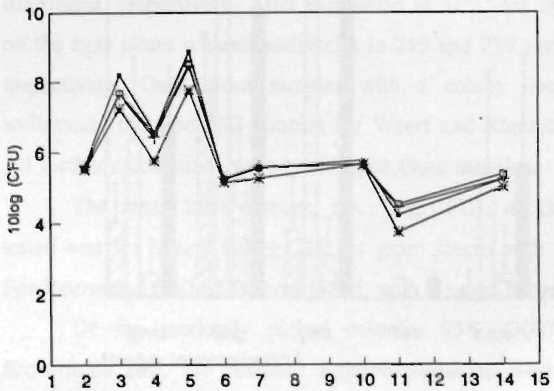


The mean percentages of the faecal samples with a relatively low degree of resistant Enterobacteriaceae varied in Weert from 22% for oxytetracycline to 0.1% for ciprofloxacin; in Roermond from 27% for sulphamethoxazole to 0% for ciprofloxacin (Figure 1). The mean percentages of a high degree of resistance varied in Weert from 8% for sulphamethoxazole to 0% for ciprofloxacin, nalidixic acid and nitrofurantoin, in Roermond from 14% for sulphamethoxazole to 0% for ciprofloxacin and nitrofurantoin (Figure 1).

Significant differences in the degree of resistance between Weert and Roermond were observed for trimethoprim (i.e. high degree) and for sulphamethoxazole (i.e. high as well as low degree) ($P < 0.05$, two-tailed).

During the 15-week period the individual variation in the number of Enterobacteriaceae present in the faecal samples was $\pm$ 1.25 ^{10}log and $\pm$ 1.36 ^{10}log for Weert and Roermond, respectively. Testing 15 samples from one subject consecutively, the total variation in the results observed is considered to be due to the measurement error variation (S.D.) and the biological variation (S.D. individual). Although variations in the prevalence and the degree of resistance did occur, in those samples with a high number of Enterobacteriaceae/g faeces the high degree of resistance over time was more or less constant (Figure 2).

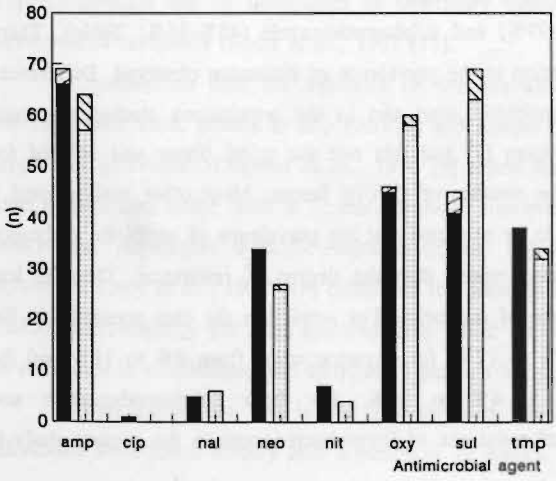
Figure 2. The ^{10}log (CFU) of bacteria within one person during a period of 15 weeks.
-•- Blank; -+- ampicillin; -✳- oxytetracycline; -□- sulphamethoxazole.



Ampicillin resistance was found in 69/91 individuals from Weert, resistance to sulphamethoxazole was observed in 69/96 individuals from Roermond (Figure 3). The prevalence of resistance for sulphamethoxazole in Roermond was significantly higher than in Weert ($P<0.05$, two-tailed). The number of individuals with a relatively low proportion of resistant Enterobacteriaceae in the faecal samples, varied in Weert from 66 for ampicillin to one for ciprofloxacin (Figure 3). For Roermond the numbers varied from 69 for sulphamethoxazole to none for ciprofloxacin (Figure 3). A high degree of resistance to ampicillin was observed in three and seven individuals from Weert and Roermond, respectively. To ciprofloxacin, nalidixic acid and nitrofurantoin no high degrees of resistance were found for either population (Figure 3).

In Roermond the low degree of resistance to sulphamethoxazole was significantly different to that in Weert ($P<0.05$, two-tailed). The percentages of a high degree of resistance to either ampicillin, oxytetracycline, sulphamethoxazole or trimethoprim in the populations studied, can be predicted to be 29% and 35% for Weert and Roermond, respectively ($P<0.05$, two-tailed).

Figure 3. Frequency of low degree of resistance in faecal samples for Weert (■ ,number of individuals=91) and Roermond (□ ,n=96); and frequency of high degree for Weert (▨ ,n=91) and Roermond (▧ ,n=96). For legend see Figure 1.



DISCUSSION

This study shows a relatively high prevalence of resistance of Enterobacteriaceae to ampicillin, oxytetracycline and sulphamethoxazole in healthy individuals from two relatively small cities in the south of the Netherlands. The prevalence of antibiotic resistance was more or less constant during the 15-week study period.

It was quite remarkable that 28% of the samples, both for Roermond and Weert, yielded no growth of Enterobacteriaceae on the agar plates without antibiotics. It is not very likely that this finding was due to the method used: as soon as the samples were delivered to the laboratory a 1:10 dilution was made in glycerol (20%(w/v)) and kept at -20°C until analysis, which was performed with a spiral plater. The lowest detection limit using this apparatus was 10^3 CFU/g faeces, thus no growth means that the sample contained less than 10^3 CFU/g faeces. Moreover, no growth inhibitory factors could be demonstrated in the 50 randomly selected faecal samples yielding no growth. It is also unlikely that freezing the 1:10 dilution of specimens affected growth as for positive samples no appreciable difference between fresh and frozen samples was found. Although it is still not clear why 28% of the samples analysed contained less than 10^3 CFU/g faeces it can be assumed that samples yielding no growth are a random part of the total number of samples collected. All data were calculated on the basis of those samples that yielded Enterobacteriaceae, thereby precluding any implications that samples yielding no growth could have on the results.

A wide range of prevalence percentages have been described in different studies, especially for ampicillin (25%-99%) and sulphamethoxazole (45%-86%; Table). There are several reasons for the variation in the prevalence of resistance observed. Differences in selective concentration of antibiotic used and in the populations studied are most important. In the studies of Bonten [7] and this one the spiral plater was utilized for quantitative determination of the number of CFU/g faeces. Most other studies used a semi-quantitative method. It is to be expected that the prevalence of antibiotic resistance is influenced by the method used more than the degree of resistance. There is less variation between the high degree of resistance. For ampicillin the data presented in the different studies varied from 5% to 17%, for oxytetracycline from 6% to 19% and for sulphamethoxazole from 8% $\pm$ 4% to 37%. For both sulphamethoxazole and oxytetracycline the prevalence of resistance of Enterobacteriaceae in the present study is

much lower than that found by Bonten *et al.*,1990 [7] and Degener *et al.*, 1990 [9].

The tendency to a decrease in prevalence and in a high degree of resistance to sulphamethoxazole may be due to the decrease in the use of this agent since 1980. Very little sulphamethoxazole was prescribed by the general practitioners in Weert and Roermond in 1991 (personal communication). Trimethoprim was prescribed twice as frequently in Weert compared to Roermond, and sulphamethoxazole-trimethoprim ten times more frequently. However, in Roermond norfloxacin was prescribed eight times as often compared to Weert. It is very likely that the general practitioners in Roermond prescribed norfloxacin instead of trimethoprim due to the high degree of resistance to sulphamethoxazole and trimethoprim. For tetracycline the explanation for the percentages found is less self-evident. The population tested in this study lives in an area with many stock-farms, while the populations tested by other authors were mainly city-dwellers. In veterinary medicine large amounts of tetracycline are used for therapy and prophylaxis as well as for growth promotion, therefore a higher prevalence may be expected in this study compared to others (Table). However, our values are of the same order of magnitude as those found by Levy *et al.*, 1988 [18] and Degener *et al.*, 1990 [9].

In this study no ciprofloxacin-resistant *Escherichia coli* were found. At present most Enterobacteriaceae are highly susceptible to the fluoroquinolones although the use of enrofloxacin in poultry has caused resistance in *Campylobacter* spp. (Endtz *et al.*, 1991 [11]. Large-scale use of quinolones in veterinary medicine may further increase the prevalence of resistance (Endtz *et al.*, 1991 [11].

At present no data are available on the prevalence of resistance in the same individual over time. Bonten *et al.*, 1990 [7] and Amyes *et al.*, 1992 [3] tested only one sample per individual. Degener *et al.*, 1990 [9] tested the same population (but not the same individuals) twice with a 5 year interval. However, the study does not present census and population dynamic information on individuals of the original study population. Levy *et al.*, 1988 [18] compared the prevalence of antibiotic resistance in the aerobic Gram-negative gut flora cultured from faecal samples from medical students with the resistance in hospitalized and ambulatory patients ten years earlier.

To our knowledge this is the first study in which faecal samples of the same individuals were tested weekly over a period of 15 weeks. The data clearly showed that a

high degree of resistance in the individuals remained more or less constant over time (Figure 2). For the low degree of resistance more variation was observed (data not shown). Consequently testing only once is, as expected, less accurate. The most important cause of the variations observed in the prevalence and the degree of resistance was biological variation, rather than measurement error variation. In other studies, based upon one sample per individual, one does not take into account the biological variation. In those studies the variation in the data mentioned is also at least $\pm$ 1.25 ^{10}log.

Testing over a period of time gives the possibility to predict the prevalence of resistance in the near future. This calculated future prevalence for ampicillin, oxytetracycline, sulphamethoxazole and trimethoprim in the population studied is 26% and 29% for Weert and Roermond, respectively. Whether these values correspond with the actual prevalence warrants surveillance of resistance of the faecal flora of healthy individuals. This surveillance of resistance will provide general practitioners with essential data for their choice of empirical therapy [22]. The Infectious Disease Society of America stressed the importance that hospital formularies are based on antibiotic susceptibility patterns of the own hospital isolates. General practitioners also have to base their empirical therapy on susceptibility data of outpatient isolates. Taking into account that the bowel flora are considered to be an important reservoir for antibiotic resistance genes [12], especially outside hospitals, knowledge about the antibiotic resistance of these isolates from healthy individuals will contribute to an educated guess of the empirical therapy.

Table. Comparison of data from different studies.

Ampicillin :							
	(1)	(2)	(3)	(4)	(5)	(6)[a]	
	1978-80	1987	1988	1990	1992	1992	
						W[b]	R[b]
(mg/L)	40	40	30	25	10	25	25
prevalence (%)	25	27	35	76	99	28±12	33±12
high degree (%)	5	11	17	8	-	7±5	9±5
Oxytetracycline :							
	(1)	(2)	(3)	(4)	(5)	(6)[a]	
	1978-80	1987	1988	1990	1992	1992	
						W	R
(mg/L)	30	30	10	25	-	25	25
prevalence (%)	42	20	30	47	-	25±7	31±7
high degree (%)	12	6	19	11	-	3±3	5±4
Sulphamethoxazole :							
	(1)	(2)	(3)	(4)	(5)	(6)[a]	
	1978-80	1987	1988	1990	1992	1992	
						W	R
(mg/L)	100	100	-	100	-	100	100
prevalence (%)	45	46	-	86	-	28±10	41±7
high degree (%)	19	21	-	37	-	8±4	14±4

[a] (1) Degener *et al* '78-'80; (2) Degener *et al* '87; (3) Levy *et al* '88; (4) Bonten *et al* '90; (5) Amyes *et al* '92; (6) present study.

[b]W=Weert and R=Roermond.

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CHAPTER IV

CARRIAGE OF ANTIBIOTIC-RESISTANT *ESCHERICHIA COLI* BY HEALTHY VOLUNTEERS DURING A 15-WEEK PERIOD.

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SUMMARY

Escherichia coli strains (n=678 and n=670) isolated from faecal samples from 90 and 93 healthy volunteers of two cities, Weert and Roermond respectively, were analysed for their susceptibility to 12 antimicrobial agents, during a 15-week period. Significant differences between both cities in the distribution of the MIC values were observed for apramycin, chloramphenicol, kanamycin, neomycin, nitrofurantoin, sulphamethoxazole and trimethoprim. For Weert (n=678) the antibiotic resistance percentages varied from 0.4% for nalidixic acid to 26.7% for sulphamethoxazole. For Roermond (n=670) the figures varied from 0.6% for nitrofurantoin to 37.5% for sulphamethoxazole. Resistance to amoxycillin/clavulanate was not found in either city.

The most frequent pattern was resistance to sulphamethoxazole only, followed by resistance to oxytetracycline, streptomycin and sulphamethoxazole.

In each individual there was only a small variation in resistance patterns of the isolates, i.e. the majority had one (n=51) or two (n=63) patterns with a maximum of five during the 15-week period. A fully susceptible pattern was found in the strains from 38 individuals.

INTRODUCTION

Bacterial resistance to antimicrobial agents is a problem of worldwide concern [18,23,27]. Also to recently introduced antibiotics, such as the newer fluoroquinolones [14,35] and the third generation cephalosporins [11,16,28] bacterial resistance does occur. In contrast to the extensive data available on antibiotic susceptibilities of hospital isolates [6,7,9,24,25,30] less information is available on the incidence of antibiotic-resistant strains in general practice [2,10,34] or in healthy volunteers [1,21]. Knowledge on the antibiotic resistance of strains colonising healthy people is important for several reasons. First, antibiotic-resistant strains are considered to be the largest reservoir of resistance genes [13]. Second, the mechanism of resistance in these isolates may be different from that in hospital strains and subsequently the therapy for them may be different as well [8]. Finally the correlation between antibiotic resistance of colonising strains and the resistance of infecting microorganisms as clearly demonstrated by Lester *et al.* [19] stressed the

importance of ongoing antibiotic surveillance of colonising strains in healthy individuals to predict resistance in future infecting isolates and to support the antibiotic choices of empiric therapy.

Therefore a 15-week surveillance period was started during which faecal samples from about 100 healthy volunteers in each of two cities in the same region were collected weekly. One of the aims was to determine the antibiotic resistance patterns of the faecal *Escherichia coli* from all samples and to analyse the variation in antibiotic resistance of the isolates from each individual over the 15-week period. Also the mechanism of resistance to one of the antibiotics, trimethoprim, was tested.

MATERIALS AND METHODS

Sample population

Faecal samples of healthy volunteers of two cities and their rural surroundings, Weert (n=91) and Roermond (n=96), were collected weekly from 25 March to 1 July 1991 as described previously [22].

Sample processing

Faecal dilutions (1:10 to $1:10^5$) were applied onto Levine agar plates (Oxoid CM69, Ltd., Basingstoke, England) without antibiotics using a spiral plater (Lameris Laboratorium B.V., Breukelen, the Netherlands). Colonies with the colonial morphology of *Escherichia coli* were randomly picked out from each agar plate without antibiotics, one colony per faecal sample, and tested for indole reaction and growth at 42°C. Only isolates yielding positive results for both tests were considered to be *Escherichia coli*. The identification of 50 randomly chosen *Escherichia coli* strains, identified as mentioned above, were confirmed using the API 20E system to be *Escherichia coli*.

Susceptibility tests

The antibiotic susceptibility was determined using a microbroth dilution method in Iso-Sensitest Broth (Oxoid CM473). An inoculum of 5×10^5 CFU/ml was used. The plates were incubated for 18-24 hours at 37°C, the MIC being defined as the lowest antibiotic concentration completely to inhibit growth. *Escherichia coli* ATCC 25922 and *Escherichia*

coli ATCC 35218 were used as reference strains. The antimicrobial agents used are shown in Table 1. The breakpoints for resistance were those recommended by the guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing [17]. For apramycin the breakpoint of resistance was >16 mg/L [15].

Characterisation of trimethoprim resistant strains

Plasmid DNA was isolated as described by Sambrook *et al.* [29] Two probes encoding trimethoprim resistance were used : one was a 0.49 kb *KpnI/BamHI* fragment of pLKO627 encoding the *dhfrI* gene and the other was a 0.49 kb *HpaI* fragment of pLKO2-2A encoding the *dhfrV* gene [32,33]. The DNA probes were purified by extraction with diatoms earth from agarose gels and labeled with digoxigenin according to the manufacturer's instructions (Boehringer Mannheim Biochemica, Mannheim, Germany). The strains were screened by making colony blots on nylon membranes (Nytran) according to the manufacturer's instructions (Nytran, Schleicher & Schuell, Den Bosch, the Netherlands). As positive controls the strains containing the plasmids pLK0627 and pLKO22A were used, as negative control *Escherichia coli* K-12. The probes for type I and V hybridised only with their own positive control strains; no other cross-hybridizations were detected. Prehybridization and hybridization were performed under stringent conditions (50%(v/v) formamide and 5xSSC [1xSSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 42°C, and washes in 2xSSC plus 0.1%(w/v) SDS and 0.1xSSC plus 0.1%(w/v) SDS at 68°C. The detection of digoxigenin-labelled nucleic acids was performed by chemoluminescence on radiographic films (Kodak X Omat AR).

Statistics

The F-test, chi-square test, Mann-Whitney test and hierarchical cluster analysis by average linkage (UPGMA) and Squared Euclidian distances (i.e. Distance $(X,Y)=\Sigma(X_i-Y_i)^2$) were used to analyse whether there were significant differences ($P<0.05$, two-tailed) between the cities Weert and Roermond.

RESULTS

Antibiotic resistance

In faecal samples from one individual from Weert and three individuals from Roermond, *Escherichia coli* was not detected. Finally, a total of 678 and 670 *Escherichia coli* strains for Weert and Roermond respectively were analysed for their susceptibility to 12 antimicrobial agents. Significant differences in the distribution of the MIC values were observed for apramycin, chloramphenicol, kanamycin, neomycin, nitrofurantoin, sulphamethoxazole and trimethoprim ($P < 0.05$) (Table 1).

For further analysis all values were first classified by hierarchical cluster analysis. Consequently, slight differences between the frequencies found in Table 1 and Table 2 might occur. The frequency of resistance to the antimicrobial agents tested is shown in Table 2. The highest frequency of resistance was found for sulphamethoxazole, 26.7% and 37.5%, followed by streptomycin (20.5% and 26.9%) and oxytetracycline (17.3% and 24.6%). Resistance to amoxycillin/clavulanate (2:1) was not observed in the samples of either city. High MIC values, i.e. MIC $\geq$ 512 mg/L, for apramycin were observed for three isolates of Roermond, which were isolated from one individual. All three were also resistant to gentamicin (MIC $\geq$ 8 mg/L). High level resistance to nalidixic acid (MIC $\geq$ 128 mg/L) was observed for three and ten isolates from Weert and Roermond respectively, isolated from three and six individuals. None of these strains were resistant to ciprofloxacin (MIC $\leq$ 1 mg/L).

Table 1. Distribution of MIC values for isolates from Weert (n=678) and Roermond (n=670).

Agent[a]	City	Number of strains inhibited by concentration (μg/ml)														
		0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024
AM	W[b]				0	1	22	365	210	23	1	1	3	0	52	
	R[b]				1	4	5	404	158	13	1	0	0	3	81	
AP	W				0	0	31	345	244	46	11	1	0	0	0 *	
	R				0	1	36	382	201	41	6	0	0	0	3	
AM/CL	W				0	1	14	296	283	84	0	0	0	0	0	
	R				0	3	8	294	269	96	0	0	0	0	0	
CH	W					0	9	101	485	49	1	3	30 *			
	R					1	6	170	404	27	2	1	59			
KA	W						212	385	42	15	1	8	3	12 *		
	R						253	338	44	10	2	6	1	16		
NA	W					7	109	501	58	0	0	0	3			
	R					3	137	477	42	1	0	2	8			
NE	W					327	282	36	13	2	8	5	5 *			
	R					374	216	49	8	4	1	12	6			
NI	W					4	5	27	390	221	25	4	2 *			
	R					6	1	50	438	151	19	4	1			
OX	W						8	142	392	19	1	1	3	112		
	R						10	176	310	9	2	2	10	151		
ST	W					0	36	365	122	17	21	35	82			
	R					1	29	342	94	21	48	41	94			
SU	W							0	248	162	41	28	19	25	37	118 *
	R							1	209	110	49	28	17	6	18	232
TR	W	2	58	247	285	34	1	1	0	21	0	1	1	0	27	0 *
	R	18	112	238	200	32	6	4	0	6	0	0	0	1	51	2

[a] AM=amoxycillin; AP=apramycin; CH=chloramphenicol; CL=clavulanate; KA=kanamycin; NA=nalidixic acid; NE=neomycin; NI=nitrofurantoin; OX=oxytetracycline; ST=streptomycin; SU=sulphamethoxazole; TR=trimethoprim.

[b] W=Weert and R=Roermond *P<0.05

Table 2. Antibiotic resistance of isolated *Escherichia coli* from Weert (n=678) and Roermond (n=670) to 12 antimicrobial agents.

Antimicrobial agent[a]	Number (percentage) of resistant isolates for	
	Weert	Roermond
AM	57 (8.4)	83 (12.4)*
AP	15 (2.2)	8 (1.2)
AM/CL	0	0
CH	59 (8.7)	68 (10.1)
KA	24 (3.5)	26 (3.9)
NA	3 (0.4)	10 (1.5)
NE	18 (2.7)	19 (2.8)
NI	5 (0.7)	4 (0.6)
OX	117 (17.3)	165 (24.6)*
ST	139 (20.5)	180 (26.9)*
SU	181 (26.7)	251 (37.5)*
TR	51 (7.5)	65 (9.7)

[a] For abbreviations, see Table 1

* $P < 0.05$, two-tailed

Antibiotic resistance patterns of *Escherichia coli*

Multiple resistance, i.e. resistance to two or more antibiotics, was found in 202/670 strains from Roermond and 150/678 from Weert ($P < 0.05$, two-tailed).

The resistance pattern most frequently observed for Weert and Roermond was resistance to sulphamethoxazole only, n=80 and n=89, respectively (Table 3). With a distinctly lower frequency resistance to oxytetracycline, streptomycin and sulphamethoxazole was observed in both groups (n=22 and n=25), followed by streptomycin alone in Weert (n=20) and oxytetracycline and streptomycin in Roermond (n=25). Susceptibility to all agents tested was found for 395 (58.3%) and 330 (49.3%) isolates from Weert and Roermond, respectively (Table 3).

The majority (i.e. 568/678 and 551/670) of the faecal isolates of both cities had a resistance pattern belonging to one of the patterns shown in Table 3. In addition 23 patterns, i.e. only 42/678 strains were unique for Weert and 35 patterns, only 66/670 strains

for Roermond, giving rise to 51 and 63 different patterns for Weert and Roermond, respectively. Testing faecal samples from each individual consecutively during a 15-week period, individual variation in resistance patterns could be analysed. One to seven different patterns in each individual were observed.

Table 3. The ten most frequent patterns of resistance for *Escherichia coli* isolates from Weert (n=678) and Roermond (n=670).

Weert		Roermond	
Pattern[a]	(n)	Pattern	(n)
--[b]	395	--	330
SU	80	SU	89
OX-ST-SU	22	OX-ST-SU	25
ST	20	OX-ST	25
0X	14	ST	16
AM-CH-OX-ST-SU-TR	13	OX-SU	15
CH-OX-ST	9	AM-OX-ST-SU-TR	13
AM-OX-ST-SU	8	OX	10
AM	7	ST-SU	10
AM-OX-ST-SU-TR	6	AM-CH-OX-ST-SU-TR	9
CH-OX-ST-SU-TR	6	CH-OX-SU-TR	9
	568/678		551/670

[a] For abbreviations, see Table 1

[b] -- = no resistance.

The majority of the isolates had one (n=51) or two (n=63) different resistance patterns (Table 4).

Table 4. The number of resistance patterns present in each individual from Weert (n=90) and Roermond (n=93).

Number of patterns	Number of individuals	
	Weert	Roermond
1	27	24
2	27	36
3	17	20
4	14	4
5	4	6
6	0	3
7	1	0

Because there were no significant differences in the number of patterns in the individuals of both cities, for further analysis all individuals were grouped together.

The number of patterns in relation to the number of strains tested per individual is given in Table 5. Because one strain from each sample was picked out, the number of strains corresponded to the number of samples tested. The number of samples (i.e. strains) analysed per individual ranged from one to 13, whereas the number of different patterns observed in most individuals during the study period was not more than five. Only four individuals showed during 8,10 and 11 weeks, six and seven different patterns. A fully susceptible pattern during the 15-week period was found in 38/51 individuals. Thus the isolates from these individuals had only one pattern (i.e. susceptible to all agents) during the whole period.

Trimethoprim resistance

Resistance to trimethoprim was observed in 116 isolates, from which 81 were resistant to high levels of trimethoprim (MIC $\geq$ 512 mg/L). These strains were isolated from 29 individuals. After screening for the presence of the most frequently occuring dihydrofolate reductase genes, i.e. *dhfrI* and *dhfrV* , all individuals except one were posi-

tive for *dhfrI*, while 9/29 individuals were positive for *dhfrV*.

Table 5. The number of patterns in relation to the number of strains tested per individual.

Number of samples	Number of individuals	Number of patterns						
		1	2	3	4	5	6	7
1	10	10						
2	11	4	7					
3	14	7	5	2				
4	9	2	3	3	1			
5	14	3	5	3	3			
6	11	5	3	2		1		
7	12	4	4	1	2	1		
8	22	3	11	4	3		1	
9	26	6	9	6	3	2		
10	17	2	7	4	2	1	1	
11	16	2	5	3	1	3	1	1
12	11	1	2	5	2	1		
13	10	2	2	4	1	1		
		---	---	---	---	---	---	---
		51	63	37	18	10	3	1

DISCUSSION

The main feature of the study was the high frequency of resistance to sulphamethoxazole, streptomycin and oxytetracycline of the *Escherichia coli* strains (n=678 and n=670) isolated from faecal samples of 90 and 93 healthy volunteers from Weert and Roermond, respectively. Moreover the differences in resistance to these compounds as well as to amoxycillin were significant ($P<0.05$). In addition, our data during the 15-week period strongly suggest that the pool of antibiotic-resistant strains is relatively constant over time and is maintained in the faecal flora of healthy individuals who have not taken any antibiotics recently.

The ten most frequent resistance patterns in this and in other studies are shown in

Table 6. Although some differences did occur the majority of the resistance patterns observed in the studies mentioned were quite similar despite the different populations studied. The population analysed by Linton *et al.* [21] and in this study consisted of healthy people; Møller *et al.* [25] studied in- and outpatients and Levy *et al.* [20] analysed healthy people, laboratory workers and hospital patients. In the present study, resistance to chloramphenicol was always (except for two strains) correlated with resistance to other antimicrobial agents. The most frequent pattern was resistance to chloramphenicol, oxytetracycline, streptomycin and sulphamethoxazole. Often resistance to amoxycillin and trimethoprim was present as well. Chloramphenicol and tetracycline do select for the chromosomal Mar-system which confers also resistance to structurally unrelated agents, including nalidixic acid, fluoroquinolones, rifampin, penicillins and cephalosporins. Isolates being resistant to oxytetracycline, chloramphenicol and nalidixic acid are more likely to select for this Mar-system and could therefore be a direct potential clinical problem in the hospital environment [5,12].

Resistance to apramycin, an aminoglycoside used only in veterinary medicine, was observed in 23 *Escherichia coli* strains, three of which were resistant to high levels of apramycin (MIC $\geq$ 512 mg/L). This high level apramycin-resistance confers also resistance to gentamicin, which is probably due to production of plasmid-mediated aminoglycoside acetyltransferase 3-IV (AAC(3)-IV). Notably, of the 187 individuals tested who did not have a history of recent hospital stay or antibiotic use, in one individual this type of resistance was present. It is to be expected that in the hospital environment a higher prevalence and spread of this plasmid will occur [3,4].

The presence of both *dhfrI* and *dhfrV* in one strain was also observed by Singh *et al.* [31]; of all colony isolates 21% hybridised under stringent conditions, and all of these were with type I (17%) or type V (4%); of the type V positive ones, two were also positive for type I. Resistance to trimethoprim was often correlated with resistance to amoxycillin, oxytetracycline, streptomycin, sulphamethoxazole and chloramphenicol.

In the present study, in the majority of the individuals only one or two different resistance patterns, with a maximum of five, could be observed, which were independent of the number of strains tested for that individual. In contrast, Levy *et al.* [20] found that 90% of all individuals showed a gain (47.6%) and/or a loss (65.7%) of one or more detectable resistances when analysing two samples obtained from individuals of the

ambulatory group off antibiotics. No distinct explanation could be given for the differences in antibiotic resistance between the two populations studied. Because both cities have the same socio-economic level, and are only 30 km apart, it is not very likely that environmental circumstances could explain the differences observed. Only one person reported having taken antibiotics, i.e. tetracycline, during the study period so the influence of individual recent antibiotic use on the resistance percentage observed was negligible. Moreover differences in antibiotic prescriptions by the general practitioners during the year preceding the start of the surveillance could not explain the differences in resistance observed. Sulphamethoxazole is hardly used in either city. Amoxycillin has been prescribed slightly more in Weert compared to Roermond, whereas for the antibiotic resistance the reverse was found (8.4% and 12.4%), Quinolones (ciprofloxacin and norfloxacin) were eight times more frequently used in Roermond. In contrast, trimethoprim alone and in combination with sulphamethoxazole was used twice and eight times as much in Weert compared to Roermond. However the differences in antibiotic prescriptions were not reflected in the antibiotic resistance. Further studies to analyse factors influencing antibiotic resistance in healthy individuals remain to be performed.

Table 6. Comparison of the ten most frequent patterns of resistance for four different studies.

Weert[a]	Roermond[a]	(2)	(3)	(4)
SU[b]	SU	OX	OX	OX-ST
OX-ST-SU	OX-ST-SU	AM	ST-SU	AM-OX-ST
ST	OX-ST	AM-OX	OX-ST-SU	AM-KA-OX-ST
OX	ST	OX-ST-SU	AM	AM-OX
AM-CH-OX-ST-SU-TR	OX-SU	ST-SU	AM-ST-SU	AM-ST
CH-OX-ST	AM-OX-ST-SU-TR	AM-ST-SU	ST	KA-OX-ST
AM-OX-ST-SU	OX	AM-OX-ST-SU	CH-ST-SU	AM-KA
AM	ST-SU	OX-ST	OX-ST	KA-ST
AM-OX-ST-SU-TR	AM-CH-OX-ST-SU-TR	ST	AM-CH-KA-OX-SU	AM-KA-ST
CH-OX-ST-SU-TR	CH-OX-SU-TR	CH-OX-ST-SU	OX-SU	KA-OX

[a] present study; (2) Linton *et al.* [21]; (3) Mϕller *et al.* [25]; (4) Levy *et al.* [20]

[b] For abbreviations, see Table 1

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CHAPTER V

EFFECT OF ANTIBIOTIC THERAPY ON THE ANTIBIOTIC RESISTANCE OF FAECAL *ESCHERICHIA COLI* IN PATIENTS ATTENDING GENERAL PRACTITIONERS.

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SUMMARY

To analyse the influence of antibiotic therapy on the faecal flora of patients from general practice with complaints of a respiratory tract infection (RTI), 189 paired faecal specimens were collected, before and after completing antibiotic therapy (n=129) and symptomatic therapy (n=60). Faecal specimens were examined for the prevalence and degree of resistance to amoxycillin, apramycin, ciprofloxacin, nalidixic acid, neomycin, nitrofurantoin, oxytetracycline, sulphamethoxazole and trimethoprim. In the antibiotic-treatment group a significant increase in the prevalence of resistance to amoxycillin post-treatment from 50% to 64% ($P<0.05$, Wilcoxon) was observed. In the symptomatic treatment group no significant differences in the prevalence of resistance were found. Using discriminant analysis, amoxycillin and doxycycline therapy contributed to an increased prevalence of resistance to amoxycillin and oxytetracycline, respectively. In the antibiotic-treated group *Escherichia coli* isolates post-treatment had a significantly increased resistance rate to amoxycillin (15% - 23%) and to neomycin (2% - 6%) ($P<0.05$, Wilcoxon). Logistic regression analysis showed a cross resistance to neomycin and kanamycin, and for kanamycin cross-resistance to apramycin, neomycin and streptomycin occurred.

INTRODUCTION

Antimicrobial agents are one of our major resources for the prevention and therapy of infectious diseases. However, use of antibiotics selects for antibiotic-resistant strains, for example in the faecal flora [3,14,19]. Most studies dealing with the relation between antibiotic consumption and antibiotic resistance have been performed in hospitals and describe a positive correlation between usage and resistance [2,8]. In Finland the increased resistance of *Escherichia coli* to trimethoprim from 8% to 35% paralled the increased consumption of trimethoprim and co-trimoxazole between 1971 and 1984 [7]. Mouton *et al.*, 1990 [15] found a positive correlation between antibiotic consumption of flucloxacillin, cephalosporines and gentamicin and prevalence of methicillin-resistant coagulase negative staphylococci. In contrast, Rosendal *et al.*, 1977 [17] showed a rapid decrease in the percentage of methicillin-resistant *Staphylococcus aureus* from 1969 to

1974, despite an increase in the usage of methicillin. Sometimes a decline in usage of one compound is not only related to a decreased resistance to that agent but also to a non-related antibiotic [13]. Only limited data are available on antibiotic resistance in general practice patients [1,4,20,21]. General practitioners prescribe antimicrobial agents mostly for the treatment of urinary tract infections (UTI) and respiratory tract infections (RTI). This study describes the effect of antibiotic treatment of RTI on the antibiotic resistance of faecal isolates of general practice patients.

MATERIALS AND METHODS

Sample population

During a six-week period, November to December 1992, patients in the south of the Netherlands, who attended their general practitioner with RTI were requested to participate in the study. Regardless of the therapy prescribed, they were asked to send in one faecal specimen before starting therapy and one just after completion treatment. In addition they were asked to fill in a questionnaire that included questions on the current therapy prescribed (antibiotic or symptomatic treatment), antibiotic use and/or hospitalization in the previous three months, keeping domestic animals, occupation (slaughter or stock-farmer or others). Patients were excluded if the first faecal sample was collected more than 24 hours after start of the therapy. In addition faecal samples had to be received and processed not more than 48 hours after collection and only those specimens which showed growth on the agar plates without antibiotics were included in the final analysis. Other exclusion criteria were antibiotic use and/or hospitalization in the previous three months.

Specimen collection and processing

One gram of fresh faeces was diluted 1:10 in 0.9%(w/v) NaCl supplemented with 20%(v/v) glycerol and stored at -20°C until assayed. The methods used for the analysis were as previously described [11]. Briefly, after thawing the suspensions, serial ten-fold dilutions were made up and 0.037 mL were inoculated on to Levine agar plates (Oxoid CM 69, Ltd., Basingstoke, England) with and without antibiotics using a spiral plater (Lameris Laboratorium B. V., Breukelen, the Netherlands). On Levine agar plates

Escherichia coli has a purple appearance with a black centre and a green metallic sheen. The antimicrobial agents and the concentrations used are shown in Table II. The total number and number of resistant Enterobacteriaceae were recorded after incubation at 37°C for 18-24 hours.

The prevalence of antibiotic-resistance in the faecal specimens tested was defined as the number of specimens with resistant Enterobacteriaceae divided by the total number of specimens tested multiplied by 100%. The degree of antibiotic resistance in each sample was defined as the percentage ratio between the number of colonies grown on the agarplates with antibiotics and without antibiotics. If the ratio was ≥50% the degree of resistance was defined as high, and a ratio between 0% and 50% was defined as low. From each sample one colony with the colonial morphology of *Escherichia coli* was randomly picked out from the agar plate without antibiotics and tested for indole reaction and growth at 42°C. Only isolates yielding positive results for both tests were considered to be *Escherichia coli*. Identification was confirmed by random selection of 50 *Escherichia coli* using the API 20E system.

Susceptibility tests

The antibiotic susceptibility was determined for all *Escherichia coli* strains, isolated from the agar plates without antibiotics, using a microbroth dilution method in Iso-Sensitest Broth (Oxoid CM 473) and an inoculum of $5x10^5$ CFU/mL. The plates were incubated for 18-24 hours at 37°C, with the MIC defined as the lowest concentration to completely inhibit growth. *Escherichia coli* ATCC 25922 and ATCC 35218 were used as reference strains. The breakpoints for resistance of the antimicrobial agents (Table IV) were those recommended by the guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing [9]. For apramycin the breakpoint of resistance was >16 mg/L [6]. Analysis of the antibiotic susceptibility of the *Escherichia coli* strains isolated before and after therapy was performed firstly using percentages of sensitive and resistant strains, according to these breakpoints, and secondly as a classification of the MIC values into two categories. This classification is based on the assumption that MIC values within one doubling-dilution step are considered to be the same. Category I includes strains with the same MIC value (± one dilution) or a decreased MIC value of more than one dilution,

and category II includes strains with an increased MIC value of more than one dilution.

Statistics

The chi-square test, Wilcoxon Matched-Pairs test and Mann-Whitney U-test were used to analyse whether there were significant differences in antibiotic-resistance pre-and post-treatment. Discriminant analysis was used to analyse the relationship of questionnaire answers to antibiotic resistance. These were considered to be dichotomised variables. When analysing resistance to one particular agent, pre- and posttreatment, the prevalences and degree of resistance to the other agents were also considered as variables. When analysing the changes in antibiotic resistance of the post-treatment-group, the prevalences and degree of resistance of all agents from the pre-treatment period were analysed together with the other variables. Logistic regression was used to analyse the contribution of questionnaire answers to the ratio between the categories I and II. The ratios of the other agents were also considered as variables.

$P < 0.05$ was considered significant.

RESULTS

Sample population

The total number of pre-treatment faecal specimens was 325. Excluded from further analysis were those patients who used any antibiotics, or were hospitalized in the previous three months (n=25 and n=2, respectively), or from whom the first faecal sample was collected more than 24 h after start of the therapy (n=10). This left 288 pre-treatment samples, including 91 from symptomatic-treatment patients. Post-treatment 189 faecal specimens were collected, including 60 from symptomatic-treatment patients. Only paired samples, (n=189) were included in the final analysis. The majority of the antibiotic-treatment patients received amoxycillin (n=68) or doxycycline (n=54). Only six patients received erythromycin and one co-trimoxazole. As symptomatic therapy mostly analgesic and mucolytic agents were used. The characteristics of the symptomatic- and the antibiotic-treatment patients were similar (Table I).

Table I. Characteristics of the patient population.

	symptomatic therapy (n=60)	antibiotic therapy (n=129)
Mean age/range (years)	37.9/2-74	35.3/1-82
Males (n)	29	64
Hospitalization of family member (n)	9	18
Domestic pets (n)	32	68
Slaughterer/stock-farmer (n)	7	3

Prevalence of antibiotic-resistant Enterobacteriaceae

The mean $\log^{10}$(CFU) of Enterobacteriaceae per gram of faeces, in the specimens pre- and post-treatment was similar, regardless of the therapy prescribed. For the pre-treatment specimens of the antibiotic-treatment and the symptomatic-treatment groups the mean $\log^{10}$(CFU) was 7.60 (range 3.43 - 10.37) and 7.49 (range 4.13 - 10.00), respectively. For the post-treatment specimens the corresponding figures were 7.49 (range 3.43 - 10.12) and 7.40 (range 3.43 - 10.06). As shown in Table II, no significant differences were observed in the prevalence of resistance to any of the antibiotics tested before and after symptomatic treatment. In the antibiotic-treated group the prevalence of resistance to amoxycillin increased significantly (from 50% to 64%, $P<0.05$, Wilcoxon). The observed increases in resistance to oxytetracycline and sulphamethoxazole were not significant. Between the antibiotic-treatment and the symptomatic-treatment group, a difference in prevalence of resistance to neomycin was observed both pre- and post-treatment. The increased prevalence of antibiotic resistance post- versus pre-treatment was related to the antibiotic therapy prescribed (Table III). Amoxycillin therapy contributed significantly to the increased prevalence of resistance post-treatment not only to amoxycillin but also to oxytetracycline. Similarly, doxycycline therapy resulted in an increased prevalence of resistance post-treatment to oxytetracycline as well as to amoxycillin.

Using discriminant analysis, antibiotic therapy as well as pre-treatment resistance were important factors contributing to the resistance post-treatment (data not shown).

Table II. Prevalence of resistance of the symptomatic-treatment group (n=60) and the antibiotic-treatment group (n=129) pre- and post-treatment.

Antibiotic (mg/L)	Symptomatic-treated group prevalence		Antibiotic-treated group prevalence	
	pre (%)	post (%)	pre (%)	post (%)
Amoxycillin (25)	55[1]	48	50[12]	64[2]
Apramycin (32)	2	2	0	1
Ciprofloxacin (4)	0	0	0	0
Nalidixic acid (32)	2	2	1	2
Neomycin (8)	3[3]	5[3]	13[3]	19[3]
Nitrofurantoin (50)	3	0	2	2
Oxytetracycline (25)	37	43	38	49
Sulphamethoxazole (100)	40	46	44	52
Trimethoprim (8)	19	25	25	26

[123]$P < 0.05$

Table III. Relation between amoxycillin / doxycycline therapy and resistance.

Agent	Kind of therapy	Pre- / post-	Susceptible	Low degree	High degree	Number of patients
Amoxycillin	amoxycillin	pre-	34	20	14	68
		post-	20	29	19	68
	doxycycline	pre-	47	18	10	75
		post-	44	23	8	75
Oxytetra-	amoxycillin	pre-	31	21	9	61
cycline		post-	26	24	11	61
	doxycycline	pre-	33	13	8	54
		post-	22	19	13	54

Susceptibility testing

From eight out of 60 and 13 out of 129 patients no *Escherichia coli* could be isolated either from the first or the second faecal sample, resulting in totals of 52 and 116 *Escherichia coli* strains, respectively, for susceptibility testing to 12 antimicrobial agents. As shown in Table IV, for the antibiotic-treatment patients a significant increase in resistance post-treatment was observed for amoxycillin (from 15% to 23%) and for neomycin (from 2% to 6%). For the symptomatic-treatment patients a significant increase in resistance post-treatment was only observed for sulphamethoxazole (19% to 39%).

Table IV. Antibiotic resistance of *Escherichia coli* isolates from the symptomatic-treatment group (n=52) and the antibiotic-treatment group (n=116) to 12 antimicrobial agents.

Antibiotic	Breakpoint (mg/L)	Percentages of resistant isolates symptomatic (n=52)		antibiotic (n=116)	
		pre-	post-	pre-	post-
Amoxycillin	16	6	15	15[a]	23[a]
Apramycin	16	0	0	1	0
Amoxycillin/ clavulanic acid	16	0	0	0	0
Chloramphenicol	8	2	6	3	9
Kanamycin	16	2	2	4	7
Nalidixic acid	8	0	0	3	2
Neomycin	16	2	2	2[a]	6[a]
Nitrofurantoin	32	0	2	0	3
Oxytetracycline	16	19	25	19	22
Streptomycin	16	17	27	27	28
Sulphamethoxazole	128	19[a]	39[a]	27	27
Trimethoprim	2	4	8	10	10

[a] $P<0.05$

After antibiotic treatment, the ratio between the number of strains in category I and II for neomycin and kanamycin was significantly higher than after symptomatic treatment. For sulphamethoxazole, the opposite was observed (Table V). Logistic regression analysis showed a positive correlation between the number of strains with an increase in neomycin resistance and kanamycin resistance (category II), and also between kanamycin and apramycin, neomycin and streptomycin resistance (data not shown).

Table V. Distribution of strains among the categories I and II (see text) for the symptomatic-treated group (n=52) and the antibiotic-treated group (n=116).

	Symptomatic-treated number of strains in category:		Antibiotic-treated number of strains in category:	
Antibiotic	I	II	I	II
Amoxycillin	43	9	99	17
Apramycin	51	1	113	3
Amoxycillin/ clavulanic acid	44	8	105	11
Chloramphenicol	48	4	108	8
Kanamycin	51	1*	107	9*
Nalidixic acid	50	2	109	7
Neomycin	49	3*	101	15*
Nitrofurantoin	48	4	106	10
Oxytetracycline	45	7	100	16
Streptomycin	44	8	98	18
Sulphamethoxazole	33	19*	90	26*
Trimethoprim	43	9	96	20

* $P < 0.05$

DISCUSSION

The main finding of the present study was the increased prevalence of antibiotic resistance in patients faecal flora receiving amoxycillin and/or doxycycline therapy for the treatment of RTI in general practice. In 1991 in the same region, the antibiotic resistance

of isolates from faecal specimens from healthy volunteers was studied [11]. The prevalences of resistance to oxytetracycline, sulphamethoxazole and trimethoprim were similar to those observed in the present study. The prevalence of resistance to amoxycillin was higher in the faecal flora of patients in the present study compared with healthy volunteers (approximately 20% increase). Seasonal variations in antibiotic resistance may occur. The study with healthy volunteers was performed in spring, and the present one in early winter. However, in the study of Vorland *et al.*, 1985 [21] the resistance percentages of antibiotic-resistant UTI isolates was constant throughout the year. Although in the present study the prevalence of antibiotic resistance in each group was similar (50% and 55%), the difference observed was significant. The antibiotic susceptibility of *Escherichia coli* isolates, pre- and post-treatment, was significantly different. For the antibiotic-treated group, amoxycillin resistance increased from 15% to 23%. Remarkably, for the symptomatic-treatment group an increase in resistance to sulphamethoxazole from 19% to 39% was observed. However, in our previous study of healthy volunteers the frequency of resistance to sulphamethoxazole ranged from 26.7% to 37.5% [12]. Therefore, we suggest that the observed increase in resistance is artefactual, reflecting the low prevalence found pre-treatment. Rosner, 1985 [18] showed that *Escherichia coli* K-12 strains become significantly tolerant to chloramphenicol, tetracycline, ampicillin and nalidixic acid in the presence of chemorepellents such as acetylsalicylate (aspirin), and that this occurred regardless of the different modes of action of the antibiotics, and the lack of relation between the structures of the agents and the chemorepellents. Whether other analgesic or mucolytic agents can cause an increase in the prevalence of antibiotic resistance needs further study.

Another striking observation was the higher prevalence of resistance to neomycin in the antibiotic-treatment group pre-and post-treatment (Table II) and the increased resistance to this agent of the *Escherichia coli* isolates (Table IV). In the Netherlands neomycin is mainly used in veterinary medicine for the treatment of diarrhoea in young pigs, and the use in human medicine is limited to local treatment with skin ointments. Although the questionnaire did not include questions about use of such local treatment, it is not likely that the use in skin ointment could cause the increased resistance to neomycin of faecal flora. The influence of veterinary antibiotic use on the bacterial resistance in

humans to antibiotics in general requires further study.

We conclude that in addition to antibiotic therapy pre-treatment, antibiotic resistance positively contributed to resistance post-treatment. Ongoing surveillance of antibiotic-resistance in the flora of healthy people should occur, because resistant bacteria in the community represent the largest reservoir of resistant genes [5], and because of the correlation between antibiotic-resistance of colonizing and infecting bacteria [10]. Knowledge of the prevalence of antibiotic-resistance provides general practitioners with essential data for their choice of empiric therapy [16].

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CHAPTER VI

COMPARISON OF VIRULENCE FACTORS OF URINARY AND FAECAL *ESCHERICHIA COLI* ISOLATED FROM THE SAME PATIENT

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(submitted for publication)

SUMMARY

The degree of correspondence between urinary and faecal *Escherichia coli* isolated from the same individual was assessed in twenty-seven non-pregnant women with urinary tract infection. Strains were biotyped, analysed for O:K serotypes, hemolysin production, P fimbriation, transfer of antibiotic resistance, plasmid content, outer membrane protein profile and presence of the aerobactin iron uptake system.

Cluster analysis revealed similar *Escherichia coli* isolates in the corresponding urine and faecal samples in 18 of 27 patients. Similar plasmid patterns and outer membrane protein profiles were obtained in 13 and 19 of the combinations tested, respectively. The genes for both the aerobactin-iron receptor and production were present in 9 urinary and 8 faecal strains. Resistance to amoxycillin, oxytetracycline, streptomycin and sulphamethoxazole in various combinations occurred most frequently in both groups of strains.

O1 was mainly associated with K1 antigen and P fimbriation, O2:K? and O6:K2 with P fimbriation and hemolysin production.

The data in this study underscore the importance of the faecal flora as a reservoir and source of potentially uropathogenic bacteria in women.

INTRODUCTION

Of all Gram-negative bacteraemias *Escherichia coli* accounts for 30 to 45% of these episodes with an overall mortality of about 33% [25]. Despite the availability of new antimicrobial agents the mortality did not significantly reduce over the past twenty years [8]. Since, inadequate therapy will result in an increase in mortality. For optimal antibiotic therapy knowledge as to the antibiotic susceptibility of the microorganisms to be expected is essential.

The primary site of infection for *Escherichia coli* bacteraemia is the urinary tract followed by the gastrointestinal tract [25]. Urinary tract infection (UTI) is the most common form of extraintestinal *Escherichia coli* infection in women, and *Escherichia coli* is the pathogen most commonly isolated in UTI [13]. Since the faecal and periurethral flora are considered to be the reservoir for these bacteria, several studies have been

performed to examine the correlation between urinary and periurethral *Escherichia coli* [2], urethral and faecal *Escherichia coli* [23] or urinary and faecal *Escherichia coli* [3,5,18,21,22,29].

Although several studies stressed the importance of the faecal flora as the most important reservoir of potentially uropathogenic bacteria [10,17], none of them analysed extensively virulence factors including antibiotic resistance of the uropathogen and the corresponding faecal isolate of the same patient. In the present study we provide ecidence as to the similarity between urinary tract and faecal isolates of the same patients using hierarchical cluster analysis.

MATERIALS AND METHODS

Patients and strains

During a six-week period adult, non-pregnant, female patients 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 or had known structural abnormalities of the urinary tract. Patients were also excluded if they had received immunosuppressive drugs or antibiotics, had been hospitalized or had a known urinary tract infection within the past three months. In addition to a clean voided urine specimen for standard quantitative and qualitative bacterial culture patients were asked to send in one faecal sample before starting antibiotic therapy. Only those patients were included if they had a bacterial colony count in the urine specimen $\geq 10^5$ CFU/ml of one single species and from whom a faecal sample was received and processed within 24 hours after receiving the urine specimen. For isolation and identification of the microorganisms standard bacteriological methods were used that included the API-20E biochemical system (API System, La Balme Les Grottes, Montalieu-Vercieu, France). From both urine and faecal sample one *Escherichia coli*-like colony was randomly picked out for comparative analysis. The study was approved by the Ethical Committee of the Maastricht University Hospital. All patients received oral and written information and all gave informed consent.

Serotyping of *Escherichia coli*

Escherichia coli serotyping (for O and K antigens) was performed at the National Institute for Public Health and Environmental Protection (RIVM), Bilthoven, the Netherlands on those isolates from patients whose urine and faecal samples yielded *Escherichia coli*. The O antigens were identified by bacterial agglutination using 170 anti-O sera. Non-agglutinating strains were defined as O-nontypeable (ONT), spontaneous agglutination as SA. Strains without a detectable K-antigen were defined as K-, strains not agglutinating with any of the antisera related to a specific O group were defined K?

Hemolytic activity

Hemolysin production was determined on nutrient agar plates (Oxoid CM3) containing 5%(v/v) sheep blood. The presence of a hemolytic zone larger than the overlaying colony was considered positive.

P Fimbriation

The strains were subcultured twice on colonization factor antigen (CFA) agar plates [6] to stimulate P fimbriae expression. P fimbriation was assayed by indirect immunofluorescence with monoclonal antibodies raised against various serotypes of P fimbriae, i.e. $F7_1$, $F7_2$, F8, F9, F11, F12 or *pap* (pyelonephritis-associated pilus). The same monoclonal antibodies were used as in the study of de Ree [27].

Susceptibility testing

The antibiotic susceptibility was determined using a microbroth dilution method in Iso-Sensitest broth (Oxoid CM 473) and an inoculum of 5×10^5 CFU/ml. After incubation at 37°C for 18-24 hours the MIC was defined as the lowest concentration that completely inhibited growth. *Escherichia coli* ATCC 25922 and ATCC 35218 were used as reference strains. The breakpoints for resistance were those recommended by the guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing [15]. For apramycin the used breakpoint was > 16 mg/L [12].

Resistance transfer

Broth culture mating was performed using *Escherichia coli* K-12 (nalidixic acid-resistant) as recipient. In short, overnight cultures of recipient and donor were diluted 1:10 in fresh Brain Heart Infusion broth (Oxoid CM 225) and incubated at 37°C for 2 h. Then they were mixed in the ratio 1:1 in fresh BHI and incubated at 37°C for another 2 h. Mixtures were inoculated on the surface of Iso-Sensitest agar (Oxoid CM 471) plates containing nalidixic acid (32 mg/L) and one of the following antibiotics : amoxycillin (32 mg/L), chloramphenicol (32 mg/L), oxytetracycline (32 mg/L), streptomycin (20 mg/L) or sulphamethoxazole (256 mg/L). The plates were incubated overnight and then examined for growth. Control experiments were performed with donor or recipient and 0.9%(w/v) NaCl as recipient or donor, respectively.

Plasmid studies

Plasmid profile analysis was done using the method of Kado and Liu [14]. Plasmid DNA was visualised by staining with ethidium bromide and visualised with ultraviolet light. As size markers (4.4, 5.8, 7.6, 39 and 91 kb) plasmids isolated from a *Salmonella typhimurium* strain (kindly provided by N.van Leeuwen, the National Institute of Public Health and Environmental Protection) were used.
Gels were photographed with a Polaroid camera with a red filter using a type 667 Kodak film.

Characterization of amoxycillin-resistant *Escherichia coli* strains

For all amoxycillin-resistant *Escherichia coli* strains sonicated extracts from 4h cultures in Isosensitest broth were used for analytical isoelectric focusing (IEF) using a pH range of 3-10 and 4-6.5 [26]. *Escherichia coli* strains producing either the TEM-1 or the TEM-2 ß-lactamase were used as reference strains.

Analysis of outer membrane protein profile

Overnight cultures in Iso-Sensitest broth derived from single colonies were centrifuged (15 min, 6000 *g*, 4°C), resuspended in 7 ml 50 mM Tris-2 mM EDTA buffer pH 8.5 and lysed by ultrasonication five times during 20s at 0°C, with a 20s cooling period between treatments. After centrifugation (20 min, 2000 *g*, 4°C), the supernatants

were cleared by centrifugation (60 min, 26500 *g*, 4°C), the pellets resuspended in 5 ml 2 mM Tris buffer pH 7.8 + 0.5 ml $MgCl_2$ and incubated for 60 min at 4°C. After 15' at roomtemperature 0.5 ml 20%(v/v) Triton X-100 was added and another 45 min incubated at the same temperature. Then the suspensions were centrifuged (60 min, 140000 *g*, 15°C) and the resultant pellet resuspended in 1 ml 2 mM Tris buffer pH 7.8 and stored at -20°C.

The protein content was estimated by the method of Bio-Rad with bovine serum as the standard (Bio-Rad Laboratories). For SDS-PAGE analysis, 10 μg protein per sample was separated in 11% polyacrylamide gels (acrylamide/bisacrylamide ratio 30% / 2.67%) according to the method of Laemmli [16]. Gels were stained with Coomassie brilliant blue R-250. As size standards the low molecular weight standards from Bio-Rad (Bio-Rad Laboratories) were used.

Detection of genes coding for aerobactin production and aerobactin-iron receptor function

The aerobactin biosynthesis probe was a 2 kb *Ava I* fragment prepared from plasmid pABN5 [1] and the aerobactin-iron receptor probe was a 2.3 kb *Pvu II* fragment prepared from plasmid pABN1 [4], both kindly provided by K.G.Wooldridge. The DNA probes were purified by extraction with diatoms earth from agarose gels and labeled with digoxigenin according to the manufacturers instructions (Boehringer Mannheim Biochemica, Mannheim, Germany). The strains were screened by making colony blots on nylon membranes (Nytran) according to the manufacturers instructions (Nytran, Schleicher & Schuell, Den Bosch, the Netherlands). As positive controls the strains containing the plasmids pABN5 and pABN1 were used, as negative control *Escherichia coli* K-12. Prehybridization and hybridization were performed under stringent conditions (50%(v/v) formamide and 5xSSC [1xSSC is 0.15M NaCl plus 0.015M sodium citrate] at 42°C, and washes in 2xSSC plus 0.1%(w/v) SDS and 0.1xSSC plus 0.1%(w/v) SDS at 68°C). The detection of digoxigenin-labeled nucleic acids was performed by chemoluminescence on radiographic films (Kodak X Omat AR).

Statistics

Hierarchical cluster analysis by single linkage and Squared Euclidian distances (i.e. Distance $(X,Y)=\Sigma(X_i-Y_i)^2$) was used to determine the similarity between the urinary and faecal *Escherichia coli*.

RESULTS

During the six-week period 83 female patients presented to their general practitioner with complaints of UTI. Excluded from further analysis were patients who had used antibiotics (n=8) or had being hospitalized in the previous three months (n=6). From five patients the faecal sample was not received duly in time. Another 27 women were excluded because of a colony count of the urine of less than 10^5 CFU/ml. In nine cases another species than *Escherichia coli* was isolated. In one faecal sample no *Escherichia coli* could be isolated and therefore also excluded from further analysis. Finally a total of 27 patients with a bacterial count of 10^5 CFU/ml *Escherichia coli* in urine specimens and from whom also a faecal sample was received were studied. The mean age of the patients was 46.8 years with a range between 17 and 78 years. The characteristics of the *Escherichia coli* isolates in the corresponding urine and faecal samples are shown in the Table.

Biotyping of *Escherichia coli*

The API 20E-profile most often found was 5144572, i.e for the urinary and faecal strains ten-and nine-times, respectively. The same profile was found in 17 of 27 pairs tested.

O antigens and their association with K antigen

In 19 of 27 urinary isolates O antigen was detectable. O6 was the most common type (5 of 27), followed by O2 (4 of 27) and O1 (3 of 27). Of the faecal isolates 21 of 27 were O-groupable. The most common types were O1 and O2 (each 3 of 27), both types were mainly combined with K1 antigen. Spontaneously agglutinating (SA) strains were found in 5 urinary and 4 faecal strains, respectively. The same combination of O and K antigens was found in 14 of 27 pairs of urinary and faecal *Escherichia coli*.

Hemolysin production

Seven urinary and 5 faecal strains were positive, which corresponded with 5 pairs of isolates. Hemolysin production was most often associated with O6 (6 of 12) and O2 (4 of 12) antigen, respectively.

P fimbriation

Four pairs of urinary and faecal strains were P fimbriated. Serotype F9 was present in 4 strains (i.e. 2 pairs) and associated with O1:K1 but not with hemolysin production. The other two serotypes, F12 and $F7_2$, found were associated either with O2:K? or O6:K2 and hemolysin production.

Antibiotic resistance

Resistance to amoxycillin, oxytetracycline, streptomycin and sulphamethoxazole in various combinations occurred most frequently. In total 19 of the 54 strains were resistant to any (or combinations) of the antibiotics tested. In 12 of these 19 isolates the resistance was transferable to *Escherichia coli* K-12 (underlined in Table). Resistance to amoxycillin was in the 8 amoxycillin-resistant isolates due to TEM-1 ß-lactamase. Similar antibiotic resistance patterns were observed in 7 of 27 pairs of urinary and faecal strains, while susceptibility to all agents tested was found in 16 of 27 pairs.

Plasmid profiles

Similar plasmid profiles were observed in 13 of 27 pairs of isolates. Most profiles consisted of one or two large plasmids and several smaller ones. No plasmids could be isolated from 4 urinary and 4 faecal strains, respectively.

Outer membrane protein profiles

Analysis of the outer membrane proteins (OMPs) yielded 14 different profiles, which could be distinguished from each other by the various combinations of ten particular protein bands. Pattern 5 and 8 were observed 15- and 12-times, respectively. Similar OMPs were observed in 19 of 27 pairs of urinary and faecal strains.

Aerobactin production and aerobactin-iron receptor

Thirteen urinary *Escherichia coli* strains possessed the genes for the aerobactin-iron receptor; another 13 strains the genes for the aerobactin production. In 9 isolates both kind of genes were present. For the corresponding faecal strains the figures were 11, 11 and 8, respectively. Positive results for both the receptor and the production of aerobactin were obtained for 5 pairs of isolates.

Cluster analysis using all characteristics tested revealed similar *Escherichia coli* isolates in the corresponding urine and faecal samples in 18 of 27 combinations tested. The dendrogram is shown in the Figure.

Figure. Dendrogram: The numbers placed vertically correspond with the pairnumbers used in the Table. Horizontally the rescaled distance cluster combine is shown.

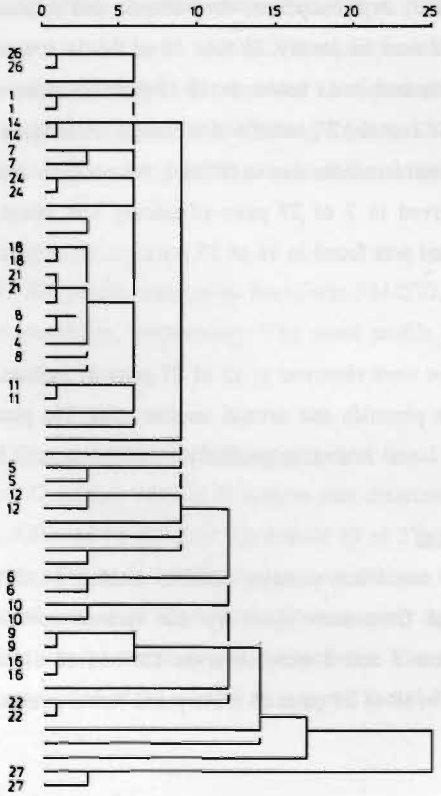

Table. Prevalence of virulence factors in urinary and faecal *Escherichia coli* of 27 patients.

Source[1]		API 20E	Serotype	HA[2]	Pf[3]	R-type[4]	Plasmids (kb)	OMP[5]	Aerobactin Synt[6]	Aerobactin Rec[6]
1*	U	5044552	O91:K-	-	-		24	1	-	-
	F	5044552	O15:K?	-	-		-	1	-	-
2	U	5144552	O6:K-	+	-		-	2	+	+
	F	5044152	O107:K?	-	-		21,19,3	3	-	+
3	U	5044532	O7:K1	-	-		20,2.8	4	+	-
	F	5144572	O149:K?	-	-	OxStSu	39,4.6	1	-	-
4*	U	5144572	O2:K?	-	-		71,9.1,4.3	5	+	+
	F	5144572	O50:K?	-	-		71,9.1,4.3	5	+	-
5*	U	5144542	O2:K?	+	-		56,3.9	6	+	+
	F	5144552	O2:K?	+	-		21	6	+	+
6*	U	5044552	O1:K1	-	F9		20,17,8.7,5.2,4.4	7	+	+
	F	5044552	O1:K1	-	F9		20,17,8.7,5.2,4.4	7	+	-
7*	U	5144512	O6:K	-	-		8.3,4.1	8	-	+
	F	5144512	O126	-	-		8.3,4.1	8	+	+
8*	U	5144572	O2:K?	-	-		71,21,8.7,4.1	5	-	+
	F	5144572	O2:K1	-	-		71,21,8.7,4.1	5	+	+
9*	U	5144532	ONT	-	-	AmSu[7]	71,21,11,9.8,4.8	9	+	+
	F	5144532	ONT	-	-	AmSu	71,21,11,9.8,4.8	9	+	+
10*	U	5144572	O102:K?	-	-	AmChOxStSu	112,39,9.3,5.5	5	+	-
	F	5144572	O102:K?	-	-	AmChOxStSu	112,39,19,9.3,5.5	5	+	+
11*	U	5144552	SA	-	-		18	8	-	-
	F	5144552	SA	-	-		18	8	-	-
12*	U	1044152	SA	-	-	StSu	63,8.5,7.6,5,4.4	8	-	+
	F	1044152	SA	-	-	StSu	63,8.5,7.6,5,4.4	8	-	-
13	U	5144572	ONT	+	-		14	9	-	-
	F	5044572	SA	-	-		100,63	8	-	-
14*	U	5144572	O2:K?	+	F12		63,3.9	5	+	+
	F	5144572	O2:K?	+	F12		63,3.9	5	+	+
15	U	1044552	O18AB:K?	-	-		100,19,3.6	8	-	-
	F	5144512	O74:K-	-	-		-	5	-	-

16*	U	7144572	O14:K?	-	-	AmOxSu	23,7.9,6.6,4.6,3.1	9	+	+
	F	5144572	O14:K?	-	-	AmOxSu	23,7.9,6.6,4.6,3.1	9	+	+
17	U	5144572	ONT	-	-	StSu	40,18	9	+	-
	F	5144552	08-like	-	-		18,8.9,4.7	5	-	-
18*	U	5044572	O11:K-	-	-		-	5	-	-
	F	5044572	O11:K-	-	-		16	5	-	-
19	U	5044552	SA	-	-	St	100,8.3	8	+	+
	F	5044552	SA	-	-	OxStSu	100,8.3	8	-	+
20	U	5144572	O6:K-	+	-		-	10	-	-
	F	5144552	O22:K13	+	-		12,6.2	1	-	-
21*	U	5144572	SA	-	-		17,6.8	5	-	-
	F	5144572	O135:K?	-	-		17,6.8	5	-	-
22*	U	5144552	O6:K2	+	$F7_2$	Am	17,2.5	8	-	+
	F	5144552	O6:K2	+	$F7_2$	Am	17,2.5	8	-	+
23	U	5144152	SA	-	-	OxStSu	91,50,7.8,4.9,4.6	11	+	-
	F	5144572	O25:K-	-	-		112	5	-	-
24*	U	5144552	O1:K1	-	-		112,6.2	1	+	+
	F	5144552	O1:K1	-	-		112	1	+	+
25	U	5144572	O150:K-	-	-		71	12	-	-
	F	1044552	ONT	-	-		4.9,4	13	-	-
26*	U	5144572	O6:K-	+	-	Ox	-	1	-	-
	F	5144572	O6:K-	+	-	Ox	-	1	-	-
27*	U	4044102	O1:K1	-	F9	ChOxStSu	50,4.9,4.4	14	-	-
	F	4044102	O1:K1	-	F9	ChOxStSu	-	14	+	-

[1] U = urinary and F = faecal isolate; [2] HA = hemolysin activity; [3] Pf = Serotype of P-fimbriae; [4] R-type = Antibiotic resistance pattern : Am=amoxycillin, Ch=chloramphenicol, Ox=oxytetracycline, St=streptomycin and Su=sulphamethoxazole, " underlining = transferable; [5] OMP = outer membrane protein profile; [6] Synt=aerobactin production and Rec= aerobactin-iron receptor

* urinary and faecal sample similar obtained by hierarchical cluster analysis

DISCUSSION

This study showed the similarity between urinary and faecal *Escherichia coli* isolated from the same patient as to the prevalence of virulence factors as well as the antibiotic resistance tested. Using cluster analysis the majority of the isolates (i.e. 18/27 pairs) was similar for all characteristics tested. In most other studies dealing with the similarity of urinary and faecal isolates, strains were derived from different patient populations [9,21] or in the case the urinary and the faecal isolates were derived from the same patient a limited number of virulence factors were compared, i.e. O-serotyping only [29]. In the study of Lidin-Janson *et al.* [18] more virulence factors were analysed but only from asymptomatic schoolgirls. In the present study the uropathogens were isolated from symptomatic UTI in adult female patients and the virulence factors studied included the presence of the aerobactin system as well as plasmid profiles.

In general the prevalence figures of the virulence factors for the urine and faecal isolates were quite similar. However some differences did occur. For the uropathogens the O6-serogroup producing hemolysin was the most prevalent one.Of the K antigens the combination O1K1, P-fimbriae positive and hemolysin negative was found most frequently. Similar data were found by Evans *et al.* [6] and Hughes *et al.* [11]. A relation between antibiotic resistance and serogroup O8 and O77 was described by Lidin-Janson *et al.*[19]. In contrast, serogroup O1, O2 and O6 as well as K1 antigen were not related to antibiotic resistance. Also in the present study only three pairs of antibiotic-resistant urinary and faecal isolates belonged to the O1K1 or O6-serogroup. However, it is known from other studies that plasmids, i.e. $F_I$me plasmids, can code for both antibiotic resistance and for the production of the hydroxamate siderophore aerobactin, so it is possible that antibiotic resistance can be present as a result of a plasmid also coding for another virulence factor [28]. Of the remaining nine combinations the urinary and faecal strains were distinctly different. This might be explained by the fact that in this study only one single, randomly selected colony has been used for analysis. Although this single colony would have represented the dominant biotype in 86% of the specimens, it is to be expected that testing more colonies would result in a higher percentage of similarity [20].

In the present study the highest prevalence of resistance was found to sulphamethoxazole (urinary and faecal 26%), streptomycin (urinary 22%, faecal 19%), oxytetracy-

cline (urinary 19%, faecal 22%) and amoxycillin (urinary and faecal 15%). No strains were found resistant to trimethoprim, nitrofurantoin or nalidixic acid. These percentages are similar to those we found in a previous study of the antibiotic resistance of faecal *Escherichia coli* isolated from healthy volunteers living in the same region [24].

According to a recnt study of Eykyn *et al.* [7] episodes of bacteraemia are either hospital acquired (60%) or community acquired (40%). Although Enterobacteriaceae were usually hospital acquired, *Escherichia coli* was considered community acquired in more than 40%, with as primary site of infections the urinary or the gastrointestinal tract [25].

Thus optimal antibiotic therapy requires data as to the antibiotic resistance of the microorganism to be expected, especially *Escherichia coli* from urine or faecal specimen. The high similarity between urine and faecal isolates as shown in the present study gives us the possibility to obtain these data by monitoring antibiotic resistance of faecal isolates. Therefore ongoing surveillance of antibiotic resistance of faecal isolates is needed [10,17].

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CHAPTER VII

A PIPERACILLIN-TAZOBACTAM RESISTANT *ESCHERICHIA COLI* STRAIN ISOLATED FROM A FAECAL SAMPLE OF A HEALTHY VOLUNTEER.

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(submitted for publication)

SUMMARY

As part of a surveillance program of the prevalence of antibiotic resistance, the faecal bacteria of healthy people (n=1348) were examined and the antibiotic resistance of the *Escherichia coli* strains determined. One strain out of 142 amoxycillin-resistant isolates, *Escherichia coli* strain 1662, was also resistant to piperacillin-tazobactam but susceptible to amoxycillin-clavulanic acid. The piperacillin-tazobactam resistance determinant was transferable to standard *Escherichia coli* strains by conjugation. However, the strain produced a β-lactamase with several characteristics very similar to those of the TEM-1 β-lactamase, i.e. pI of 5.4, an M_r value of 22000 and a comparable substrate profile. The enzyme was as efficiently inhibited by clavulanic acid and tazobactam as the TEM-1 and TEM-2 β-lactamases but more than the amoxycillin-clavulanic acid-resistant TRC-1 enzyme. The transferable resistance to piperacillin-tazobactam appears to be mediated by a novel resistance mechanism that has previously not been described.

INTRODUCTION

Bacterial resistance to β-lactam antibiotics is due mainly to the production of β-lactamases and, by far the most common of the plasmid-mediated enzymes is the TEM-1 β-lactamase [6]. In order to overcome the action of these ubiquitous enzymes, β-lactamase inhibitors, such as clavulanic acid or tazobactam, have been developed and used in combination with amoxycillin and ticarcillin or piperacillin, respectively [8]. The latter combination shows particular promise *in vitro* and *in vivo*, especially with all genera of the Enterobacteriaceae except *Enterobacter* spp. [4]. Recently some reports mentioned the isolation of *Escherichia coli* strains with increased resistance to the combinations amoxycillin-clavulanic acid and/or piperacillin-tazobactam [1,11,12] and, up until now, all these strains were clinical isolates. In this study some characteristics of an amoxycillin-clavulanic acid susceptible, piperacillin-tazobactam-resistant faecal *Escherichia coli* strain, isolated from a healthy person who had not undergone any antibiotic therapy in the previous three months, are described. The data are compared to the strain producing the TRC-1 β-lactamase, an amoxycillin-clavulanic acid-resistant variant of the TEM-1 enzyme identified in a clinical isolate [10].

MATERIALS AND METHODS

As part of a surveillance program investigating the prevalence of antibiotic resistance in healthy people, *Escherichia coli* from 1348 faecal specimens were examined and their antibiotic resistance profiles determined. Amoxycillin resistance was observed in 142 of the strains isolated. Four of these strains were also piperacillin-tazobactam resistant; however only one of these showed a stable resistance towards that combination (strain 1662).

Susceptibilities to amoxycillin (AM), amoxycillin/clavulanic acid (XL), piperacillin (PP) and piperacillin/tazobactam (PTc) were determined by the E-test (AB Biodisk, Solna, Sweden) both with an inoculum of 0.5 McFarland and 0.5 McFarland 1:100 diluted (i.e. $5x10^5$ CFU/ml). *Escherichia coli* ATCC 25922 and *Escherichia coli* ATCC 35218, were used as reference strains.

The MICs of aminoglycosides, cephalosporines, oxytetracycline, streptomycin, sulphamethoxazole, tazobactam and trimethoprim were determined by the microdilution method with an inoculum of $5x10^5$ CFU/ml. The breakpoints for resistance were those recommended by the guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing [3].

Transferability of antibiotic resistance from *Escherichia coli* 1662 to *Escherichia coli* K-12 (nalidixic acid resistant) was tested in broth mating experiments. Transconjugants were selected on IsoSensitest agar plates containing nalidixic acid (32 mg/L) plus one of the following antibiotics: amoxycillin (32 mg/L), oxytetracycline (32 mg/L), streptomycin (20 mg/L), sulphamethoxazole (256 mg/L) or trimethoprim (4 mg/L) after 2h mixed incubation of donors with the recipient strain *Escherichia coli* K-12, *nal*^{R}. Control experiments were performed with donor or recipient and 0.9%(w/v) NaCl as recipient or donor, respectively. Co-transfer of piperacillin-tazobactam resistance with amoxycillin resistance determinant was analysed by replica plating.

Plasmid DNA from donors and amoxycillin or piperacillin / tazobactam transconjugants was extracted by the method of Kado & Liu [2], separated by agarose gel electrophoresis and visualised by straining with ethidium bromide and viewing with UV light.

The molecular masses (M_r) of the outer membrane proteins of the strains were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

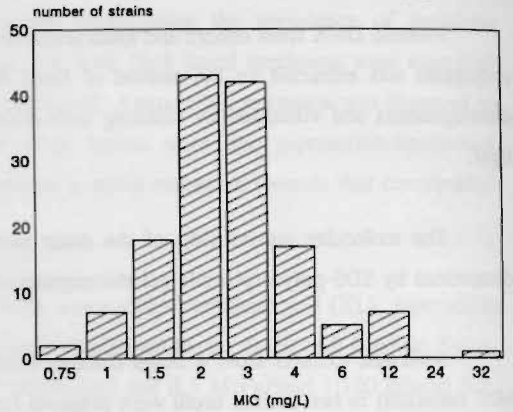
Sonicated extracts from 4 hours cultures (with or without prior induction by 1/2 MIC cefoxitin) in IsoSensitest broth were prepared from strain 1662 and from *Escherichia coli* strains producing the TEM-1, TEM-2 and TRC-1, β-lactamases. The enzymes were characterised by analytical isoelectric focusing (IEF) over the pH ranges 3 - 10 and 4 - 6.5 [5]. β-lactamase activity, substrate profile and the inhibition by clavulanic acid or tazobactam (ID_{50}) were determined spectrophotometrically. The degree of purity of the enzymes were analysed both by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and IEF. Both kind of gels were stained with Coomassie Brilliant Blue (CBB).

RESULTS

Antibiotic susceptibility of *Escherichia coli* strains

The MICs of amoxycillin (>256 mg/L), piperacillin (>256 mg/L), amoxycillin-clavulanic acid (8 mg/L), piperacillin-tazobactam (32 mg/L) and tazobactam alone (>128 mg/L) were determined for *Escherichia coli* strain 1662. The MIC of piperacillin-tazobactam was determined several times by using the E-test and ranged from 32 mg/L to 128 mg/L with an inoculum of 0.5 McFarland and 0.5 McFarland 1:100 diluted. The MIC distribution of all 142 amoxycillin-resistant strains is shown in Figure 1. When the TRC-1 producing strain was examined under the same conditions, MICs of amoxycillin (>256 mg/L), piperacillin (>16 mg/L), amoxycillin-clavulanic acid (24 mg/L) and piperacillin-tazobactam (4 mg/L) were found. Both strains remained fully susceptible to second and third-generation cephalosporins.

Figure 1. The MIC distribution of all 142 amoxycillin-resistant strains for piperacillin-tazobactam by using the E-test.



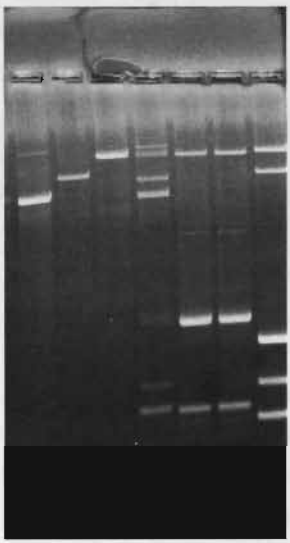
Escherichia coli strain 1662 was also resistant to oxytetracycline (MIC >256 mg/L), streptomycin (MIC >128 mg/L), sulphamethoxazole (MIC >1024 mg/L) and trimethoprim (MIC >512 mg/L), but was susceptible to apramycin, neomycin, kanamycin and gentamicin. Resistance to amoxycillin, streptomycin, sulphamethoxazole and trimethoprim was transferable to *Escherichia coli* K-12. The transfer frequencies (Table 1) show that the amoxycillin resistant determinant was not closely linked with oxytetracycline, streptomycin, sulphamethoxazole and trimethoprim resistance genes and was thus located on a different plasmid. However, in most cases, transfer of amoxycillin resistance was accompanied by co-transfer of piperacillin-tazobactam resistance. For these transconjugants the MIC of amoxycillin was >256 mg/L, piperacillin-tazobactam 32 mg/L and tazobactam alone was >128 mg/L.

Table 1. Transfer frequencies for *Escherichia coli* strain 1662.

Antimicrobial agent	Selecting concentration (mg/L)	Transfer frequency per donor cell
Amoxycillin	32	5.4×10^{-3}
Oxytetracycline	32	0
Streptomycin	20	4.6×10^{-5}
Sulphamethoxazole	256	1.9×10^{-4}
Trimethoprim	4	5.2×10^{-6}

The plasmid profiles of *Escherichia coli* 1662 and its transconjugants together with the strains producing the TEM-1, TEM-2 and TRC-1 β-lactamases, respectively, are shown in Figure 2.

Figure 2. Plasmids in the strains producing TEM-1 (a), TEM-2 (b), TRC-1 (c), *Escherichia coli* strain 1662 (d), *Escherichia coli* K-12 amoxycillin transconjugant (e) and *Escherichia coli* K-12 piperacillin-tazobactam transconjugant (f). As molecular weight standards plasmids of 91, 39, 7.8, 5.6 and 4.4 kb from *Salmonella typhimurium* ST13 (g) were used. Strains were grown in Luria broth and analysed in 0.7% agarose gels.



A B C D E F G

Characteristics of β-lactamase

The β-lactamases produced by *Escherichia coli* strain 1662 and its transconjugants were not inducible, had a similar specific activity towards nitrocephin (Table 2) and focused at pI 5.4, similar to the TEM-1 β-lactamase. The substrate profiles of the enzymes from strain 1662, TEM-1 and TEM-2 are shown in table 3. No hydrolysis of cefazolin, cefipime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, aztreonam and imipenem was detected by spectrophotometric assay.

Table 2. Specific activities (nmol nitrocephin hydrolysed/min/mg protein) of ß-lactamases 1662, TEM-1, TEM-2 and TRC-1.

ß-lactamase	Specific activity
1662	3110
TEM-1	2210
TEM-2	9270
TRC-1	1800

Table 3. [a]Relative rates of hydrolysis (%) of ß-lactamases 1662, TEM-1 and TEM-2.

ß-Lactam substrate	1662	TEM-1	TEM-2
Nitrocephin[a]	100	100	100
Penicillin	> 100	> 100	> 100
Amoxycillin	37	26	27
Piperacillin	33	37	52
Ticarcillin	58	100	56
Cephaloridine	17	7	18

[a] Rate for nitrocephin = 100%

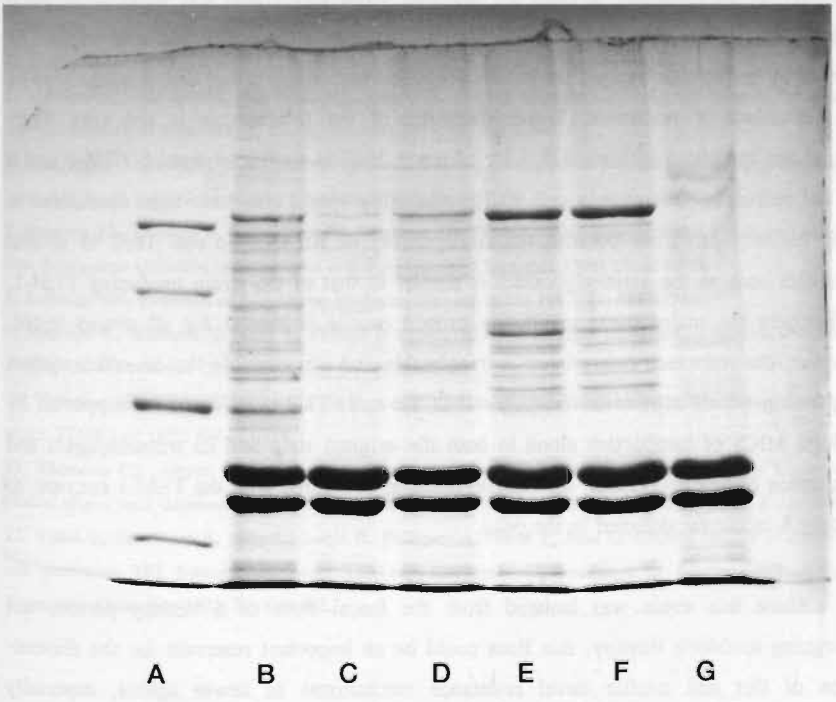
The concentrations of clavulanic acid and tazobactam required to inhibit enzyme activity by 50% (ID_{50}) were quite similar for the 1662 enzyme and TEM-1, TEM-2. These concentrations were distinctly lower than those required for the inhibition of the TRC-1 enzyme (Table 4).

Table 4. Inhibitory dose (μg/L) of clavulanic acid and tazobactam required to inhibit enzyme activity by 50% (ID_{50}) when nitrocephin was used as the substrate.

Inhibitor	1662	TEM-1	TEM-2	TRC-1
Clavulanic acid	16-32	16-32	32	256-512
Tazobactam	1-2	1-2	2	16-32

The molecular mass of the β-lactamase was estimated by Native-PAGE to be approximately 22,000, similar to that of the TEM-1 enzyme. There were no visual differences in major outer membrane protein profile between *Escherichia coli* strains 1662 and its transconjugants and the *Escherichia coli* strains producing the TEM-1, TEM-2 or TRC-1 β-lactamases (Figure 3).

Figure 3. SDS-polyacrylamide gel electrophoresis patterns of cell envelopes of the strains producing TRC-1 (b), TEM-2 (c), TEM-1 (d), *Escherichia coli* K-12 piperacillin-tazobactam transconjugant (e), *Escherichia coli* K-12 amoxicillin transconjugant (f) and *Escherichia coli* strain 1662 (g). In lane (a) molecular weight standards, i.e. 97.4, 66.2, 45 and 31 kD are shown. 11% gel.



DISCUSSION

The piperacillin-tazobactam resistant *Escherichia coli* strain described in this study was isolated from a faecal sample of a healthy person who had not taken any antibiotics in the previous three months before sampling. The piperacillin-resistant determinant was usually co-transferable with the amoxycillin resistance gene. The β-lactamases produced by strain 1662 and its transconjugants were indistinguishable from the TEM-1 β-lactamase for all characteristics tested. Remarkably the β-lactamase from strain 1662 was as efficiently inhibited by clavulanic acid and tazobactam as the TEM-1 enzyme. However, the MIC of piperacillin-tazobactam for the original strain and its transconjugants is ≥ 32 mg/L and both had a high MIC of tazobactam on its own.

Besides alterations in the β-lactamase produced by the cell [10], resistance to the combinations of β-lactams with β-lactamase inhibitors has previously been explained either as a result of hyperproduction of the β-lactamase [9] or as a decrease in the permeablility of the inhibitor through the bacterial outer membrane, i.e. absence of porin OmpF, [7]. Unless the alterations in the β-lactamase are very subtle and cannot be detected by convention assays, the results presented here suggest that this is unlikely to be the mechanism of resistance. Hyperproduction of the β-lactamase is not very likely because the specific β-lactamase activity of strain 1662 is similar to that of TEM-1 and it was not inducible. Decrease in cell wall permeability would also have been considered to be an unlikely candidate because the susceptibility of *Escherichia coli* 1662 to several antibiotics such as the aminoglycosides is similar to that of the strain producing TEM-1. Furthermore the major outer membrane protein profile is similar for all strains tested. However, the resistance determinant is transferable and it is possible that an efflux system is operating which exports tazobactam out of the cell. This view could be supported by the high MICs of tazobactam alone in both the original stain and its transconjugant and the absence of any decrease in tazobactam binding, compared with the TEM-1 enzyme, to the only ß-lactamase detected in the cells.

Since this strain was isolated from the faecal flora of a healthy person, not undergoing antibiotic therapy, this flora could be an important reservoir for the dissemination of this and similar novel resistance mechanisms as newer agents, especially containing β-lactamase inhibitors, are introduced.

ACKNOWLEDGEMENTS

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CHAPTER VIII

GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis describes the prevalence and degree of antibiotic resistance in healthy humans, i.e. pig farmers, abattoir workers and (sub)urban residents and, in particular, discusses the reproducibility of sampling and the intra-individual variation in resistance over time. Attention has been paid to the effect of antimicrobial chemotherapy on the prevalence of antibiotic resistance in the faecal flora of general practice patients. Evidence was provided as to the usefulness of monitoring antibiotic resistance of faecal *Escherichia coli* to predict antibiotic resistance of uropathogenic *Escherichia coli* in primary health care patients. The mechanisms of resistance of a faecal *Escherichia coli* strain resistant to the recently introduced combination of piperacillin and tazobactam, and isolated from a healthy individual not using any antibiotic therapy in the previous three months, were analysed.

Prevalence of antibiotic resistance in healthy humans

The highest prevalence of antibiotic resistance in the faecal samples of three populations of healthy volunteers studied was found for the pig farmers, the lowest for the (sub)urban residents. No significant differences in high degree of resistance to any of the antimicrobial agents tested were found, except for neomycin which was highest for the pig farmers.

The variations observed in the prevalence and the degree of resistance in faecal samples per individual over time could be explained by biological variation (i.e. $\pm$ 1.25 ^{10}log CFU) and measurement error variation (i.e. 0.5 ^{10}log CFU). The high degree of antibiotic resistance per individual was rather constant over time, but more variation was observed for the low degree of resistance.

The small variation in antibiotic resistance holds true not only for the high degree of resistance but also for the antibiotic resistance pattern of the faecal *Escherichia coli* isolates. In Chapter IV the antibiotic susceptibility of 1348 faecal *Escherichia coli* isolated during the 15-week period, was determined. The majority of the isolates per individual had only one or two different antibiotic resistance patterns, with a maximum of five. The number of different patterns was independent of the number of strains per individual.

The prevalences of antibiotic resistance in the faecal samples of both abattoir workers and (sub)urban residents were of the same order of magnitude. Higher prevalence percentages were found for the pig farmers. Although pig farmers and abattoir workers do have regular contact with pigs/carcasses and pig faeces, this factor is of minor importance as no significant differences are observed between these abattoir workers who have intensive contact and these in whom the contact is less intensive. Eight percent of the abattoir workers and 5% of the pig farmers mentioned personal antibiotic consumption recently, whereas none of the (sub)urban residents mentioned this. However, this antibiotic usage could not explain the differences found between the three populations. It is believed that exposure to low concentrations of antibiotics used in animal feed and for therapy of pigs is the most important factor for the higher percentages found in the pig farmers.

A wide range of prevalence percentages were found when the results of the 15-week follow-up study were compared with earlier studies performed in the Netherlands. The high degree of resistance ($\geq$50% of *Escherichia coli* resistant) showed less variation, as shown in the Table.

In comparing these data one has to take into account that the studies mentioned did not all use the same methodology. Although Degener *et al.* [6] used a higher selective concentration for ampicillin (40 mg/L) and tetracycline (30 mg/L) and a semi-quantitative method for determining the numbers of resistant *Escherichia coli*, the percentages found for the prevalence and high degree of resistance were quite similar to those found in the present study.

In the studies of Bonten *et al* [2,3] and our own studies the same methods and the same concentrations were used, and only the populations studied were different. The prevalences and degrees of resistance found, however, were quite different. These data suggest that several other factors such as differences in overall use of antibiotics in the populations studied and socio-economic variations are of influence.

Table. Comparison of % Prevalence (% High Degree) of resistant *Escherichia coli* from different studies in the Netherlands.

study/year (city)	amoxycillin			oxytetracycline			sulphamethoxazole		
	mg/L	P	H.D	mg/L	P	H.D	mg/L	P	H.D
Degener[6]/1978-80 (Zoetermeer)	40	25	5	30	42	12	100	45	19
Degener[6]/1987 (Zoetermeer)	40	27	11	30	20	6	100	46	21
Bonten[2]/1990 (Maastricht)	25	76	8	25	47	11	100	86	37
Bonten[3]/1992 (Maastricht)	25	62	12	25	68	21	100	71	19
Bonten[3]/1992 (Zwolle)	25	89	13	25	49	14	100	49	15
London/1993 (Weert)	25	28±12	7±5	25	25±7	3±3	100	28±10	8±4
London/1993 (Roermond)	25	33±12	9±5	25	31±7	5±4	100	41±7	14±4

A high degree of resistance means that the majority ($\geq$50%) of the isolated faecal *Escherichia coli* are (multi-)resistant. About 10^6 to 10^8 *Escherichia coli* are generally found per gram of human faeces. Given about 100 grams of faeces produced daily per person, an individual with a high degree of resistance to an antimicrobial agent excretes at least 5×10^7 to 5×10^9 resistant bacteria per day. Therefore such people represent a large source of resistant bacteria and resistance genes [9].

Persons with a low degree of resistance are important as well. Shortly after an antimicrobial agent for which resistant *Escherichia coli* are present in the gastrointestinal tract has been taken, the resistant bacteria will be selected and excreted in high numbers. Moreover, both groups of humans have more chance of an infection caused by resistant microorganisms in the future [8].

The small variations in resistance patterns in each individual as found in our study are in contrast with those found by Levy *et al.* [9] who observed that 90% of all

individuals showed a gain (47.6%) and/or a loss (65.7%) of one or more detectable resistances. However, his findings were based upon the two first faecal samples per individual while in our study one to 13 samples per individual were analysed during a 15-week period. Consequently testing more than two faecal samples per individual is, as expected, more accurate than testing only two samples.

Effect of antibiotic therapy on antibiotic resistance

The data in chapter V demonstrate that amoxycillin and/or doxycycline therapy results in an increase in prevalence of resistance post-treatment to oxytetracycline as well as amoxycillin in faecal *Escherichia coli*. These data are in line with those from other studies showing that antimicrobial agents can select for antibiotic-resistant strains, especially in the faecal flora [5,7,11].

As the faecal flora is considered to be the reservoir and source not only of potentially pathogenic bacteria, but also of resistance genes, data concerning the antibiotic resistance of the faecal *Escherichia coli* will predict the antibiotic resistance of these potentially pathogenic microorganisms and will provide essential information for effective empirical therapy.

Chapter VII describes the isolation and characterization of a faecal isolate (*Escherichia coli* 1662) resistant to a recently introduced antimicrobial agent, i.e. piperacillin-tazobactam. The strain was isolated from a healthy person who had not received any antibiotics in the previous three months. Until then, piperacillin-tazobactam-resistant strains were either *in vitro* isolated mutants or in a few cases clinical isolates. The mechanism of resistance of *Escherichia coli* strain 1662 seems to be different from other piperacillin-tazobactam-resistant strains as isolated so far.

GENERAL CONCLUSIONS AND RECOMMENDATIONS

In the past 15 years in the Netherlands several studies have been performed regarding the prevalence and degree of antibiotic resistance of faecal *Escherichia coli* in healthy humans. The differences observed in the studies performed could be explained only in part by differences in methodology and it is very likely that differences in the populations studied have contributed to the observed variation. Differences in the population include differences in socio-economic circumstances as well as differences in direct (i.e. personal) antibiotic usage and indirect antibiotic usage (i.e. antibiotic usage in the population, degree of direct exposure to antibiotics, e.g. in animal feeds, to animals and animal waste or products).

In general, no steady increase in antibiotic resistance in the open Dutch population in the past decade has been observed. These data are in line with a survey of nearly one million hospital isolates in the New York area collected over a 10-year period which did not show any increase in resistance over time [1]. The authors stated that although local outbreaks with multi-resistant microorganisms do occur the overall situation is not too worrying.

It should be a public health goal that a low level of carriage of antibiotic-resistant strains is maintained in the near future [8,10]. To achieve this active surveillance of antibiotic resistance of extramural isolates has to be performed. These include establishment of national monitoring stations, which at regular intervals collect a sufficient number of strains from healthy persons to study antibiotic susceptibilities [4] and antibiotic resistance patterns as well as the mechanisms of antibiotic resistance.

Knowledge of the prevalence and degree of resistance present in the bacterial flora of the healthy human population will provide general practitioners with essential data for their choice of empirical therapy and could alert them to optimalize their prescribing practices.

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SUMMARY

Emergence of antibiotic-resistant microorganisms continues to be an important problem for the treatment of bacterial infections. There is general agreement that antibiotic use in general medical practice and in veterinary medicine is responsible in part for the development and selection of antibiotic-resistant bacteria. However, it is still not clear whether antibiotic use in humans or animals has contributed most to the environmental pool of resistant microorganisms.

Up to now many studies have examined the prevalence of antibiotic resistance in hospitalized patients. In contrast, less information is available on the incidence of antibiotic resistance outside the hospital. In general, the faecal flora is considered the most important reservoir of antibiotic-resistant microorganisms. However, in the Netherlands no recent data are available about the prevalence of antibiotic-resistant bacteria in the faecal flora of non-hospitalized patients. Therefore, several studies analysing the prevalence of antibiotic-resistant faecal *Escherichia coli* of healthy volunteers and of patients from general practice with complaints of an urinary tract infection or a respiratory infection are presented in this thesis.

In chapter I, after a short introduction, a review of the literature concerning the prevalence of antibiotic-resistant microorganisms in different populations, i.e. pig farmers, abattoir workers and general practice patients is given. Furthermore the effect of antibiotic therapy on the selection of antibiotic-resistant bacteria from human and animal origin is described as well as possible routes (contact, foodchain) by which these animal bacteria can reach man and may cause resistance are discussed. Finally the prevalence of antibiotic resistance in humans outside the hospital, i.e. general practice patients and (sub)urban residents, is presented. As described in several studies the prevalence percentages observed are strongly influenced by the method used also data concerning the high degree of resistance, i.e. the proportion of faecal samples with a ratio $\geq$ 50%, thus the majority of the faecal *Escherichia coli* showing resistance to that agent, are given.

In chapter II the prevalence of antibiotic resistance in three populations, i.e. pig farmers, abattoir workers and as a control group (sub)urban residents, all living in the same region in the South of the Netherlands, is described. The highest prevalence percentages were found for the pig farmers, indeed, for amoxycillin, neomycin,

oxytetracycline, sulphamethoxazole and trimethoprim significantly higher percentages than for the abattoir workers and (sub)urban residents were found. It was quite remarkable that only the high degree of resistance to neomycin significantly differed for the three populations tested, which was again highest for the pig farmers. The differences in prevalence of resistance observed between pig farmers and abattoir workers could not be explained by their (intensive) contact with pigs only. Also personal antibiotic consumption, 5% for the pig farmers and 8% for the abattoir workers, could not explain the differences found between the three populations. It is to be expected that direct contact with antibiotic-supplemented pig feed, i.e. mass medication, is an important factor for the higher percentages found in the pig farmers.

Chapter III describes the individual variation regarding the presence of antibiotic resistance over time. The same group of individuals, already described in chapter II, were followed up during a 15-week period and their faecal samples analysed weekly. The high degree of resistance to an antimicrobial agent was more or less constant over time. Another striking observation were the significant differences in resistance found between individuals living in the regions Weert and Roermond, of which the last group showed a higher prevalence and degree of resistance to some of the agents tested compared to the first group. Although on one hand these results emphasize regional differences in prevalence of antibiotic resistance, it was on the other hand quite remarkable that these differences already occurred within such a short distance of 30 km.

The small individual variation in antibiotic resistance was confirmed by the relatively small number of antibiotic resistance patterns per individual as found in the faecal *Escherichia coli* strains (chapter IV). The faecal *Escherichia coli* from 51 out of the 183 individuals studied showed only one antibiotic resistance pattern, from 63 individuals two patterns were found. The resistance patterns most frequently observed were resistance to sulphamethoxazole alone, followed by resistance to sulphamethoxazole in combination with oxytetracycline and/or streptomycin.

There is general agreement that antibiotic use is an important risk factor for selection of antibiotic-resistant bacteria not only to the agent used but also to other (un)related agents. Chapter V describes the effect of antibiotic therapy on the antibiotic resistance of faecal *Escherichia coli* from patients attending their general practitioners with complaints of a respiratory tract infection. Amoxycillin and doxycycline therapy

resulted in an increased prevalence of faecal *Escherichia coli* resistant to both agents.

The faecal flora is not only considered to be the reservoir for antibiotic-resistant *Escherichia coli* but also for uropathogenic *Escherichia coli*. This last item is elucidated in chapter VI in which 27 paired isolates from faeces and urine were analysed. Based on data concerning several virulence factors including hemolytic activity, P fimbriation and aerobactin production an overall degree of correspondence between urine and faecal *Escherichia coli* could be shown in 66% of the patients.

In chapter VII the mechanism of resistance against a recently introduced combination of a ß-lactam antibiotic, piperacillin and a ß-lactamase inhibitor, tazobactam, is analysed. It was quite remarkable that the piperacillin/tazobactam-resistant *Escherichia coli* was isolated from a healthy person who had not undergone any antibiotic therapy recently. Up until now, all piperacillin/tazobactam-resistant strains were clinical isolates.

In the concluding chapter (chapter VIII) recommendations are made as mentioned in this summary. It is strongly recommended to establish monitoring stations to analyse at regular intervals antibiotic susceptibilities of faecal isolates from non-hospitalized patients in order to provide essential data for a correct choice of empiric therapy.

SAMENVATTING

Het voorkomen van antibiotica-resistente microörganismen vormt nog steeds een belangrijk probleem bij de behandeling van bacteriële infecties. Algemeen wordt aangenomen dat het (dier)geneeskundig gebruik van antibiotica bijdraagt aan de ontwikkeling en selectie van antibiotica-resistente bacteriën. Het is echter nog steeds niet duidelijk of het gebruik in de geneeskunde of juist in de diergeneeskunde het meest heeft bijgedragen aan de omvang van het resistentie probleem.

Tot nu toe is veel onderzoek beschreven betreffende het voorkomen van antibiotica resistentie bij patiënten in ziekenhuizen. Daarentegen is nog relatief weinig bekend over het resistentie probleem buiten het ziekenhuis. Algemeen wordt de faeces beschouwd als het reservoir van antibiotica-resistente microörganismen. Echter recente gegevens betreffende het voorkomen van antibiotica-resistente bacteriën in de faeces van personen buiten het ziekenhuis zijn in Nederland niet voorhanden. In dit proefschrift worden de resultaten van faecesonderzoek betreffende de antibiotica-resistente faecale *Escherichia coli* bij gezonde vrijwilligers en bij patiënten welke de huisarts bezoeken met klachten van een urineweginfectie of een luchtweginfectie beschreven.

In het eerste hoofdstuk wordt, na een korte inleiding, een overzicht van de literatuur gegeven met betrekking tot het voorkomen van antibiotica-resistente microörganismen in diverse groepen personen, zoals varkenshouders, slachthuispersoneel en huisartspatiënten. Vervolgens wordt het effect van het antibioticagebruik op selectie van antibiotica-resistente bacteriën bij mens en dier beschreven en worden de mogelijkheden bediscussieerd (contact, voedselketen) hoe de bij dieren aanwezige resistentie kan leiden tot antibiotica resistentie bij de mens.

Tenslotte wordt het voorkomen van antibiotica resistentie bij personen buiten het ziekenhuis besproken, te weten bij patiënten welke een huisarts bezoeken en bij stedelingen. Aangezien de prevalentiepercentages sterk afhankelijk zijn van de gehanteerde methode worden ook gegevens vermeld betreffende de hoge mate van resistentie, dat wil zeggen het percentage van de onderzochte personen waarvan het merendeel ($\geq 50\%$) van de faecale flora resistent is tegen het onderzochte antibioticum.

In hoofdstuk 2 worden de prevalenties van antibiotica-resistentie in drie verschillende populaties, te weten varkenshouders, slachthuispersoneel en gezonde stedelingen, allen afkomstig uit de regio Weert en Roermond, beschreven. De hoogste prevalentiepercentages werden bij varkenshouders vastgesteld, welke voor amoxycilline, neomycine, oxytetracycline, sulphamethoxazole en trimethoprim significant hoger waren dan bij slachthuispersoneel en stedelingen. Opvallend was dat ten aanzien van de hoge mate van resistentie alleen die voor neomycine significant verschilde tussen de drie populaties. Deze was het hoogst voor de varkenshouders. Het verschil in antibiotica resistentie tussen de varkenshouders en het slachthuispersoneel kon niet alleen verklaard worden door het (intensieve) contact met varkens. Het eigen antibioticumgebruik, bij de varkenshouders 5% en het slachthuispersoneel 8%, kon ook niet de verschillen tussen de drie populaties verklaren. Het wordt aangenomen dat het contact met antibioticum-gesupplementeerd varkensvoer een belangrijke rol speelt bij de hogere prevalentiepercentages bij de varkenshouders.

Hoofdstuk 3 beschrijft de individuele variatie in antibiotica resistentie in de tijd. Van de in hoofdstuk 2 beschreven groep gezonde personen werden gedurende 15 weken faecale monsters geanalyseerd. De hoge mate van resistentie tegen een bepaald antibioticum vertoonde weinig variatie tijdens de periode van onderzoek. Opvallend waren de significante verschillen waargenomen tussen de regio's Weert en Roermond, waarbij Roermond een hogere prevalentie en mate van resistentie vertoonde dan Weert. Hoewel enerzijds deze resultaten regionale verschillen in antibiotica resistentie benadrukken is het opvallend dat ook binnen een afstand van $\pm$ 30 km reeds verschillen waarneembaar zijn.

De geringe individuele variatie in antibiotica resistentie bleek ook uit het relatief beperkte aantal resistentie patronen van de faecale *Escherichia coli* per individu (hoofdstuk 4). Van de 183 onderzochte personen vertoonden de *Escherichia coli* isolaten van 51 van hen éénzelfde antibioticum resistentie patroon, bij 63 personen werden twee patronen vastgesteld. De meest voorkomende resistentie betrof resistentie tegen sulfamethoxazole alleen, gevolgd door resistentie tegen sulfamethoxazole in combinatie met oxytetracycline en streptomycine.

Algemeen wordt aangenomen dat gebruik van antibiotica een belangrijke risicofactor is voor de selectie van bacteriën resistent tegen het gebruikte middel maar mogelijk ook tegen (niet-)verwante antibiotica. Hoofdstuk 5 beschrijft het effect van antibioticum therapie in de eerste lijn bij patiënten met klachten van een luchtweginfectie op het voorkomen van antibiotica-resistente faecale *Escherichia coli*. Amoxycilline en doxycycline therapie leidde tot een toename in percentage *Escherichia coli* resistent tegen beide antibiotica.

De faecale flora wordt niet alleen beschouwd als reservoir voor antibiotica-resistente *Escherichia coli* maar ook als reservoir voor uropathogene *Escherichia coli*. Dit laatste aspect wordt in hoofdstuk 6 nader toegelicht aan de hand van 27 gepaarde isolaten van faeces en urine. Op basis van verschillende virulentiefactoren zoals hemolytische activiteit, P-fimbriae en aerobactine-produktie kon bij 66% van de patiënten overeenkomst tussen faecale en uropathogene *Escherichia coli* worden vastgesteld.

In hoofdstuk 7 wordt het mechanisme van resistentie tegen een recent geïntroduceerde combinatie van een ß-lactam antibioticum, piperacilline en een ß-lactamase remmer, tazobactam nader geanalyseerd. Opvallend was dat de piperacilline-tazobactam resistente *Escherichia coli* geïsoleerd was bij een persoon die vermeldde recent geen antibioticum gebruikt te hebben. De tot nu toe beschreven piperacilline/tazobactam resistente isolaten waren allen klinische isolaten.

Tenslotte worden in hoofdstuk 8 aanbevelingen gedaan voor een continue monitoring van antibiotica resistentie in de niet-ziekenhuis populatie.
Gepleit wordt regelmatig vast te stellen het resistentie niveau in de open populatie via resistentie peilstations om zodoende een bijdrage te kunnen leveren aan een juiste keuze van empirische therapie.

DANKWOORD

In september 1989 begon ik met mijn stage bij de vakgroep Medische Microbiologie van de Rijksuniversiteit Limburg. In augustus 1990 werd het stagecontract omgezet in een tijdelijk contract voor de duur van één jaar. Het jaar was voor driekwart ten einde toen Ellen mij de vraag stelde of ik geen interesse had in een baan als AIO op een door het Preventiefonds gesubsidieerd onderzoek voor een periode van ongeveer drie jaar. Alhoewel het antwoord eigenlijk al meteen "ja" was, werden alle mogelijke positieve en negatieve kanten van het AIO zijn eerst goed overdacht.
Nu dat het onderzoek bijna afgerond is besef ik dat ik toen wel degelijk de juiste beslissing heb genomen maar ook dat ik dit onderzoek nooit tot een goed einde had kunnen brengen zonder de hulp en het medeleven van een aantal mensen die ik hier met name wil noemen.

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De medewerkers van de vakgroep Medische Microbiologie, met wie het fijn werken was.

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CURRICULUM VITAE

De schrijfster van dit proefschrift is op 19 februari 1968 in Maastricht geboren. In 1986 behaalde zij het Atheneum B diploma aan het Stedelijk Lyceum te Maastricht. In hetzelfde jaar begon zij aan de HLO-biochemie opleiding aan de Hogeschool Heerlen te Sittard. In 1990 behaalde zij haar examen en kreeg ze een baan bij de vakgroep Medische Microbiologie. Vanaf september 1991 werkte ze aan het onderzoek, dat in dit proefschrift beschreven is.

Vormgeving/Druk : Unigraphic