Public health aspects of bacterial resistance in food animals

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*Public health aspects
of bacterial resistance
in food animals*

Volksgezondheidsaspecten van bacteriële resistentie bij nutsdieren

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op
vrijdag 22 december 2000 om 16.00 uur

door

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Prof. dr. P.G. Knipschild

Prof. dr. J.A. Knottnerus

Prof. dr. K.M.L. Leunissen

**“Vanitas est praesentem vitam solum attendere,
et quae futura sunt non praevidere”**

*“Ijdelheid is alleen acht slaan op het heden
en niet aan de toekomst denken”*

Thomas à Kempis, *De imitatione Christi* 1.1.4

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	The prevalence of antimicrobial resistance of indicator bacteria: <i>Escherichia coli</i> and enterococci, in pig faecal samples collected at 5 Dutch abattoirs. A.E. van den Bogaard, N. London and E.E. Stobberingh. <i>Report Dutch Ministry of Agriculture, Nature Management and Fisheries</i> , August 1998.	
	and	
	Antimicrobial resistance in pig faecal samples from the Netherlands (five abattoirs) and Sweden. A.E. van den Bogaard, N. London and E.E. Stobberingh <i>Journal of Antimicrobial Chemotherapy</i> 2000; 45: 663-671.	
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	and	
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Introduction

“Predicting things is hard, especially about the future”

attributed to *Yogi Berra*

Over the last 60 years, antibiotics have largely changed the prognosis of many bacterial diseases. Their success originates in the combination of their high antimicrobial activity, low toxicity for the host and the ease with which they can be administered. This explains the huge quantities that are used for therapy and control of bacterial diseases in humans and animals, for growth promotion in animal husbandry, for crop protection in agriculture and even for conservation of food and wood²⁰. This high efficacy and low toxicity, however, might also have provoked lavish use and even abuse. These life-saving drugs have been and are still being used with unrestrained enthusiasm far surpassing the needs of the management and control of bacterial infections and often only for mere economic reasons. The other side of the coin is that resistance is an inevitable sequel of antibiotic use, which endangers the efficacy of these drugs. Immediately after the introduction of the first antibiotics emergence of resistance has been described^{1,18}, but, because of the euphoria and the regular introduction of new drugs at that time, this warning was neglected. In recent years, however, antibiotic resistance has been increasing at an alarming rate world-wide. Some authorities are referring to an approaching “post-antimicrobial era” in which bacterial infections will again be a common cause of death like before the introduction of antibiotics in clinical medicine⁴.

Are things really so bad? At present, no. Indeed sixty years of antibiotic usage have provided an unfortunate example of a massive experiment in microbial evolution under intense selection. The bacteria found in many hospitals today are very different from those of the pre-antibiotic era, as increasingly antibiotic resistant strains of the perennially important pathogens have replaced their antibiotic-susceptible forebears²¹.

However, despite the increased resistance in bacteria all over the world, most infections requiring antimicrobial therapy remain amenable to the common antibiotics that have become the mainstay of the formularies for treatment of bacterial infections in humans and animals. Indeed we have now in some countries reached the unacceptable situation that many hospital isolates of Gram-negative enteric bacteria, staphylococci and enterococci are now resistant to most, formerly very effective, antibiotics². This hampers empirical therapy, which causes therapy failures resulting in increased morbidity and even mortality. In addition it requires, apart from necessary extra laboratory tests, regular use of, always more expensive and often more toxic new antibacterial agents. Similar problems with antibiotic resistance are increasingly found with many of the community-acquired pathogens^{7,24} and with bacteria pathogenic for animals^{23,29,37}. Until recently, such new antibiotics have always appeared just in the nick of time, before resistance problems got out of hand. However, now the increasingly rapid pace at which bacteria develop or acquire new drug resistance profiles seems to outpace the rate at which the pharmaceutical industry is able to develop and market new antibiotics. In the near future few really new antibiotics, that are not analogues of existing drugs, might be expected to become available for clinical use.

With any new antibiotic becoming available it is not a question of whether resistance to the new drug will emerge (it will!), but how fast and how extensive. Moreover newly developed antibiotics will be mainly used against multiple resistant pathogens. This practise will virtually ensure that eventually resistance will appear in the currently multiple resistant species. Also other new strategies for infection intervention which might arise from the rapidly increasing knowledge of

bacterial genomics are not likely to become available to us fast³.

Despite the massive usage of antibiotics the microbial ecosystems seem not to have been much affected, at least quantitatively. Bacteria are still present in high numbers in the human and animal environment. For instance, the intestinal tract of humans and animals are still populated by huge numbers of bacteria - roughly ten times more bacterial cells than the eukaryotic cells of which the body of the host consists i.e. 10^{12} bacteria and 10^{11} body cells per kg bodyweight - just as it was before the introduction of antibiotics into clinical medicine¹¹. Resistance, however, is very common among the bacteria that populate the intestinal tract of humans and animals living today and, although historical comparisons are missing, it is very likely that the endogenous flora is now more resistant to antibiotics than previously. Results from studies of bacteria collections from before the introduction of antibiotics^{8,15} and in populations of humans and animals, who had not been exposed to modern medical and veterinary practices²⁵ seem to confirm this hypothesis. Also nowadays large differences in the prevalence and degree of resistance between human and animal populations with a high respectively low antibiotic usage are observed. The intestinal flora of populations with a high antibiotic consumption harbours significantly more resistant bacteria than that of populations exposed to less antibiotics^{19,39}. It has been shown that these resistant bacteria in the intestinal flora of man and other animals form a reservoir of resistance genes for pathogenic bacteria¹⁹. From this point of view the prevalence of resistance in pathogenic bacteria could be considered as only the top of the resistance iceberg.

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance in bacteria may emerge by several pathways. A bacterium of a normally susceptible species might become resistant by mutation or acquisition of new genes.

Genetic mutations

Changes in only a few base pairs causing substitution of one or a few amino-acids in a crucial target (enzyme, cell structure or function, or the cell wall) can affect

chromosomal structural or control genes leading to new resistant strains, that might be able to inactivate a whole class of antibiotics, such as the penicillins. Subtle mutations in an existing resistance gene can have major effects on an organisms resistance profile. So the change of a single amino-acid in the protein sequence of the TEM or SHV β -lactamases can extend their spectrum of activity from mainly inactivation of aminopenicillins to almost all existing β -lactam antibiotics^{14,16,35}.

Acquisition of genetic material

Resistance can be acquired by a susceptible strain from other bacteria by transfer of genetic bacterial i.e. DNA from one bacterium to another via transformation, transduction or conjugation. Many of the resistance genes are on transposons that can jump between the bacterial chromosome and plasmids present in the bacterial cell. These plasmids can, and do, transfer themselves to other bacteria belonging to the same or a different species or genus⁹. Even exchange of genes between Gram positive and Gram negative bacterial species has been reported⁶. An example of clinical importance is the movement of genes encoding for β -lactamase and high level gentamicin resistance from staphylococci to enterococci which leaves only vancomycin or another glycopeptide available for therapy of serious enterococcal infections³¹. Of greatest concern presently is the risk that transfer of vancomycin resistance genes from enterococci to staphylococci will occur *in vivo*, as already has happened under laboratory conditions³³. This could make methicillin resistant *Staphylococcus aureus* strains, which are usually multiresistant, virtually impossible to treat with antibiotics clinically available at this moment.

Mutations and acquisition of new resistance genes occur spontaneously and at random in bacteria and are in most cases not influenced by the presence of antibiotics. Resistant bacteria have been found in soil bacteria, bacteria of the intestinal flora and clinical isolates before antibiotics became clinically available, but in very low numbers^{8,15}.

*Introduction of resistant bacteria into
a previously susceptible population*

Apart from mutations or gene acquisition resistant strains can be introduced in a population from the outside. Examples are vancomycin resistant enterococci brought into a hospital by a new patient who carries it in his intestinal flora or a fluoroquinolone resistant *Escherichia coli* strain from food animals which might reach the human intestinal tract via meat(products).

SELECTION OF RESISTANCE

Due to mutations, acquisition of resistance genes or introduction of a single or a few resistant strains a minority of strains resistant to a certain antibiotic may be present in a population.

Whether these resistant strains survive and replicate depends in large degree on the selective pressure placed on them. Mostly they will disappear as spontaneously as they emerged, but in a niche characterised by large quantities of antibiotics they might proliferate because the majority of susceptible bacteria are killed by the use of that antibiotic or a related one allowing the resistant subset of resistant strains to grow at expense of susceptible organisms. Within a short time all susceptible bacteria will be eradicated and replaced by resistant bacteria; a typical example of the Darwinian principle of survival of the fittest. Moreover the large numbers and the high diversity of bacterial species present in the intestinal tract further facilitate the transfer of resistance among bacteria. After discontinuation of antibiotic use the susceptible bacteria might be gradually reintroduced, but certainly after repeated exposure to antibiotics a certain percentage will stay resistant. After prolonged or continuous use of antibiotics in a population even the majority of bacteria present may stay resistant. This effectively means that the use of antibiotics, both in people and in animals, will "amplify" the numbers of resistant bacteria present, which also increases the chance of transfer of their resistance genes to bacteria of other species e.g. (opportunistic) pathogens.

DISSEMINATION OF RESISTANCE

The increase in numbers of resistant bacteria by selection under antibiotic pressure means also that they are

excreted in much larger numbers into the environment enhancing the chance of colonisation and/or infection of other individuals. This can be either by direct contact between individuals or indirectly among patients via health care workers or medical equipment in hospitals or via foods of animal origin from animals to humans.

RESERVOIRS OF RESISTANCE

The extend of antibiotic resistance in a population is strongly correlated with the level of antibiotic usage and the ease with which bacteria can disseminate: crowding and poor sanitary levels. Hospitals are traditionally the major breeding grounds and reservoirs of resistant bacteria^{13,26,28}, however other important reservoirs are for the same reasons day care centres and nursing homes^{30,32}. Moreover, as fortunately many more people live outside than inside hospitals, more than 80% of all antibiotics consumed by humans are used in the open population. Therefore carriage of resistant bacteria by healthy persons is another source of resistance^{22,24}. Outside the human population intensive farming units in which large numbers of animals are closely housed together with no restrictions to oral faecal contact and where, in addition to the high antibiotic use for treatment and control of bacterial infections, antibiotics are continuously fed to these animals for growth promotion are an ideal place for selection and dissemination of resistant bacterial strains. This might not only endanger the efficacy of antibiotics in veterinary medicine, but also affect public health. As we now begin to appreciate more fully the interrelatedness of resistance in bacteria of animal origin: zoonotic bacteria and faecal flora, and resistance encountered in bacteria isolated from humans, measures to curb bacterial resistance in the human populations must extend beyond human medicine, and also include antibiotic usage in animals.

CONTROL OF RESISTANCE

Prevention of dissemination of resistant strains population by hygienic measures in populations is an important way in the control of bacterial resistance. Infection control, strict hygiene and barrier isolation of patients

infected with resistant organisms have been very successful in preventing acquisition of resistant micro-organisms from exogenous sources in hospitals²⁷. However, for prevention of infections by resistant bacteria, which are endogenous to the patient i.e. originating from the patient's own intestinal flora, strict adherence to these procedures is not very successful. As it is obvious that the selective pressure of antibiotic usage is a factor of major importance, - according to Gaynes "amplification of resistance by antibiotic use is the major factor"¹², decreasing antibiotic use is a cornerstone in dealing with antimicrobial resistance.

CONCLUSIONS

Antibiotic resistance in pathogenic bacteria is only the tip of the iceberg and resistant bacteria and resistance genes are to be found in the intestinal flora of man and other animals.

Antibiotic resistance in the intestinal flora is high in populations where antibiotic consumption is large. It is not only a source of resistance genes for pathogens, but can also spill over to other populations. Hence food animals might be a source of resistant bacteria and resistance genes for the human intestinal flora and for (human) pathogenic bacteria. To prevent the emergence of resistance in human pathogenic bacteria it has been suggested that a low degree of resistance in the intestinal flora of humans should be considered a public health goal like low cholesterol levels and normal blood pressure¹⁹. In food animals this should be considered as a safety and quality bench mark, as this will not only safeguard the future efficacy of antibiotics in veterinary medicine, but also decreases public health risks⁴⁰. In food animals where prevention of dissemination of bacteria among animals is impossible this can only be achieved by using less antibiotics. This means that these agents should only be used, if indicated, for treatment and control of bacterial infections and not for economic reasons as growth promoters. The structural dependence on antibiotics in intensive farming should be reduced by infection control measures and improving animal husbandry.

AIMS OF THE STUDY

The risk of transfer of resistant bacteria other than zoonotic pathogens, or resistance genes from food animals to humans is now generally accepted, but the extent to what this contributes to resistance problems encountered in human medicine is still much debated.

Therefore the literature on transfer of resistant bacteria and resistance genes from animals to humans is reviewed. As antibiotic use in animals is the major factor for resistance encountered in bacteria of animal origin the phasing out of the usage of antibiotic for mere economic reasons as growth promoters has been suggested^{5,10,17,36}, but a ban on the veterinary use of antibiotics is out of the question. We are as humans responsible for the welfare of animals under our care. Therefore a proposal was made for a veterinary antibiotic policy. This is a set of rules and guidelines for veterinary surgeons to optimise the treatment of bacterial infections in animals ensuring at the same time an adequate veterinary therapy and minimising resistance development. Surveillance of resistance is an essential part of a veterinary antibiotic policy. Therapy advice and eventual interventions have to be based on recent susceptibility data.

A feasibility study was performed whether it was possible to detect differences in antibiotic consumption in pig populations based on the basis of the prevalence and degree of resistance of bacteria in faecal samples of pigs collected in slaughterhouses, using *E. coli* and enterococci as indicator bacteria.

To detect if and to what extent, antibiotic resistance bacteria and resistance genes from animal bacteria can be transferred to the intestinal flora of humans we studied the resistance in bacteria from faecal samples of poultry, poultry farmers and poultry slaughterers. The use of antibiotics in poultry is in the Netherlands higher than in other animal species and in man. This is mainly due to the use in broilers and turkeys. In laying hens producing eggs for human consumption, antibiotic use is, at least during their productive life, low. The prevalence and degree of resistance was determined in indicator bacteria *E. coli* and enterococci from faecal samples of animals with a high exposure to antibiotics: broilers and turkeys, and with a relatively low exposure: laying hens.

In addition we compared five populations with a different intensity and frequency of contact with poultry or poultry products:

- a. broiler farmers and turkey farmers, who have daily intensive contact with these animals, that are continuously fed antimicrobial growth promoters and in which therapeutic antibiotics are regularly used for control of bacterial infections
- b. laying hen farmers, who have daily intensive contact with chickens with a low antibiotic consumption.
- c. broiler and turkey slaughterers, who have daily contact with carcasses or meat from poultry that have been heavily exposed to antibiotics.

The effect of addition of an *in vitro* "plasmid curing" antibiotic to food animal feeds on the prevalence and degree of resistance in the faecal flora of exposed animals was studied on a commercial pig farm as an additional possibility to control antimicrobial resistance in the faecal flora of food animals.

Finally to evaluate the public health effect of an intervention measure: the suspension by the European commission in 1997 of the use of avoparcin as antimicrobial growth promoter in food animal feeds, in 1999 the prevalence and degree of resistance of enterococci was determined in faecal samples of broilers and pigs, collected after slaughtering, and of healthy human volunteers.

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OUTLINE OF THE THESIS

This thesis attends to several aspects of antimicrobial use in animals, the consequences for public health and possible intervention methods.

In *chapter I*, entitled “Antibiotic Usage in Animals; Impact on bacterial resistance and public health” the literature on usage of antibiotics in and the consequences for public health of antimicrobial resistance in food animals is reviewed with special emphasis on antimicrobial growth promoters.

In *chapter II*, entitled “A veterinary antibiotic policy; a personal view on the perspectives in the Netherlands” ways to minimise selection and dissemination of bacterial resistance against antibiotics by enhancing optimal use and selection of antibiotics for the treatment and control of bacterial infections in animals are discussed. A policy and the development of therapeutic guidelines for use and selection of antibiotics in veterinary medicine is proposed.

In *Chapter III*, entitled “Antimicrobial resistance in pig faecal samples from five abattoirs in the Netherlands and from Sweden” the feasibility of monitoring resistance in faecal indicator bacteria: *E. coli* and enterococci, in faecal samples of pigs collected at abattoirs is examined. As no antibiotic consumption data could be obtained from the different Dutch pig populations studied the results are compared with those from Swedish pig samples.

In *Chapter IV*, entitled “Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the South of the Netherlands: evidence for transmission of

vancomycin resistance from animals to humans?” the public health risk of addition of a glycopeptide antibiotic: avoparcin, as growth promoter to turkey feeds was studied.

In *Chapter V* entitled “Faecal carriage of antibiotic resistant enterococci by poultry, poultry farmers and poultry slaughterers in the Netherlands” the prevalence and degree of resistance of faecal enterococci of broilers and laying hens, and of broiler farmers, laying hen farmers and poultry slaughterers are compared and analysed.

In *Chapter VI*, entitled “Prevalence of resistance in faecal *E. coli* of poultry, poultry farmers and poultry slaughterers in the Netherlands” the prevalence and degree of resistance of faecal *E. coli* of poultry: turkeys, broilers and laying hens, and of poultry farmers and poultry slaughterers are compared and analysed with special emphasis on fluoroquinolone resistance.

In *Chapter VII*, entitled “The effect of flavophospholipol on the prevalence and degree of resistance of indicator bacteria; *E. coli* and enterococci, in the intestinal flora of fattening pigs under field conditions” the effect of adding flavophospholipol, a “plasmid curing” antibiotic, to the finisher feed of fattening pigs on the prevalence and degree of resistance of faecal *E. coli* and enterococci from pigs is studied.

In *Chapter VIII*, entitled “Results of decreasing antibiotic usage in animals on resistance in the intestinal flora of food animals and healthy humans”, the effect is shown of the suspension of the use of growth promoters on the prevalence and degree of resistance of indicator bacteria: *E. coli* and enterococci, in faecal samples of pigs and poultry and healthy humans with special emphasis on vancomycin resistant enterococci.

Chapter I

Antibiotic usage in animals

Antibiotic Usage in Animals

Impact on Bacterial Resistance and Public Health

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Abstract

Antibiotic use whether for therapy or prevention of bacterial diseases, or as performance enhancers will result in antibiotic resistant micro-organisms, not only among pathogens but also among bacteria of the endogenous microflora of animals. The extent to which antibiotic use in animals will contribute to the antibiotic resistance in humans is still under much debate. In addition to the veterinary use of antibiotics, the use of these agents as antimicrobial growth promoters (AGP) greatly influences the prevalence of resistance in animal bacteria and a poses risk factor for the emergence of antibiotic resistance in human pathogens. Antibiotic resistant bacteria such as *Escherichia coli*, *Salmonella* spp., *Campylobacter* spp. and enterococci from animals can colonise or infect the human population via contact (occupational exposure) or via the food chain. Moreover, resistance genes can be transferred from bacteria of animals to human pathogens in the intestinal flora of humans.

In humans, the control of resistance is based on hygienic measures: prevention of cross contamination and a decrease in the usage of antibiotics. In food animals housed closely together, hygienic measures, such as prevention of oral-faecal contact, are not feasible. Therefore, diminishing the need for antibiotics is the only possible way of controlling resistance in large groups of animals. This can be achieved by improvement of animal husbandry systems, feed composition and eradication of or vaccination against infectious diseases. Moreover, abolishing the use of antibiotics as feed additives for growth promotion in animals bred as a food source for humans would decrease the use of antibiotics in animals on a worldwide scale by nearly 50%. This would not only diminish the public health risk of dissemination of resistant bacteria or resistant genes from animals to humans, but would also be of major importance in maintaining the efficacy of antibiotics in veterinary medicine.

Since the 1970s many reports have been published about multiresistant bacteria, but it has only been recently reported that organisms resistant to all clinically available antibiotics have emerged.^[1,2] Despite the fact that hospitals are considered to be major breeding grounds of resistant bacteria, it is becoming accepted that the much larger populations of healthy people living outside hospitals might be an important reservoir of resistant genes

as well. Most antibiotics are used outside hospitals, and not only in humans but also in animals.^[3] Antibiotic use in animals, as in humans inevitably selects for resistant bacteria. The proliferation of antibiotic resistance genes on transposons and plasmids in animal populations is a direct result of the widespread use of antibiotics in animals and is increased by the ease of dissemination of resistant strains between animals via faecal contact, espe-

cially among animals housed together in large groups, as with intensive farming units.^[4,5]

In domestic animals antimicrobial agents are used for 3 major purposes: therapy to treat an identified bacterial infection, prevention of bacterial infections in animals at risk, or as feed additives to enhance performance. Veterinary antibiotic therapy involves treatment of an individual animal or a group of sick animals with one or more antibiotics during a defined period of time and, in most Western countries, only upon prescription from a veterinarian. Similarly, antibiotics can be prescribed by a veterinarian for a defined period of time to prevent bacterial infections in a group of animals at risk or to prevent the spread of an existing infection in a herd. In the situations when antimicrobials are used as therapy or for prevention of disease in a group of animals, antibiotics are mostly dissolved in the drinking water or milk, or mixed in the feed. This is called group or mass medication.

Despite the fact that much attention is being paid to hygiene and prevention of infectious diseases, intensive animal production depends heavily on the usage of antimicrobial agents for veterinary purposes. Apart from veterinary use, antibiotics are added continuously to the feed of animals used as a food source (i.e. 'food animals') for humans (e.g. pigs, poultry and nonruminating veal calves), to enhance their performance and increase growth. In this situation they are called antimicrobial growth promoters (AGP). The term growth promoter is used for feed additives, other than dietary nutrients, which increase growth rate and/or improve feed efficiency in healthy animals fed a balanced diet.^[6] AGP are more effective in young than in older animals and the general opinion is that the observed growth and feed efficiency responses to the use of AGP are lower under optimal hygienic and animal husbandry conditions compared with poorer environments.^[7] However, even under optimal conditions, the positive effects on growth rate and feed efficiency are between 2% and 4%.^[8] As a consequence of the improved feed efficiency, the amounts of waste products excreted in faeces and

urine by the animals are lowered in proportion to the decreased amount of feed consumed by the animals, that is, by approximately 3 to 4%.^[9]

From an economic point of view, responses in feed efficiency are of greater importance than growth improvement or decreased waste production. The mode of action of AGP has been the subject of numerous scientific papers, but most of these deal with effects rather than modes of action. An early and fundamental finding was that germ-free animals, that grow approximately 20% faster than conventionally-reared animals, do not respond to dietary inclusion of AGP.^[10] Therefore, the growth promoting activity of AGP seems to be related in one way or another to effects on or mediated by modulation of the intestinal flora of the exposed animals. Recently, several reviews on the possible modes of action of AGP have been published.^[7,11,12] Other names for AGP are antimicrobial performance enhancers, antimicrobial feed additives, feed savers, digestion enhancers or intestinal flora modulators.

The use of antibiotics for veterinary purposes is strictly regulated in most countries in a similar way to pharmaceuticals for humans. AGP, however, are regulated separately and differently, even if exactly the same drug is also registered for therapeutic use. Despite the fact that APG are not intended for and not registered for prevention of bacterial diseases, a part of their positive effect is most likely caused by prevention or suppression of bacterial infections as was claimed already by the discoverer of the growth promoting effect of antibiotics.^[13] This might be an explanation of why they are more effective in young animals and when used under sub-optimal conditions. As many people are not very familiar with the use of antibiotics as growth promoters and with the special agents used for this purpose, special attention will be paid in this review to the use of antibiotics as AGP, that is, the compounds used and their antibacterial activity, the effect of their use on the resistance of bacterial flora of animals, and the risk of transfer of resistant bacteria or their genes from animals to humans.

1. Antimicrobial Growth Promoters

Originally, therapeutic antibiotics like tetracyclines and penicillins were used as AGP, but in lower doses than those required for therapeutic use.^[14] In the US, oxytetracycline is still used, but for therapeutic applications it is mixed in pig feeds in concentrations between 200 to 800 mg/kg. For AGP use, it is administered in concentrations between 50 to 200 mg/kg. The quinoxaline antibiotics, carbadox and olaquinox, are used in the US in pig feeds at doses of 50 to 55mg per kg for therapy, and at doses of 10 to 25mg per kg as AGP.^[15] Therefore, the use of antibiotics as AGP is sometimes referred to as the 'subtherapeutic use of antibiotics'. In contrast, in the European Union (EU) and many other countries, drugs that have been registered for therapeutic use in humans and/or animals are not allowed to be used as AGP. However, many of the compounds used as AGP are analogues of, and show cross resistance with, therapeutic antibiotics. Table I shows the most important compounds authorised as AGP in the EU, together with the concentrations in which they are added to animal feeds and the minimum inhibitory concentration (MIC) range of susceptible indicator bacteria, namely, *Enterococcus* spp., *Clostridium perfringens* and *Escherichia coli*.^[15-18]

For AGP that are not used for therapy, no official breakpoints to differentiate between resistant and susceptible strains have been established. As cross resistance to therapeutically used antibiotics occurs, the break-points of the therapeutic counterpart have been used. Adsorption from the intestinal tract of most of these drugs is very limited or non-existent, therefore, the concentrations used are high enough to ensure that the levels of AGP reached in the intestinal tract of exposed animals are inhibitory to susceptible bacteria, as shown in table I. This inhibitory effect is confirmed by the preventive effect of AGP against certain intestinal diseases in animals such as necrotic enteritis in poultry (which is caused by *Clostridium perfringens*), swine dysentery (a contagious diarrhoea in pigs caused by *Serpulina hyodysenteriae*), and weaning diarrhoea in swine caused by *E. coli*.^[15,19]

In Sweden, which prohibited the use of AGP in 1986, a higher incidence of bacterial infections in poultry and pigs was observed immediately after the ban, with a resulting increase in the veterinary use of antibiotics by approximately 20%.^[20] However, improvement of hygiene and animal husbandry systems, and reformulation and modification of feeds has solved nearly all of these problems. Consequently, the incidence of clostridia enteritis in poultry in Sweden is now much

Table I. Authorised antibacterial performance enhancers

Compound			Minimum inhibitory concentration range (mg/L)	
Name	Chemical group	Dose in animal feeds (mg/kg)	Clostridia	Enterococci
Avilamycin	Evernimycins	18-40	<0.25-0.5	0.06-0.075
Avoparcin ^a	Glycopeptide	5-100	0.5-2	1-2
Bacitracin	Polypeptides	5-100	<1-4	<0.5-16
Flavomycin	Bambermycins	1-25	<1-8 ^b	0.25-4
Monensin	Ionophore	10-40	0.5-4	1-2
Spiramycin	Macrolide	5-80	0.25-8	0.5-4
Tylosin	Macrolide	4-40	<1	1-4
Virginiamycin	Streptogramins	5-80	0.25-1	0.25-8
Salinomycin	Ionophore	15-60	No data	0.5
				<i>Escherichia coli</i>
Carbadox	Quinoxaline	20-50	≤0.25	≤2
Olaquinox	Quinoxaline	15-50	No data	≤16

a Registration temporarily suspended.

b *Clostridium perfringens* is naturally resistant.

lower than before the ban on AGP. On pig farms, mainly those with a suboptimal management, there are still problems with bacterial infections in recently weaned pigs. Zinc oxide is now added routinely to the feed of young pigs for a short period after weaning for the control of these infections. As a result, the total amount of antibiotics used in animals is now (10 years after the ban on AGP), 55% lower than that before the ban. Furthermore, the total amount of antibiotics used for veterinary prescriptions decreased by 33%, despite an increase in the total number of animals.^[20] This decrease in antibiotic use was reflected in a recent study in which faecal samples of Swedish pigs were compared with samples of Dutch pigs for the prevalence and degree of resistance of faecal indicator bacteria: enterococci and *E. coli*. The prevalence and degree of resistance was not only significantly lower for AGP, but also for veterinary administered antibiotics.^[21]

Despite their undisputed economic value in animal husbandry, there is much concern about the effect of AGP on the selection of resistant bacteria and resistance genes, not only in zoonotic pathogens, but also in the endogenous microflora of animals, as these bacteria might reach humans either directly or via foods of animal origin.^[22] According to the advice of the Swann committee in 1968^[23] (adopted by most European countries at that time), no antibiotics should be used as AGP which are also (or closely related to) therapeutically used antibiotics. In the EU, this is only true for monensin, salinomycin, flavomycin and quinolones. Other compounds such as spiramycin and bacitracin are used in human medicine, and

avoparcin, tylosin and virginiamycin are closely related analogues of valuable human therapeutic antibiotics: vancomycin, erythromycin and pristinamycin, respectively.

Moreover, older agents, rejected for therapy and presently used as AGP, are being used as templates for the development of new antibiotics for the treatment of infections with multiresistant bacteria.^[24] For example, everninomycin (SCH-27899) is a new antibiotic being developed for treatment of multiresistant cocci, derived from avilamycin by chemical modification of the molecule.^[25,26] The mode of action of these new human therapeutics is similar to the AGP ancestor molecules and they show complete cross resistance. As mentioned before, the concentrations in which AGP are used, are high enough to reach levels well above their MIC in the gut. Therefore, the use of AGP results in bacterial subclones or subpopulations resistant against these AGP and related therapeutic compounds.

2. Amount of Antibiotics Used in Animals

Accurate figures on the antibiotic use in humans or animals are hard to obtain. Most data on antimicrobial use in humans and animals are based on the monetary value of sales of antibiotics per annum and show that use in humans is considerably higher.^[27] However, as antibiotic use in humans is more expensive per gram of active compound than veterinary antibiotics and certainly than AGP, this might be very misleading.^[28] It has also been speculated that the overall use in humans is at least 4 times that in animal husbandry and veterinary medicine. This assumption is based on figures that, in

Table II. Veterinary use of antibiotics in the Netherlands in 1990 (300 000 kg/year)

Animals	% of total	No. of animals ($\times 10^6$)	Total weight of animals (kg $\times 10^6$)	Total amount of antibiotics used ^a (mg/kg/year)
Pigs	42	15	1000	125
Poultry	26	100	180	430
Cattle	30	5	1800	55
Average ^b				100
Other domestic species	2	No data presented	No data presented	No data presented

a Active substance.

b Average of antibiotic usage in food animals.

humans, most antibiotics are used in individuals below the age of 15 years and above 65 years,^[29] and that the older age group does not exist in food animals but is only limited to pet animals. Therefore, it was thought likely that antibiotic use in humans contributes more to resistance in the bacterial population than does nonhuman or veterinary use.^[30] However, compared on a bodyweight basis the antibiotic consumption by animals might be as high or higher, because the lifespan of food animals is short, for example, for broilers approximately 7 weeks, and for fattening pigs 6 months. This means that most of the animals present in a population belong to the young age group that is more susceptible to bacterial infections, similar to humans below the age of 15 years. Moreover, in small animals like poultry, the dosage per kg bodyweight is much higher than that in humans.

According to the European Federation of Animal Health Industries (FEDESA),¹ the World Animal Health Product Market was, at manufacturers prices, 11 billion Euro in 1995, of which 44% were therapeutic pharmaceutical products and 41% feed additives. The European market was 3.3 billion Euro of which 48% were therapeutics and 37% feed additives. In 1993, it was estimated that 42% of all pharmaceuticals used in animals worldwide were AGP and 18% therapeutic antibiotics.^[31] Prescott^[32] considered that some 40% of the total antibiotic production in the US was for animal production, and even 55 to 60% of the production of benzylpenicillin (penicillin G) and tetracyclines was used in food animal feeds. Levy^[33] agreed with the figure of 40% of total production, which he considered to be 23 million kg. He also reported that more than 80% of all tetracycline and benzylpenicillin use were in subtherapeutic doses mixed in animal feeds as AGP.

In the EU and Switzerland in 1997,² 10493 tons of antibiotics (active ingredient at 100% purity) were used and of this amount nearly 50% was used

in animals: 3474 tons (33%) were used in animals for therapy and prevention, and 1590 tons (15%) as AGP. However, large differences in antibiotic use exist between countries. In Belgium, Denmark, France, Ireland and the Netherlands (countries with a large intensive animal farming industry), the use of AGP was of the same size of order as the veterinary use of antibiotics. In Austria, AGP use was 3 times higher than the therapeutic use in animals. In Sweden and Finland, no AGP (<1%) were used, and in Germany, Greece, Italy, Portugal, Spain and the UK, AGP use was considerably lower (15 to 50%) than veterinary use.

These figures are not stable, but tend to change over time. In Finland in 1995 for example, 14 tons of antibiotics were used as AGP and 14 tons for therapeutic reasons in animals (approximately 30% of the human use). In Denmark in 1995, these figures were 94 and 50 tons, respectively.^[34] In Norway, figures were 25 000kg in humans and 50 000kg in animals. In 1990 in the Netherlands, 80 000kg of antibiotics were used on medical prescription for a population of approximately 15 million people. This is equivalent to 60 million defined daily doses or 100mg active substance/kg bodyweight/year.^[28] The veterinary usage was 300 000kg, which also equated to 100mg active substance/kg bodyweight/year, as presented in table II. The additional use of antibiotics as AGP in animal feeds was estimated (no official figures could be obtained) to be approximately 300 000kg.^[28] These figures show that the selection pressure exerted by the veterinary use of antibiotics on the animal bacterial population in the Netherlands is of the same order as medical usage. However, this selection pressure is more than doubled in intensively reared animals because of the use of antibiotics as AGP.

The literature on resistance against AGP is very limited as most of these compounds are not used for therapy, susceptibility testing is not regularly performed, and no recognised breakpoints to differentiate between susceptible and resistant microorganisms exist. Therefore, AGP that are not com-

1 Fedesa Animal Health Dossier 14. Facts and figures about the European Animal Health Industry 1997.

2 Pres. release FEFANA at the invitational EU-conference 'The Microbial Threat' Copenhagen, Denmark, 1998.

monly used in human or veterinary medicine will be discussed briefly.

3. Antimicrobial Growth Promoters

3.1 Avoparcin and Ardacin

Avoparcin and ardacin are glycopeptides and closely related to vancomycin and teicoplanin. Avoparcin has been widely used as AGP in the EU, but has never been registered in the US. However, the EU has suspended the use of avoparcin and ardacin, which was just being introduced, in 1997. Both agents are active against most aerobic and anaerobic Gram-positive bacterial species, but not against most Gram-negatives as these drugs are unable to pass through the outer membrane of these bacteria.^[35]

Glycopeptides are compounds of 2 or more substances with similar molecular structures, produced by a variety of soil bacteria. Lactobacilli are intrinsically resistant to glycopeptides. The use of avoparcin selects for an acquired high level resistance in enterococci, that is mostly mediated by the *VanA*-gene cluster located on a transposon Tn 1546 or a self transferable plasmid.^[35-37] Transfer of the *VanA*-gene cluster has been shown *in vitro* from *Enterococcus faecium* to *Listeria monocytogenes*, *Staphylococcus aureus* and several *Streptococcus* spp. with frequencies ranging from 10^{-6} to 10^{-9} . Transfer from *E. faecium* to *E. faecium* was more efficient with a frequency of $1 : 10^{-4}$.^[38] The *VanB*-gene cluster is transferable either via Tn 1547 or via plasmids at a low frequency,^[39-41] and is induced by vancomycin, but not by teicoplanin or avoparcin,^[42] which is consistent with the fact that there are no reports that avoparcin use selects for *VanB* resistance genes. Transfer *in vivo* of glycopeptide, erythromycin and chloramphenicol resistance on the skin of nude mice from *Enterococcus faecalis* to *S. aureus* has been described by Noble et al.^[42]

Use of avoparcin results in a wide spread prevalence of enterococci with high level *VanA*-mediated resistance to vancomycin (VRE) and other glycopeptides in the enterococcal flora of exposed

animals and their environment.^[43-45] However, the lack of earlier data precludes conclusions on whether the resistance trait was present in animal populations before or at the time of introduction of avoparcin in animal husbandry,^[19] but in countries where avoparcin has never been used, such as the US, or where its use has been suspended for several years as in Sweden, no VRE have been found in the intestinal flora of food animals or healthy people outside the hospital environment.^[46,47] The presence of *VanC*, conferring low level resistance is species-specific for *E. gallinarum* and *E. casseliflavus*.^[48] More recently, the *VanD* gene which confers low level transferable resistance to vancomycin has been described in enterococci.^[49]

3.2 Tylosin, Spiramycin and Virginiamycin

Tylosin and spiramycin, are macrolide antibiotics, while virginiamycin belongs to the streptogramins. Both groups of antibiotics are produced by *Streptomyces* spp. Macrolides and streptogramins are not chemically related, but are often grouped together because of similar antibacterial spectra and mode of action. The lincosamides (linco- and clindamycin) are often included for the same reason, leading to the acronym MLS antibiotics (macrolides, lincosamides and streptogramins). They all inhibit protein synthesis by binding to the 50S subunit of the bacterial ribosome and are active against a wide range of aerobic Gram positive bacteria and obligately anaerobic bacteria.^[15] Most aerobic Gram-negative bacteria are not susceptible because their outer membrane is not permeable for MLS antibiotics.

Lincosamides are not used as AGP. Tylosin and spiramycin, are commonly used as therapeutic agents in veterinary medicine, and as AGP. They are in contrast to most other AGP readily absorbed from the gut. Virginiamycin is not absorbed and in most countries only used as AGP, but in a few countries it is utilised for therapy in animals or humans. An analogue compound, however, quinupristin-dalfopristin has recently been introduced as an alternative for treatment of multiresistant and/or glycopeptide-resistant *E. faecium*. *E. faecalis* is in-

trinsically resistant to streptogramins,^[50] and another new streptogramin efepristin (RPR106972) is currently being evaluated for oral medication in humans.^[51] Virginiamycin is effective for therapy or prevention of clostridial infections in animals.^[15] All streptogramins (also called pristinamycins) are a mixture of 2 different groups of molecules: A and B, which are both bacteriostatic, but act synergistically to express bactericidal activity. Acquired resistance to MLS antibiotics is due to alteration of the ribosomal target, enzymatic inactivation and active efflux.^[52,53] Two genes *SatA* and *vgb* encoding streptogramin resistance in *E. faecium* have been detected in streptogramin resistant clinical isolates of *E. faecium*^[54] and in faecal isolates from healthy individuals and food animals.^[55] *SatA* encodes for resistance to the streptogramin A components while *vgb* confers resistance against the streptogramin B components. *Erm*-genes not only confer resistance to streptogramin B but also to all MLS antibiotics.^[56,57]

Transfer of pIP811, a self transmissible plasmid carrying the *ermB*-gene, and also resistance to chloramphenicol, tetracycline and streptomycin from *L. monocytogenes* to *E. faecalis* and vice versa has been observed.^[58] Moreover, transfer of Tn 1545, a chromosomally located transposon carrying resistant determinants to macrolides, lincosamides, streptogramin B (*ermB*), kanamycin (*aphA3'*) and tetracycline (*tetM*) from *E. faecalis* to *L. monocytogenes* has been described both *in vitro* and *in vivo* in mice.^[59]

3.3 Avilamycin

Avilamycin, a mixture of oligosaccharides of the orthosomycin group produced by *Streptomyces viridochromogenes*, is mainly active against Gram-positive bacteria by binding to the 30S subunit of the bacterial ribosomes.^[60] Other members of this group include curamycin and the everninomycins. Avilamycin has never been used for therapeutic purposes, but a related compound everninomycin (Ziracin®) is being developed for therapeutic use in humans.^[25,26] No data have been published on resistance mechanisms against everninomycins,

but increased MICs for avilamycin and bacteria resistant to everninomycin have been shown.^[61]

3.4 Bacitracin

A mixture of cyclic polypeptides produced by *Bacillus subtilis* and *Bacillus licheniformis*, bacitracin is bactericidal against Gram-positive bacteria and shows limited activity against Gram-negatives.^[15] Bacitracin interferes with the formation of bacterial cell wall peptidoglycans. As AGP it is mostly used in complex with zinc which stabilises the molecule. Bacitracin, in concentrations used as AGP, also showed preventive and therapeutic effects on necrotic enteritis in poultry.^[62,63] In humans, bacitracin is mainly used topically and for intestinal decontamination in elective colorectal surgery.^[64] It is expected that future bacitracin use in humans will increase because of its effectiveness against VRE.^[65,66] No information about cross resistance with other antibiotics exists, but several resistance mechanisms of which at least one is plasmid mediated, have been described.^[67]

Bacitracin has been shown to induce the expression of the *VanA*-gene in enterococci.^[68,69] It has been claimed that bacitracin as AGP 'cures' resistance against other antibiotics^[70-73] but because of weaknesses in study design this claim cannot be considered to be substantiated.

3.5 Flavophospholipol

Flavophospholipol, a complex of very similar phosphorus-containing glycolipids, produced by a group of *Streptomyces* spp. is also known as flavomycin, bambermycins or moenomycin. It is used for growth promotion only and is effective against Gram-positive organisms, by inhibiting bacterial cell wall synthesis. However, it is not active against Gram-negative bacteria as it cannot penetrate the lipid membrane of these organisms.^[74] Nevertheless, some activity against enterobacteriaceae has been observed.^[22,75-77] In some studies a plasmid clearing effect and a decrease in transfer frequency of some R-plasmids has been described, but an increase in frequency transfer of other plasmids has also been observed.^[78-82] No reports on resistance

mechanisms against flavophospholipol have been found. Flavophospholipol has been shown to induce VanA resistance in enterococci.^[68] Currently, insufficient data are available to assess the risk of flavomycin use as AGP on the prevalence and spread of resistance of this compound and related substances in humans.

3.6 Monensin and Salinomycin

Monensin, like salinomycin, is a carboxylic ionophore antibiotic produced by *Streptomyces cinnamomensis*. Both drugs are bactericidal by altering the membrane permeability which causes passive transport of potassium ions out of the bacterial cell. The drop in intracellular pH as a result of their replacement by hydrogen ions kills the bacterial cells. They are active against Gram positive bacteria, *Campylobacter* spp., *Serpulina* spp., coccidia and *Toxoplasma*. These drugs are used in poultry feeds for the prevention of clinical coccidiosis in chickens.^[15]

In contrast to other AGP that are only used in pigs, poultry and nonruminating veal calves, monensin and salinomycin are also used to enhance feed conversion in ruminants by modulation of the rumen flora. Both are toxic (at the recommended dosages for pigs and poultry) for several other mammalian species such as horses and dogs. Even in poultry and pigs, it has a small tolerability margin and its toxicity is increased by a number of other drugs.^[15] No information could be found on resistance mechanisms, transfer and prevalence of resistance in bacteria.

3.7 Carbadox and Olaquinox

Carbadox and olaquinox are quinoxalines, which are synthetically produced compounds with activity against obligately anaerobic bacteria such as *Clostridium* spp., and Enterobacteriaceae and staphylococci.^[15,83] In contrast to most other AGP they are readily absorbed from the intestinal tract. Quinoxalines are not used in human medicine. Despite their toxicity they are used therapeutically in some countries against *Salmonella*, *E. coli* and *Serpulina* infections in pigs. Quinoxalines inhibit

DNA synthesis and denature preexisting DNA. Resistance to quinoxalines is caused by target alterations, cell wall impermeability or increased efflux of the drug. Plasmid mediated resistance has been described.^[84,85]

4. Resistance in Animals

4.1 Animal Pathogens

Many retrospective and prospective studies have been performed concerning the emergence and selection of resistance in bacteria from animals following antibiotic use. Despite large differences in methodology, most results show that after the introduction of an antibiotic in veterinary practice the resistance in pathogenic bacteria and/or the commensal intestinal microflora increases, as in human medicine. However, some bacteria, such as most Enterobacteriaceae, staphylococci and *Pasteurella* spp. become more readily resistant to certain antibiotics than other bacteria such as *Clostridium* spp. and streptococci, of which animal isolates are still fully susceptible to benzylpenicillin.

In veterinary medicine the problem of resistant bacteria is less severe than in human medicine. Broad-spectrum β -lactamases or bacteria that are multiresistant, because of a broad spectrum efflux pump mechanism,^[86,87] coded for by a single gene and conferring resistance to several unrelated antibiotics, have never been found in veterinary pathogens. This difference is most likely due to the fact that immunocompromised patients and the accumulation of these patients in special wards in hospitals are uncommon in veterinary medicine. Recently, however, for the first time the first well documented cases of methicillin-resistant *S. aureus* infection in animals have been published, namely, postoperative wound infections in a horse and in dogs.^[88,89]

4.2 Faecal Flora

Antibiotic use not only selects for resistance in the target pathogenic bacteria, but also causes the emergence of resistant populations of bacteria in the endogenous flora of exposed individuals and

populations. Resistance genes are mostly associated with plasmids or transposons located on plasmids or on the chromosome and can easily be transferred to susceptible micro-organisms. The high density of bacteria in the gut facilitates this transfer between bacteria.

Transfer of resistance has been described between bacteria belonging to the same species, and the gene pool of Gram-positive cocci has extended to Gram-negative bacteria as well.^[90,91] Under antibiotic pressure, resistance genes tend to accumulate in multiresistant plasmids. However, not only does clonal spread of bacteria occur but also resistant bacteria from animal origin may transfer these resistance genes either to bacteria pathogenic for humans or to the endogenous flora of humans. Conjugal transfer of plasmid pAT 191 DNA which confers resistance against kanamycin, from *E. faecalis* to *E. coli* in the digestive tracts of gnotobiotic mice have been found with a frequency of 3×10^{-9} .^[92] Furthermore, *in vitro* conjugative transfer of VanA vancomycin resistance between Enterococci and listeriae of different species have been found.^[93]

Co-transfer or linked transfer, that is, the simultaneous transfer of different genes located on the same mobile element, has been described for genes encoding for different antimicrobial agents and for genes encoding for antibiotic resistance and virulence genes. The *E. coli* plasmid pNV 13 encodes for resistance to carbadox, streptomycin, spectinomycin and ampicillin.^[84,85] The transposon Tn 1545 carries resistance genes to kanamycin, macrolides and tetracyclines.^[59] Furthermore, co-transfer of antibiotic resistance and virulence genes has been described for enteropathogens.^[94] Macrolide resistance and toxin A production, encoded by Tn 5398 can be transferred from *Clostridium difficile* to *B. subtilis*.^[95] The greater the number of resistant intestinal micro-organisms in the intestinal flora the greater the chance of transfer of their resistance genes to pathogenic bacteria and dissemination in the environment.

Comparing the populations of 3 cities in 3 different countries, Lester found that the prevalence

of resistance in the intestinal flora of each population was not only strongly correlated with the amount and types of antibiotics used, but also indicative of resistances observed in pathogenic bacteria isolated from infections in that particular population.^[96] This clearly demonstrates that the endogenous flora is a reservoir for resistance genes and that resistance in pathogenic bacteria is only the 'tip of the resistance iceberg'. Because intensively reared food animals are mostly treated as a group, continuously with AGP and temporarily (but regularly) for therapeutic reasons or for prevention, the endogenous flora of these animals contains a relatively high proportion of resistant bacteria. Furthermore, since hygienic measures in intensive farming are only directed against the introduction of pathogenic micro-organisms from outside the stable or farm, and not against the prevention of spread within a stable, newly introduced resistant bacteria or emerged resistance genes will spread very fast through the whole herd by faecal contact. These resistant micro-organisms can reach humans either directly through direct contact or indirectly via meat products.

As bacteria of the endogenous flora of food animals contaminate foods of animal origins, they might either colonise humans or transfer resistance genes to human endogenous flora and so superimpose an additional load to the reservoir of resistance genes already present in humans. Therefore, it has been proposed that a low level of resistance in the intestinal flora of food animals should be considered as a safety and quality mark for these animals.^[24,28] Several studies have shown a correlation between the amount of antibiotics used and the prevalence of resistance in the faecal flora of animals.

In the Netherlands, enrofloxacin, a methylester of ciprofloxacin, is extensively used for therapy in turkeys, but its use in pigs is very limited. In a point prevalence study the prevalence of ciprofloxacin resistant faecal *E. coli* in turkey flocks was 49% and in pig herds 2%.^[97] For tetracycline, ampicillin and furazolidone, which are used regularly for therapy in both animal species, the prevalence of

resistance was, for both animal populations, in the same order of approximately 98%, 98% and 16%, respectively. The literature on resistance against AGP is very limited as most of these compounds are not used for therapy and therefore susceptibility testing is not regularly performed.

In a surveillance study on the resistance against AGP of *C. perfringens* from various animal sources conducted from 1979 until 1992 no increase in resistance to AGP was observed.^[116] However, Linton et al.^[98] found a significant increase in the prevalence of resistance against tylosin and bacitracin in faecal enterococci of pigs and poultry fed these compounds. Virginiamycin use did not result in an increase in resistance. Thal et al.^[99] found an increase over time in resistance against quinupristin/dalfopristin in faecal *E. faecium* in turkey flocks given virginiamycin as AGP. After the introduction of olaquinox in 1982 on farms using olaquinox as AGP the prevalence of resistance in faecal *E. coli* in pigs increased from 0.004% to 6% in 3 years, whereas on farms not using olaquinox the prevalence of resistance increased by a significantly lesser degree, suggesting dissemination of resistant clones.^[100]

Ohmae et al.^[84,85] noticed an increase of resistance against carbadox in faecal *E. coli* isolates in pigs after its introduction as AGP. All resistant isolates from 6 farms that fed carbadox continuously to pigs either as AGP or for prevention of swine dysentery, carried the same transferable plasmid conferring carbadox resistance. Carbadox is not used in poultry and cattle, and no carbadox resistance was found in *E. coli* isolates from these animal species in the same region. Mills and Kelly^[101] also reported an increase in resistance of *Salmonella* isolates from 37% to 61% after the introduction of carbadox. Carbadox, however, was not only used as an AGP, but also for the prevention of swine dysentery and therapy of salmonellosis. Hedges and Linton^[102] showed that not only the prevalence but also the degree of resistance increased by olaquinox use. In herds not exposed to the AGP, the degree of resistance varied from 0 to 0.02%,

while in herds given olaquinox it varied from 1.3 to 6.5%.

4.2.1 Vancomycin-Resistant Enterococci

Interest in the selection of resistance by AGP increased after the emergence of vancomycin resistant enterococci (VRE) in human infections. It was soon recognised that avoparcin, a glycopeptide like vancomycin and until recently commonly used as AGP in most EU-member states, selects for VRE in the intestinal flora of animals.^[44,103] In countries where avoparcin was used as AGP, VRE were not only found in food animals fed avoparcin, but also in the faecal flora of healthy humans and pet animals.^[43,103-106] Furthermore, resistance against MLS antibiotics such as erythromycin and the streptogramins is common in enterococci from animals fed related antibiotics as AGP, such as tylosin (a macrolide) or virginiamycin (a streptogramin).^[106] Similar figures have been found in other European countries like Denmark,^[34] where in 1995 the prevalence of resistance in enterococci isolated from faecal samples of pigs and poultry was 21% and 56% against vancomycin, 91% and 59% against erythromycin, and 53% and 37% against pristinamycin, respectively. In Finland, however, where tylosin is not used as AGP and only limited to veterinary purposes, the prevalence of erythromycin resistance in enterococci is significantly lower, being 18% and 9%, respectively.^[107]

Since 1986, Sweden has banned the use of AGP in animal feeds. The prevalence of resistance against AGP or related compounds in faecal samples of Swedish pigs was significantly lower than that in Dutch pigs in 1997. In Sweden and the US, where avoparcin has never been used, no high level VRE (VanA resistance) have been found in faecal samples of food animals or healthy humans outside hospitals.^[46] Following the suspension of the use of avoparcin in Denmark in 1995 the prevalence of vancomycin-resistant *E. faecium* in broilers decreased from 5.2% in 1995 to 1.2% in 1997.^[34]

In the Netherlands, a significantly higher percentage of VRE per gram of faeces (10 to 100%) was observed in veal calves from farms using avoparcin as an AGP than in faecal samples of calves

fed bacitracin as AGP (≤ 1 to 10%).^[108] The prevalence of VRE in turkey flocks fed avoparcin was 60% in contrast to 8% in flocks not exposed to avoparcin,^[109] and the relative odds ratio was 7.5. In Denmark, Bager et al.^[110] found a high correlation between the use of avoparcin on a farm and the prevalence of VRE in the intestinal flora of animals. The likelihood of isolation of VRE from faecal samples of animals (pigs and poultry) was 3 times higher in animals fed avoparcin than in other animals. The relative odds ratio for the use of avoparcin on the presence of VRE in the faecal flora of these animals was 2.9 for poultry and 3.3 for pigs.

It can be concluded that the use of antibiotics for growth promotion in animals, as with veterinary antibiotic use, selects for resistance among susceptible micro-organisms, not only in pathogens but also in bacteria belonging to the commensal intestinal microflora of animals, such as enterococci and *E. coli*. This has been shown for avoparcin, bacitracin, tylosin, virginiamycin, carbadox and olaquinox.

5. Transfer of Resistant Bacteria from Animals to Humans

5.1 Zoonotic Bacteria

Most investigations on the transfer of resistant bacteria from animals to humans concern Gram-negative food infections caused by bacteria like *Salmonella* spp., *Campylobacter* spp. and *Yersinia* spp. Transfer of resistant *Salmonellae* from animals to humans has been described by several authors.^[111-114] Because the resistance of salmonella isolates from humans and animals has been monitored for many years, the emergence and dissemination of resistance in this species is very well documented. Before the introduction of antibiotics, salmonella isolates and other Enterobacteriaceae were susceptible to most antibiotics despite the prevalence of plasmids.^[115] Humans become infected with salmonella isolates from animals by direct contact with infected animals or animal fae-

ces, but the most important source of human infections are food products of animal origin.

Asymptomatic salmonella infections and carriers are common in food animals in intensive animal husbandry. The salmonellae in the intestinal tract of these animals contaminate during slaughtering and processing of the carcasses, and meat and meat products. Humans can then become infected via the meat and meat products, and also via eggs etc. Humans do not always become ill after a salmonella infection. Deleener and Haebaert^[116] showed that the frequency and variation of the different isolated salmonella-serotypes from asymptomatic carriers in a meat packing plant corresponded with the serotypes isolated from the supplied meat and from the produced meat products.

Despite the fact that since the introduction of antibiotics in clinical medicine resistance in human and animal isolates increased in general, the majority of clinical salmonella isolates are still susceptible to most antibiotics.^[117] In the Netherlands, the prevalence of tetracycline resistance in human and animal salmonella isolates increased clearly until the ban on tetracycline as AGP,^[118] when it started to gradually decline.^[119-122] In Great Britain, after the ban on tetracycline as AGP, tetracycline-resistant *Salmonella typhimurium* isolates from calves fell from 60% in 1970 to 8% in 1977.^[123] However, the spontaneous ending of epidemics by virulent tetracycline resistant *S. typhimurium* clones might have contributed to this decrease as well.^[124] In most EU-member states *Salmonella enteritidis* is the most commonly isolated serotype from human infections, as a result of its extensive dissemination among poultry since 1980. Because this serotype does not, in most cases, cause clinical symptoms in affected flocks, the animals are not treated with antibiotics. Therefore, the selection pressure is low and generally isolates are still susceptible to most antibiotics.

Sporadically however, epidemics of salmonella clones with an enhanced virulence and pathogenicity for animals occur, such as *S. typhimurium* phage type 29 from 1963 to 1969, definitive type (DT)

204 in 1977, and DT 204 and DT 193 in 1980.^[123] The primary reservoir of *S. typhimurium* is calves, but sheep, goats, pigs, poultry and horses can also become infected. During all these epidemics the same phage type with identical resistance profiles was isolated from animal and human infections. Because these strains cause serious disease in affected animals, these animals are treated with antibiotics and as a result of the selection pressure these strains tend to become (multi)resistant. Since 1994, *S. typhimurium* DT 104 has caused an epidemic. This strain was, from the start, resistant to most of the antibiotics normally used to treat enteric infections in animals, but it has acquired additional resistance against trimethoprim and fluoroquinolones,^[117,125,126] most likely because affected groups of animals could only be treated with these antibiotics.

The most important reservoir for human campylobacter infections is poultry. Endtz et al.^[127] observed that the emergence of fluoroquinolone-resistant *C. jejuni* infections in humans in the Netherlands coincided with the introduction of enrofloxacin for poultry therapy in spring 1987. Enrofloxacin and ciprofloxacin (the latter was introduced in October 1988 for human therapy in the Netherlands), are fully cross-resistant. In 1989, 14% of poultry and 11% of human isolates of *C. jejuni* were resistant to ciprofloxacin. Experimentally, it was shown that in flocks only colonised with ciprofloxacin-susceptible *C. jejuni*, after therapy with enrofloxacin mutants resistant to ciprofloxacin emerged.^[128] In Great Britain, enrofloxacin was registered for veterinary use in 1993, and in that year 14% of *C. jejuni* isolated from poultry carcasses imported from the Netherlands were fluoroquinolone resistant compared with 1% from locally raised broilers.^[129] In 1997, the percentage of fluoroquinolone resistant *C. jejuni* from English broilers had approached a continental level of about 10%. Transfer of chloramphenicol resistant *Yersinia enterocolitica* strains from animals to humans has been described by Perez Trallero.^[130]

6. Disturbance of Colonisation

Another aspect of the use of antibiotics in animals is disturbance of the colonisation resistance of the intestinal flora of animals exposed to certain antibiotics.^[131,132] In cases of reduced colonisation resistance not only are the minimal infectious or colonisation doses of pathogenic or resistant bacteria considerably lower, but animals also excrete these bacteria in higher numbers and over a longer period of time compared with animals with an intact colonisation resistance. This enhances not only dissemination of salmonellae or resistant bacteria within a group of animals, but also increases the contamination of carcasses with these bacteria during slaughter. This effect has been clearly demonstrated for most broad-spectrum antibiotics^[133] and for certain AGP: avoparcin^[134-136] and to a lesser extent, virginiamycin and tylosin. Avilamycin and bacitracin do not appear to disturb the colonisation resistance in the dosages used for growth promotion.^[137-141] Furthermore, flavomycin has been shown to provide a certain degree of protection against salmonella infections.^[142]

6.1 Indicator Bacteria

As a result of exposure to antibiotics, the level of resistance against antibiotics among bacteria belonging to the normal intestinal flora of humans and animals increases. These bacteria constitute an enormous reservoir of resistance genes for (potentially) pathogenic bacteria.^[4] Moreover, the level of resistance in the endogenous flora is considered a good indicator for the selection pressure exerted by antibiotic use in that population and for resistance problems to be expected in pathogens.^[96] Resistant bacteria from the intestinal flora of food animals contaminate, like zoonotic bacteria, the carcasses of slaughtered animals and reach the intestinal tract of humans via the food chain.

Investigation of the prevalence of resistance of certain indicator bacteria like *E. coli* and enterococci in the intestinal tract of different populations of animals and humans makes it feasible to compare the prevalence of resistance in different pop-

ulations and to detect a possible transfer of resistant bacteria from animals to humans and vice versa. Because of the inevitable high use of antibiotics in hospitals, selection and dissemination of resistant clones and resistance genes is high in this environment. The emergence of new resistances due to the acquirement of new genes or gene clusters like the *VanA*-gene cluster are not likely to occur in hospitals but must have been introduced from somewhere outside into the hospital setting. Therefore, healthy individuals in the community are a source of resistant bacteria and resistance genes, and can be considered to be a suitable population to study with regard to the possibility of transfer of resistance from animals to humans.

Corpet^[143] showed that the prevalence and degree of resistance in faecal *E. coli* flora in humans who used only sterilised food decreased significantly. Furthermore, Nijsten found significantly more resistant *E. coli* in the faecal flora of pig farmers than in faecal (sub)urban residents.^[144,145] However, the personal antibiotic use of the farmers was much higher than that of the (sub)urban residents.

Comparison of the prevalence of ciprofloxacin resistant *E. coli* in faecal samples of turkeys and turkey farmers with pig and pig farmers clearly indicated transfer of ciprofloxacin-resistant *E. coli* strains from turkeys to turkey farmers.^[97] In the Netherlands, enrofloxacin is commonly used in turkeys but not in pigs because no oral formulation for pigs was available at the time of study. Tetracyclines are extensively used in both animal species, similar to the use of furazolidone in the past. The prevalence of ciprofloxacin resistant *E. coli* was not only significantly higher in turkey farmers and turkeys than in pig farmers and pigs, but also *E. coli* strains were isolated from farmers and turkeys which were completely identical in pulsed field gel electrophoresis (PFGE) after *XbaI* digestion. None of the turkey farmers and urban residents in this study had used antibiotics in the 3 months prior to the study. For the turkey slaughterers the infection risk seemed much lower, despite the fact that ciprofloxacin-resistant *E. coli* strains

had been isolated from the turkey carcasses after slaughtering.^[109]

In contrast, there was no difference between the prevalence of furazolidone or tetracycline resistant *E. coli* between the 2 animal populations and between the 2 groups of farmers. For furazolidone, as with the fluoroquinolones, horizontal transfer of resistance via plasmids or transposons is not important for dissemination of resistance, but only clonal spread of resistant strains. These results therefore suggest transfer of resistant strains from animals to humans. The extent of transfer seems to be correlated with the prevalence of resistance in the animal population, which is positively correlated with the amounts of antibiotics to which the animal population is exposed. In the same study, VRE were isolated from a turkey farmer and his turkeys which were not only identical using PFGE after *SmaI* digestion, but also had a *VanA*-gene with a unique mutation.^[105] This again strongly indicates transfer of resistant strains from animals to humans.

In Sweden, no VRE were found in the faecal flora of healthy humans and animals, and no VRE could be detected in stool samples of healthy volunteers after taking a course of oral vancomycin.^[146] However, in Belgium in a similar experiment, all volunteers in which no VRE were found in their stool samples before the study, became positive.^[147] This is in concordance with the results of Quednau et al.,^[148] who were able to isolate VRE from Danish but not Swedish meat products, and a Dutch study in which VRE could only be isolated from faecal samples of carnivorous humans but not from vegetarians.^[149]

7. Transfer of Resistance Genes from Animals to Humans

In 1976 in a prospective study, Levy observed transfer of tetracycline resistance genes between chicken *E. coli*-strains, from chicken to chicken and from chicken to humans, in chickens fed tetracycline.^[150,151] A wide dissemination of a tetracycline resistance gene (*tetQ*) was observed by Nikolich et al.^[152] They found identical *tetQ* genes

in host-specific intestinal flora bacteria; *Bacteroides* spp. and *Prevotella intermedius* from humans, and *P. ruminicola* from bovines.^[152,153] The relationship between the use of an antibiotic and the dissemination of bacterial resistance from animals to humans has been described in detail by Hummel et al.^[154]

In 1982 in the former German Democratic Republic (DDR) nourseotricin, a streptotricin antibiotic, was introduced as an AGP for pigs. Streptotricin antibiotics have not been used in human medicine and do not show cross-resistance with other antibiotics. Resistance to nourseotricin became, within 1 year after its introduction, common in faecal *E. coli* from pigs fed this antibiotic. The resistance genes were located on transposon Tn 1825 and within 2 years this transposon was found in faecal isolates from pig farmers and their family members, in urban residents, and in *E. coli* isolated from urinary tract infections in humans. A few years later it was also found in pathogenic bacteria, not only in zoonotic bacteria like *Salmonella* spp. but also in *Shigella* spp., which only affect humans and do not have an animal reservoir. Outside the DDR, nourseotricin resistance has never been found.

Other examples of the dissemination of resistance genes from animals to humans are the dissemination of the *aacC4*-gene (apramycin resistance) and *hphB*-gene (hygromycin resistance) from animals to human bacteria. These genes are co-transferred. Despite the fact that apramycin is only used in animals, these genes have been found in animal isolates or zoonotic bacteria isolated from humans, in Enterobacteriaceae in the environment, the intestinal flora of farmers, and in hospital isolates.^[155-158] A high degree of similarity between plasmids encoding for *aacC4* and *hphB* from these sources has been demonstrated.^[159,160] A question regarding the similarity of the *VanA*-gene cluster from animals and humans has been addressed in several studies. In a study from Norway, horizontal transfer of the gene cluster was strongly suggested, that is, 9 out of 12 human and 7 out of 10 poultry isolates were identical.^[161] In the Netherlands, sim-

ilarity between a VRE isolated from a turkey and a turkey farmer was demonstrated.^[105] Furthermore, in a study from the UK, the novel insertion sequences in the *VanA* genes from VRE isolated from poultry were not found in hospital isolates.^[162]

8. Conclusions

In animals, as with humans, the use of antibiotics causes an increase of resistance both in pathogenic bacteria, and in the endogenous flora of these animals. Resistant bacteria from animals, that is, zoonotic bacteria or intestinal flora, can infect or reach the human population by direct contact, and also via food products of animal origin. These resistant bacteria can colonise humans and/or transfer their resistance genes to other bacteria belonging to the endogenous flora of humans. Moreover, the greater the number of resistant bacteria in the intestinal flora, the greater the likelihood that genes encoding resistance will be transferred to (potentially) pathogenic bacteria, and disseminated into the environment from animals to foods of animal origin. In this respect, one might consider the resistance observed in zoonotic and nosocomial pathogens to be just the tip of the iceberg.

As bacteria from the human flora cause not only infections in immunocompromised hosts, but are also considered an important reservoir of resistance genes for human pathogens, it has been proposed that a low level of carriage of resistant strains by humans should be a public health goal in much the same way as a normal blood pressure and a low serum cholesterol level are public health goals.^[96] Despite the fact that it is not yet clear to what extent the use of antibiotics in animals contributes to the resistance problems in human medicine, it cannot be disputed that there is a definite link. Because we are now encountering multiresistant micro-organisms in the clinical setting which are difficult to combat with currently available antibiotics, every source of resistance must be controlled as efficiently as is feasible. Consequently, a low level of resistance in the intestinal flora of food animals should be thought of as a distinguishing safety and quality mark for food animals.^[24,28]

Moreover, this will not only protect public health, but also safeguard the future efficacy of antibiotics in veterinary medicine.

This goal can only be achieved by reducing the amounts of antibiotics used in animals. The requirement for antibiotics in veterinary therapy and bacterial infection prevention should be minimised by improving methods of animal husbandry, disease eradication, optimal use of existing vaccines and the development of new vaccines. If antibiotics have to be used, the use of agents with a narrow spectrum of activity should be preferred and this use should be according to a sensible veterinary antibiotic policy.^[163] Discontinuing the practice of routinely adding AGP to animal feeds would reduce the amount of antibiotics used for animals in the EU by a minimum of 30% and in some countries by up to 50%. In this case, the public health risks should be weighted against the economic profits. The Swedish have shown that modern and profitable animal husbandry without AGP is feasible.^[20] A ban on the use of AGP would encourage research into alternatives like pre- and probiotics.

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Chapter II

A veterinary antibiotic policy: a personal view on the perspectives in the Netherlands

A veterinary antibiotic policy: a personal view on the perspectives in the Netherlands

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ABSTRACT

To reach a more adequate and responsible use of antibiotics in veterinary medicine a broad approach is required. The growing interest in the optimum use of antibiotics in veterinary medicine is stimulated by awareness of the necessity to prevent the emergence of resistance and the spread of resistant bacteria. This is a matter of primary importance to safeguard the efficacy of antibiotic therapy in animals now and in the future, but will also minimise possible public health risks. As resistance develops in rough relation to antibiotic use, the best way to minimise emergence of resistance, without interfering with optimal veterinary care, is to rationalize the use of antibiotics by, or under responsibility of, a veterinary surgeon. A second further improvement can be reached by developing a veterinary antibiotic policy to prevent emergence and spread of resistance as much as possible. Measures that must be taken in combination are:

1. Establishing a policy for the use of antibiotics jointly by veterinary practitioners and experts resulting in a formulary.
2. Systemic registration and analysis of patterns of bacterial resistance (not only of pathogens but of faecal flora as well) and the use (qualitative and quantitative) of antibiotics in veterinary medicine.
3. Regular feedback of these data to the veterinary practitioners and consultation about the efficacy of the formulary guidelines in daily practise.
4. Systemic monitoring and evaluation the impact of the antibiotic policy and, if indicated, updating of the formulary.
5. Continuous education of veterinary surgeons about infectious diseases and antimicrobial therapy and development of a veterinary audit system.

The first step to rationalize the veterinary use of antibiotics was taken by publishing a veterinary antibiotic formulary in 1987, compiled by a group of experts. Despite the fact that this was a pure top down approach and bottom up input from veterinary practitioners was lacking, this first formulary was very well received. However the impact of this formulary on the veterinary use of antibiotics and prevalence of resistance has not been evaluated at this moment. Until now regular and nationwide systemic monitoring of resistance and registration of veterinary use of antibiotics is not practically feasible in the Netherlands. A new formulary, based on a veterinary antibiotic policy, will be published by the end of 1992. However, to be able to evaluate the impact of this policy on the prevalence of resistance and, if necessary, to adapt the policy the development of facilities for resistance epidemiology is urgently needed.

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INTRODUCTION

Antibiotics are among the most used drugs in veterinary medicine and there is no field of veterinary therapy, which more directly invites intervention from outside than does antimicrobial therapy. The main reason is that the use of antibiotics inevitably leads to the emergence of resistance against the used antibiotics and spread of resistant bacteria, not only in animals but in their environment as well.

Compared with most other drugs of similar potency, antibiotics are in general remarkably safe and effective, because they are directed against invading bacteria, instead of, like most other drugs, altering a pathophysiological process in the host. This safety may have provoked liberal, even lavish use in man and animals arguing, if it doesn't do any good, it will do no harm either. Antibiotics, however, are not only effective against pathogenic bacteria, but also against the normal bacterial flora of the host. In addition the selective pressure of antibiotics, promotes the emergence of resistant bacteria among the endogenous flora. These, mainly enteric, microorganisms may colonize other animals and/or transfer their resistance plasmids to either the enteric flora of the animals treated with antibiotics or after excretion to the environmental bacterial population. The spread of these microorganisms can diminish the effectiveness of previously useful antibiotics, enforcing the use of more expensive or less safe alternatives.

On the other hand the resistance problem should not be overstated. Most specific infections have remained susceptible to antibiotics available for many years, while new and useful drugs are being marketed regularly. Therapeutic failure today is seldom due to the acquired resistance of bacteria to all the drugs available for treatment. The contribution of veterinary usage of antibiotics to the human load of resistant bacteria is still unclear. However the major selective pressure leading to resistant microorganisms in human patients is the use of antibiotics in human medicine. Nevertheless, like with all chemotherapeutics the benefit of treating animals must be weighted against the risk of unwanted side-effects. The veterinary profession in the Netherlands is well aware of the fact, that how well we use antibiotics, is a strong indicator of how effective we are in fulfilling our responsibility as veterinary surgeons. Therefore the Dutch Koninklijke Maatschappij voor Diergeneeskunde (Royal Society for Veterinary Medicine: KNMvD) has decided to develop a veterinary antibiotic policy to safeguard the continuous efficacy of veterinary antibiotic therapy now and in the future and to take responsibility for public health.

Rationale

As resistance to antibiotics develops in rough relation to the extent of use, emergence and spread of resistance should be prevented primarily by limiting

the amounts of antibiotics used (Greenwood, 1989). However, our responsibility as veterinary surgeons requires that we do so without hampering optimal treatment of sick animals. Hence, antibiotics should be used by veterinary surgeons only if indicated: that is to cure or prevent infectious diseases in animals, and then in an optimal way. The necessity is certainly not to prohibit the use of certain antibiotics, but to encourage rational and responsible use of antibiotics.

Methods by which this may be achieved

It is often claimed, that veterinary surgeons and doctors alike use a lot of antibiotics inappropriately. This may be attributed to the complexity of decision making. Clinical diagnosis of infectious diseases in veterinary practice hinges on the predictive value of symptoms–sign complexes and the epidemiological situation or on the basis of the results of cultivation. When a bacterial infection is suspected, initial antibiotic therapy is in first instance empirically chosen on an “educated guess” basis. This is necessary when considering outbreaks of serious infections, where the initial antimicrobial therapy must be effective against all the likely pathogenic bacterial species before results of laboratory tests are available. The first objective is then to ensure effective care. Because antibiotic therapy is more effective if started early in the disease, it is very unrealistic to expect veterinary surgeons to wait until culture and susceptibility testing results are available, before initiating antibiotic treatment. Also the often-criticized use of antibiotics for so called trivial bacterial infections or for the prevention of secondary infections, may, especially in animals housed closely together, prevent much of these infections developing beyond the trivial.

It is also important to appreciate that the choice of antibiotics is to a large extent dependent on patient and farm factors. This requires not only extensive knowledge about pathology and antibiotics, but also up to date information on the disease history of the farm, drug susceptibilities in previous cases on that farm and in the neighbourhood. Moreover special care and nutrition, and other supportive therapeutic measures are also essential for the effective treatment of bacterial diseases. The only person who is qualified to advise on treatment and able to make the decision that antibiotics are indicated and to make a choice is the veterinary practitioner of that farm. Moreover, each time that the use of antibiotics is necessary, this should be considered as an abnormal situation that should have been prevented. So professional advice on other measures, which might prevent new infections in the future, such as changes in management, improving hygiene, housing or vaccination are indicated as well. When it has been decided that antibiotic treatment is indicated the efficacy of the treatment should always be the overriding aim of choice of antibiotic. Apart from the choice of antibiotic the correct dosage, route, frequency and time-span of administration are equally important. Under-

treatment may be due to too low dosing, but also be caused by an inadequate route of application or pharmaceutical formulation. This may result not only in treatment failure, but might also enhance development of resistance. Overtreatment, however, is much more common. Either an antibiotic is being used when none is needed or a sledgehammer combination to crack a nut, or, worst of all, as a substitute for a professionally sound diagnosis. Again, the only person, qualified to make a diagnosis and to advise on proper use of antibiotics and prevention of future infections is the veterinary practitioner of that farm.

His or her main difficulty is, however, to make the appropriate choice among the many possibilities. This is becoming so for a number of reasons: firstly, because the choice of antibiotics is so bewilderingly large, that veterinary practitioners, who have to be experts in many other areas, can rarely keep up with all the new developments in antibiotic therapy. Moreover, clinicians and (in some countries like the Netherlands) even farmers, receive a great deal of confusing and sometimes biased "guidance" from the pharmaceutical industry. These promotional efforts of the pharmaceutical industry, the desire of veterinary surgeons to use the best drug available and client pressure, lead to a trend to use new antibiotics, when older drugs may be as effective. As it is difficult enough for specialists to assess the claims made for the many antibacterial products on the market, this is almost impossible for most practitioners. Therefore expert advice on rational antibiotic use is needed and welcomed. However, even in relatively straightforward clinical situations there are often several equally effective agents which might be used. Choice may then be determined by an agreed antibiotic policy.

Antibiotic policy

An antibiotic policy has been defined as a set of rules, which superimposes on the definition of rational treatment other considerations, being the most important: protection of the population and environment of the emergence and spread of resistant bacteria, which lead to a decision which agent or agents, among the several that are rational, should actually be used (Greenwood, 1989). The aim should be to achieve three goals:

1. Control of antibiotic resistance.
2. Optimal patient treatment and protection of animals at risk.
3. Treatment should be practical, affordable and avoid risks of residues in or damage to animal products for human consumption.

Prevention of drug resistance is the major consideration in an antibiotic policy, but should not interfere with the other two objectives.

Tactics to prevent emergence of resistance are (Gruneberg, 1980; Selkon, 1980):

1. Restriction of antibiotics mainly to oral or parenteral routes, unless specifically indicated, such as for treatment of mastitis or otitis.

2. Diversification: for example betalactam antibiotics may be used primarily for respiratory tract infections and quinolones for enteric infections.
3. Usage of a combination of antibiotics when there is sufficient scientific evidence that it improves efficacy of treatment or diminishes the risk of resistance emergence.
4. Preventing, if possible, the situation where groups of animals are treated several times during their lives with the same antibiotic. So when a second condition occurs, which indicates the use of antibiotics, a drug should be selected which does not show crossresistance with the formerly used antibiotics.
5. Rotation. As soon as resistance emerges or on a regular time base the first choice antibiotic changed for another rational choice.

Antibiotic formulary

Application of these principles should result in a preferred and limited list of antibiotics for more than 90% of the conditions, commonly presented to a practising veterinary surgeon and provide a practical and rational treatment choice for these cases based on scientific data and practical experience. Such a list is called a formulary and includes data on appropriate dosage, method and time-span of application, possible side effects or interactions and tissue damage or residue problems (withdrawal times). If several antibiotics can be used, the formulary must differentiate between first, second and third choice antibiotics.

First choice antibiotics are the preferred antibiotics for a certain condition. Second choice antibiotics might be used if the responsible veterinarian has a good reason not to use a first choice antibiotic. In most cases this will be a resistance problem on a particular farm. Third choice antibiotics should be only used after susceptibility testing and/or consultation of a specialist. Consultation of a specialist is an effective means of controlling usage of third choice antibiotics while at the same time assuring that sick animals will be treated effectively and appropriately. Clinical consultations on diagnosis and management should be provided by experts in infectious diseases: veterinary microbiologists or species specialists.

Newer antibiotics should not be first choice antibiotics, except when there is overwhelming evidence of their advantages over established drugs.

A first veterinary antibiotic formulary, edited by W.D.J. Kremer was in 1987 published in the Netherlands and was very well received by the veterinary profession. It gave, however, mainly guidelines for rational usage of antibiotics and it was compiled completely by experts. A danger of such a formulary, could be that it is not always practical in all situations, that might occur in daily veterinary practice.

Ample experience with therapeutic formularies for family doctors has

shown, that to be accepted and thus effective, formularies must be developed from the basis (bottom up) and cannot be designed from an "ivory tower" (top down) (Baker, 1988). In contrast to the hospital situation, where adherence to a formulary can be more or less enforced by the hospital pharmacy, this is not feasible with general medical and veterinary practitioners. The basis for an effective antibiotic policy in these populations is consensus among and agreement on the formulary by the practitioners. Even in a hospital setting the consensus model has been shown to be much more effective (Wyatt et al., 1990). A new updated and extended version of the Dutch veterinary formulary, that differentiates between first, second and third choice antibiotics, will be published by the end of 1993. As this formulary is compiled by the steering group for veterinary antibiotic therapy of the KNMvD with the help of specialists, it must again be considered as a top down approach. On the other hand a formulary does not dictate but merely advise on antibiotic treatment and force veterinary surgeons to consider and encourage discussions about the use and choice of antibiotics among themselves (veterinary audit) and with specialists. As a formulary must be updated regularly every new edition can be more influenced by general practitioners and becoming more and more the result of a bottom up approach, with advice of experts. This is the only way to ensure that in the long run a veterinary antibiotic policy will be effective.

Laboratory testing and advice

Provided antimicrobial susceptibility data should correlate with the policy. An important step is to give as little laboratory encouragement as possible to inappropriate use of antibiotics. A report on susceptibility data should only be sent to a veterinary practitioner when a pathogenic species has been isolated and than so censored that only first and second choice drugs listed in the formulary for that condition are included. Only when an organism is multiply resistant is full information given. This discourages the use of third choice drugs, as susceptibilities are seldomly reported. Reporting of sensitivities directly to the farmer, which unfortunately is becoming a more and more common policy among laboratories in the Netherlands, seems undesirable. It encourages self-vetting by farmers, inevitably resulting in inappropriate use of antibiotics.

The second step is to have a good network providing regular information about trends in resistance development. Careful record keeping of all pathogens, complete with antibiotic susceptibilities, is of crucial importance.

However, as culturing and susceptibility testing is requested mainly in cases of treatment failure, use of these data alone, can easily lead to an exaggerated view on resistance prevalence in a population. Therefore it may be as important to monitor regularly the prevalence of resistance of the faecal flora (e.g. *Enterobacteriaceae*) of groups of animals to follow the effect of antibiotic usage

in that species. The results of such a surveyance system might predict the emergence of resistance in pathogens and be used to rotate the selection of first and second choice antibiotics.

A regular computer analysis of resistance patterns in faecal flora and of all pathogenic isolates by animal species, age group and organ system involved, should be made for regions and nationally and send to all involved in antibiotic policy-making. On a more confidential basis this could also be done for individual farms and for veterinary practices.

This gives early warning of emergence of resistance and/or cross-infection and clustering of resistant strains, which may need special attention. Moreover, since bacterial infections, as said previously, require immediate treatment, knowledge of local resistance trends may for practitioners even be more important than the results of susceptibility testing of single cases.

Standardisation among and automation of the laboratories of the Dutch animal health services by the laboratory information system (LIS) makes this feasible. Private laboratories doing bacteriological investigations for veterinary practitioners should be supervised by a certified veterinary microbiologist for quality assurance and also be obliged to make their susceptibility reports available.

Pharmacovigilance

However extensive and thorough clinical trials may have been performed, the true benefits of a new antibiotic as well as the possible hazards, are only confirmed by extensive clinical use. Realisation of this fact has lead to a post-marketing surveillance of drugs in human medicine. In the Netherlands there are also plans for a central office for the study of side-effects of veterinary drugs. In contrast to drugs intended for use in human beings, the risk of toxic side effects can be much better evaluated in preclinical studies in animals. As emergence and spread of resistance is the most serious side-effect of antibiotics intended for use in animals, the development of facilities to monitor and analyse regularly the prevalence and patterns of resistance should be the most important item for post-marketing surveillance by the pharmaceutical industry and pharmacovigilance by the government and funds should be made available for this purpose.

Recording of antibiotic usage

Recording, qualitative and quantitative, of the antibiotics used in veterinary medicine is of paramount importance, not only to monitor the impact of the antibiotic policy, but also to look for a possible correlation between the usage of a certain group of antibiotic(s) and the emergence of resistance (resistance-epidemiology).

At this moment it is very difficult to obtain data on the amount of antibiotics used in veterinary medicine in the Netherlands. Most pharmaceutical companies are very reluctant to make these figures officially available even

confidentially and for scientific purposes, The new Dutch Diergeneesmiddelenwet (Veterinary Medicines Act) requires, however, registration of the amounts of veterinary medicines sold by each company. Moreover, the Veterinary Medicines Act and the development of an integrated quality control (IQC) system in different sectors of animal production (pigs, calves and poultry) require the recording of use of veterinary drugs and the use of pre-medicated feeds by means of a logbook on each farm. This proposed recording system of health status and use of medicines from the breeding phase to the finishing and slaughterhouse phase will make it not only feasible to monitor the usage of antibiotics on a farm and per veterinary surgeon, but also the indications and efficacy under practice conditions. In fact reports of lack of efficacy are at least as important for an antibiotic policy as those recording adverse reactions or resistance spread. Negative reactions, however, often tend to go unnoticed or unrecorded. Such a farm logbook makes control on the appropriateness of antibiotic use by individual veterinarians also possible. So we can learn, who is using what drugs, for which indications, in which kind of animals and with what effect. This knowledge can be used to design and target education programs, reevaluate the advice in the formulary and to single out veterinary surgeons, who do not comply with the antibiotic policy on a regular basis. If an investigation proves that they do not have valid reasons to do so the Dutch law regulating veterinary medicine (wet op de uitoefening van de diergeneeskunde) makes it possible for the profession to take disciplinary measures. Again funds have to be found to effect these possibilities.

Education and postgraduate training

Veterinary students in the Netherlands are trained to use the veterinary antibiotic formulary. Postgraduate courses on antibiotic therapy are very popular among veterinary surgeons in the Netherlands. Although decision-making is a preeminent function of veterinary surgeons, veterinary (postgraduate) education mostly concentrates only on the methods of veterinary knowledge acquisition. This is due to the fact that to teach veterinary decision-making processes in their complexity is not possible by traditional forms of teaching. To improve the teaching process in this direction, the Dutch animal health services organised problem-based postgraduate courses on the rational use of antibiotics. In three sessions small groups of practitioners discussed a collection of four carefully constructed problems, together with relevant literature which they had received by mail a week before. These problems consisted of real life phenomena, derived from daily practice and adapted for educational purposes. The task of the students was to discuss these problems, find explanations and formulate ways to manage these problems, i.e. to solve them. The explanations and solving of the problems by the different groups were discussed by all students together and commented on by experts at the end of each session. This approach also stimulated exchange of

ideas between teachers and students, the latter being often very experienced practitioners.

In this way practitioners can compare their way of using antibiotics with that of their colleagues and trying by criticizing each other to reach consensus among peers. Doing this, one of the best educational methods is practised. Moreover, it is another way to find out which antibiotics are mainly used for certain indications and why. By using, in such courses, the formulary as the standard, acceptance of the formulary is improved, but also weaknesses or impracticabilities in the formulary that need improvement can be revealed. In this way postgraduate training becomes an essential part of an antibiotic policy and it provides a vehicle for one of the most important functions of an antibiotic policy: ensuring that regular discussion between all concerned in optimal veterinary use of antibiotics does take place. It might also reduce the influence of pharmaceutical representation and advertising, which seem by now far too powerful determinants of selection.

Veterinary audit

These problem-based courses could be the start of developing a veterinary audit system, in which veterinary surgeons regularly measure the quality of the care and advice they provide, by discussing real cases and problems among peers. This will provide a regular and systematic evaluation of the quality and effectiveness of veterinary services to the public and should be regarded as an important professional obligation.

Conclusion

Effective use of (limited) resources, such as antibiotics is a professional duty of the veterinary surgeon. An antibiotic policy with a formulary will assist veterinary surgeons in providing optimal therapy and prevention of bacterial infections with minimal risk of emergence and spread of resistance. The first formulary, that only tried to rationalize veterinary usage of antibiotics, was very well received by the Dutch veterinary surgeons, but it has not been feasible to evaluate its effect either on veterinary professional behaviour or on the bacterial population. However a recent study showed that pigfarms supervised by a veterinary surgeon on a regular basis (weekly or twice monthly) had not only better technical results but also used less antibiotics than farms on which a veterinarian was only consulted when this was thought necessary (Elbers, et al., 1990; Elbers, 1991).

It is of paramount importance that the introduction of the new extended formulary, which includes advice on optimal veterinary use of antibiotics, is followed by the development of facilities for monitoring resistance. This may then be related to the quantitative and qualitative use of antibiotics. Moreover correlation of these data with information on prevalence of resistance in the open (not hospital!) human population and the use of antibiotics by doc-

tors, might give an indication of possible public health risks of veterinary use of antibiotics. All this is crucial information for a further development of a scientifically based veterinary antibiotic policy and regular adaptation of the guidelines in the formulary.

Regular consultation between veterinary practitioners and veterinary specialists on infectious diseases about the clinical efficacy of the recommendations in the formulary, laboratory procedures and resistance, as well as continuous education are an essential part of an antibiotic policy. It should be realised that a formulary, as essential part of an antibiotic policy, is by no means a way of simplifying veterinary practice and can therefore never be a substitute for or used without sound professional knowledge, experience and advice. Therefore a veterinary surgeon familiar with infectious disease problems on a farm and in the region is the only person qualified and able to decide if and what antibiotic is indicated. To strengthen his or her position and to decrease client pressure, which is one reason of misuse of antibiotics, pharmaceutical companies should not directly advertise to farmers and people other than qualified veterinarians, such as advisers from feedmills etc., should refrain from giving advice on the use of antibiotics. In this way we can improve animal health and decrease emergence of antibiotic resistance and spread of resistant strains of bacteria in a responsible way. And there is no reason to prohibit the use of certain antibiotics by qualified veterinary surgeons, provided the veterinary profession takes severe disciplinary measures against the very few who cannot, or are not, willing to behave professionally and take their responsibility.

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Chapter III

Antimicrobial resistance in faecal samples of pigs from five abattoirs in the Netherlands and from Sweden

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and
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ABSTRACT

The prevalence and degree of antibiotic resistance of faecal indicator bacteria, *Escherichia coli* and enterococci, were determined in 1321 faecal samples collected from pigs at five abattoirs in the Netherlands and in 100 samples from Swedish pigs. In the Dutch samples a high prevalence of resistance was observed in *E. coli* for three commonly used antibiotics in pig medicine, amoxycillin (70-94%), oxytetracycline (78-98%) and trimethoprim (62-96%). Also, the prevalence for chloramphenicol (55-67%) and neomycin (38-67%) was relatively high. For the other compounds tested the prevalence was less than 10%. The percentage of samples with a high degree of resistant *E. coli* showed the same tendency in all Dutch abattoirs although significant differences between the abattoirs were observed. The percentage of Swedish samples with a high degree of resistant *E. coli* was significantly lower for all antibiotics except nitrofurantoin, gentamicin, flumequin and ciprofloxacin. All enterococci were susceptible to amoxycillin and high-level resistance to gentamicin was observed in 4-6% of the Dutch samples. A high prevalence of resistance and a high degree of resistance was found for erythromycin and oxytetracycline. The prevalence of resistance to dalfopristin-quinupristin ranged from 6 to 8% and for vancomycin from 24 to 46%. Significant differences between the abattoirs were found for all compounds tested except amoxycillin. In the Swedish population both the prevalence and degree of resistance in enterococci were significantly lower except for amoxycillin and gentamicin. This point prevalence study showed that the prevalence and degree of antibiotic resistance in indicator bacteria, *E. coli* and enterococci, in faecal samples from pigs differed between two countries and reflected differences in antibiotic usage in pigs. To analyse the differences observed between the slaughterhouses, additional information about the farms of origin and antibiotic consumption is necessary.

INTRODUCTION

The prevalence and extent of antibiotic resistance in a population are strongly correlated with antibiotic usage, as selection and dissemination of resistant bacteria are heavily augmented under selective pressure caused

by antibiotics. As a consequence, resistance is most common where there is heavy use of antibiotics and appreciable host-to-host contact, as in hospitals, day-care centres for children and nursing homes.^{1,2} Intensive farming units also represent a large reservoir of antibiotic-resistant bacteria.² Here resistance is selected and perpetuated not only by the regular veterinary use of antibiotics, but also by the continuous feeding of antibiotics to enhance growth of animals. Moreover, resistant microorganisms are very easily disseminated within units via faecal contact. Many studies on bacterial resistance have been published, but mainly of hospital and animal pathogens and based on data generated to support therapy. Therefore non-therapeutic antibiotics such as antimicrobial growth promoters (AMGP) have seldom been studied. Moreover, the selection of clinical cases from which samples are tested represents a worst-case scenario. Knowledge of the transfer of resistance via plasmids, phages, transposition and 'free' DNA³ has increased considerably since the 1970s and 1980s, when transfer was considered to be uncommon, it was thought that only zoonotic bacteria from animals could infect humans and transfer of resistance genes from animal bacteria to human bacteria was thought to be rare.⁴⁻⁸ The fear now is that antibiotic-resistant bacteria, pathogenic or non-pathogenic to humans, are selected in the intestinal flora of animals, contaminate foods of animal origin and transfer their resistance to other bacteria in the human gut.^{9,10} Humans and animals live in close association with large numbers of bacteria, of which the majority are found in the large intestine, where they are exposed to antimicrobial agents, exchange genetic material with other bacteria and, on excretion, contaminate the environment or colonize other animals and humans. Hence, the intestinal flora of healthy animals and humans is considered to be the most important reservoir of resistant bacteria and resistance genes.¹¹⁻¹³ As contamination of carcasses with faecal flora during slaughtering inevitably occurs, food of animal origin may serve as a vehicle to transport resistant bacteria and resistance genes between animals and humans. The prevalence and degree of antibiotic resistance found in indicator bacteria in the faecal flora of humans and animals are considered to be a good indicator of the selective pressure of antibiotic usage.¹⁴⁻¹⁸ They correlate with

the amounts and types of antimicrobial agents consumed by these populations¹⁹⁻²¹ and changes in resistance can be considered as an early warning system for resistance to be expected in potentially pathogenic bacteria.²⁰⁻²¹ Therefore it has been suggested that a low level of carriage of resistant intestinal (indicator) bacteria in humans should become a public health goal, as have normal blood pressure and low serum cholesterol levels.²¹ In food animals a low prevalence and degree of antibiotic resistance in the intestinal flora should be considered a distinguishing quality and safety mark.^{22,23} Indicator bacteria such as *Escherichia coli* and enterococci are organisms that constitute a natural part of the intestinal flora of humans and animals. Using these species makes it feasible to compare levels of resistance between populations. The aim of the present study was to determine and compare the prevalence and degree of resistance to antibiotics commonly used in pig medicine and AMGP in indicator bacteria, *E. coli* and enterococci, in faecal samples from pigs slaughtered in five regions in the Netherlands and in Sweden. In Sweden the use of AMGP was banned in 1986.

MATERIALS AND METHODS

Collection of faecal samples

Fresh faecal samples were collected in November and December 1995 at the slaughterhouse in Weert and in March and April 1996 at the other four slaughterhouses (Table 1 and 3) by inspectors from the RVV (Rijkskeuringsdienst voor Vee en Vlees: Dutch National Inspection Service for Livestock and Meat). The slaughterhouses were widely located in the Netherlands. The Swedish samples were collected during September 1997. To prevent multiple sampling from pigs from the same farm, faeces (approximately 20 g in a plastic vial) were collected from every 300th or more pig after evisceration at the slaughtering line. The samples were stored at 4°C and sent weekly to the bacteriological laboratory. On the day of arrival at the laboratory they were diluted (10^{-1}) in 0.9% saline containing 20% (v/v) glycerol and stored at -20°C until examined. The faecal samples collected in the slaughterhouse in Weert and in Sweden were frozen immediately after collection and stored at -20°C until examined. All faecal samples were analysed within 61 days of collection.

BACTERIOLOGICAL ANALYSIS

After thawing, the samples were inoculated on selective media with and without antibiotics. For *E. coli*, 40 µL of 10^{-2} and 10^{-4} dilutions in 0.9% saline were inoculated on Levine agar plates (BBL 11221; Becton Dickinson BV, Etten-Leur, the Netherlands) with a spiral plater (Salm en Kip, Utrecht, the Netherlands). For enterococci, KF-Streptococcus agar plates (Oxoid CM701; Basingstoke, UK) were inoculated with 40 µL of the 10^{-1} and 10^{-3} dilutions with a spiral plater. If no vancomycin-resistant enterococci (VRE) were detected on the vancomycin agar plate a new vancomycin agar plate was inoculated by swabbing 0.5 mL of the 10^{-1} dilution. For trimethoprim testing 5% lysed horse blood was added to the agar. The antibiotics tested and concentrations used are given in Tables 1 and 2. *E. coli* isolates from plates with chloramphenicol were tested for resistance to florfenicol (MIC > 8 mg/L) by broth microdilution. The antibiotics were selected because they, or related antibiotics, are or have been until recently regularly used in pigs, either on veterinary prescription or as AMGP. *E. coli* grows on Levine agar as purple colonies with a black centre and metallic shine. Only these colonies were counted after 18-24 h incubation at 37°C. The minimum detection level by this method was 300 cfu/g faeces and it has been shown that more than 95% of the presumptively identified colonies are *E. coli*.²⁴ Previous studies also showed that all isolates from antibiotic-containing plates had MICs identical to or higher than the concentration of the antibiotic in the selective plate from which they had been isolated.²⁵⁻²⁷

Enterococci appear as typical red or pink colonies on KF-Streptococcus agar. After 48 h incubation at 42°C only the typical pink colonies were counted. At random, typical colonies collected from 100 KF-Streptococcus agar plates with and without antibiotics (five colonies per plate) were subcultured on blood agar (Oxoid CM854 with 5% sheep blood) and identified on the basis of tolerance for bile, aesculin hydrolysis, growth on 6.5% NaCl (w/v) and a positive pyrrolidonyl-arylamidase reaction (pyrrolidonyl-arylamidase tablets, Rosco, Taastrup, Denmark). In total 500 colonies were identified, and all belonged to the genus *Enterococcus*. The minimum detection level was

Table 1. Prevalence of resistant *Escherichia coli* and percentages of samples with a high degree of resistance in pig faecal samples collected in the Netherlands (five abattoirs) and Sweden.

Antibiotic (Concentration in µg/ml/L)	Slaughterhouses										the Netherlands		Sweden	
	Apeldoorn	Boxtel	Meppel	Olst	Weert	Total								
	(n = 249) ¹	(n = 265)	(n = 254)	(n = 253)	(n = 299)	(n = 1320)		(n = 100)						
	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD
Amoxycillin (25)	89	12	94*	17	92	20*	84	8*	70**	14	85+	13**	51	3
Oxytetracycline (25)	98	45	97	44	98	44	96	39	78**	30**	93+	40+	69	6
Chloramphenicol (25)	64	2	65	3	67	3	55*	0.4**	NT ²		63 ³⁺	3	3	0
Nitrofurantoin (50)	12**	0	0.8**	0	2	0	0	0	3	0	3	0	0	0
Trimethoprim (8)	96**	27	91	19	94	31*	88	14*	62**	15*	85+	21+	46	1
Neomycin (32)	67*	5	61*	4	67	5	52	2	38**	3	56+	4	17	3
Gentamicin (16)	3	0	2	0	2	0	2	0	0	0	2	0	0	0
Flumequin (16)	2	0	0.8*	0	6*	0	4	0	3	0	3	0	1	0
Ciprofloxacin (4)	0.4	0	0.4	0	2	0	4	0	1	0	1	0	0	0

¹ number of samples tested.* significantly different from the total of all data $p < 0.05$.² not tested.** significantly different from the total of all data $p < 0.005$.³ n = 1022.+ significantly different from Sweden $p < 0.001$.** significantly different from Sweden $p = 0.005$.

10^2 cfu/g faeces. For the prevalence of resistance to dalfopristin-quinupristin (SynercidTM) a specimen was considered positive only if at least one *Enterococcus faecium* was isolated and identified from the dalfopristin-quinupristin agar plate. For this reason the degree of resistance could not be calculated. For identification to species level, acid production from different sugars was determined.²⁸ Isolates from a previous study had all MICs identical to or higher than the concentration of the antibiotic in the selective plate from which they had been isolated.²⁹

The samples from the abattoir in Weert were processed first and susceptibility was tested against a smaller number of antibiotics than the other samples.

STATISTICAL ANALYSIS

A one-way analysis of variance was used to estimate overall differences between the group means. Group means were compared pairwise using *t*-tests controlled for overall error rate (Bonferroni test) where $p < 0.05$ was regarded as statistically significant.³⁰

DEFINITIONS

Prevalence of antibiotic resistance

The prevalence of resistance (%) for either *E. coli* or enterococci in the population for an antibiotic is given by the number of samples showing growth on the plates containing that antibiotic, divided by the total numbers of samples tested $\times 100$.

Degree of antibiotic resistance

The degree (%) of antibiotic resistance in each faecal sample to each antimicrobial agent tested was calculated as the number of cfu growing on the plate containing that agent divided by the total number of typical colonies on the antibiotic-free control plate $\times 100$.

Two degrees of antibiotic resistance to a particular antibiotic could be distinguished: low degree, i.e. less than 50% of the indicator microorganisms resistant; and high degree, 50% or more (thus the majority) of the indicator microorganisms being resistant to that agent.

The prevalence of high degree of resistance (%) is the number of samples with a high degree of resistance to a

particular agent divided by the total number of samples tested $\times 100$.

RESULTS

E. coli

No significant differences in the population of samples from which *E. coli* were isolated and the total numbers of *E. coli* per positive sample were observed between the slaughterhouses or between the Swedish and Dutch samples (data not shown). The mean $\pm$ S.D. number of *E. coli* ($\log_{10}$ cfu/g faeces) was 6.4 ± 1.1 for the Dutch samples and 6.2 ± 0.9 for the Swedish samples. The prevalence and percentages of samples with a high degree of antibiotic-resistant *E. coli* in samples collected at the slaughterhouses in the Netherlands and from Sweden are depicted in Table 1. A high prevalence of resistance in the samples from all slaughterhouses was observed for amoxycillin, oxytetracycline and trimethoprim, and a relatively high prevalence for chloramphenicol and neomycin. For the other compounds the prevalence was less than 10%. Florfenicol resistance was present in only two samples from Meppel. The percentage of samples with a high degree of resistance showed the same trend in all abattoir populations and was always highest for oxytetracycline followed by trimethoprim and amoxycillin. Significant differences ($P < 0.05$) in prevalence of resistance and prevalence of a high degree of resistance between samples received from the various slaughterhouses were observed for all compounds tested except gentamicin and ciprofloxacin. The prevalence of resistance to trimethoprim and nitrofurantoin in the samples from Apeldoorn was significantly higher than the mean of all Dutch samples tested. In contrast, the prevalence of resistance and/or a high degree of resistance to amoxycillin, trimethoprim and neomycin in the samples from Olst and Weert were significantly lower than the mean. Compared with the total Dutch population, and also with each of the abattoir populations, both the prevalence and degree of resistance in the Swedish population were significantly lower ($P < 0.005$) for amoxycillin, oxytetracycline and trimethoprim. For chloramphenicol and neomycin, only the prevalence was significantly lower in the Swedish population. No differences were found for nitrofurantoin, gentamicin,

Table 2. Proportion (%) of resistant *E. coli* of the total *E. coli*-population in the faecal flora of pigs with high degree ($\geq 50\%$) and low degree ($< 50\%$) of resistance.

Antibiotic	High degree		Low degree	
	mean $\pm$ S.E.M.	(n)	mean $\pm$ S.E.M.	(n)
Amoxycillin	74 $\pm$ 16	(185)	12 $\pm$ 0.5	(941)
Oxytetracycline	76 $\pm$ 1	(516)	22 $\pm$ 0.5	(683)
Chloramphenicol	77 $\pm$ 4	(21)	4 $\pm$ 0.3	(620)
Trimethoprim	79 $\pm$ 1	(280)	13 $\pm$ 0.5	(845)
Neomycin	76 $\pm$ 3	(44)	5 $\pm$ 0.3	(699)
Gentamicin	0		0.2 $\pm$ 0.1	(24)
Ciprofloxacin	0		1 $\pm$ 0.5	(14)
Flumequin	0		1 $\pm$ 0.3	(29)
Nitrofurantoin	0		3 $\pm$ 1	(40)

flumequin and ciprofloxacin. For all tested antibiotics combined, the proportion of resistant *E. coli* in faecal samples with a high degree of resistance was 76% (range 74-79%) and for low-degree samples 7% (range 0.2-22%), as shown in Table 2. In Figure 1 the bimodal distribution of the samples with a high and low degree is visualised for amoxycillin and oxytetracycline respectively.

ENTEROCOCCI

No significant differences in the percentage of samples (91-100%) from which enterococci were isolated and the total numbers of enterococci per positive sample were observed between the slaughterhouses or between the Swedish and the Dutch samples (data not shown). The mean $\pm$ S.D. number of enterococci ($\log_{10}$ cfu/g faeces) was 5.1 ± 1.0 for the Dutch samples and 5.1 ± 1.3 for the Swedish samples. No amoxycillin-resistant enterococci were found. The prevalence of antibiotic-resistant enterococci and the prevalence of high-degree antibiotic resistance in enterococci in the various populations of pig faecal samples is presented in Table 3. In the abattoir groups the prevalence of high-level gentamicin resistance ranged from 1 to 6%. A high prevalence of resistance was found for erythromycin and oxytetracycline. The prevalence of resistance to dalbopristin-quinupristin ranged from 62 to 82%, and for vancomycin from 24 to 46%. Overall, a high degree

Figure 1. The percentage (mean $\pm$ S.E.M.) of amoxycillin: A, and oxytetracycline: B, resistant *E. coli* of the total *E. coli* population in faecal samples of Dutch pigs with a low degree (< 50%) or a high degree ($\geq$ 50%) of resistance.

Figure 2. The percentage (mean $\pm$ S.E.M.) of vancomycin: A, and oxytetracycline: B, resistant enterococci of the total enterococcal population in faecal samples of Dutch pigs with a low degree (< 50%) or a high degree ($\geq$ 50%) of resistance.

of resistance was found for erythromycin and oxytetracycline: 70% and 46%, respectively. Significant differences in prevalence and high degree of resistance between the slaughterhouses were found for all compounds tested except amoxycillin (no resistance). As shown in Table 3 significantly lower prevalences of resistance and high degree of resistance were found in the Swedish samples for oxytetracycline and erythromycin, and of resistance for dalfopristin-quinupristin either compared with the total population or with the various slaughterhouse populations separately ($P < 0001$). No VRE were found in the Swedish

samples. The percentages of resistant enterococci in faecal specimens with low and high degree of resistance showed a bimodal distribution as depicted in Figure 2 for vancomycin and oxytetracycline respectively. In samples with a high degree of resistance approximately 80% (range 73-86%) of all the enterococci were resistant, whereas in samples with a low degree of resistance this varied from 2% to 28% (mean 17%) as shown in Table 4.

Table 3. Prevalence (Prev.) of antibiotic resistant enterococci and percentage of samples with a high degree (HD) of resistance in pig faecal samples collected at five abattoirs in the Netherlands and in Sweden.

Antibiotic (Concentration in agarmg/L)	Slaughterhouses										the Netherlands		Sweden	
	Apeldoorn		Boxtel		Meppel		Olst		Weert		Total			
	(n = 249) ¹		(n = 265)		(n = 254)		(n = 253)		(n = 299)		(n = 1320)		(n = 100)	
	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD	Prev.	HD
Amoxycillin (25)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gentamicin (500)	6	0	3	0	1*	0	2	0	NT ²	NT	3 ³	0	0	0
Oxytetracycline (25)	96*	48	93	55*	92	35*	86*	47	NT	NT	93 ³⁺	46 ⁺	67	6
Erythromycin (10)	98*	67	93	69	97	71	93	61**	94	82**	95 ⁺	70 ⁺	63	3
Vancomycin (10)	46	1	24*	0.4**	58*	3	36	0.4**	34	3	39 ⁺	2	0	0
Dalfopristin-														
Quinupristin ⁴ (8)	72	NT	62**	NT	82**	NT	71	NT	76	NT	72 ⁺	NT	45	NT ²

¹ number of samples tested.* significantly different from the total of all data ($p < 0.05$).² not tested.** significantly different from the total of all data $p < 0.005$.³ $n = 1022$.+ significantly different from Sweden $p < 0.001$.⁴ only *E. faecium*.**Table 4.** Proportion (%) of resistant enterococci of the total enterococcal population in the faecal flora of pigs with high degree ($\geq 50\%$) and low degree ($< 50\%$) of resistance.

Antibiotic	High degree		Low degree	
	mean $\pm$ S.E.M.	(n)	mean $\pm$ S.E.M.	(n)
Oxytetracycline	79 $\pm$ 0.8	(471)	24 $\pm$ 0.7	(471)
Erythromycin	86 $\pm$ 0.5	(872)	26 $\pm$ 0.8	(314)
Vancomycin	73 $\pm$ 4	(19)	6 $\pm$ 0.4	(492)
Gentamicin	0		2 $\pm$ 1	(29)

DISCUSSION

Given the large number of samples tested and their geographical distribution of origin, the resistance percentages found in the present study can be considered to be representative of resistance in the faecal flora of pigs in the Netherlands. As the population of positive samples and the total numbers of bacteria isolated did not significantly differ between the populations, the different methods of storage of samples before testing did not seem to have had any influence on the results. All the antibiotics for which the prevalence of resistance in *E. coli* was determined are used only for therapy on veterinary prescription. The overall high prevalence of *E. coli* resistant to oxytetracycline and

trimethoprim is in accordance with the amounts of these compounds used in pig medicine. Approximately 50% of all antimicrobial agents used in pigs are tetracyclines and 30% a combination of trimethoprim with a sulphonamide.³¹ In the Dutch veterinary antibiotic guidelines both agents are recommended as first choice antibiotics for many indications for therapy in fattening pigs.³² Only 4% of the antibiotics used on veterinary prescription are penicillins, of which more than 90% are ampicillin or amoxycillin for oral medication.³¹ The observed high prevalence of resistance to amoxycillin can therefore not be explained by a high usage. It has been recognized, however, that these broad-spectrum penicillins disturb the colonization resistance of the intestinal tract profoundly,³³⁻³⁵ facilitating overgrowth by and increasing excretion of resistant bacteria. The most important indication for mass medication of pigs with broad-spectrum penicillins is therapy and prevention of *Streptococcus suis* and clostridial infections. For both indications the treatment of choice is an acid fast small-spectrum penicillin. As no oral formulation of penicillin-V has been registered in the Netherlands for use in pigs, it is strongly recommended to make such a product available. An additional explanation is that the genes encoding β -lactamase production are often located on the same

Table 5. Increase of the prevalence (%) of resistance in the faecal *E. coli* population of fattening pigs in the present study compared with previous studies.

Year of study (reference)	1956 (50)	1972 (50)	1979 (50)	1980 (51)	1987 (52)	1992 (49)	1995 (this study)
Number of samples	100	100	100	100	47	291	1321
Antibiotic							
Ampicillin	NT	30	50	74	89	98	85
Tetracycline	53	93	99	97	98	100	93
Sulphonamide	8	75	91	97	NT	94	NT
Trimethoprim	NT	0	42	69	NT	88	85
Furazolidone or Nitrofurantoin	NT	8	12	25	NT	17	3
Flumequin or Nalidixic acid	NT	0	0	0	NT	2	3

Table 6. Prevalence (%) and percentage of samples with a high degree of resistant *Escherichia coli* in pig faeces collected in 1992 (n = 291) and in 1995 (n = 299) at the abattoir in Weert.

Antibiotic	mg/L*	Prevalence (%)		High degree	
		1992	1995	1992	1995
Amoxycillin	25	84	70	12	14
Apramycin	32	1	0	0	0
Ciprofloxacin	4	0	0	0	0
Flumequin	16	1	3	0	0
Neomycin	8	73	38	2	3
Nitrofurantoin	50	4	1	0	0
Oxytetracycline	25	89	78	45	32
Trimethoprim	8	77	62	3	20

* mg/L: Antibiotic concentrations in the agar plates.

plasmid as the genes for tetracycline and chloramphenicol resistance.³⁶ However, it is most likely that, as a result of co-selection, the prevalence of resistance to broad-spectrum penicillins (only limited) and chloramphenicol (use prohibited for more than 5 years) is higher than would be expected in comparison with the amounts of these drugs used in the pig population. In contrast, the observed resistance to nitrofurantoin was much lower. The use of nitrofurans in food animals has also been prohibited for more than 5 years and resistance to this class of agents seems to be disappearing from the population. This process might be faster than with other antibiotics because no transferable resistance to nitrofurans exists. Aminoglycosides represent only 2% of the veterinary use of antibiotics

and neomycin is the most widely used agent of this group. For gentamicin and fluoroquinolones such as ciprofloxacin no oral formulations for mass medication of pigs are available in the Netherlands and therefore these agents have only limited use in pigs. In contrast, enrofloxacin accounts in the Netherlands for 14% of all veterinary antibiotic use in poultry and the prevalence of fluoroquinolone resistance in faecal *E. coli* from poultry is 50%.^{19,37} If these molecules would become available for in feed medication an increase in resistance can be expected. Florfenicol is a structural analogue of chloramphenicol that is not inactivated by chloramphenicol acetyltransferase.³⁸ In the Netherlands it is used only for parenteral therapy of bacterial respiratory tract infections in cattle. Nevertheless in

two samples florfenicol resistant *E. coli* were found. Eventual use of florfenicol in pigs, would therefore be most likely result in a dissemination of resistant clones or genes conferring resistance to florfenicol in the exposed pig population. Also in the past introduction of a new antibiotic for usage in pigs has always been followed by an increase of resistance against that antibiotic in the faecal *E. coli*-population of fattening pigs. Sulphonamides became available in veterinary medicine direct after the war, and tetracycline in the early fifties. Around 1970 ampicillin and trimethoprim were introduced on the veterinary market and around 1980 flumequin. The results of several previous studies regarding the prevalence of resistant *E. coli* in faecal samples of healthy fattening pigs show all, despite the differences in methodologies used, the same trend: shortly after introduction of a new antibiotic for usage in pigs resistance against that antibiotic appeared as shown in Table 5. Comparison of the data on the prevalence and degree of resistance of faecal *E. coli* in this study with a similar study performed at the same abattoir in 1992⁴⁹ showed a significant ($p < 0.05$) decrease in prevalence of resistance for neomycin and an increase of the prevalence of high degree samples for trimethoprim from 3% to 20% (Table 6). This might be due to the fact that in the Dutch antibiotic guidelines for pigs of which the first edition was published in 1994, neomycin is only for neonatal diarrhoea recommended as a first choice antibiotic. This might have caused a decline in use of neomycin, but as in the present study neither the farms of origin were known, nor data on antibiotic use on the different farms were available, further analysis was not possible. The significant differences observed between the studied slaughterhouses underscore the need for information on the farm of origin as well as reliable data on antibiotic usage on farms.

In the present study no differentiation was made between *Enterococcus* spp., except for resistance to dalfopristin-quinupristin, because *Enterococcus faecalis* is considered intrinsically resistant to pristinamycins such as dalfopristin-quinupristin. The highest prevalence and degree of resistance was found for erythromycin and oxytetracycline. The resistance to tetracyclines was in line with the extensive usage of these drugs in pigs. Despite the relatively low susceptibility of enterococci to amoxycillin, with MICs of most

E. faecalis of 1-8 mg/L and even higher for *E. faecium*,³⁹ no amoxycillin-resistant enterococci (MIC > 25 mg/L) were found. In recent clinical studies the majority of *E. faecium* isolates had MICs lower than 25 mg/L and resistant isolates with higher MICs were related to hospital stay.⁴⁰⁻⁴² The samples collected at the various slaughterhouses showed significant differences in resistance for most antibiotics tested. As most slaughterhouses tend to collect pigs from a particular region the differences observed are probably due to differences in antibiotic usage, qualitatively and quantitatively, between these regions. However, the influence of differences in husbandry systems, stocking densities and density of animal populations between regions should also be considered.

In enterococci tylosin use is probably responsible for the high prevalence and degree of resistance to erythromycin, and virginiamycin use as AMGP for the observed prevalence of resistance to dalfopristin-quinupristin in *E. faecium*. In this study the prevalence of VRE ranged from 24% in Boxtel to 46% in Apeldoorn, with a mean prevalence of 39%. Similar⁴³ or lower⁴⁴ prevalences of VRE in pigs have been reported from other European countries that used avoparcin as AMGP. In the USA, where avoparcin has never been licensed for use in animals, no VRE have been isolated from food animals.^{45,46} A high degree of vancomycin resistance was observed in only 2% of the samples, which might reflect the fact that use of avoparcin, a glycopeptide like vancomycin, as AMGP has been banned in the EU recently, because a relationship between avoparcin use and the prevalence of VRE in the faecal flora of food animals and humans was suggested.^{47,48}

The observed differences in resistance to antimicrobial growth promoters between abattoirs is probably a regional difference, because one or two feedmills provide pig feeds to the majority of the farms in a region and feedmills tend to use the same AMGP for a long time. Most striking was the difference in prevalence of VRE between the Dutch (39%) and Swedish (0%) population. Avoparcin, tylosin (a macrolide like erythromycin) and virginiamycin (a mixture of two pristinamycins like dalfopristin-quinupristin) were at the time of the study extensively used as AMGP in the Netherlands, but not in Sweden. The observed differ-

ences are probably a result of the Swedish ban on AMGP in 1986, as before that year AMGP were also commonly used in pig feeds in Sweden. Unfortunately, no data on prevalence of resistance to AMGP from before 1986 are available.

Prevalence and degree of resistance to antibiotics in veterinary use was also significantly lower in Sweden than in the Netherlands. This contradicts the common opinion that a prohibition of the use of AMGP inevitably must lead to an increase in veterinary use of antibiotics. As the prevalence and degree of resistance in indicator bacteria is strongly correlated with the amounts of antibiotics used in a population, the present data show clearly that the prohibition of AMGP in Sweden has not increased the therapeutic usage of antibiotics in pigs in Sweden to such an extent that the selection pressure on the intestinal flora was higher than in a country using AMGP. The lower prevalence and degree of resistance to most antibiotics in *E. coli* and enterococci strongly suggests that veterinary use in Sweden is even lower. The observed prevalence of resistance to dalfopristin-quinupristin in *E. faecium* in the Swedish population is probably due to veterinary usage in Sweden of virginiamycin or macrolides such as tylosin, which show cross resistance and select for the same *erm* genes.

The marked bimodal distribution of the proportion of resistant bacteria in the indicator populations studied for all antibiotics was unexpected. There was a clear difference between low- and high-degree resistance in the faecal samples studied. In the majority of positive samples only a low degree of resistance was found. For example, the mean $\pm$ s.e.m. of the proportion of VRE in the total enterococcal population in samples ($n = 492$) with a low degree of resistance was $6\% \pm 0.4\%$, but in samples ($n = 24$) with a high degree of resistance the proportion of VRE was $73\% \pm 4\%$. Therefore at random susceptibility testing of one to three isolates from faecal specimens as is commonly done inevitably results in a serious underestimation of the prevalence of resistance of indicator bacteria in a population, because mainly isolates of samples with a high degree of resistance will be found positive.

In this study, current frequent usage of a particular antibiotic in a pig population seemed to correlate not only with a high prevalence of resistance to that antibi-

otic, but also with a higher prevalence of animals with a high degree of resistance in the population. Similarly frequent usage of an antibiotic in the past was associated with a relatively high prevalence of resistance in the population, but with a low prevalence of animals with a high degree of resistance. Increasing usage of an already commonly used antibiotic, against which there was a high prevalence of resistance in the population, was reflected in an increase in the prevalence of faecal samples with a high degree of resistance to that antibiotic.

In conclusion, because of the increasing problem of bacterial resistance, a rational usage of antibiotics including the implementation of a veterinary antibiotic policy is of utmost importance to safeguard the efficacy of veterinary antibiotic therapy for the future and to minimize public health risks from veterinary use. Surveillance is an essential part of an antibiotic policy and should consist of the regular or continuous monitoring not only of antimicrobial resistance, but also of antibiotic usage in the population under study or at risk, followed by analysis and dissemination of the results. Differences between countries, regions, farms and veterinary practices can be observed and investigated, and therapeutic recommendations adapted when resistance exceeds threshold levels. Moreover, emergence and spread of new resistance genes, resistant clones or new resistance profiles can be detected early and action taken to prevent further dissemination.

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Chapter IV

Vancomycin-resistant enterococci in turkeys and farmers

and

**Enterococci with glycopeptide resistance in turkeys, turkey
farmers, turkey slaughterers, and (sub)urban residents in the
south of the Netherlands: evidence for transmission of
vancomycin resistance from animals to humans?**

Chapter IVa

Vancomycin-resistant enterococci in turkeys and farmers

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VANCOMYCIN-RESISTANT ENTEROCOCCI
IN TURKEYS AND FARMERS*To the Editor:*

In enterococci vancomycin resistance of the *VanA* phenotype is usually due to the presence of seven genes organized in a cluster on a transposon (Tn1546)^{1,2}. The recent emergence of vancomycin-resistant enterococci is believed to be due to the vertical clonal spread of resistant strains and the horizontal spread of the transposon itself among different strains of enterococci in a selective environment. Use of avoparcin – a glycopeptide, like vancomycin – as an antimicrobial growth promoter in animal feeds creates such a selective environment and causes the selection of vancomycin-resistant enterococci in the intestinal flora.

To determine whether vancomycin-resistant enterococci from animals can cause colonization in humans, we collected fecal samples from turkeys at 47 farms and from 47 turkey farmers. In addition, fecal samples from 48 turkey slaughterers and 188 healthy persons living in the same area were screened³. Vancomycin-resistant enterococci were isolated from 50 percent of the samples from the turkeys, 39 percent of the samples from the turkey farmers, 20 percent of the samples from the turkey slaughterers, and 14 percent of the samples from area residents. The prevalence of vancomycin-resistant enterococci in 12 turkey flocks not receiving avoparcin was 8 percent, as compared with 60 percent in flocks fed avoparcin ($p < 0.001$).

Almost all the vancomycin-resistant enterococci were *Enterococcus faecium*, and they were highly resistant to vancomycin (minimal inhibitory concentration >64 mg per liter). The resistance to teicoplanin varied (minimal inhibitory concentration, 0.5 to 8 mg per liter).

Phenotypically identical strains were further analyzed by pulsed-field gel electrophoresis after digestion with *Sma*I. Most isolates showed variations in patterns. Only in samples from one farmer and his turkey flock were indistinguishable strains of vancomycin-resistant enterococci isolated with an identical pattern of the 17 bands. Moreover, genetic characterization of the *vanA* gene cluster by the polymerase chain reaction and hybridization showed that the two strains were identical

in the tested areas containing a unique genetic element; this element proved to be different from the published *vanA* gene clusters in BM4147^{1,2,4} in that there was an insertion in the intergenic area between the *vanX* and the *vanY* gene and a deletion in the right end of the gene cluster that removed the *vanZ* gene and created a larger positive band when a *vanX* probe was used, as shown in Table 1.

Table 1. Phenotypic and genetic characteristics of vancomycin-resistant enterococci in fecal isolates from humans and turkeys in the Netherlands and in reference strain BM4147*.

Characteristic	Human Isolates	Turkey Isolates	Reference Strain (Human MB4147)
Phenotype			
Vancomycin (MIC)	>64	>64	>64
Teicoplanin (MIC)	8	≤ 8	>64
		base pairs	
PCR			
<i>vanA</i>	535	535	535
<i>vanR</i>	409	409	409
<i>vanX</i>	423	423	423
<i>vanX</i> mutation†	C	C	C
<i>vanZ</i>	–	–	323
<i>vanXY</i>	1350	1350	750
<i>vanYZ</i>	335	335	335
Insertion sequences‡			
<i>IS1251</i>	Absent	Absent	Absent
<i>IS1216-IS3like</i>	Absent	Absent	Absent
		kilobase pairs	
Hybridization§			
<i>vanA</i>	50	50	35
<i>vanX</i> (right end)	>23	>23	3.5
<i>vanZ</i> (right end)	–	–	3.5

* The dashes indicate that no polymerase-chain-reaction (PCR) amplicon or hybridization signal was obtained. MIC denotes minimal inhibitory concentration (milligrams per liter).

† C indicates a base-pair substitution: a single nucleotide variation (C to A) was found in the *vanX* gene at position 8234.

‡ PCR amplification was performed to determine the presence or absence of previously published insertion sequences (IS) in Tn1546.

§ Hybridization was performed after *Sma*I digestion for *vanA* and *Bam*HI-*Kpn*I digestion for *vanX* and *vanZ*.

Given the wide use and the selective pressure created by antibiotics and the differences in the prevalence of vancomycin-resistant enterococci in the populations studied, the most plausible explanation for these findings is the spread of a vancomycin-resistant enterococcal strain from the turkeys to the farmer. The absence of the *vanZ* gene may contribute to the observed low resistance to teicoplanin as compared with vancomycin². These findings confirm the high prevalence of vancomycin-resistant enterococci in healthy persons living in areas where avoparcin is used as an antimicrobial growth promoter. Our study also shows that humans and animals can carry the same clone of vancomycin-resistant enterococci.

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Chapter IVb

**Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of the Netherlands:
evidence for transmission of vancomycin resistance from animals to humans?**

Enterococci with Glycopeptide Resistance in Turkeys, Turkey Farmers, Turkey Slaughterers, and (Sub)Urban Residents in the South of The Netherlands: Evidence for Transmission of Vancomycin Resistance from Animals to Humans?

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The number of vancomycin-resistant enterococci (VRE) relative to the total number of enterococci was determined in fecal samples from turkeys and three human populations in 1996, each with a different level of contact with turkeys, i.e., turkey farmers, turkey slaughterers, and (sub)urban residents. The percentage of VRE relative to the total enterococcal population (i.e., the degree of resistance) was low (2 to 4%) in all groups (except in six samples). No difference was observed between farmers who used avoparcin and those who did not. The pulsed-field gel electrophoresis (PFGE) patterns of the VRE isolates from the different populations were quite heterogeneous, but isolates with the same PFGE pattern were found among animal and human isolates, in addition to the isolates which were described previously (A. E. van den Bogaard, L. B. Jensen, and E. E. Stobberingh, *N. Engl. J. Med.* 337:1558–1559, 1997). Detailed molecular characterization of *vanA*-containing transposons from different isolates showed, that in addition to a previously reported strain, similar transposons were present in VRE isolates from turkeys and turkey farmers. Moreover, similar VanA elements were found not only in isolates with the same PFGE pattern but also in other strains from both humans and animals.

Vancomycin-resistant enterococci (VRE) were isolated for the first time in 1986 in Europe and in 1987 in the United States. Since then VRE infections have been reported from all over the world. In 1993 enterococci accounted for 12% and VRE accounted for 1% of all hospital infections in the United States (10). In Europe the rate of fecal carriage of VRE in the community is much higher (e.g., 2 to 28%) than that in the United States, where VRE seem to be more or less absent outside hospitals (10, 17). In Europe a diversity of VRE types has also been isolated from sewage, animal waste, meat and meat products, and feces of healthy persons, suggesting a heterogeneous pool of VRE outside hospitals (7, 8, 25, 26). The observation that in the United States VRE have not been isolated from animal sources (6, 10) might be due to the fact that in the United States glycopeptides were never approved for use in animal feeds as antimicrobial performance enhancers (APEs) for growth promotion (28). Until recently, avoparcin, a glycopeptide that shows complete cross-resistance with vancomycin, was commonly used in The Netherlands and in most other countries of the European Union for this purpose (23). The use of avoparcin as an APE has been suggested as a risk factor for carriage of VRE in food animals, as the usage of any antibiotic produces a selective pressure in favor of resistance (5, 12, 26, 27, 29, 34, 39). Therefore, its use as an APE was suspended in the European Union in 1996.

Four main types of resistance to glycopeptides have been described: VanA, VanB, VanC, and VanD (4, 18, 30, 31). The

vanA gene cluster is the one most commonly found and encodes inducible high-level resistance to both vancomycin and teicoplanin. Dissemination of vancomycin resistance can occur through both clonal expansion of resistant enterococci and horizontal transmission of resistance genes (21, 22). Horizontal transfer of vancomycin resistance is explained by the fact that the genetic determinant for the VanA type of vancomycin resistance typically resides on a mobile DNA element such as Tn1546 (3), which can be transferred to enterococci by plasmids. Recently, in several studies, the heterogeneity of the VanA resistance determinant was described. In those studies, indistinguishable Tn1546-like elements could be found in VRE isolated from farm animals and humans, suggesting transfer of vancomycin resistance elements from farm animals and humans (31, 38, 40).

In a previously published report of a study in which the prevalence of VRE in turkeys and farmers was assessed, we found that VRE could be detected in 50% of the fecal samples from turkeys ($n = 47$) and in 39% of the fecal samples from turkey farmers ($n = 47$) (33). Furthermore, VRE were isolated from 20% of the fecal samples from turkey slaughterers ($n = 48$) and 14% of the fecal samples from (sub)urban residents from the same area ($n = 117$). In addition, an indistinguishable VRE and a *vanA*-containing element were found in one sample from a turkey and one from a turkey farmer, suggesting animal-to-human transmission of vancomycin resistance as a likely route (33).

In the present study we analyzed in more detail the epidemiology of VRE in the context of the influence of avoparcin use in turkey feed. The relative amount of VRE in fecal samples from turkeys and three groups of healthy volunteers with different risks of exposure to fecal bacteria from turkeys, i.e., turkey farmers, turkey slaughterers, and a control group of

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(sub)urban residents, was determined by calculating the number of VRE relative to the total number of enterococci. The VRE that were isolated were identified and classified with respect to the type of vancomycin resistance. In addition, the antibiotic susceptibilities to several antimicrobial agents of enterococci isolated from vancomycin-free and vancomycin-containing selective agar plates were determined. Possible sharing of VRE between turkeys and humans was assessed by genotyping by pulsed-field gel electrophoresis (PFGE). In addition, the similarity of vancomycin resistance elements found in animal and human isolates was assessed by comparing Tn1546 derivatives of isolates of VRE from turkeys and the three groups of healthy human volunteers.

(Parts of this study were presented at the 37th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 28 September to 1 October 1997.)

MATERIALS AND METHODS

Collection of fecal samples. In 1996, the major turkey farmers in The Netherlands ($n = 81$) were asked to collect one fresh fecal sample from themselves and one mixed fecal sample from three randomly chosen turkeys at their farms close to the time of slaughter. Approximately 100 workers on a plant that processed the turkeys from these farms and workers who handled turkeys or turkey products on a daily basis were also requested by letter to provide a fecal sample from themselves. (Sub)urban residents ($n = 200$) were randomly selected from the telephone directory of two cities in The Netherlands, Weert and Roermond (approximately 40,000 inhabitants each), and were asked to participate in the study. Most participants lived in the same area, i.e., the province of Limburg in the southern part of The Netherlands, and all samples were collected within 3 months. The participants were asked to complete a questionnaire about recent hospital stays, whether they kept food or pet animals, and antibiotic use by themselves, their family members, or their animals during the 3 months preceding the sample collection. Turkey farmers were also asked which APE was incorporated into the feed of their animals.

Isolation of (vancomycin-resistant) enterococci. The fecal samples and the questionnaire were, immediately after collection, sent to the bacteriological laboratory, where, on the same day, they were diluted (10^{-1}) in 0.9% (wt/vol) NaCl containing 20% (vol/vol) glycerol and were stored frozen at -20°C until they were examined. After thawing of the fecal suspensions, 0.04 ml of these dilutions were inoculated on KF Streptococcus agar (Oxoid CM701; Oxoid) with ($10 \mu\text{g/ml}$) and without vancomycin (Eli Lilly & Company, Indianapolis, Ind.) with a spiral plater (Spiral Systems; Lameris Laboratorium BV, Breukelen, The Netherlands). The plates were incubated at 42°C for 48 h. The lowest detection level is about 250 CFU of VRE/g of feces. KF Streptococcus agar is a selective medium for enterococci, and all enterococci of group D and group G except *Enterococcus cecorum* grow on it. For enrichment of VRE, 1 ml of 10^{-1} -diluted feces was added to 9 ml of KF Streptococcus broth supplemented with $10 \mu\text{g}$ of vancomycin per ml. After incubation at 42°C for 24 h, $100 \mu\text{l}$ was subcultured on the same medium with agar. The lowest detection level is 10 CFU/g. Strains were presumptively identified as enterococci by colony morphology. One typical red or pink colony was randomly picked from each KF Streptococcus agar plate with and without vancomycin and was subcultured onto blood agar, identified, and tested for susceptibility.

Identification and antibiotic susceptibility of the enterococcal isolates. For the (sub)urban residents group, one colony or, when different colony types were present, more colonies per fecal sample were randomly picked from the vancomycin-free and vancomycin-containing KF Streptococcus agar plate. For the other groups, only one colony per fecal sample was tested. The isolated colonies were identified by using tolerance to bile, esculin hydrolysis, growth in 6.5% (wt/vol) NaCl, and a positive pyrrolidonylarylamidase reaction (Wellcome). Identification to the species level was performed by testing for arginine dehydrolase and for acid production from 1% L-arabinose, sucrose, melibiose, raffinose, mannitol, α -methyl-D-glycoside, raffinose, sorbitol, and L-sorbose in purple base broth. Reactions were observed after 24 h of incubation at 37°C . Motility was determined as described previously (13). Yellow pigmentation was scored on tryptone soy agar (CM131; Oxoid) after overnight incubation at 37°C and for 24 h at room temperature. Tellurite reduction was read on blood agar after overnight incubation at 37°C .

The antibiotic susceptibility was determined by a broth microdilution method in Iso-Sensitest Broth (CM473; Oxoid) with an inoculum size of 5×10^5 CFU/ml. The antimicrobial agents tested and the breakpoint concentrations used to define resistance in this study were as follows: amoxicillin, $16 \mu\text{g/ml}$; chloramphenicol, $32 \mu\text{g/ml}$; ciprofloxacin, $4 \mu\text{g/ml}$; erythromycin, $8 \mu\text{g/ml}$; oxytetracycline, $16 \mu\text{g/ml}$; quinupristin-dalfopristin, $8 \mu\text{g/ml}$; teicoplanin, $32 \mu\text{g/ml}$; and vancomycin, $32 \mu\text{g/ml}$. Also, three growth promoters, i.e., avoparcin ($8 \mu\text{g/ml}$), tylosin ($8 \mu\text{g/ml}$), and virginiamycin ($16 \mu\text{g/ml}$), were included in the tests. The breakpoints for the growth promoters tested were based on those reported in the literature

(14, 16). The plates were incubated for 18 to 24 h at 37°C . *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used as reference strains.

Detection of *vanA*, *vanB*, and *vanC* genes. The *vanA*, *vanB*, and *vanC* genes were detected by hybridization with specific probes. The *vanA* probe was a 390-bp *Bam*HI-*Pst*II fragment (9), the *vanB* probe was a 581-bp *Eco*RI-*Hind*III fragment (18), and the *vanC* probe was a 690-bp *Eco*RI-*Hinc*II fragment (15). The *E. coli* strains used for isolation of the probes were kindly provided by P. Courvalin. The DNA probes were isolated from 1% low-melting-point agarose gels and were labelled 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; Schleicher & Schuell, Den Bosch, The Netherlands) according to the manufacturer's instructions.

Enterococci containing the different *van* genes were used as positive controls, i.e., *Enterococcus faecium* BM4147 (*vanA*) (9), *E. faecalis* V583 (*vanB*) (18), and *Enterococcus gallinarum* BM4174 (*vanC*) (15). *E. faecalis* JH2-2 was used as a negative control (20). Prehybridization and hybridization were performed under stringent conditions (50% [vol/vol] formamide and $5\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 42°C), and the probes were washed in $2\times$ SSC plus 0.1% (wt/vol) sodium dodecyl sulfate at room temperature and $0.1\times$ SSC plus 0.1% (wt/vol) sodium dodecyl sulfate at 68°C . The detection of digoxigenin-labelled nucleic acids was performed by chemiluminescence on radiographic films (X-Omat AR; Kodak).

PFGE analysis of VRE. The PFGE analyses performed with enterococci isolated from the agar plates with vancomycin were as described previously, with minor modifications (35). To assess the similarities of the different patterns, the criteria of Tenover et al. were used (32). If an isolate differed from a main type by only three or fewer bands, it was considered a subtype.

Molecular characterization of Tn1546 derivatives. Characterization of the *vanA*-containing transposon was performed by means of combination of restriction fragment length polymorphism analysis and DNA sequencing of Tn1546-specific PCR products and classified by type as described previously (38). PCR primer sequences and their positions relative to the Tn1546 sequence used in this study are listed in Table 1. All VRE were analyzed for the presence of point mutations at positions 1226, 4847, 7658, 8234, and 9692 as described previously (38). To determine the DNA sequence of the left end of the truncated *vanA*-containing transposon derivatives, type A2, E4, E8, and E10 DNA fragments were amplified with Tn1546 primer 184.R or 4511.R in combination with IS1216V primer IS1216V.E and IS1216V.F. The exact integration site and orientation of the IS1216V element downstream of *vanX* was determined by amplifying a DNA fragment with primers 7875.F and 9580.R, and the sequence was determined with the primers IS1216V.E and IS1216V.F.

RESULTS

Characterization of the (vancomycin resistant) enterococcal population. Forty-seven fecal samples were received from turkey farmers and their turkeys. The turkey farmers that responded comprised about 50% of all major turkey farmers in The Netherlands. In addition, 47 fecal samples were received from turkey slaughterers and 117 samples were received from the (sub)urban residents. Thirty farms used avoparcin, and 12 farms used virginiamycin; information on the use of APEs was not received from 5 farms.

None of the human participants or their family members were admitted to a hospital or had used antibiotics in the 3 months preceding the start of the study. About 50% kept pet animals, mainly dogs or cats.

The mean number of enterococci found was 6.9 ± 1.2 (mean $\pm$ standard deviation) $\log_{10}$ CFU per g of feces for the turkey samples and $5.6 \pm 1.6 \log_{10}$ CFU/g for the human samples. No differences were observed between VRE-positive and VRE-negative samples or between samples from animals fed avoparcin or not. The degree of resistance, i.e., the number of VRE relative to the total number of enterococci, was $3\% \pm 3\%$ for the turkey samples, $2\% \pm 4\%$ for the turkey farmers, and $4\% \pm 10\%$ for the turkey slaughterers. Again, no differences were found between samples from farms that used avoparcin and those that did not. In samples from two turkeys, one farmer, one turkey slaughterer, and two (sub)urban residents, more than 50% of the total number of enterococci were resistant to vancomycin.

A total of 283 colonies from vancomycin-free medium and 59 colonies from vancomycin-containing agar plates were fur-

TABLE 1. PCR primers used in this study

Primer group and primer ^a	Sequence	Position ^b
Tn1546 primers		
22.F	5'-GGATTTACAACGCTAAGCC	22-40
184.R	5'-ACCATATGTCGCCCTTAG	184-167
1890.F	5'-TAAATAATCATAGTCGGCAGG	1890-1910
1913.R	5'-CGTCCTGCCGACTATG	1913-1898
3514.F	5'-ACTGTAATGGCTGGTGTAAAC	3514-3534
3560.R	5'-TATCCGAATAAGATCTCGCT	3560-3542
3940.R	5'-ATTTATCAGATTATAGGGCCG	3940-3920
3992.F	5'-TTATTGTGGATGATGAACATG	3992-4012
4511.R	5'-TCGGAGCTAACCACATTG	4511-4494
5235.F	5'-ATATCACGTTGGACAAAGC	5235-5253
5374.R	5'-TTCATCGGTCATCTGCAC	5374-5357
6964.F	5'-AAAGGAGACAGGAGCATG	6964-6981
7035.R	5'-TTACGTCATGCTCCTCTGAG	7035-7017
7875.F	5'-CCGCATTGTACTGAACG	7875-7891
7986.R	5'-CAAGCGGTCAATCAGTTC	7986-7969
8544.F	5'-GCATATAGCCTCGAATGG	8544-8561
8691.R	5'-TTACATACGTCGGGTTTCC	8691-8673
8969.R	5'-GATTGTGCCGTTTTCG	8969-8954
9580.R	5'-TCGTCAAGCTTGATCCTAC	9580-9562
9970.R	5'-GCCATCCTTACCTCCTTG	9970-9953
10716.R	5'-TTTTCCCTCACTTCACAC	10716-10698
IS1216V primers		
IS1216V.E.	51-AGCTTAAATCATAGATACCGTAAGG	913-935
IS1216V.F	51-TTCATCGTATTCTCCTCCTCG	243-225

^a The names of the Tn1546 primers indicate the position of the first nucleotide and the orientation of the primer (F, forward; R, reverse).

^b The positions of the Tn1546 primers are based on the sequence of Tn1546 (GenBank and EMBL accession no. M97297). The positions of the IS1216V primers are based on the sequence of IS1216V (GenBank and EMBL accession no. L40841).

ther identified. No additional VRE were isolated after enrichment. All colonies belonged to the genus *Enterococcus*. Complete identification to the species level mainly yielded *E. faecium*, followed by *E. faecalis*.

The percentages of single isolates from the vancomycin-free and vancomycin-containing agar plates resistant to the antimicrobial agents tested are shown in Table 2. Among the isolates from the (sub)urban residents, one isolate was lost during culture; i.e., 161 enterococcal colonies were tested for this group. No resistance to amoxicillin was observed in any of the

isolates tested. The percentages of resistance to avoparcin, vancomycin, and teicoplanin among the enterococci isolated from the plate without vancomycin were quite similar, ranging from 0 to 14% for the four groups of isolates tested. In contrast, the percentages of resistance to tylosin, erythromycin, and oxytetracycline were significant lower for the isolates from turkey slaughterers and (sub)urban residents than those from turkeys and turkey farmers.

Almost all enterococci isolated from turkeys and the three groups of humans with a selective concentration of 10 µg of

TABLE 2. Antibiotic resistance patterns of enterococci isolated from fecal samples from turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents

Antibiotic (breakpoint for resistance [µg/ml])	% Antibiotic resistance among enterococci isolated from ^a :							
	Vancomycin-free plates (n = 282)				Vancomycin-containing plates (n = 59)			
	T (46)	TF (41)	TS (34)	UR (161)	T (23)	TF (16)	TS (6)	UR (14)
Avoparcin (8)	4	7	0	14	87	88	100	100
Vancomycin (32)	4	7	0	2	91	81	100	86
Teicoplanin (32)	2	5	0	0	48	13	0	21
Tylosin (8)	41	42	24	23	70	75	67	93
Erythromycin (8)	37	42	21	28	70	75	67	93
Virginiamycin (16)	13	0	3	2	13	13	0	29
Quinupristin-dalfopristin ^b (8)	13	5	3	NT ^c	17 ^d	13 ^c	0	NT
Amoxicillin (16)	0	0	0	0	0	0	0	0
Chloramphenicol (32)	9	5	0	4	9	6	0	7
Oxytetracycline (16)	96	71	21	31	87	88	100	93
Ciprofloxacin (4)	28	2	12	6	35	13	0	0

^a T, turkeys; TF, turkey farmers; TS, turkey slaughterers; UR, (sub)urban residents. Numbers in parentheses are numbers of individuals.

^b Only *E. faecalis* isolates are not included.

^c NT, Not tested.

^d *E. faecium* (n = 2) and *E. durans* (n = 2).

^e *E. hirae* (n = 2).

FIG. 1. PFGE patterns of *E. faecium* strains isolated from turkeys (T), turkey farmers (TF), and turkey slaughterers (TS) after digestion of total DNA with *Sma*I. Lane 1, molecular weight marker; lane 2, TF6, pattern N7; lane 3, TF16, pattern N; lane 4, TF17, pattern N1; lane 5, TF46, pattern N1; lane 6, TS19, pattern N2; lane 7, TS31, pattern N3; lane 8, TF20, pattern D; lane 9, TF26, pattern D; lane 10, T10, pattern D; lane 11, T44, pattern W; lane 12, TF8, pattern W; lane 13, T5, pattern B; lane 14, TF5, pattern B.

vancomycin per ml were resistant to avoparcin and vancomycin (MICs, >32 $\mu\text{g/ml}$). The percentage of resistance to teicoplanin was significantly lower, i.e., from 0% for the isolates from the turkey slaughterers to 48% for the turkey isolates. Two and three enterococci isolated from turkeys and turkey farmers, respectively, lost their vancomycin resistance after storage at -70°C and were susceptible to vancomycin. By using colony blots, the *vanA* gene could not be demonstrated in these isolates. The presence of the *vanA* gene in the vancomycin-resistant isolates from turkeys ($n = 21$), turkey farmers ($n = 13$), turkey slaughterers ($n = 10$), and (sub)urban residents was confirmed ($n = 14$) by using colony blots. A high percentage of resistance to tylosin and erythromycin among these enterococci from turkeys and humans was observed, ranging from 67% among isolates from turkey slaughterers to 93% among isolates from (sub)urban residents. The same holds true for the resistance to oxytetracycline: from 87% among isolates from turkeys to 100% among isolates from turkey slaughterers. Resistance to ciprofloxacin was observed among isolates from turkeys (35%) and turkey farmers (13%) but was absent from isolates from turkey slaughterers and (sub)urban residents.

PFGE of VRE. The PFGE patterns of the VRE from the different populations were quite heterogeneous. Representative PFGE patterns from VRE from turkeys, turkey farmers, and slaughterers are shown in Fig. 1. Among the 13 VRE isolated from turkeys, 12 different PFGE types could be discriminated. The nine VRE from turkey farmers contained six different PFGE types, while among the VRE isolated from

turkey slaughterers ($n = 4$) and (sub)urban residents ($n = 14$), three and five different PFGE types, respectively, were found. PFGE type B and type D were found among isolates from both turkeys and turkey farmers (Table 3).

Tn1546 types among different VRE isolates. Analysis of the Tn1546-like elements from the different VRE isolates showed that the 14 isolates from the (sub)urban residents almost exclusively contained the previously described Tn1546 of types A1 and A2 (38). Only one isolate contained a type E transposon, E4 a subtype transposon, which is characterized by a 5' end deletion that encompasses the entire transposase gene and the 5' end of the resolvase gene (36). Furthermore, this type contains an *IS1216V* insertion in the *vanX-vanY* intergenic region. The four isolates from turkey slaughterers contained transposons of types A1 and A2. In contrast, the majority of the turkey isolates (10 of 13) contained a Tn1546 type E transposon (Table 3). Four different E subtypes, subtypes E8, E9, E10, and E11, were distinguished in these isolates; these subtypes differed in the sizes of the 5' end deletion and in the sizes of small deletions surrounding the *IS1216V* insertion site (Fig. 2). In addition, Tn1546 subtypes E9, E10, and E11 contained a right-end deletion that included deletion of the *vanZ* gene, which has been shown to be involved in teicoplanin resistance (2).

For two isolates, T5 and TF5, the insertion of *IS1216V* in the *vanX-vanY* intergenic region and the deletion of the *vanZ* gene have been described previously (33). The remaining three turkey isolates were type A1. The finding of isolates containing a VanA transposon from which the *vanZ* gene was missing may explain the unexpected observation of VanA-positive isolates which were sensitive to teicoplanin (Table 3). Almost half (four of nine) of the turkey farmer isolates also contained a type E, subtypes E9 and E11, transposon. The other five isolates contained a type A transposon, four isolates contained a type A2 transposon, and one contained a type A1 transposon. In three cases an identical VanA transposon was found in a turkey and turkey farmer isolate from the same farm. In the samples that contained isolates T5 and TF5, as published previously (33), and in more that contained isolates T20 and TF20, isolates with transposon type E9 were found in both the turkey and turkey farmers, and in the samples that contained isolates T32 and TF32, isolates of type A1 were found in both the turkey and the turkey farmer. Two different transposons were present only in the combination T36 and TF36.

DISCUSSION

In a previously published study on the dissemination of VRE from farm animals to humans, we showed high prevalences of VRE in turkey, turkey farmers, turkey slaughterers, and area residents (50, 39, 20, and 14%, respectively). Furthermore, in samples from one farmer and his turkey flock, an indistinguishable VRE strain and *vanA*-containing transposon were identified. This demonstrates that, at least in one case, an animal and human carried the same VRE clone (33). In order to extend this study on the controversial issue of spillover of VRE or vancomycin resistance genes between animals and humans, we have determined the relative number of VRE in feces isolated from turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents. Furthermore, we have compared the PFGE types of a number of VRE isolated from turkeys and the three groups of human volunteers and finally characterized in detail the *vanA*-containing transposon of VRE isolated from the different sources.

Surprisingly, no significant difference in the number of VRE relative to the total number of enterococci was observed be-

TABLE 3. Characteristics of VRE from different sources

Source	Species	Strain no.	MIC (μ g/ml)		PFGE type	Tn546 type
			Vancomycin	Teicoplanin		
Turkey	<i>E. faecium</i>	T3	64	4	A	A1
	<i>E. faecium</i>	T5	32	1	B	E9
	<i>E. faecium</i>	T8	128	64	C	A1
	<i>E. faecium</i>	T10	32	4	D	E9
	<i>E. faecium</i>	T14	32	0.5	E	E11
	<i>E. faecium</i>	T20	64	0.5	D1	E9
	<i>E. faecium</i>	T24	32	0.5	F	E11
	<i>E. hirae</i>	T28	128	4	G	E11
	<i>E. faecium</i>	T32	128	32	H	A1
	<i>E. hirae</i>	T36	64	4	I	E10
	<i>E. faecium</i>	T38	128	32	J	E8
	<i>E. faecium</i>	T39	32	0.5	K	E11
	<i>E. faecium</i>	T47	32	0.5	L	E11
Turkey farmer	<i>E. hirae</i>	TF2	32	4	M	E11
	<i>E. faecium</i>	TF5	16	0.5	B	E9
	<i>E. faecium</i>	TF16	128	8	N	A2
	<i>E. faecium</i>	TF17	128	8	N1	A2
	<i>E. faecium</i>	TF20	64	1	D	E9
	<i>E. faecium</i>	TF26	64	2	D	E9
	<i>E. faecium</i>	TF32	64	4	O	A1
	<i>E. hirae</i>	TF36	128	0.5	P	A2
	<i>E. faecium</i>	TF46	64	8	N1	A2
Turkey slaughterer	<i>E. faecium</i>	TS19	128	8	N2	A1
	<i>E. faecium</i>	TS31	128	8	N3	A2
	<i>E. faecium</i>	TS32	128	8	Q	A2
	<i>E. hirae</i>	TS36	64	0.5	R	A1
(Sub)urban residents	<i>E. faecium</i>	W31	32	32	S	E4
	<i>E. faecium</i>	H10	16	2	N	A2
	<i>E. faecium</i>	H15	32	16	N	A2
	<i>E. faecium</i>	H17	128	16	N	A2
	<i>E. faecium</i>	M1	128	32	N4	A1
	<i>E. faecium</i>	M13	128	16	N	A2
	<i>E. faecium</i>	M27	128	32	T	A2
	<i>E. faecium</i>	R2	128	4	N	A2
	<i>E. faecium</i>	R13	32	4	N	A2
	<i>E. faecium</i>	R19	64	4	U	A1
	<i>E. faecium</i>	R20	64	8	V	A2
	<i>E. faecium</i>	R24	64	8	N	A2
	<i>E. faecium</i>	R25	128	16	N5	A2
	<i>E. hirae</i>	R27	16	0.5	N6	A1

tween the farmers and the turkeys on farms where avoparcin was and was not used. This might be explained by the fact that farmers have been exposed to VRE in the recent past by former flocks fed avoparcin as an APE. If this were true, it would suggest that even in the apparent absence of exposure to VRE or glycopeptides, farmers can carry VRE for long periods of time.

The distribution of the different species in the present study differed from those described by Welton et al. (37). They studied different age groups of three flocks of turkeys from two different farms and found that the most prevalent species in the oldest age group was *E. faecalis* (51%), followed by *E. faecium* (22%). In our study, in which the samples were obtained from turkeys close to the time of slaughter, the reverse was found; i.e., *E. faecium* was the most prevalent (74%), followed by *E. faecalis* (22%). *E. gallinarum*, which was isolated from 8 of 41 samples from the oldest group 5 in the study of Welton et al. (37), was found only twice in our study and was found in (sub)urban residents. *Enterococcus casseliflavus* which was not isolated from turkeys in age group 5, was found in the

samples from turkey farmers ($n = 1$), turkey slaughterers ($n = 1$), and (sub)urban residents ($n = 8$). In both studies, *E. faecium* and *E. faecalis* were the most prevalent species.

In general, the PFGE patterns of VRE isolated from animal and human sources were different. PFGE pattern type N was the most prevalent type among human isolates, whereas the PFGE patterns of the isolates from turkeys were heterogeneous. These results are in contrast to those described by van den Braak et al. (35), who found dominant PFGE types among poultry strains derived from throughout The Netherlands. Our results suggest that, in general, different enterococcal strains are found in turkeys and turkey farmers and that the clonal dissemination of turkey strains and colonization of human is less common. In addition to the previously described similarities between one isolate from a turkey farmer and isolates from his turkeys (33), similar PFGE types (types D and D1) were also found in isolates T20, TF20, T10, and TF26. In addition to these similar PFGE types found in turkeys and turkey farmers, similar *vanA*-containing transposons were also found in turkeys and turkey farmers. In five of the nine turkey

FIG. 2. Genetic maps of Tn1546 and six Tn1546 derivatives. The thick horizontal lines represent Tn1546 (type A1) (3) and Tn1546 types A2 (36), E4 (36), and E8 to E12. The positions of genes and open reading frames (orf's) and the directions of transcription are depicted by open arrows. Dotted boxes represent insertion sequence (IS) elements. The positions of the first nucleotide upstream and downstream from the insertion sequence insertion sites are depicted. Filled arrows indicate the transcriptional orientation of inserted insertion sequence elements. Deletions have been indicated by dotted lines. The position of the base pair mutation in type A2 is indicated above the transposon type: position 8234, G → T (K → N).

farmer isolates, transposon-like elements identical to those in turkey isolates, i.e., types A1, E9, and E11, were found. Furthermore, in three cases (isolates T32 and TF32, T20 and TF20, and T5 and TF5) in which a VRE was isolated from a turkey farmer and a turkey of the same farm, an identical *vanA*-containing transposon was found (Table 3). The present data for strains T32, TF32, T20, and TF20 confirm previously published results (33). The most likely route of spread of vancomycin resistance from turkeys to turkey farmers is by horizontal dissemination of derivatives of the vancomycin resistance transposon Tn1546. The finding of type A2 as the predominant transposon type in (sub)urban residents analyzed in this study is in agreement with previously published results, in which this type seemed to be common among human VRE from The Netherlands (36), and suggests that the frequency of transmission of vancomycin resistance from turkeys to the general population in The Netherlands is low but that transmission may occur. However, we must realize that only one isolate was tested from each fecal sample, and therefore, the method used is likely to have a very low sensitivity. Testing of more isolates was not possible because of the workload involved. Also, studies from others point to a similarity between *vanA*-containing elements from animals and humans and suggest the spread of vancomycin resistance between the human and animal bacterial flora (1, 24, 31, 38, 40), with the most likely way being food-borne transmission. However, more information on the prevalence of different PFGE types among VRE in meat prod-

ucts in Weert and Roermond and the share of turkey meat consumption in relation to total meat consumption is needed before any conclusion can be drawn.

In the present study not only was resistance to vancomycin in the different populations observed, but resistance to quinupristin-dalfopristin, a combination of streptogramins B and A which is not approved for human use in The Netherlands, was also found. Resistance to this combination was not only found in *E. faecalis*, which is considered intrinsically resistant, but was also found in *E. faecium*, *Enterococcus durans*, and *Enterococcus hirae* (Table 2). These data are consistent with previously published results by Welton et al. (37), in which resistance to this combination among *E. faecium* isolates was described, with the prevalence of resistance, depending on the age of the turkeys, being up to 100%. The interesting finding of both studies is the isolation of *E. faecium* strains resistant to quinupristin-dalfopristin before the compound has been used therapeutically in humans. The use of virginiamycin as an APE, consisting of virginiamycin M (a streptogramin A type antibiotic) and virginiamycin S (a streptogramin B type antibiotic), might be responsible for the resistance observed among isolates from animals, and the dissemination of enterococcal strains or resistance determinants from animals to humans may account for the observed resistance to quinupristin-dalfopristin in humans (37).

There is still much debate on the hazard of the use of APE in food animals and on the possible dissemination of resistant strains or genes from animals to humans or vice versa. In a recent review, Corpet (11) discussed some examples suggesting that the use of APEs such as carbadox, nourseothricin, and streptothricin is hazardous for humans. However, reports with circumstantial evidence were also discussed in the same review, suggesting that the APEs allowed in Europe are not a threat to human health. On the basis of the prevalence of identical vancomycin resistance transposon types in human and animal isolates described in this and other (35, 24, 38, 40) studies, one might conclude that an animal source is likely to serve as a reservoir for the VanA type of resistance in humans in Europe and that the horizontal transmission of the resistance genes is probably more important than clonal spread in the dissemination of vancomycin resistance.

In conclusion, as was discussed 15 years ago, the relative contribution of both APE use and human use of antimicrobial agents to antibiotic resistance in humans is still not known (19). Monitoring of antibiotic resistance, antibiotic resistance transfer, and antibiotic use (including APE use) and studies on the dissemination of antibiotic resistance genes in both animals and humans is essential to obtaining consistent and reliable data on the epidemiology of resistance of isolates from animals and humans.

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Chapter V

Fecal carriage of antibiotic resistant enterococci by poultry, poultry farmers and poultry slaughterers in the Netherlands

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ABSTRACT

The numbers of enterococci resistant to antibiotics commonly used in poultry for therapy or as antimicrobial growth promoter (AMGP), relative to the total number of enterococci were determined in fecal samples from two poultry populations. One population were broilers in which use of therapeutic antimicrobials and AMGP use is common and the other was a population of laying hens with a low antibiotic usage. In addition fecal samples were examined from three human populations: broiler farmers, laying hen farmers and poultry slaughterers. From each fecal specimen the MIC for an extended panel of antibiotics of a single randomly selected enterococcal isolate was also determined. For all antibiotics tested the percentage of samples containing resistant enterococci was higher in broilers than in laying hens. The percentage of resistant enterococci, relative to the total enterococcal populations (i.e. the degree of resistance) was significantly higher in broilers than in laying hens for tetracycline, erythromycin, vancomycin and quinupristin-dalfopristin. For almost all antibiotics tested resistance in fecal enterococci of broiler farmers was more common than in laying hen farmers and poultry slaughterers. The overall resistance of enterococci from broilers correlated with the resistance of enterococci from broiler farmers and from poultry slaughterers. The pulsed-field gel electrophoreses (PFGE) patterns of vancomycin resistant enterococci (VRE), isolated from the five populations, were quite heterogeneous, but *Enterococcus hirae* isolates with the same or a closely related PFGE pattern were isolated at two farms from both the broiler farmer and from broilers. Molecular characterisation of *vanA*-containing transposons of these isolates showed that similar transposon types, predominantly found in poultry, were present. Moreover, similar *vanA*-elements were not only found in isolates with the same PFGE pattern but also in other VRE isolated from both humans and chickens. The results of this study strongly indicated transmission of antibiotic resistance in enterococci from poultry to man. For VRE this might reflect transfer of resistant clones of animal origin, but inter-strain transfer of resistance elements on transposons seems to be more common.

INTRODUCTION

Acquired resistance against commonly used antibiotics has been observed since these agents were introduced in human and veterinary medicine. However, the rate of development of resistance appears to have accelerated in the past decade⁷⁸ and today multiple antibiotic resistant bacteria constitute a global problem^{55,57,74,82}. In modern poultry industry antibiotics are used in large quantities not only for therapy and prevention of bacterial diseases, but also as antimicrobial growth promoters (AMGP) in animal feeds. In 1990 in the Netherlands 80,000 kg of antibiotics (active substance) were used in humans and 300.000 kg on veterinary prescription in animals⁹². In both humans and animals this is equivalent to approximately 100 mg of active substance/kg body weight/year. However about 26% of the antibiotics used in the veterinary sector were intended for poultry, mainly broilers, resulting in a yearly exposure of around 430 mg of antibiotics/kg/year for poultry. This was considerably higher than the antibiotic usage in other food animal populations. In addition to these therapeutic antibiotics, food animals received an approximately equivalent amount of antibiotics in their feed as antimicrobial growth promoters (AMGP). Similar figures have been published for 1997⁹⁵ indicating that these amounts have not changed significantly in subsequent years. This high level of antibiotic use in poultry may compromise veterinary therapy but is also a public health concern. Antibiotic use selects for resistance, not only in pathogenic bacteria, but also in the endogenous flora of exposed animals. Enterococci are a component of the endogenous flora of man and other animals and are intrinsically resistant to a number of antibiotics including cephalosporins, penicillinase resistant penicillins, and clinically achievable levels of lincosamides and aminoglycosides. In addition to this intrinsic resistance, enterococci have acquired resistance to many other antimicrobial agents and have a propensity to acquire multiple antibiotic resistance traits by various mechanisms of conjugation: broad host-range plasmids⁵³, conjugative transposons and for *E. faecalis* pheromone responsive plasmids. As intestinal bacteria of food animals inevitably contaminate food products derived from these animals, animal enterococci can

reach humans via the food chain and colonize the intestinal tract or transfer resistance genes to enterococci of the human endogenous flora.

Enterococci are not important pathogens for animals. In humans, however, enterococci have been recognized as a cause of infective endocarditis and urinary tract infections for nearly a century⁵⁴. In the past decade, enterococci have emerged as major nosocomial pathogens. Around 12% of all nosocomial infections in the United States are caused by enterococci^{20, 21}. The emergence of enterococci as a nosocomial pathogen may be attributed to their intrinsic resistance to many antibiotics commonly used in hospital practice in the last decade, in particular the third and fourth generation cephalosporins, and by the increasing numbers of immunocompromised patients. In the USA the observed increase in nosocomial enterococcal infections has been accompanied by the occurrence of β -lactamase producing²⁵ multiresistant strains that are also resistant to vancomycin and have high level resistance to aminoglycosides^{45,52,53,75,88}. In the United States the vancomycin resistant enterococci (VRE) have increased from 0.3% of hospital isolates in 1989 to more than 20% in 1997^{34,50}, however the occurrence of VRE is mainly limited to hospitals and the *vanA* and *vanB* type of resistance are both commonly encountered in this setting²³.

In Europe VRE are much less frequently isolated from patients with hospital acquired infections (1-4%)^{12,22,24,63,73}. However *vanA* mediated resistance is common in enterococci isolated from the fecal flora of healthy people in the community, from companion and food animals^{17,27,41,94,100} and from raw meat and meat products^{10,43,61,97}. Frequent isolation of VRE from these sources is thought to be related to the use of the glycopeptide antibiotic: avoparcin, as an antimicrobial growth promoter (AMGP) in food animals. Avoparcin, has never been licensed for use in the United States and VRE have not been isolated from healthy humans outside hospitals nor from poultry or other food animals in the USA^{15,51,76,83,98}. Within the European Union the use of avoparcin was suspended in April 1997.

In addition to the observed differences in VRE prevalence between Europe and the USA, there are also differences in the resistance profile of VRE isolates

from these regions. VRE isolates of European origin from outside of the hospital setting are highly polyclonal and glycopeptide resistance of the *vanA* type typically occurs as an isolated acquired resistance mechanism. Infection with such VRE can be effectively treated with the combination of amoxycillin and gentamicin. VRE isolates from European hospitals share the same characteristics of polyclonality and isolated glycopeptide resistance observed in VRE isolated from the fecal flora of healthy animals and humans, raw meat and sewage. By contrast VRE isolates from US hospitals generally exhibit multiple acquired resistance mechanisms and show a limited genetic variability. To date, *vanB* resistance has not been found in non-human enterococci. Therefore it seems likely that avoparcin use selects only for *vanA* resistance⁹⁶. This suggests that *VanA* resistance in VRE in Europe is not so much a result of selection in hospitals and intra- and inter hospital dissemination like in the USA, but is occasionally introduced into to hospitals from the community and that nosocomial VRE infection in Europe is mostly caused by a VRE from the patients own flora, acquired from a heterogeneous pool of VRE outside hospitals²³.

Since the emergence of multiresistant MRSA in the early 1980s and of VRE in the early 1990s the pharmaceutical industry has developed new antibiotics for treatment of serious infections due to multiresistant Gram-positive cocci. However, two of these: dalbapristin-quinupristin^{9,48} and everninomicin³³ are closely related to and show cross-resistance with virginiamycin and avilamycin respectively¹. These drugs have been used for many years as AMGP. As a result of this practice a bacterial population resistant to these compounds was present in the intestinal tract of food animals and healthy humans prior to introduction of these new antibiotics for clinical use^{4,94,98}. It has been shown that the prevalence and degree of resistance in intestinal bacteria is a good indicator for the selection pressure exerted by antibiotics^{46,47} and that the intestinal flora serves as a reservoir of resistance genes for pathogenic bacteria⁴⁶. Intestinal enterococci are therefore considered good indicator bacteria for the selection pressure exerted by antibiotics on bacterial populations. Investigation of the prevalence and degree of resistance of enteric indicator bacteria such as enterococci from different populations of humans and

animals makes it feasible to compare the prevalence and degree of antibiotic resistance in these populations. In combination with identification and genomic fingerprinting of isolates and of resistance genes it is possible to detect evidence of transfer of antibiotic resistant bacteria and/or transfer of genes encoding antibiotic resistance between the endogenous flora of animals and humans⁹⁵.

AIM OF THE STUDY

The aim of this study was to analyze the influence of exposure to antibiotics used in the Netherlands as AMGP or for veterinary therapy in poultry on the resistance of fecal enterococci recovered from poultry, poultry farmers and poultry slaughterers. In addition, the susceptibility of enterococci isolated from antibiotic free and from vancomycin and gentamicin containing agar plates to a variety of antibiotics was determined. The poultry population consisted of two populations with different antibiotic use profiles. Broilers are young chickens raised for slaughter within 8 weeks. Broilers are fed continuously with AMGP and there is a relatively high level of use of veterinary prescription antibiotics. AMGP are not used in laying hens producing eggs for human consumption and prescription only antibiotics are used infrequently. Reasons for these differences include the higher frequency of infection in broilers as with most young animals, but also reluctance on the part of farmers to administer antibiotics to laying hens as some antibiotics may cause a drop in egg production and sale of eggs for human consumption is not permitted during a specified withdrawal period after administration of antibiotics.

In addition, the prevalence and degree of resistance against the same antibiotics was assessed in fecal enterococci from three populations of humans with different risks of exposure to fecal bacteria from poultry. These populations are broiler and laying hen farmers, which have daily close contact with poultry with high and low level exposure to antibiotics respectively. The third human population are workers in a poultry processing plant, who handled broilers or broiler products on a daily basis. Finally, possible sharing of VRE between poultry and humans was assessed by genotyping of VRE by pulsed-field gel electrophoresis (PFGE).

The similarity of vancomycin resistance elements found in poultry and human isolates was evaluated by comparing Tn1546 derivatives found in poultry and human isolates of VRE.

MATERIALS AND METHODS

Collection of fecal samples

From September to December 1997 approximately 250 farmers in the south of the Netherlands keeping either broilers or laying hens were asked by letter to collect one fresh fecal sample from themselves and a mixed fecal sample from the oldest chickens at their farms. In addition 100 poultry slaughterers working at a poultry-processing plant in the same area were asked to provide one fecal specimen each. All participants were requested to send the samples on the day of collection to the bacteriology laboratory together with a completed questionnaire on recent hospital stay, antibiotic use by themselves, family members or their animals during the three months preceding sample collection and whether they kept food and/or companion animals. On the day of arrival at the laboratory the samples were diluted (10^{-1}) in 0.9% NaCl (wt/vol) with 20% (vol/vol) glycerol and stored frozen at -20°C until assayed.

Isolation of (resistant) enterococci

After thawing the samples 40 μL of 10^{-1} and 10^{-3} dilutions in 0.9% NaCl (wt/vol) were inoculated on KF-Streptococcus agarTM plates (Oxoid CM701; Basingstoke, UK) with and without antibiotics using a spiral plater (Salm en Kip BV, Utrecht, the Netherlands) as previously described⁵⁶. The antibiotics were selected because these, or related antibiotics, are (or had been until recently) regularly administered to poultry either on veterinary prescription or as AMGP (Table 1). The concentrations used in the agar were based on guidelines and modified where appropriate to make results comparable with those of previous studies^{49,79,89,90}. If after 48 hours of incubation no vancomycin resistant enterococci (VRE) were detected on the vancomycin containing agar plate, 0.5 mL of the 10^{-1} dilution was incubated overnight in nutrient broth containing 10 mg/L vancomycin and 0.4 g/L sodium azide. Next morning 0.5 mL of this broth was

plated on a vancomycin containing agar plate and checked for growth after 48 hrs of incubation.

Enterococci appear as typical red or pink colonies on KF-Streptococcus agar. After 48 hrs of incubation at 42°C only the typical pink colonies were counted. The minimum detection level, as assayed with spiked feces samples, was approximately 300 CFU/g feces.

The prevalence of antibiotic resistance (%) in a population was calculated as the number of samples showing growth of enterococci on the antibiotic containing plates, divided by the total numbers of samples tested $\times 100\%$.

The degree (%) of antibiotic resistance of each fecal sample tested is the number of enterococcal colonies on the antibiotic containing plate divided by the total number of enterococcal colonies on the antibiotic free plate $\times 100\%$. Two degrees of antibiotic resistance can be distinguished: low degree resistance, i.e. less than 50% of the total number of enterococci in a sample is resistant and high degree resistance, 50% or more (thus the majority) being resistant to that particular agent^{47,91}. The prevalence of high degree resistance (%) is the number of samples with a high degree of resistance to a particular antibiotic divided by the total number of samples tested $\times 100\%$.

Because *E. faecalis* is considered to be intrinsically resistant to dalfopristin-quinupristin, a sample was only considered to contain resistant enterococci to this drug if at least one *Enterococcus faecium* was isolated and identified from the dalfopristin-quinupristin containing agar plate. The (prevalence of high) degree of resistance against dalfopristin-quinupristin could only be calculated for the total enterococcal population in the specimen.

Identification and antibiotic susceptibility testing of enterococcal isolates

From each sample one typical enterococcal colony was randomly chosen from the antibiotic free plate for identification and determination of the MIC for an extended panel of antibiotics. MIC were determined by a microbroth dilution method in Iso-Sensitest BrothTM (Oxoid CM 473) using an inoculum size of 5×10^5 cfu/ml. The panel of antibiotics for the MIC determination (Table 3) included the same antibiotics as used in the agar plates and in addition related antibiotics of

the same chemical groups were added. If growth was observed on the vancomycin or gentamicin containing agar plate, one colony was also selected randomly for identification and MIC determinations. *Enterococcus faecalis* ATCC 29212 and *E. coli* ATCC 25922 were used as reference strains. Identification to species level was performed as described previously^{18,79}.

If VRE were recovered from a chicken and a farmer from the same farm these isolates were genotyped by PFGE, the resistance genes identified and the *vanA* transposons analyzed.

Detection of vanA, vanB and vanC genes

The *vanA*, *vanB* and *vanC* genes were detected by hybridization with specific probes as described previously⁷⁹.

Pulsed-field gel electrophoresis analysis of VRE

The PFGE analysis of the enterococci isolated from the agar plates with vancomycin was performed after DNA digestion with *SmaI* as described previously⁹⁷. To assess similarity of the different patterns the criteria of Tenover were used: if an isolate differed from a main type by only three or less bands, it was considered as a subtype.

The computer-assisted analysis of the PFGE fingerprints was done with BioNumerics (Applied Maths, Kortrijk, Belgium). Comparisons of patterns were done by the unweighed pair group method using arithmetic averages (UPGMA) clustering method by using the Dice coefficient.

Molecular characterization of Tn1546 derivatives

Characterization of the *vanA* containing transposons was performed by means of a combination of restriction fragment length polymorphism (RFLP) analysis and DNA sequencing of Tn1546 specific PCR products as described previously^{79,99}. Tn1546 derivatives were classified by type in concordance with the nomenclature used previously^{79,99}. All VRE isolates were analyzed for the presence of the point mutations at positions 1226, 4847, 7658, 8234, and 9692 for left and right end deletions and for the exact integration site and orientation of IS1216V downstream of *vanX* in the type B and E transposons.

Statistical analysis

A one-way analysis of variance was used to estimate overall differences between the group means. Group means were compared pairwise using t-tests controlled for overall error rate (Bonferroni test) where $p < 0.05$ was regarded statistically significant. Correlations between populations were calculated using the Pearson's product moment correlation coefficient test.

RESULTS

Study population

Of the persons asked to participate in the study approximately 50% of the poultry slaughterers and 30% of the poultry farmers responded. One broiler farmer had no animals on the farm at the time of sample collection so that only a human fecal sample was supplied. Of 46 fecal samples of slaughterers received 41 (89%) yielded enterococci. Enterococci were detected from all 50 samples from broilers, from all 25 samples of laying hen farmers, from 24 (96%) of 25 samples from laying hens, and from 49 (96%) of 51 stools of broiler farmers. Growth of enterococci on the vancomycin containing agar plates was detected from 65 specimens (33%) and additionally from 19 samples (10%) after enrichment (Table 1).

The mean $\pm$ SD $\log_{10}$ CFU of enterococci per gram feces in positive fecal samples was 5.4 ± 1.4 for human fecal specimens and 7.7 ± 0.4 in poultry specimens. In the three months preceding the sample collection, two poultry slaughterers and one laying hen farmer had been hospitalized. None of the laying hen farmers had received antibiotics in the preceding three months. However in four cases a member of the family has received antibiotics. Four of 51 broiler farmers (8%) and two family members of broiler farmers, and four (9%) slaughterers and two of their family members had used antibiotics in the preceding three months. In the three groups approximately 55% kept companion animals; mainly dogs and cats. Thirteen laying hen farmers (52%) and 35% of broiler farmers also kept other food animals on the farm. Other food animals were mainly pigs (32% of laying hen farmers and 29% of broiler farmers). Six of the (13%) of the slaughterers kept pigs. There were no farmers in the study that kept both laying hens and broilers. None of the poultry slaughterers

Table 1. Prevalence of antibiotic resistant enterococci and prevalence of high degree of resistance in fecal samples from broilers, laying hen, broiler farmers, laying hens farmers and poultry slaughterers.

Antimicrobial agent	Concentration mg/L	Study population (number of fecal samples)											
		Broilers		Laying hens		Broiler farmers		Laying hen farmers		Poultry slaughterers			
		(n=50)	Prev.	(n=25)	HD	(n=51)	HD	(n=25)	HD	(n=46)	HD	Prev.	HD
Amoxicillin	25	6		0	0	4	0	0	0	0	0	0	0
Ciprofloxacin	4	28		8	0	2	0	4	0	2	0	2	0
Gentamicin	500	44		40	0	10	0	0	0	2	0	2	0
Oxytetracycline	25	94		98	24	75	20	76	4	46	10		
Quinupristin-dalfopristin	8	92 ¹		12 ¹	8	37 ¹	6	8 ¹	0	15 ¹	0		
Vancomycin	10	80 (82) ¹		12	8	27 (36) ¹	0	8 (12) ¹	0	13 (17) ¹	2		
Erythromycin	8	94		98	44	67	16	64	4	50	8		

Prev. = Prevalence of resistance (%).

HD = Prevalence of high degree of resistance (%).

¹ At least one *E. faecium* was isolated from each positive sample.

() samples positive after enrichment included.

Table 2. MIC distribution of enterococci isolates from the different study populations.

Antimicrobial Agent	Population (number of isolates)	% resistant	Number of isolates with MIC of:										MIC ⁵⁰	MIC ⁹⁰	Range
			≤ 0.25	0.5	1	2	4	8	16	32	64	≤ 128			
Amoxycillin	Broilers (50)	0			43	5	2						1	2	1-4
	Laying hens (24)	0			23		1						1	1	1-4
	Broiler farmers (49)	0			47	2							1	1	1-2
	Laying hen farmers (25)	0			25								1	1	1
	Poultry slaughterers (41)	0			39	2							1	1	1-2
Vancomycin	Broilers (50)	8			35	7	2	2	2			2	1	8	1-128
	Laying hens (24)	13			12		6	3	1		1	1	1	16	1-128
	Broiler farmers (49)	2			42	2	3	1			1		1	4	1-64
	Laying hen farmers (25)	0			22	1	2						1	2	1-4
	Poultry slaughterers (41)	0			29	6	6						1	4	1-4
Teicoplanin	Broilers (50)	4		47			1			1	1		0.5	0.5	0.5-64
	Laying hens (24)	8		21				1	1	1			0.5	0.5	0.5-32
	Broiler farmers (49)	0		49									0.5	0.5	0.5
	Laying hen farmers (25)	0		25									0.5	0.5	0.5
	Poultry slaughterers (41)	0		40	1								0.5	0.5	0.5-1
Avoparcin	Broilers (50)	4	12		27	7	2			2			1	2	0.5-4
	Laying hens (24)	13	5	3		8	3	2	2	1			2	16	0.5-32
	Broiler farmers (49)	0	26	15		7	1						0.5	2	0.5-4
	Laying hen farmers (25)	0	6	8		9	2						1	2	0.5-4
	Poultry slaughterers (41)	0	5	11		15	9	1					2	4	0.5-4
Oxytetracycline	Broilers (50)	64		8				3	7	8	7	17	64	128	1-128
	Laying hens (24)	54		8					3	9	3	1	32	64	1-128
	Broiler farmers (49)	35		28					4	5	6	6	1	128	1-128
	Laying hen farmers (25)	28		15				1	2	3	3	1	1	32	1-128
	Poultry slaughterers (41)	0	40		1								0.5	0.5	0.5-1

Table 2. Continued

Antimicrobial Agent	Population (number of isolates)	% resistant	Number of isolates with MIC of:										MIC ⁵⁰	MIC ⁹⁰	Range
			≤ 0.25	0.5	1	2	4	8	16	32	64	≤ 128			
Quinupristin-Dalfopristin [*]	Broilers (30)	80			4	2	1	4	13	6			16	32	1-32
	Laying hens (21)	62	1		2	5	1	5	6	1			8	16	0.25-32
	Broiler farmers (30)	40	3		13	2	7	3			2		1	8	0.25-8
	Laying hen farmers (15)	73	1			3	2	6	3				8	16	0.25-16
	Poultry slaughterers (20)	25		3	2	11		2	2				2	16	0.5-16
Virginiamycin [*]	Broilers (30)	80			6		4	4	11	5			6	32	1-32
	Laying hens (21)	10			15	4	1	1					1	4	1-8
	Broiler farmers (30)	6			23	2	1		2	2			1	16	1-32
	Laying hen farmers (15)	0			15								1	1	1
	Poultry slaughterers (20)	15			17	1		1	1				1	4	1-16
Quinupristin-Dalfopristin ^{**}	Broilers (20)	75			5		12	2	1				4	8	1-16
	Laying hens (3)	100						1	2				116	6	8-16
	Broiler farmers (19)	47			5	5	5	8	1				4	8	25-16
	Laying hen farmers (10)	90				1	1	1	8				16	16	4-16
	Poultry slaughterers (21)	76		2	1	2	1	12	3				8	16	0.5-16
Virginiamycin ^{**}	Broilers (20)	80			7	6	7						2	4	1-4
	Laying hens (3)	33				1	1	1					4	8	2-8
	Broiler farmers (19)	47			3	7	9						2	4	1-4
	Laying hen farmers (10)	10			7	2	1						1	2	1-4
	Poultry slaughterers (21)	10			6	11	4						1	4	1-16
Chloramphenicol	Broilers (50)	2			11	21	17			1			2	4	1-32
	Laying hens (24)	42			8	4	2				1	9	2	128	1-128
	Broiler farmers (49)	4			6	22	17	2			2		2	8	1-64
	Laying hen farmers (25)	2			7	14	3					1	2	4	1-128
	Poultry slaughterers (41)	0			6	13	21	1					4	4	1-8

Table 2. Continued

Antimicrobial Agent	Population (number of isolates)	% resistant	Number of isolates with MIC of:										MIC ⁵⁰	MIC ⁹⁰	Range
			≤ 0.25	0.5	1	2	4	8	16	32	64	≤ 128			
Erythromycin	Broilers (50)	54		17	2	4		2	2		23		8	64	0.5 - 64
	Laying hens (24)	32		12	2	1		3		1	5		0.5	64	0.5 - 64
	Broiler farmers (49)	27		24	5	6	1	2	3		8		1	64	0.5 - 64
	Laying hen farmers (25)	12		14	1	4	3			1	2		0.5	32	0.5 - 64
	Poultry slaughterers (41)	24		20	3	5	3	4		1	5		1	64	0.5 - 64
Tylosin	Broilers (50)	58		1	11	9			1		28		64	64	0.5 - 64
	Laying hens (24)	33		3	7	6					8		2	64	0.5 - 64
	Broiler farmers (49)	27		7	10	15	4				13		2	64	0.5 - 64
	Laying hen farmers (25)	20		1	13	4	2	1		1	3		1	32	0.5 - 64
	Poultry slaughterers (41)	22		1	14	13	4	4			5		2	64	0.5 - 64
Ciprofloxacin	Broilers (50)	8	4	16	16	10	4						1	2	≤ 0.125 - 4
	Laying hens (24)	13	6	4	10	1	3						1	4	≤ 0.125 - 2
	Broiler farmers (49)	0	14	20	11	4							0.5	1	≤ 0.125 - 2
	Laying hen farmers (25)	12	6	8	5	3	2	1					0.5	2	≤ 0.125 - 8
	Poultry slaughterers (41)	7	8	7	13	10	3						1	2	≤ 0.125 - 4
Trimethoprim/ Sulfa- methoxazole***	Broilers (50)	4		45	2			1	1	1			0.5	0.5	0.5 - 32
	Laying hens (24)	0		20	2	1		1					0.5	1	0.5 - 8
	Broiler farmers (49)	0		43	5	1							0.5	1	0.5 - 2
	Laying hen farmers (25)	4		19	1	2	2		1				0.5	4	0.5 - 16
	Poultry slaughterers (41)	34			22	2		1	2	3	7	4	1	64	1 - 128

* *E. faecalis* excluded.** only *E. faecalis*.

*** trimethoprim and sulfamethoxazole 1 : 19. The trimethoprim concentrations are presented in the Table.

Table 3. Identification of enterococcal isolates from broilers, laying hens, broiler farmers, laying hen farmers and poultry slaughterers.

	Broilers	Laying hens	Broiler farmers	Laying hen farmers	Poultry slaughterers
Species	n = 50	n = 24	n = 49	n = 25	n = 41
<i>E. faecalis</i>	20	3	21	10	21
<i>E. faecium</i>	26 (52%)	5 (29%)	6 (32%)	8 (32%)	14 (34%)
<i>E. casseliflavus</i>	1	6	2	1	2
<i>E. mundtii</i>	1	1			1
<i>E. avium</i>	1			1	1
<i>E. hirae</i>	1		6	2	1
<i>E. durans</i>		2	1		
<i>E. gallinarum</i>		4	2		
Unidentified		3		3	1

n = number of isolates

kept poultry. There were no significant differences observed within the same group between people keeping pigs and those that did not (data not shown). Of the laying hen flocks 2 (8%) had received a course of antibiotics on veterinary prescription while on the farm, compared with 23 (46%) of the 50 broiler flocks. No information was obtained about AMGP use.

Prevalence and degree of resistance

The prevalence of resistance and the prevalence of high degree resistance for the tested antibiotics and of the populations studied are depicted in Table 1.

The highest percentage of samples with a high degree of resistance was observed in the fecal samples of broilers. The prevalence of resistance to quinupristin-dalfopristin and vancomycin was significantly ($p < 0.05$) higher in broilers than in laying hens. The prevalence of resistance to oxytetracycline, erythromycin and gentamicin was of the same order in both chicken populations, but the number of erythromycin and oxytetracycline resistant enterococci per gram feces (prevalence of high degree resistance) was significantly higher ($p < 0.05$) in broilers than in laying hens. In the human samples, amoxycillin resistance was observed only in fecal samples from broiler farmers and high level gentamicin resistance only in a few samples from broiler farmers and poultry slaughterers. Tetracycline resistance was common in all three human populations. The prevalence of resistance for vancomycin and

quinupristin-dalfopristin was higher in broiler farmers than in laying hen farmers and poultry slaughterers, but statistical significance was achieved only for vancomycin ($p < 0.05$).

In general there was a close correlation of the prevalence of resistance to all antibiotics between broilers and broiler farmers (correlation coefficient: 0.95) and between broilers and poultry slaughterers (correlation coefficient: 0.96) ($p < 0.01$). No significant correlation was observed between the prevalence of antibiotic resistance strains in laying hens and those observed in any of the three human populations studied.

MIC distributions

As shown in Table 3, *E. faecalis*, 73 (39%), and *E. faecium*, 74 (39%), were the most prevalent enterococcal species among the 189 isolates from the antibiotic free control plates. No significant differences in MIC distribution between the different populations were observed for amoxycillin, chloramphenicol, cotrimoxazole and oxytetracycline. Resistance against all three glycopeptide antibiotics was observed in both chicken populations, while only one fecal sample of a broiler farmer yielded a VRE. On average the MIC of isolates from poultry and from broiler farmers were higher than those from the laying hen farmers and poultry slaughterers. Marcolide resistance was significantly higher in broilers than in laying hens and also higher in broiler farmers and in poultry slaughterers

Table 4. MIC values of enterococcal isolates (n = 64) from vancomycin containing (10mg/L) selective agar plates.

Antibiotic /MIC (mg/L)	0.5	1	2	4	8	16	32	64	128
Vancomycin		2			3	3	8	23	25
Avoparcin	2			4	13	29	14	2	
Teicoplanin	14	6	5	11	9	11	8		

Table 5. Patterns of resistance of multiple resistant enterococcal isolates from fecal samples of broilers, laying hens, broiler farmers, laying hen farmers and poultry slaughterers.

Population	Species	n	Ery	oxy	dalfo	cipr	amo	chlor	Van	TPM/S
Broilers n = 18	<i>E. faecium</i>	8	R	R	R					
	<i>E. faecium</i>	5	R	R	R	R				
	<i>E. faecium</i>	3	R		R	R				
	<i>E. faecium</i>	1	R	R	R	R	R	R	R	
	<i>E. faecalis</i>	1	R	R	*					R
Laying hens n = 6	<i>E. faecium</i>	1		R	R				R	
	<i>E. faecium</i>	2		R	R	R				
	<i>E. durans</i>	1	R	R				R		
	<i>E. durans</i>	1	R	R	R			R	R	
	<i>E. casseliflavus</i>	1	R	R	R	R			R	
Broiler Farmers n = 5	<i>E. faecium</i>	2	R	R	R					
	<i>E. hirae</i>	1	R	R	R					
	<i>E. hirae</i>	1		R	R			R		
	<i>E. durans</i>	1	R	R	R					
Laying hen farmers n = 3	<i>E. faecium</i>	1		R	R	R				
	<i>E. faecium</i>	1	R	R	R					
	<i>E. faecalis</i>	1	R	R				R		
Poultry-slaughterers n = 2	<i>E. faecium</i>	2	R	R	R					

R = resistant.

MIC mg/L: ery = erythromycin MIC > 4, oxy = oxytetracycline MIC > 8, dalfo = dalfopristin-quinupristin MIC > 2, cipr = ciprofloxacin MIC > 2, am = amoxycillin MIC > 16, chlor = chloramphenicol MIC > 16, van = vancomycin MIC > 8 and TPM/S = trimethoprim-sulfamethoxazole MIC > 8/152.

n = number of isolates.

* = resistant but not included.

than in laying hen farmers. No amoxycillin resistant VRE were isolated from the control plates. As shown in Table 4 multiple resistance to three or more of the tested antibiotics was found in 36% (18), 25% (6), 10% (5), 12% (3) and 4% (2) of broiler, laying hen,

*broiler farmer, laying hen farmer and poultry slaughterer isolates respectively. Of these multiresistant isolates 24 (71%) were *E. faecium*. The most observed resistance pattern was resistance against erythromycin, oxytetracycline and quinupristin-dalfopristin. This

Table 6. Pulsed-field gel electrophoresis patterns and characteristics of 20 paired vancomycin resistant enterococcal isolates from fecal samples of farmers and chickens from the same farm.

Source	Species	MIC vanco (mg/L)	MIC teico (mg/L)	PFGE Pattern	Transposon type
Laying hen (2)	<i>E. faecium</i>	128	8	A	B2
Laying hen farmer (2)	<i>E. faecium</i>	128	8	B	A2
Broiler (3)	<i>E. hirae</i>	64	4	C	E11
Broiler farmer (3)	<i>E. hirae</i>	64	4	D	E11
Broiler (4)	<i>E. faecium</i>	64	8	E	ND ¹
Broiler farmer (4)	<i>E. faecium</i>	1	0.5	F	A1
Broiler (14)	<i>E. hirae</i>	64	4	G	E11
Broiler farmer (14)	<i>E. hirae</i>	64	0.5	G	E11
Broiler (31)	<i>E. hirae</i>	64	4	H	E11
Broiler farmer (31)	<i>E. hirae</i>	32	1	H1	E11
Broiler (36)	<i>E. faecium</i>	64	4	I	A1
Broiler farmer (36)	<i>E. faecium</i>	64	16	J	E7
Broiler (37)	<i>E. faecium</i>	128	32	K	A1
Broiler farmer (37)	<i>E. faecium</i>	128	16	L	A2
Broiler (39)	<i>E. faecium</i>	32	0.5	M	E19
Broiler farmer (39)	<i>E. hirae</i>	64	1	N	E14
Broiler (40)	<i>E. faecium</i>	32	1	O	A1
Broiler farmer (40)	<i>E. hirae</i>	64	0.5	G1	E11
Broiler (45)	<i>E. faecium</i>	32	2	P	ND ¹
Broiler farmer (45)	<i>E. hirae</i>	64	0.5	Q	E17

¹ND = Not determined.

pattern was found in 13 (38%) isolates, all except one *E. faecium*.

From the gentamicin containing agar plates 27 enterococci were isolated with high level resistance (HLR) to gentamicin. Three of these, isolates from broilers, were identified as *E. faecium* and were also resistant to amoxycillin and two were additionally resistant to nearly all antibiotics tested: oxytetracycline, erythromycin, quinupristin-dalfopristin, cotrimoxazole and the tested related molecules, but not to vancomycin. Of the other 24 gentamicin HLR isolates the majority was identified as *E. faecalis* except for 4 isolates from broilers and 3 from broiler farmers, which were identified as *E. faecium*.

Of 58 quinupristin-dalfopristin resistant *E. faecalis* isolates from the antibiotic free control plates the MIC for virginiamycin was more than one dilution step lower than for quinupristin-dalfopristin for 37 isolates (64%) (data not shown) and 33 (57%) isolates were

susceptible to virginiamycin. For the 64 non-*E. faecalis* quinupristin-dalfopristin resistant isolates, of which 44 were *E. faecium* these figures were 50% and 48% respectively. All except one of 119 isolates from the quinupristin-dalfopristin containing agar plate were resistant to quinupristin-dalfopristin. The MIC for virginiamycin was more than one dilution step lower than for quinupristin-dalfopristin resistance in 32 (27%) isolates. Of these 32 isolates 19 were susceptible for virginiamycin. Isolates with MIC for virginiamycin higher than for quinupristin-dalfopristin were not isolated.

The MIC for glycopeptides of isolates from the vancomycin containing plates are shown in Table 5. Only 64 isolates were tested as one was lost during storage and in all but two the *vanA* gene cluster was detected by blot hybridization. No *vanB* or *vanC* genes were found. Most *vanA* containing isolates were identi-

Figure 1. PFGE patterns and dendrogram of 10 paired vancomycin resistant enterococci isolated from fecal samples of farmers and poultry from the same farm after total DNA digestion with *Sma*I.

fied as *E. faecium* (n=37), but 5 isolates were *E. faecalis*, 15 *E. hirae* and 5 *E. durans*.

Pulsed-Field Gel Electrophoresis

The 10 paired VRE isolates from farmer and chicken recovered from the same farm were analyzed by PFGE (Fig. 1, 2 and Table 6). In general the obtained PFGE patterns were very diverse. In two cases, SK14/SKH14 and SK31/SKH31, strains isolated from broiler farmers and their broilers, all identified as *E. hirae*, were identical 100% or very similar (93%). In addition, isolate SKH40, isolated from a broiler farmer, but from a different farm, showed a high degree of similarity with isolates SK14 and SKH14. *y* with isolates SK14 and SKH14.

Tn1546 types among different VRE isolates

Eight different *vanA* transposon types were found among the 18 VRE isolated from poultry and farmers (Fig. 2). Tn types A1, A2, B2, E7 and E11 have been described previously. In short type A1 is identical to Tn1546. Type A2 is characterized by a G → T point mutation at position 8234 and an IS1216V-IS3 insertion at the left end of the transposon. Type B2 contains an IS1216V insertion in the *vanX-vanY* intergenic re-

gion. Type E7 contains also the IS1216V insertion in the same region as in type B2 as well as a large left end deletion encompassing the *orf1* gene and a part of the *orf2* gene, while type E11 contains an additional deletion at the right end of the transposon including the *vanZ* gene. Three new transposon types were found, type E14, E17, and E19. Types E14 and E17 are closely related to type E11. The only difference resides in the size of the small deletion flanking the IS1216V insertion site downstream of *vanX*. Type E19 is comparable to type E7, with differences in the size of the left end deletion and in the size of the small deletions adjacent to the IS1216V insertion site in the *vanX-vanY* intergenic region. Comparative analysis of the Tn1546-like elements of the paired VRE strains revealed that in addition to the two sets of paired strains with comparable PFGE patterns, 14 and 31, the paired strains broiler and broiler farmer 3 also contained identical transposon types. In the other cases different transposon types were present in poultry and poultry farmer isolates. In conclusion, in three of the eight paired strains which were analyzed an identical *vanA* transposon was present in both the animal and farmer isolate.

Figure 2. Genetic map of Tn1546 and four Tn1546 derivatives. The thick horizontal lines represent Tn1546 and Tn1546 types E8-112. The position of genes and open reading frames (orf's) and direction of transcription is depicted by open arrows. Dotted boxes represent IS elements. The position of the first nucleotide up and downstream from IS insertion sites are depicted. Filled arrows indicate the transcriptional orientation of inserted IS elements. Deletions have been indicated by dotted lines.

DISCUSSION

Enterococci are not important animal pathogens. As antibiotic susceptibility testing is usually performed to guide antimicrobial therapy, no data on antibiotic resistance rates in clinical isolates of enterococci from poultry were available in the literature. Until quite recently only a few studies were performed about the resistance found in the fecal enterococci of food animals, but since Bates suggested an animal reservoir for VRE in man, the issue of selection pressure exerted by the use of antibiotics on the fecal flora of exposed animals and the potential for transfer of resistant fecal bacteria from food animals to humans has contracted considerable interest. Because of differences in methodology as often only resistance in one species, mostly *E. faecium*, is investigated, direct comparison of figures and percentages of different studies is not feasible.

Comparison of the results of Tables 1 and 3 showed a difference between the quantitative determination of resistance (direct plating on antibiotic containing plates) and the qualitative determination based on the MIC of a single randomly selected isolate per sample. As expected the quantitative method was more sensitive than the qualitative method. For most tested antibiotics results obtained by the qualitative method correlated better with the prevalence of high degree of resistance in a sample than with the prevalence of resistance. Because this difference in sensitivity direct comparison of the obtained figures is not feasible. However, comparing the results of MIC of single isolates of enterococci of the populations studied to the direct plating results, the same trends were observed and therefore conclusions drawn from the results of both methods might be compared. The increase in sensitivity of VRE detection that was obtained after enrichment of fecal samples in vancomycin containing broth as compared to direct plating was relatively low and did not influence the conclusions. As AMGP are selected for addition to broiler feeds by the feed miller many broiler farmers were not even aware that they were feeding AMGP to their animals. Therefore it was not possible to acquire reliable data on AMGP usage. In broilers the prevalence of resistance for antibiotics most frequently used for therapeutic purposes was higher than in laying hens. Similarly prevalence, but also high

degree of resistance to substances (vancomycin and erythromycin) for which cross-resistance to commonly used AMGP occurs was more common in broilers than in laying hens. This corroborates with the results of another study showing that a high degree of antibiotic resistance against an antibiotic in pigs was correlated with current or recent high or continuous use of that antibiotic in these animals⁹¹. Animals with a high degree of resistance in their fecal flora excrete more resistant bacteria in their environment, which increases the risk for acquisition of these strains by humans. Moreover it has been shown that people with a high degree of resistance against a certain antibiotic carry these resistant bacteria more consistently and over a longer time span than persons with a low degree of resistance in their fecal flora^{47,49}. This might also be the case in poultry. Although the overall prevalence of antibiotic resistance was indeed lower in laying hens than in broilers, it was nonetheless unexpectedly high.

This may be associated with regular usage of AMGP and of therapeutic antibiotics like tetracyclines during the rearing period. AMGP like virginiamycin and tylosin are commonly mixed in the feeds of laying hens during the rearing period for prevention of bacterial infections. Regular antibiotic usage also selects also for the more intrinsically resistant *E. faecium* among enterococci in the fecal flora of exposed individuals. Fifty two percent of the total number of enterococci isolated from broiler fecal samples was identified as *E. faecium* compared with 30% in the other populations studied.

The overall prevalence of antibiotic resistant enterococci among broilers, broiler farmers and poultry slaughterers points to contact with broilers as a risk factor for colonization with resistant enterococci for humans. In laying hen and laying hen farmers this was the case only for tetracycline and erythromycin resistance. The prevalence of 6% amoxycillin resistant fecal enterococci in broiler flocks and the absence of amoxycillin resistant enterococci from laying hens are most likely a result of the higher overall use of antibiotics in broilers compared to laying hens. Regular antibiotic use favors the selection of intrinsically less susceptible *E. faecium* in the intestinal flora and the majority of broiler isolates were *E. faecium* compared to only one third of the laying hen isolates. All amoxycillin resistant

enterococci isolated in the study were *E. faecium*, except one *E. durans*. This is in agreement the results of a study of human isolates, where considerably less amoxycillin resistant *E. faecalis* than *E. faecium* have been found⁷³. In the USA Thal et al. found only in the feces from one of 12 healthy chickens from different farms amoxycillin resistant enterococci. In Denmark, one randomly chosen isolate from each fecal sample having been examined, the prevalence of amoxycillin resistant *E. faecium* in broilers was 2%³. In this study no amoxycillin resistant enterococci were found among the individual isolates, which corresponded with the low degree of amoxycillin resistance observed in positive samples. The presence of amoxycillin resistant enterococci only in the fecal flora of broilers and broiler farmers and not in any other of the studied populations nor in healthy (sub)urban residents in the Netherlands⁹⁴ is suggestive of a broiler to farmer transfer of resistant enterococci.

Resistance to fluoroquinolones was significantly more common in broilers than in the other populations. This is most likely due to the more common use of enrofloxacin and flumequine (a less potent fluoroquinolone than enrofloxacin), in broilers than in laying hens. The fluoroquinolone resistant enterococci isolated from laying hens might be due to use of quinolones during the rearing period. In Denmark 63% of fecal *E. faecium* from broilers were resistant to fluoroquinolones³. Resistance to fluoroquinolones is frequently observed among enterococci isolated from human infections^{72,73,87}. The prevalence of fluoroquinolone resistance in broiler farmers was, however, not different from the other populations studied. Resistance against fluoroquinolones is caused by mutations and not by transferable genes. Clonal transmission of fluoroquinolone resistant *E. coli* strains from turkeys to humans has been described⁸⁹. This indicates that colonization of humans by poultry enterococci occurs less readily than by poultry *E. coli*. In that case it is likely that for the transfer of resistance in enterococci from poultry to humans exchange of resistance genes between poultry enterococci and endogenous human enterococci is a more important phenomenon than colonization of the human intestinal tract by poultry enterococci.

The high prevalence of HLR to gentamicin in the enterococcal fecal flora of both broilers and laying hens in this study was unexpected. Thal et al. found no high level gentamicin resistant enterococci in fecal samples of poultry despite their presence in wild birds and in environmental samples⁶⁴. In Denmark only 1% of the *E. faecalis* isolated from healthy broilers at slaughter were resistant to gentamicin and no gentamicin resistant *E. faecium* were isolated³. In this study in both species HLR to gentamicin has been observed. As gentamicin is not registered for use in poultry in the Netherlands this might have been due to regular off-label use in young chickens. The most common cause for high level gentamicin resistance in enterococci is acquisition of a gene encoding for AAC6'-APH2, which is transferable between enterococcal species^{35,36,60,80}. However, other genetic determinants encoding for HLR to gentamicin in enterococci have been described⁵³. Rice et al. described a large transferable element, tentatively named Tn5385, which contains within it a conjugative transposon (Tn5381) encoding tetracycline resistance and an IS256-based composite transposon conferring resistance to gentamicin and erythromycin⁶⁵⁻⁶⁷. Tetracyclines are the most widely used antibiotics in poultry and tylosin, a macrolide and completely cross-resistant with erythromycin, was used regularly as AMGP at the time of the study and on veterinary prescription. Therefore co-selection might be another reason for the observed high prevalence of HLR to gentamicin among fecal enterococci of Dutch poultry. This is confirmed by the observation that nearly 50% of the isolates with HLR to gentamicin were also resistant to tetracyclines and macrolides. Further investigations are required to determine if an epidemic of Tn5385 or a related resistance determinant is responsible for the observed high prevalence of multiple resistance in the isolated enterococci with HLR to gentamicin. The prevalence of HLR to gentamicin of 10% in broiler farmers is most likely derived from their animals as in human medicine in the Netherlands gentamicin is used almost exclusively in hospitals and none of the farmers or family members had been in hospital in the three months preceding the study. High-level gentamicin resistance in enterococci has been reported in several studies from human infections^{25,59}. In a recent European study of more than

4208 enterococci isolated from human infections 23% of the isolates was HLR to gentamicin: 20% of the isolated *E. faecalis* strains and 14% of *E. faecium* isolates. However in fecal enterococci from healthy Dutch (sub)urban residents in 1997 no high-level gentamicin resistant enterococci were detected⁹⁴.

The high prevalence of resistance and the high percentage of fecal samples with a high degree of resistance against oxytetracycline in both chicken populations clearly reflected the large amounts of tetracyclines (approximately 60% of all antibiotics on a weight basis of pure drug) used in poultry in the Netherlands. The fact that both farmer groups had a significantly higher prevalence of resistance than the prevalence of 36% observed in fecal samples of healthy (sub)urban residents collected at the same time in the Netherlands (unpublished results) suggested direct transfer of resistant strains or genetic determinants of resistance from animals to humans. Tetracycline resistance is easily disseminated and several different transferable resistance determinants have been described^{14,55,69,86}. The *TetM* gene of the conjugative transposon Tn916 has even spread beyond the enterococci into Gram-negative bacterial species^{68,70}. In Danish study 61% of the *E. faecalis* isolates from broiler faeces were tetracycline resistant, compared to a more limited number (31%) of *E. faecium* isolates⁸. Tetracycline resistance among clinical enterococci from humans is common⁸⁷.

The cross-resistance of erythromycin with tylosin was confirmed by the MIC results as shown in Table 3. The prevalence of erythromycin resistance was in all populations high, but the proportions of erythromycin resistant enterococci per gram feces (high degree of resistance) were significantly higher ($p < 0.05$) in both poultry groups than in the three human populations. The mean percentage of erythromycin resistant enterococci in positive fecal samples of broilers was 79%. Such high numbers of resistant enterococci per gram feces facilitates transfer from broilers to humans. Several studies have shown that in chickens raised on tylosin containing feed not only the percentage resistant enterococci in the fecal flora of exposed animals increased over time but that also an selection for *E. faecium* occurred^{30,37-39}. In Finland, where tylosin is only used therapeutically when indicated and only on

veterinary prescription the prevalence of tylosin resistant fecal enterococci was 9% in broilers, whereas in Denmark where tylosin was also widely used as AMGP at the time of the study the prevalence of tylosin resistance was 59%. Similarly, in Sweden, where no AMGP have been used since 1986, the prevalence of tylosin resistant fecal enterococci from poultry was 17%²⁶, whereas in Belgium the percentages ranged from 50% - 67%. Also the percentage of erythromycin resistant enterococci isolated from meat from Danish poultry was significantly higher than from Swedish poultry meats 36% and 7% respectively⁶². Quinupristin-dalfopristin⁸ is like virginiamycin a mixture of two pristinamycins that act synergistically and cross-resistance between both drugs has been described. Virginiamycin use in human medicine has been limited to a few EU countries for therapy of staphylococcal infections. In contrast virginiamycin has been used extensively as AMGP for many years in the EU. At the time of the study quinupristin-dalfopristin was not registered for any use in the EU. The high prevalence of resistance to this agent observed in broilers is most likely due to the common use of virginiamycin in broiler feeds as a replacement for avoparcin, after the EU suspension of glycopeptide usage as AMGP. It has been shown that the prevalence of resistance to pristinamycins in chickens fed virginiamycin is significantly higher than in controls both for *E. faecalis* (46% and 25% respectively) and for *E. faecium* (88% and 26%). This increase in prevalence of resistance was associated with an increase in the numbers of *E. faecium* isolates relative to the total number of enterococci in the fecal flora of the exposed birds. In Denmark 60% of *E. faecium* isolates from fecal samples of healthy broilers were resistant to virginiamycin and 74% to quinupristin-dalfopristin. Resistance to the last compound in clinical isolates from humans in Europe was 75% for *E. faecalis* and 8% for *E. faecium* isolates. *E. faecalis* is considered intrinsically resistant against pristinamycins. The MIC of single isolates showed this to be true for the majority of *E. faecalis* isolates for quinupristin-dalfopristin, but not for virginiamycin. In this study the isolated enterococci tended to be more susceptible to virginiamycin than to dalfopristin and *E. faecalis* isolates were less susceptible to pristinamycins, but not all resistant.

VRE were most common in the fecal samples from broilers. In fecal samples of broiler farmers a significantly higher prevalence and degree of vancomycin resistance among enterococci was observed than in the other two human populations in this study and in Dutch (sub)urban residents⁹². Several studies have been performed on the prevalence of VRE in chickens. In Denmark and Germany VRE were found in the fecal flora of broilers from intensive farming units using AMGP, whereas no VRE were detected in fecal samples from laying chickens from organic farms, that do not use AMGP^{2,42}. In another Danish study (broilers), a Norwegian (broilers and turkeys) and a Dutch study (turkeys) comparing farms using and not using avoparcin the relative risk ratio for the presence of VRE in the fecal flora of the animals at these farms was 2.9, 5.4 and 3.3 respectively^{7,44,79}. In Japan fecal carriage was common in farms using avoparcin as AMGP but no VRE were detected in animals from farms not using avoparcin¹⁰³. The MIC of the isolates from the vancomycin containing agar plates showed a good correlation between vancomycin and avoparcin resistance, but not with teicoplanin. The *vanA* gene cluster, that was shown to be present in these isolates, consists of 9 genes of which 6 are involved in conferring phenotypic glycopeptide resistance. It resides on a small Tn3-like transposon, Tn1546⁵, and on elements that appear to be closely related (e.g. Tn5488, which has an insertion sequence IS1251 within Tn1546^{28,29} or is lacking *vanZ*^{79,92}. Isolates with the *vanA* gene clusters that lack a functional *vanZ* gene are susceptible to teicoplanin. In this study more than 50% of the VRE were phenotypically susceptible to teicoplanin. The most prevalent transposon variant was a Tn1546 derivative which contained a large left end deletion including the *orf1* gene, an IS1216V insertion in the *vanX-vanY* intergenic region and a deletion of the *vanZ* gene. These features found in the homologous types E11, E14, and E17 were present in half of the transposon types, which were analyzed. Interestingly, type E11 and the homologous types E9 and E10 were also predominantly (9/13) present in isolates recovered from turkeys and in almost half (4/9) of the isolates recovered from turkey farmers in the Netherlands^{79,92}. All Tn1546 variants found in the isolates from broilers and laying hens contained at position 8234 the "G" which

is consistent with the results reported previously^{31,79,99}. This variant is also found in humans although the majority of human isolates and all isolates recovered from pigs contain a "T" at this position^{31,79,99}. The finding that human isolates analyzed in this study contain *vanA* transposon types predominantly found in poultry and not the most prevalent human transposon type is again a strong indication that poultry farmers have contracted the *vanA* resistance genes from their animals. Since, the isolates recovered from poultry and poultry farmers were in most cases genetically different, it is most likely that vancomycin resistance was transmitted from farm animals to farmers through horizontal transfer of Tn1546 variants. From the fact that this was only found in three combinations of a farmer isolates and one of his poultry, does, however, not imply that such a transfer is a rarely occurring event. One must realize that only one isolate was tested from each sample and, therefore, the chance to detect such a combination is extremely low, but testing of more isolates was not feasible because of the workload involved. Other studies have also pointed to a similarity between *vanA* containing elements in animals and humans^{2,16,32,77,79,99,102}. It was interesting to note that all *E. hirae* isolates, irrespective whether they originated from animal or human sources contained the highly homologous *vanA* transposon types E11, E14, and E17. This could suggest a certain level of enterococcal species specificity of *vanA* transposon types which, to our knowledge, has never been described before. This could be observed because not only single isolates identified as *E. faecium* were tested and is consistent with the results of Butaye et al. who found also that 50% of VRE from boilers and nearly all VRE from laying hens were *E. hirae*¹³.

Since the EU suspension of the use of avoparcin in food animals significant decreases of the prevalence of vancomycin resistance in fecal enterococci have been observed in Danish broilers, on chicken meat in Germany⁴⁰ and in Italy⁵⁸ and from humans in Germany⁴⁰. In the Netherlands the prevalence in fecal samples of broilers collected at slaughter decreased significantly ($p < 0.001$) between 1997 and 1999 from 80% till 31% and in humans from 12% till 6% ($p < 0.05$). This could be considered as additional evidence that food animals are the reservoir of glycopeptide re-

sistance in fecal enterococci of healthy humans but also showed that intervention measures are effective.

In conclusion the results of this study point again to a transfer of resistant enterococci and/or enterococcal resistance genes from animals to man. Continued monitoring of antibiotic resistance and antibiotic use is necessary to study the epidemiology of resistance and to be able to evaluate the effect of intervention methods. As resistance is not restricted to *E. faecium* and also found in other enterococcal species, monitoring should not be restricted to *E. faecium* isolates alone. Transfer of resistance between different species of enterococci has been described and most human enterococcal infections are caused by *E. faecalis*.

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Chapter VI

Antibiotic resistance of faecal *Escherichia coli* from poultry, poultry farmers and poultry slaughterers

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ABSTRACT

The percentage of faecal samples containing resistant *Escherichia coli* and the numbers of resistant *E. coli* per faecal sample, relative to the total number of *E. coli* were determined in faecal samples of three poultry populations: broilers and turkeys (animals commonly given antibiotics) and laying hens (with a low usage of antibiotics). In addition faecal samples were examined from five human populations: turkey farmers, broiler farmers, laying hen farmers, broiler slaughterers and turkey slaughterers. Resistance to antibiotics commonly used in poultry medicine were tested (including MIC determinations). Ciprofloxacin resistant strains isolated from these eight populations as well as from turkey meat were genotyped by pulsed-field gel electrophoresis (PFGE) after *Sma*I digestion. The percentages of samples containing antibiotic resistant *E. coli* and the percentages of antibiotic resistant *E. coli* relative to the total *E. coli* population (i.e. the degree of resistance) was significantly higher in turkeys and broilers than in the laying hen population. The prevalence of antibiotic resistance in faecal *E. coli* isolated from turkey and broiler farmers, and from turkey and broiler slaughterers, was for nearly all antibiotics tested higher than those observed in laying hen farmers. Multiresistant isolates were common in turkey and broiler farmers but absent in the laying hen farmer population. The same resistance patterns were found in turkeys, turkey farmers and turkey slaughterers and in broiler, broiler farmers and broiler slaughterers. The PFGE patterns of the isolates from the eight populations were quite heterogeneous. However, *E. coli* with an identical PFGE pattern were isolated at two farms from a turkey and the farmer, and also from a broiler and a broiler farmer but of different farms. Moreover, three *E. coli* isolates from turkey meat were identical to faecal isolates of turkeys. The results of this study strongly indicated that transmission of resistant clones and resistance plasmids of *E. coli* from poultry to humans commonly occurred.

INTRODUCTION

Penicillin was discovered in 1928 by Sir Alexander Fleming and its clinical introduction more than 12 years later in human medicine was soon followed by

other antibiotics. The use of these antibiotics was the most significant advance in the treatment of bacterial infections not only in humans but also in animals. Unfortunately, sixty years later many of these antibiotics that were formerly very effective are becoming less potent and in some cases even inactive. We are now encountering some micro-organisms that are resistant to all clinically available antibiotics. Antibiotic usage is considered the most important factor for the emergence, selection and dissemination of antibiotic resistant micro-organisms both in veterinary and human medicine^{1,2}. Antibiotic usage not only selects for resistance in pathogenic bacteria but also in the endogenous flora of the exposed individuals (animals and humans) or populations³⁻⁸. In animals, as is the case in people, antibiotics are used for therapy as well as control of bacterial infections. In contrast to human medical therapy in intensively reared food animals antibiotics are for practical reasons usually administered to the whole flock and not just to individual animals. In addition antimicrobial agents are continuously fed to food animals, like broilers and turkeys, for economical reasons as antimicrobial growth promoters (AMGP). Therefore the pressure by this antibiotic use to select for antibiotic resistance in bacteria in these animal populations is high. Consequently the faecal flora of food animals contains a relatively high proportion of resistant bacteria⁹. Most AMGP, however, that are or were commonly used in the Netherlands until recently, are mainly effective against Gram positive bacteria. Therefore resistance found in faecal *Escherichia coli* of food animals is likely to be mainly associated with veterinary use of antibiotics. During the slaughter of food animals resistant strains from the gut readily soil poultry carcasses. As a result poultry meats are often contaminated with (multi)resistant *E. coli*¹⁰⁻¹⁶ and also eggs¹⁷. Resistant faecal *E. coli* from poultry can reach the intestinal tract of humans not only by direct contact, but also via the food chain. These resistant bacteria can either colonise the human intestinal tract or transfer resistance genes to the human endogenous flora. In both cases this superimposes an additional load to the resistance in the intestinal flora already present in man.

The importance of this dissemination of antibiotic resistance from animals to humans remains, however, a point of much debate. Colonisation of the human in-

restinal tract with resistant *E. coli* of chicken origin has been shown in volunteers¹⁸. One of the first studies that presented evidence that animals serve as reservoir for *E. coli* found in humans was performed by Cooks et al in the early seventies¹⁹. Antibiotic resistant *E. coli* from pigs and chickens, that were brought into the hospital kitchen on raw meat, were subsequently found in the faecal flora of hospitalised patients. Spread of an antibiotic resistant plasmid pSL222-6 in *E. coli* from chickens to human handlers was described by Levy et al²⁰. The plasmid encoding for resistance to chloramphenicol, tetracycline, sulphonamides and streptomycin that was originally present in chickens was also detected in two of the fourteen faecal samples from family members living on the farm and from handlers. Others studies have also presented evidence on the spread of antibiotic resistant micro-organisms from poultry to humans in different countries. Linton et al found the same O-serotype in chickens from a commercial rearing centre, in oven-ready birds and in humans^{13,18}. Oieniji described direct transmission of *E. coli* resistant to streptomycin, sulphonamide and tetracycline from poultry to poultry caretakers in Ibadan, Nigeria^{21,22}. No drug resistant micro-organisms were isolated from workers outside the poultry plant. Administration of antibiotics to animals over long periods favoured the persistence of resistant micro-organisms and resulted in a higher incidence of infections in man caused by antibiotic resistant micro-organisms^{21,22}. Chicken have also been described as a source of antibiotic resistance in humans in northern India²³, in Morocco²⁴ and in Saudi Arabia²⁵. In the last study 27% of the *E. coli* strains isolated from patients and 11% of the chicken isolates belonged to the same serotypes. As the prevalence of multidrug resistance among isolates from chickens was significantly higher than that from patients, chickens could serve as a source for antibiotic resistance in humans. The authors strongly recommended restricting the use of antibiotics in veterinary medicine to only the treatment of bacterial infections. Recently Bass et al²⁶ described a high incidence of integrons encoding multidrug resistance among chicken isolates as part of transposon Tn21. In addition, they described the dissemination of Tn21 among pathogenic poultry isolates and suggest the possibility of the mobility of Tn21 among pathogenic mi-

cro-organisms in humans as well as in poultry. In contrast to these studies, which describe poultry as a possible source for antibiotic resistance in humans, others have concluded that human and poultry isolates belong to two distinct pools of resistant *E. coli*. In one of the first studies analysing transfer of antibiotic resistance between animals and humans Smith concluded that the antibiotic resistance transfer was limited and that animal strains were poorer than the human ones at colonising the human alimentary tract. He stated that in view of the high prevalence of antibiotic resistance in humans, animals are not an important source of resistant *E. coli* in man²⁷. Shooter et al serotyped both animal and human *E. coli* isolates using 150 O-antisera. Of the animal strains tested 289 (36%) out of 798 could be serotyped, whereas all but two out of 1580 human isolates could be typed²⁸. They concluded that strains from animal origin may comprise other O groups than those present in humans. Differences in chloramphenicol and streptomycin resistance between poultry and their caretakers in Northern India has been described²⁹ i.e. 26% versus 58% for chloramphenicol and 69% versus 94% for streptomycin.

The risk for human health after exposure or prolonged contact with animals was assessed in a population of female workers at a poultry abattoir, not receiving antibiotic therapy, but who had prolonged exposure to resistant micro-organisms of animal origin. In these workers urinary tract infections were only infrequently caused by poultry isolates. Among recent antibiotic users, the resistance patterns of uropathogens, however, matched at least one of the poultry isolates. A more detailed analysis using restriction enzyme analysis of plasmid DNA showed that none of the plasmids from human isolates appeared to be related to any of the poultry isolates³⁰. Caya et al³¹ compared the phenotypes and genotypes of *E. coli* isolates from sick broilers in abattoirs in the province of Quebec with human isolates from hospitalised patients located in the same geographic area as the abattoir (3403). A total of 142 poultry isolates were compared to 91 human isolates. A higher prevalence of resistance was found among the poultry isolates especially against gentamicin, spectinomycin, tetracycline and sulfamethoxazole. Only two poultry isolates demonstrated a possible relationship with human isolates. Comparing

E. coli isolates from a poultry processing plant in Kenya and isolates from children with diarrhoea living in close contact with poultry, Kariuki et al.³² observed differences in antibiotic resistance patterns and in the levels of multidrug resistance. The authors concluded that human and poultry isolates represent two distinct pools of resistance plasmids. A similar conclusion was drawn by Nijsten et al. comparing resistance patterns of faecal *E. coli* isolates of pig farmers and their pigs³³.

In this study the relation between antibiotic use and the prevalence of resistance in faecal *E. coli* was analysed in the following populations: broilers and turkeys, (both populations with a relatively high antibiotic use) and laying hens (with a relatively low antibiotic use). To study the possible dissemination of resistant *E. coli* or resistance genes from these poultry populations to humans the farmers, participating in the study were also requested to submit their own faecal sample. In addition faecal samples of turkey and poultry slaughterers were studied. Farmers have daily contact with their animals and are directly exposed to animal faeces. Slaughterers have daily contact with poultry carcasses or meat. All faecal samples were analysed as to the prevalence and degree of resistance in *E. coli*.

Fluoroquinolones are considered as "critical", "essential" or "last line" antibiotics for human medicine i.e. no or few alternative agents to treat serious or life-threatening infections in humans are available³⁴, and are commonly used in poultry medicine. As the possibility of transfer of ciprofloxacin resistant bacteria from animals to humans, is a hotly debated issue, ciprofloxacin resistant *E. coli* isolates from poultry and humans and turkey carcasses were genotyped using pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Collection of faecal samples

Fresh faecal samples were collected from farmers in the south of the Netherlands keeping either turkeys, broilers or hens producing eggs for human consumption. The farmers were requested to provide one fresh faecal sample from themselves and a mixed faecal sample from the oldest flock of poultry at the farm and to send these on the day of collection with the completed questionnaire to the bacteriological laboratory. In a ques-

tionnaire the farmers were asked about other animals kept at the farm, recent hospital stay and antibiotic usage by themselves, family members or their animals during the three months preceding the sample collection. In addition poultry slaughterers working at a poultry-processing plant in a similar area were asked to send in a faecal specimen of themselves and to fill in the same questionnaire. The faecal samples from the turkey farmers, their turkeys and the turkey slaughterers were the same as described previously³⁵. On the day of arrival at the laboratory the samples were diluted (10^{-1}) in 0.9% NaCl, containing 20% (v/v) glycerol and stored at -20°C until assayed. In addition 50 turkey wing tips (one a day) were collected at the end of the slaughtering line immediately after slaughtering and send frozen at -20°C to the laboratory.

Bacteriological analysis

The methods used were as previously described^{36,37}. In short, after thawing the samples, 10^{-2} and 10^{-4} dilutions in 0.9% NaCl were inoculated on Levine agar plates (BBL 11221, Becton Dickenson BV, Etten-Leur, the Netherlands) using a spiral plater (Spiral Systems, Lameris Laboratory BV, Breukelen, the Netherlands). The antibiotics and the concentrations used in the Levine agar plates are mentioned in Table 1. The antibiotics were selected because they, or related cross reacting antibiotics, have been regularly used in poultry on veterinary prescription and on basis of their activity against *E. coli*. The concentrations used were based on NCCLS guidelines and modified where appropriate to make results comparable with those of previous studies³⁶⁻³⁸. For trimethoprim testing 5% lysed horse blood was added to the agar. The turkey wings were defrosted, shaken using a turrax mixer with 20 ml of peptone water and 1 ml of the peptone water was plated only onto a ciprofloxacin (4 mg/l) containing agar plate.

Susceptibility testing

From each faecal sample one *E. coli* colony was randomly chosen from the control plate without antibiotics for antibiotic susceptibility testing using a micro broth dilution method in Iso-Sensitest Broth™ (Oxoid CM473, Basingstoke, UK) using an inoculum size of 5×10^5 cfu/ml. The antimicrobial agents tested

and the breakpoints for resistance were as follows: amoxycillin (16 mg/l), chloramphenicol (16 mg/l), ciprofloxacin (4 mg/l), flumequine (8 mg/l), gentamicin (8 mg/l), neomycin (16 mg/l), nitrofurantoin (64 mg/l), oxytetracycline (16 mg/l), streptomycin (32 mg/l), sulfamethoxazole (128 mg/l) and trimethoprim (16 mg/l). *E. coli* ATCC 25922 was used as reference strain.

PULSED-FIELD GEL ELECTROPHORESIS

Ciprofloxacin resistant *E. coli* isolates were genotyped using PFGE after *Xba*I digestion. PFGE was performed as previously described with minor modifications³⁹. The criteria of Tenover were used⁴⁰ to assess similarity of the different patterns obtained.

DEFINITIONS

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

The degree of resistance of each sample was calculated as the ratio between the number of colonies on the agar plates with and without antibiotics multiplied by 100%. Two degrees were distinguished: a high degree of resistance i.e. ratio >50% being defined as the majority of the *E. coli* isolates of a sample showing resistance to a particular antimicrobial agent. A ratio of less than 50% was defined as a low degree of resistance.

STATISTICAL ANALYSIS

To assess significant difference in prevalence and high degree of antibiotic resistance between the different populations the chi-square test was used.

RESULTS

Study population

In total 47 faecal samples were received from turkey farmers (i.e. about 50% of the major turkey farmers in the Netherlands) and their turkeys and also 47 samples from turkey slaughterers. About 50% of the poultry slaughterers responded and 30% of the poultry farmers. In total faecal samples from 46 poultry slaughter-

ers, 51 broiler farmers and 50 broilers, 25 laying hen farmers and 25 laying hens were received. One broiler farmer had no broilers on the farm at the time of sample collection and only sent a faecal sample of himself. None of the turkey farmers and slaughterers, or a family member had been hospitalised or used antibiotics in the three months proceeding sample collection. Two poultry slaughterers and one laying hen farmer had been hospitalised in the last three months. Antibiotic use during that time span was mentioned by four broiler farmers and slaughterers and by two of their respective family members. Laying hen farmers had not use antibiotics themselves but a few family members had during the three month preceding the study.

Prevalence of resistance

Of the three poultry populations tested, the highest prevalence and degree of resistance (to almost all compounds tested) was observed in the faecal samples from turkeys. This was closely followed by those from broilers, but distinctly lower in the laying hen population (Table 1). The prevalence of resistance for ciprofloxacin, flumequine and neomycin was significantly higher ($p < 0.005$) in turkeys and broilers than in laying hens. Nitrofurantoin resistance was only found in turkey isolates. For amoxycillin and oxytetracycline the percentage of samples with a high degree of resistance among turkey and broiler isolates was significantly higher ($p < 0.005$), compared to laying hens.

In the human populations the same tendency was observed. Turkey farmers showed the highest resistance percentages to all agents tested both in terms of prevalence and high degree of resistance. The lowest resistance percentages were observed in the laying hen farmers. The prevalence of resistance in turkey and broiler farmers was significantly higher than in laying hen farmers for amoxycillin, ciprofloxacin, flumequine, neomycin, oxytetracycline and trimethoprim. In the human populations tested the only high degree of resistance was against quinolones in turkey and broiler farmers. The resistance percentages in turkey slaughterers and poultry slaughterers were of a similar magnitude except for resistance to neomycin, which was significantly lower in turkey slaughterers ($p < 0.05$). The observed prevalence of resistance was significantly higher in slaughterers than in laying hen farmers for

Table 1. Prevalence (Prev) and percentage samples with a high degree (HD) of antibiotic resistant faecal *Escherichia coli* from poultry, poultry farmers and poultry slaughterers.

Antimicrobial agent (concentration mg/l)	Turkeys (n = 47)		Broilers (n = 50)		Laying hens (n = 25)		Turkey farmers (n = 47)		Broiler farmers (n = 51)		Laying hen farmers (n = 25)		Turkey slaughterers (n = 47)		Broiler slaughterers (n = 46)	
	Prev	HD	Prev	HD	Prev	HD	Prev	HD	Prev	HD	Prev	HD	Prev	HD	Prev	HD
Amoxycillin (25)	87	32	82	24	68	4	66	19	57	22	32	4	45	9	48	20
Ciprofloxacin (4)	45	0	50	0	0	0	23	4	8	2	0	0	0	0	7	0
Flumequine (16)	66	0	64	0	8	0	26	4	14	2	0	0	0	0	9	0
Neomycin (32)	81	0	72	2	24	0	57	6	20	2	8	0	32	0	54	15
Nitrofurantoin (50)	13	0	0	0	0	0	2	0	2	2	0	0	0	0	0	0
Oxytetracycline (25)	87	51	78	26	76	12	79	28	61	16	36	4	55	19	43	13
Trimethoprim (8)	85	17	80	20	68	12	72	15	47	14	28	4	38	11	37	11

Table 2. Prevalence of (multi)resistant *Escherichia coli* (%) in the different populations studied.

Number of antibiotics resistant to	Turkey (n = 43)	Broilers (n = 45)	Laying hens (n = 20)	Turkey farmers (n = 45)	Broiler farmers (n = 42)	Laying hen farmers (n = 20)	Turkey Slaughterers (n = 45)	Poultry slaughterers (n = 39)
0	7 (16)	9 (20)	13 (65)	15 (33)	17 (40)	11 (55)	15 (33)	16 (41)
1	8 (19)	5 (11)	3 (15)	13 (29)	7 (17)	1 (5)	15 (33)	8 (20)
2	7 (16)	7 (16)	0	4 (9)	3 (7)	5 (25)	1 (2)	5 (13)
3	2 (5)	7 (16)	3 (15)	2 (4)	3 (7)	0	3 (7)	1 (3)
4	5 (12)	7 (16)	1 (5)	5 (11)	2 (7)	2 (10)	4 (9)	5 (13)
≥ 5	14 (32)	10 (23)	0	6 (14)	9 (22)	1 (5)	7 (15)	4 (10)

Table 3. Most prevalent resistant patterns found in *Escherichia coli* isolates in the different populations studied.

Antibiotic(s)	Turkeys	Broilers	Laying hens	Turkey farmers	Broiler farmers	Laying hen farmers	Turkey slaughterers	Poultry slaughterers
Amoxycillin	5	2	—	8	2	—	11	1
Oxytetracycline	2	2	2	3	2	—	4	1
Amoxycillin + oxytetracycline	5	—	—	2	1	—	1	1
Streptomycin + sulfamethoxazole	—	—	—	—	1	4	—	1
Amoxycillin + oxytetracycline + streptomycin + sulfamethoxazole + trimethoprim	6	2		3	1		2	1
Amoxycillin + oxytetracycline + sulfamethoxazole			2					
Amoxycillin + streptomycin + sulfamethoxazole + trimethoprim					1			2
Amoxycillin + oxytetracycline + streptomycin + trimethoprim		1			2			

neomycin, and oxytetracycline. Also the percentage of samples with a high degree of resistance was significantly higher for oxytetracycline and trimethoprim and only in the broiler slaughterer population also for amoxycillin and neomycin ($p < 0.05$).

Antibiotic susceptibility

In approximately 10% of the human and animal samples no *E. coli* grew on the antibiotic free control plate. This means that in these samples were less than 300 cfu *E. coli* were present per gram of faeces (minimum detection level).

Resistance to five or more antibiotics was found with the highest frequency among isolates from turkeys (32%), followed by broilers (23%) and broiler farmers (22%) as shown in Table 2. The majority of the isolates from laying hens and laying hen farmers were susceptible to all antibiotics tested (65% and 55% respectively). For the turkey and broiler isolates the percentage of isolates, that were susceptible to all antibiotics were significantly lower (16% and 20% respectively).

The most prevalent resistance patterns (maximally 5) of each population are depicted in Table 3, and compared with the prevalence of these patterns in other populations. The resistance patterns most frequently observed, (especially among turkeys, turkey farmers and turkey slaughterers) were resistance to amoxycillin alone and in combination with oxytetracycline, streptomycin, sulfamethoxazole and trimethoprim. Resistance to oxytetracycline alone was found in isolates of all populations studied with exception of the laying hen farmers and oxytetracycline resistance in combination with amoxycillin mainly in turkey isolates.

The most frequent resistance pattern observed from laying hen farmers i.e. resistance to streptomycin and sulfamethoxazole was not found among laying hen isolates. The resistance patterns found in turkey isolates was similar to those of turkey farmers and slaughterers. Resistance patterns of broilers isolates were in general also found in isolates from broiler farmers and slaughterers.

Pulsed-field gel electrophoresis patterns

The obtained PFGE patterns of the ciprofloxacin resistant isolates from turkeys ($n = 21$), turkey farmers ($n = 11$), broilers ($n = 25$) and broiler farmers ($n = 4$) were

Figure 1. Pulsed-field gel electrophoresis patterns of a ciprofloxacin resistant *E. coli* isolated from Turkey (T) and Turkey Farmers (TF) and Turkey Wings (TW) after digestion of total DNA with *Xba*.

Lane 1: molecular weight marker. Lane 2: T1, Lane 3: TF1, Lane 4: T12, Lane 5: TF12, Lane 6: T47, Lane 7: TF47, Lane 8: T27, Lane 9: T24, Lane 10: TF26, Lane 11: TF29, Lane 12: TF45, Lane 13: T28, Lane 14: TF10, Lane 15: TF28, Lane 16: TW, Lane 17: TW, Lane 18: TW

quite heterogeneous. Of the isolates from the turkey and turkey farmer populations 27 patterns could be discriminated. Five patterns were found both in turkeys and turkey farmers. The patterns of three farmer isolates were identical to those of the turkeys of the same farm. The three ciprofloxacin resistant *E. coli* isolated from the turkey wings showed the same pattern as a farmer isolate (Figure 1). Also 2 broilers and broiler farmer isolates showed similar patterns but the isolates were not from the same farm.

DISCUSSION

The major factor selecting for antimicrobial resistance in bacteria is antibiotic use. For dissemination of resistant bacteria and resistance genes in populations, in addition to antibiotic consumption, important factors are crowding and poor sanitation. These three factors are typical features of intensive farming units. The prevalence and degree of resistance among bacteria of the intestinal flora of intensively reared animals is high⁹. Ojeniyi found all 3444 *E. coli* isolates from battery

hens to be multiresistant to antibiotics compared to none of 2284 isolates from free ranging chickens²¹. Antibiotics are seldom given to laying hens producing eggs for human consumption. This is because in these adult animals less bacterial infections occur than in young animals like broilers and also farmers are reluctant to give antibiotics because the possibility of antibiotic residues in eggs. Therefore the major difference with respect to factors favouring selection and dissemination of antimicrobial resistance between the turkey and broiler population compared to the laying hen population, was that laying hens received few antibiotics. However, during the rearing period of these animals antibiotics are also commonly used and it has been shown that resistance in *E. coli* in the avian intestinal tract may persist for a long time even in the absence of selection by antibiotic use⁴¹. For the five tested human populations these three key factors were, however, not different, i.e. similar antibiotic consumption in each group.

Significant higher prevalences and degrees of antibiotic resistance for nearly all antibiotics tested were observed in the turkey and broiler populations, compared to the laying hens. Resistance to 5 or more antibiotics occurred commonly in turkey and broiler isolates but not in laying hen isolates. These differences clearly reflected more recent antibiotic usage in broilers and turkeys.

Similarly, a higher prevalence of resistance was found among faecal samples and single isolates from both turkey and broiler farmers and slaughterers compared to those from laying hen farmers. Moreover the same resistance patterns were found in turkeys, turkey farmers and slaughterers and in broilers, broiler farmers and broiler slaughterers. Dissemination of resistant bacteria and/or resistance plasmids from turkeys and broilers to their respective farmers is the most likely explanation for the high antimicrobial resistance observed in the faecal *E. coli* of the farmers and slaughterers as there had been no difference of any importance in antibiotic consumption among these populations and the use of antibiotics was also very low. Both the prevalence and high degree of resistance to neomycin was very high in the broiler slaughterers compared to other human populations. As neomycin resistance was higher in turkeys than in broilers, it is unlikely that this

was caused by direct contact with poultry meat products. However, the same phenomenon has been observed in another study in pig slaughterers⁴². The common use in that specific abattoir of neomycin containing ointments for treatment of minor cuts and skin diseases, both occupational diseases for slaughterers, could have provoked this high resistance. No information, however, about the use of ointments could be obtained from the two abattoirs in the present study.

A striking observation was the difference in resistance to ciprofloxacin i.e. respectively 45% and 50% for turkey and broiler isolates, 25% and 8% for their respective farmers and 7% for broiler slaughterers. These relatively high percentages especially among the animal isolates were very likely due to the therapeutic use of fluoroquinolones in poultry. The use of flumequine and enrofloxacin accounts for 14% of all antibiotic use in poultry, especially in turkeys and broilers⁴³. Enrofloxacin is a methylester of ciprofloxacin and both molecules are completely cross resistant. Flumequine, the first fluoroquinolone developed, might select for low level resistance against ciprofloxacin just as nalidixic acid does^{44,45}. In the Netherlands fluoroquinolones usage in humans is low and resistance in faecal *E. coli* in the open population is nearly absent^{38,42,46}. Similarly, because no formulation for mass medication of pigs is on the market in the Netherlands, enrofloxacin use in pigs is low and the prevalence of resistance of faecal *E. coli* in pigs is approximately two percent, in pig farmers one percent and in pig slaughterers less than one percent^{37,42,47}.

As resistance to fluoroquinolones occurs by chromosomal mutations, all sufficiently large populations of bacteria will probably contain small numbers of spontaneously resistant mutants, which may then undergo clonal expansion under the selective pressure of fluoroquinolone use resulting in a increased prevalence of resistance. This will in first instance only occur within the population, but as the numbers of resistant bacteria within that population (degree of resistance) increase the chance of spilling over to other populations became larger. This is what likely has occurred in the Dutch poultry and poultry farmer populations. Absence of resistance among poultry and turkey slaughterers suggested that daily intensive contact with animals and their faeces, like farmers have, is a higher risk

factor for transfer of resistant clones than handling poultry carcasses and meat.

A similar course of events have been described in Spain, where the increased use of fluoroquinolones for therapy in humans, was followed by increase in resistant *E. coli* from urinary tract infections up to 18%⁴⁸,⁴⁹. Prior exposure of a patient to a fluoroquinolones was the single most important risk factor for an infection, mostly urinary tract infections, with a fluoroquinolones resistant *E. coli*. Further analysis as to the prevalence of fluoroquinolone resistant *E. coli* in the intestinal tract of healthy persons showed a prevalence of 24% in adults and of 26% in children. Carriership in the healthy population was not correlated with previous quinolone use, which constituted a strong argument for the pre-existence of resistant strains in the community.

As the prevalence of fluoroquinolone resistance in faecal *E. coli* of Spanish chickens and pigs is very high: 90% and 45% respectively, they postulated that food animals were the primary reservoir for fluoroquinolone resistance in humans. Fluoroquinolone use in humans might have caused a further selection in the human intestinal tract and a secondary dissemination in the human population. However, as they did not genotype the resistant isolates, they could not prove that clonal transmission had occurred.

In this study PFGE of ciprofloxacin resistant isolates from turkey and turkey farmers, and broiler and broiler farmers showed a variety of patterns but in two combinations of a turkey farmer and his turkeys and in one broiler farmer and a broiler identical patterns were observed, which showed that identical clones were present in humans and in poultry. Moreover, the ciprofloxacin resistant *E. coli* isolates from the turkey wings tips showed similar patterns as in those from turkey farmers.

Because of the relatively low sensitivity of the method used in this study (only one *E. coli* was tested from each sample), it is possible that clonal transmission of resistant bacteria from animals to humans is occurring much more frequently than the data in this study suggest. The results in the present study strongly suggest a spread of antibiotic resistant *E. coli* from animals to people. High antibiotic use in turkeys and broilers appears to be the cause for the high resistance

against antibiotics in the faecal flora of these animals and appears to be a risk for the respective farmers. Most likely these resistant bacteria have also been disseminated but at a lower scale to the consumers of poultry meats and therefore are present in the Dutch human population at a low level. Therefore it is likely that further increase of fluoroquinolone use in human primary health care will not only be followed by a clonal expansion of these resistant strains, that are already present in the human intestinal flora, but that, as a result the isolation of fluoroquinolone resistant *E. coli* from infections in humans will increase, as already has been noticed among uropathogenic *E. coli* isolates⁵⁰.

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Chapter VII

The effect of flavophospholipol on the prevalence and degree of resistance of the faecal flora of fattening pigs under field conditions, using *Escherichia coli* and enterococci as indicator bacteria

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ABSTRACT

A "plasmid curing effect" of multiresistant *Escherichia coli* by flavophospholipol, an antibiotic used as antimicrobial growth promoter (AMGP) in animal feeds, has been described to occur *in vitro* and *in vivo*. To study this effect of flavophospholipol under field conditions the prevalence and degree of resistance (proportion of resistant strains to the total numbers present per g faeces) of indicator bacteria: *E. coli* and enterococci, was determined in faecal samples of three groups of pigs originating from the same breeder and fattened at the same commercial farm in three different compartments under identical conditions. During the last 12 weeks before slaughtering the pigs in compartment A were fed a commercial finisher feed without any AMGP (negative control group), in compartment B the same feed with 9 mg/kg flavophospholipol (study group) and in compartment C with 15 mg/kg avoparcin (positive control). Faecal samples of each pig were collected at the start and at the end of the study and assessed for the prevalence and degree of resistance against antibiotics either commonly used for therapy in pig medicine or as AMGP. Before the start of the study all pigs were colonised with multiresistant *E. coli* by mixing three resistant pig isolates through their feed after a three day course with lincomycin and spectinomycin. Antibiotic use either for prevention or for therapy in the three groups of pigs was similar during the study period.

At the end of the study no significant difference in performance (technical results) between the three groups were observed. The overall prevalence and degree of resistance of *E. coli* in the faecal flora increased significantly in groups A and C, but remained at the same level as at the start of the study in group B. Before the study the numbers of vancomycin resistant enterococci (VRE) in all three groups were below the detection limit of the laboratory methods used. At the end of the study the prevalence of vancomycin resistance was 44% and 41% in group A and B respectively but only very low numbers of VRE per g faeces were found. In the avoparcin fed group the prevalence was 72% and in 57% of the samples more than 50% of all enterococci present were vancomycin resistant. Only in the flavophospholipol exposed group the prevalence of

resistant *E. faecalis* increased from 23% before the start of the study to 43% at the end of the study. It was concluded that flavophospholipol effectively suppresses the augmentation and dissemination of multiresistant *E. coli* in the intestinal flora of fattening pigs and that avoparcin use strongly selects for VRE. Because flavophospholipol nor any related molecule is used therapeutically, no cross-resistance with therapeutic antibiotics exists and no transmissible resistance has been shown, the benefit of a major decrease of resistance in intestinal *E. coli* of flavophospholipol fed animals seems to outweigh the small increase of the risk of transfer of flavophospholipol resistant *E. faecalis* from animals to humans via the food chain.

INTRODUCTION

The interest in antimicrobial resistance has increased considerably in recent years, mainly because of resistance problems in hospitals^{25,44,46}. Despite the fact that these problems are largely caused by the inevitable high usage of antibiotics in hospital settings, it has become clear that the selective pressure of antibiotic use in other populations e.g. healthy people outside hospitals and animals, also contributes to the problem^{37,38,53}. Many hospital infections these days are so called endogenous infections, which means that immunocompromised patients are infected by their own intestinal flora. The prevalence and degree of resistance found in indicator bacteria of the intestinal flora: *Escherichia coli* and enterococci, of different human populations are proportional to the amounts of antibiotics used by these populations and correlate with the classes of antibiotics used. Moreover they are predictive of the resistances to be found in human pathogens isolated from the same populations^{37,39}. Therefore it has been proposed that a low level of resistance in the endogenous flora of man should be considered a public health goal like a normal blood pressure and low cholesterol levels³⁷.

Humans in close contact with food animals like farmers have a higher prevalence and degree of resistance in their faecal flora, closely related to the resistances found in their animals⁶⁹. Molecular microbiological techniques have made it feasible to trace the transfer of resistant bacteria and resistance genes from

these animals via the food chain to the human endogenous flora and finally to human pathogens. This has been shown by several authors^{30,31,66}.

Therefore, a low level of resistance in the intestinal flora should be considered as a safety and benchmark for food animals⁶⁹. Moreover, not only will this protect public health, but it might also help to safeguard the future efficacy of antibiotics used in animals on veterinary indication.

The most effective way to decrease the prevalence and degree of resistance in faecal indicator bacteria is to reduce the amount of antibiotics used in a population. In food animals antibiotics are not only used for therapy and prevention of bacterial infections, but are also added continuously to animal feeds as antimicrobial growth promoters (AMGP) to enhance growth, improve feed conversion and to decrease waste production. In Europe more than 30% of all antibiotics used in animals are used for growth promotion, but in some countries this percentage is even higher e.g. in the Netherlands nearly 50%⁶⁸. This continuous feeding of antibiotics to animals causes a strong selection force for resistance against these and related antibiotics in particular (cross-resistance) and via co-selection for multiresistance against other antibiotics for which the resistance genes are located on the same plasmid. This is the reason that it has been proposed from several sides to phase out the usage of antibiotics as AMGP^{12, 32,51,58,64}.

The most important way of emergence and dissemination of resistance in bacteria in the gut, is the spread of plasmids carrying resistance genes among and between the many species of bacteria in the intestinal flora of animals. So if there would be a safe substance that could be fed to animals and either eliminates resistance plasmids from bacteria or prevents transfer of these plasmids between intestinal bacteria, the prevalence and degree of resistance of bacteria in the intestinal flora of these animals might be reduced significantly.

A plasmid curing effect in *E. coli* and other enterobacteriaceae has been described for mitomycin, rifampicin and flavophospholipol, and, for non antibiotic substances such as acridine orange, acriflavine and nitroacridine II^{9,14}. Of these molecules only flavophospholipol has been licensed for use in pigs, calves

and poultry. Further research, however, has shown that flavophospholipol, as described for another phospholipid antibiotic macarbomycin⁴¹ had no plasmid curing effect but suppressed plasmid transfer depending on the plasmid involved and selectively inhibited the growth of bacteria harbouring certain plasmids^{36, 59}. *In vitro* flavophospholipol decreased the transfer frequency of some R-plasmids in *E. coli*, but had no effect on the transfer of other types of R-plasmids. It also inhibited the growth of bacteria harbouring certain R-plasmids but not had no effect on other strains^{23, 57,77}. These differences are most likely caused by an increased susceptibility for flavophospholipol of bacteria with sex pili and pilin protein precursors in the bacterial cell wall⁷⁷. Corpet, using a mouse model showed that animals given flavophospholipol in their drinking water, had significantly less tetracycline resistant *E. coli* in their faeces than control animals¹³.

Also in studies with limited numbers of pigs and calves, addition of flavophospholipol as AMGP to the feed caused a decrease in resistance against ampicillin, tetracyclines, streptomycin and/or sulphonamides in faecal *E. coli* and/or salmonellae^{15-17,56,60,61}. Rifampicin has also been shown to decrease the prevalence of multiresistant *E. coli* in the faecal flora of pigs³³.

Flavophospholipol, also known as bambermycins or moenomycin, is a phosphorus containing glycolipid antibiotic in which the lipid moiety (moenocinol) is a C-25 compound structurally analogous to undecaprenylphosphate⁵⁴ and is produced by a group of *Streptomyces* spp. including *S. ghanaensis*^{14,29,63,76}. It inhibits the peptidoglycan synthesis in the cell wall by inhibiting peptidoglycan polymerases through impairment of the transglycolase activities of penicillin binding proteins. (PBPs)^{28, 73, 74}. There is, however, no cross resistance between β -lactam antibiotics and flavophospholipol, as they act on different PBPs⁵⁰.

Flavophospholipol is mainly effective against Gram positive bacteria, because it cannot penetrate the outer membrane of Gram-negative bacteria. However, some activity against *Pasteurella* spp. and *Brucella* spp. has been reported²⁸. Its spectrum is similar to that of penicillin and to some extends to that of macrolides. It covers staphylococci and streptococci. Enterobacteriaceae such as salmonellae and *E. coli*, are only slightly susceptible³⁶.

As flavophospholipol is not used therapeutically no official breakpoints for resistance have been determined and very few data on acquired resistance against flavophospholipol are available. *Clostridium perfringens* and *E. faecium* are considered intrinsically resistant^{18,21,22} but some susceptible strains of *E. faecium* have been reported recently². Differences in PBPs between the different species of enterococci might explain the different susceptibilities among enterococcal species⁷⁸. In a large collection of *Staphylococcus aureus* isolates from chickens and chicken slaughterers, no resistance against flavophospholipol was detected²⁷.

No publications about genes conferring resistance to flavophospholipol, transfer of resistance or other resistance determinants in bacterial hosts were found. So far no cross-resistance with other non phosphorolipid class antibiotics has been shown.

At the moment it is used as AMGP in pig, calf and poultry feeds only. Neither flavophospholipol nor any related molecules are used for therapy in human or veterinary medicine. It is not absorbed to any great extent from the gut after oral administration^{6, 52, 76} and when parenterally administered it is excreted unchanged via the urine but at a very slow rate⁵². It has, therefore, a very prolonged activity in blood⁷⁶. In chickens given feed containing 20 mg per kg flavophospholipol no residues were detected in tissues and organs⁴³.

Limited information is available on the effect of flavophospholipol containing feeds on the intestinal flora of food animals and the results of studies are variable. In studies with pigs and calves the duration and the numbers of salmonellae excreted were reduced¹⁵⁻¹⁷, but in other studies using chickens a decrease⁷, no effect²⁴ (Humpert 1991 #84), or a slight increase in excretion was observed⁵⁵. Flavophospholipol has been shown to reduce the numbers of *C. perfringens* in the faecal flora of chickens despite its unsusceptibility *in vitro*^{7, 62}.

AIM OF THE STUDY

This study was performed to study the effect under field conditions of the use of flavophospholipol (9 mg/kg FlavomycinTM, Intervet International, Wiesbaden, Germany) as AMGP in pig feed on the preva-

lence and degree of antibiotic resistance of *E. coli* and enterococci in the faecal flora of fattening pigs.

MATERIALS AND METHODS

Animals

Three groups of fattening pigs, originating from the same breeder were individually weighed at arrival and, based on weight basis and gender, equally divided in three groups of 56 animals. Each group was housed in a separate compartment of the same pig stable under identical conditions. The animals had free access to feed and water was supplied twice daily. During the first four weeks after arrival at the farm all pigs were fed a starter pig diet containing 100 mg/kg tylosin (Tylan-100 premix 10%TM, Elanco Animal Health, Nieuwegein, the Netherlands) and from week 5 until slaughtering at week 17 the same commercial finisher pig feed with the exception of differences in inclusions of AMGP. The pigs in compartment A (negative control) received feed containing no AMGP, in compartment B (study group) feed containing 9 mg/kg flavophospholipol and in compartment C (positive control) feed with avoparcin (15 mg/kg).

Once daily the animals were observed by an animal caretaker for general health status and for any clinical symptoms of disease, to insure constant feed and water supply and check the proper functioning of heating and ventilation systems. The amounts fed to the pigs were recorded separately for each compartment. During the loading of the truck for transport to the abattoir all pigs were individually weighed again.

COLONISATION OF THE ANIMALS WITH MULTIRESISTANT *E. COLI*

Immediately after arrival all animals were treated orally with a lincomycin-spectinomycin combination (Lincospectin 12.5%TM, Pharmacia & Upjohn Animal Health, Woerden, the Netherlands) for three days to disturb the intestinal flora and facilitate colonisation by multiresistant *E. coli* strains. Lincospectin 12.5%TM (240 g) was dissolved in 2400 ml water. One hundred ml of this solution was thoroughly mixed through 7 kg of pig feed and the feeding trough of each pen was filled with this mixture on day 1, 2, and 3. Cultures of three

multiresistant non-pathogenic *E. coli* strains, originally isolated from pig faeces in a former study⁴⁹ were prepared by growing them overnight in 800 ml of brain heart infusion (CM225 Oxoid, Basingstone, UK). The next morning the three bottles were mixed together resulting in 2400 ml of an *E. coli* mixture containing approximately 0.5×10^9 CFU of each strain per ml. Of this mixture 13 ml was mixed through 1 kg of pig feed. After emptying the troughs, if necessary, in the morning of day three, 7 kg of feed, mixed with the bacterial suspension containing 4×10^{11} CFU *E. coli* and the antibiotic solution, was given to each pen containing 7 animals. On day 4 and 5 again a freshly prepared culture identical to that used on day 3 was mixed through the feed, but without the antibiotic solution and again 7 kg were supplied to each pen.

FAECES SAMPLING

During week 4 individual faecal samples of approximately 25 g from all pigs present in the three compartments were collected directly from the anus in plastic containers marked with the identification code of the pig and stored in an icebox for transport to the laboratory. At the day of collection the faecal samples were diluted 1:10 in peptone water containing 20% (v/v) glycerol, kept overnight at +4°C, then frozen at -70°C and stored subsequently at -20°C until assayed. This was done in the laboratory. The same procedure was repeated in week 10 of the study and just before slaughtering.

BACTERIOLOGICAL ANALYSIS

After thawing the samples were inoculated on selective media with and without antibiotics. For *E. coli* 37 µL of 10^{-1} , 10^{-3} and 10^{-5} dilutions in 0.9% sodium chloride solutions were inoculated on Eosin Methylene Blue AgarTM (EMBA) plates (CM 69 Oxoid) using a spiral plater (Salm en Kip, Utrecht, the Netherlands); for enterococci KF-Streptococcus agarTM plates (Oxoid CM701) with the 10^{-1} , 10^{-2} and 10^{-3} dilutions were used. For trimethoprim testing 5% lysed horse blood was added to the agar. The antibiotics tested and concentrations used are shown in Tables 2 and 5. These antibiotics were selected because of common use

in human and veterinary medicine or as AMGP or cross resistance with AMGP.

E. coli grows on EMBA as purple colonies with a black centre and metallic shine. Only these colonies were counted after 18 - 24 hrs incubation at 37°C. The minimum detection level was 300 colony forming units (cfu) per gram faeces and it has been shown that more than 95% of the in this way presumptively identified colonies are *E. coli*⁴⁸. Previous studies have also shown that all isolates from antibiotic containing plates had MICs identical or higher than the concentration of the antibiotic in the selective plate from which they had been isolated^{8,40,49}.

Enterococci appear as typical red or pink colonies on KF-Streptococcus agarTM. After 48 hrs incubation at 42°C only the typical pink colonies were counted. One randomly sampled typical colony was collected from all KF-Streptococcus agar plates without antibiotics, subcultured on blood agar (Oxoid CM854 with 5% sheep blood) and identified using tolerance for bile, aesculin hydrolysis, growth on 6.5% NaCl (w/v) and a positive pyrolidonyl-arylamidase reaction (pyrolidonyl-arylamidase tablets, Rosco, Denmark). All isolates proved to belong to the genus *Enterococcus*. The minimum detection level was 300 cfu/g faeces. For the prevalence of resistance against flavophospholipol a specimen was only considered positive if at least one *Enterococcus faecalis* was isolated and identified from the flavophospholipol containing agar plate. For this reason, for flavophospholipol only the prevalence of resistance could be calculated. In a previous study using the same methodology all enterococci had MIC identical or higher than the concentration of the antibiotic in the selective plate from which they had been isolated⁶⁷.

IDENTIFICATION AND MIC DETERMINATIONS OF ENTEROCOCCAL ISOLATES

From each antibiotic free control plate one colony was randomly picked and identified using API-strepTM (Api-Benelux, Den Bosch, the Netherlands). If necessary in addition acid production from different sugars was determined according to the criteria of Devriese^{19, 20}.

The MIC of the isolates were determined using two fold dilutions of antibiotics incorporated in Iso-

Sensitest agarTM (CM 471, Oxoid). Using a Steers inoculator plates were inoculated (droplets of approximately 2 μ L and 52 droplets per plate) with an overnight culture of the individual isolates in brain heart infusion (CM225, Oxoid) diluted to an optical density of approximately 0.5 McFarland and read after 24 hrs incubation at 37°C. *E. faecalis* (ATCC 51299) was used as control strain.

STATISTICAL ANALYSIS

To estimate the differences in prevalence and percentage of high degree of resistance between the groups a Pearsons Chi-square and, where appropriate, a Fishers exact test was used. For the degree of resistance a one-way analysis of variance was used to estimate overall differences between the group means. Group means were compared in pairs using the Student t-tests to control for overall error rate (Bonferroni test) and $p < 0.05$ was regarded as statistically significant.

DEFINITIONS

The prevalence of resistance (%) in the population for a certain antibiotic is calculated as the number of samples showing growth of *E. coli* or enterococci on the plates containing that antibiotic, divided by the total numbers of samples tested $\times 100\%$.

The degree of resistance (%) of each faecal sample to each of the antimicrobial agents tested was calculated as the number of cfu growing on the plate containing that agent divided by the total number of typical colonies on the antibiotic free control plate $\times 100\%$.

Two degrees of antibiotic resistance to a particular antibiotic could be distinguished:

low degree of resistance, i.e. less than 50% of the total number of the indicator micro-organisms present per gram faeces was resistant and

high degree of resistance, i.e. 50% or more (thus the majority) of the indicator micro-organisms being resistant to that agent. The prevalence of high degree (%) is the number of samples with a high degree of resistance to a particular antibiotic divided by the total number of samples tested $\times 100\%$.

RESULTS

During the study six animals died: one in group A, 2 in group B and 3 in group C, mostly of endocarditis. Apart from some respiratory problems in individual animals, a few abscesses and a broken pelvis, there were no serious health problems observed during the study. Numbers of individual antibiotic treatments for either respiratory problems or severe wounds caused by fighting were 4 injections in group A, 8 in group B and 8 in group C. There was no statistically significant difference in performance between the three compartments.

E. coli

The total numbers of *E. coli* in the faecal samples of all the pigs collected at the start of the study and of the pigs in the different compartments at the end of the study are depicted in Table 1. At the start of the study there were no differences between the three compartments (data not shown). In compartment B the total numbers of *E. coli* were significantly lower at the end of the study than at the start and in compartment A the total number was higher ($p < 0.05$). For compartment C there was no difference. At the end of the study the total numbers of *E. coli* in compartment A were significantly higher than in compartments B and C ($p < 0.05$).

The prevalence of resistant *E. coli* and the percentage of samples with a high degree ($> 50\%$) of resistant *E. coli* in the faecal samples of the pigs collected at the start of the study and at the end of the study are shown in Table 2. No differences between the three compartments were observed at the start of the study (data not shown). There was no difference in prevalence of resistance at the start compared with the prevalences found at the end of the study in all three compartments for ampicillin, streptomycin, oxytetracycline, trimethoprim, sulfamethoxazole and the combination of ampicillin and oxytetracycline. The prevalence of chloramphenicol resistance increased significantly ($p < 0.001$) during the study in all three compartments; neomycin resistance, however, increased only in compartments A and C ($p < 0.001$) and gentamicin resistance only in compartment A ($p < 0.001$).

At the end of the study the prevalence of resistant *E. coli* in faecal samples of compartment B was significantly lower for neomycin and gentamicin as com-

Table 1. Total numbers of *E. coli* ($10^{\log}$) per gram faeces of all pigs at the start of the study and of the different compartments A, B and C at the end of the study.

Compartment	Mean	SEM ¹	Range
Start total of all pigs (n = 168)	6.50	0.10	3.94-9.42
End compartment A (n = 55)	6.87	0.10	5.43-8.63
End compartment B (n = 54)	6.24	0.10	4.96-7.66
End compartment C (n = 53)	6.41	0.08	4.90-7.48

¹Standard error of the mean**Table 2.** Prevalence (Prev) of resistant *E. coli* and percentage of samples with high degree (HD) of resistance in faecal samples of pigs.

Antibiotic	Concentration in agar mg/L	Start of study		End of study					
				Compartment A		Compartment B		Compartment C	
		n* = 165		n = 55		n = 54		n = 53	
		Prev	HD	Prev	HD	Prev	HD	Prev	HD
Ampicillin	50	99	11	100	89	100	7	100	81
Streptomycin	25	99	29	100	73	100	26	100	74
Neomycin	25	36	0	98	0	44	0	66	0
Gentamicin	25	21	0	78	0	13	0	17	0
Oxytetracycline	25	99	43	100	89	100	20	100	85
Chloramphenicol	25	66	2	100	2	96	0	98	2
Trimethoprim	8	99	14	100	40	98	17	100	42
Sulfamethoxazole	150	99	38	100	78	100	39	100	62
Amoxy/oxytetra	50/25	99	4	100	75	100	4	100	70

* number of samples

pared to compartments A and C ($p < 0.001$) and for neomycin in compartment C in comparison with compartment A ($p < 0.001$).

For all antibiotics with a high degree of resistance in the faecal *E. coli* of the pig samples in this study the percentages of high degree samples were lower in compartment B than in the compartments A and C. The high degree percentages in compartment B were not significantly different from those found in the faecal samples of all pigs at the start of the study except for oxytetracycline which were significantly lower ($p < 0.05$). In contrast all percentages of samples with a high degree of resistance in compartments A and C were significantly ($p < 0.001$) higher compared with the percentages at the start of the study with the exception of chloramphenicol, gentamicin and neomycin. This was significant for ampicillin, streptomycin, oxytetracycline,

trimethoprim, sulphamethoxazole and the combination of ampicillin and oxytetracycline ($p < 0.001$).

Similar differences were obtained comparing the degree of resistance found before the start of the study and in the three different groups at the end of the study (Table 3).

As a typical example the differences in ampicillin and oxytetracycline resistant *E. coli* in faecal samples of all pigs at the start of the study and at the end in the compartments A, B, and C is shown in Figure 1 by means of a boxplot.

ENTEROCOCCI

The total numbers of enterococci in the faecal samples of pigs collected at the start and at the end of the study are presented in Table 4. At the start of the study there were no differences between the three compartments

Table 3. Mean resistant strains of *E. coli* in positive samples at the start of the study (Total of compartment A+B+C) and at the end of the study in the separate compartments.

Antibiotic	Total of A+B+C (%)	Compartment A (%)	Compartment B (%)	Compartment C (%)
Ampicillin	15	82	18	73
Streptomycin	66	64	36	62
Neomycin	<1	2	2	<1
Gentamicin	<1	1	<1	<1
Oxytetracycline	45	89	31	78
Chloramphenicol	2	6	2	9
Trimethoprim	17	49	27	43
Sulfamethoxazole	36	71	43	59
Ampicillin/oxytetracycline	8	68	10	65

(data not shown). At the end of the study the total numbers of enterococci in all three compartments were significantly higher than at the start ($p < 0.05$). At the end of the study the total numbers of enterococci in faecal samples of the compartments A and B were significantly ($p < 0.05$) lower than in compartment C, but no significant difference was found between compartments A and B.

The prevalence of resistant enterococci and the percentage of samples with a high degree ($> 50\%$) of resistant enterococci in the faecal samples of pigs collected at the start and at the end of the study are shown in Table 5.

No differences between the three compartments were observed at the start of the study (data not shown). There was no significant difference in prevalence of resistance found in samples taken before the start and at the end of the study for ampicillin and erythromycin. In all the compartments the prevalence of gentamicin resistance was lower at the end of the study than at the start ($p < 0.001$), but in compartment A also significantly lower than in the two other compartments ($p < 0.01$). The prevalence of vancomycin resistance increased significantly in all three compartments ($p < 0.001$). The prevalence in group A and B increased till approximately 44% but only one animal with a high

Figure 1. Degree of resistance of *E. coli* before and after the study. **Figure 2.** Degree of resistance of faecal enterococci before and after the study.

Table 4. Total number of enterococci ($10^{\log}$) per gram faeces at the start of the study in comparison to the total number at the end of the study in the compartments A, B and C.

Compartment	Mean	SEM ¹	Range
Start total of all pigs	5.04	0.07	3.21-7.24
End compartment A	5.49	0.12	3.83-7.36
End compartment B	5.43	0.12	3.73-7.20
End compartment C	6.00	0.10	3.98-7.18

¹ Standard error of the mean.**Table 5.** Prevalence (Prev) of resistance in faecal enterococci and percentage of samples with high degree (HD) of resistance in faecal samples of pigs.

Antibiotic	Concentration in agar mg/L	Start of study		End of study					
				Compartment A		Compartment B		Compartment C	
		n* = 165		n = 55		n = 54		n = 53	
		Prev	HD	Prev	HD	Prev	HD	Prev	HD
Gentamicin	1000	63	3	7	0	26	0	25	0
Ampicillin	25	0	0	4	0	0	0	0	0
Erythromycin	8	99	80	100	85	100	98	100	96
Flavophospholipol	8	23**	(9)	13*	(0)	43*	(2)	28*	(0)
Vancomycin	8	0	0	44	0	41	2	72	57

* number of samples.

** only *Enterococcus faecalis*.

() all enterococci.

Table 6. Percentage of enterococci in faecal samples resistant at the start of the study and at the end of the study in the compartments A, B and C.

Antibiotic	Total of A+B+C	Compartment A	Compartment B	Compartment C
	(%)	(%)	(%)	(%)
Gentamicin	6	<1	<1	<1
Ampicillin	<1*	<1	<1	<1
Erythromycin	80	102	101	163
Flavophospholipol	21	2	6	1
Vancomycin	<1	1	2	71

* below detection level.

degree vancomycin resistance was observed in compartment B and the proportion of VRE on the total number of enterococci in positive animals remained low, approximately 2%. In compartment C, however, the prevalence of VRE was significantly ($p < 0.01$) higher than in A and B, 57% of the animals had a high degree of resistance and the proportion of VRE was 71% as shown in Table 5, Table 6 and Figure 2. The prevalence of flavophospholipol resistant *E. faecalis* de-

creased during the study in compartment A and increased significantly ($p < 0.05$) in compartment B.

IDENTIFICATION AND MIC DISTRIBUTION

The identifications of the single isolates of faecal samples collected before and at the end of the study are shown in Table 7. There were no significant differences in species and MIC distribution between the three compartments at the start of the study (data not shown).

Table 7. Identification of faecal enterococci from pigs before (A+B+C) and at the end of the study (compartments A, B and C).

	A+B+C	A	B	C
Species	n* = 168	n = 55	n = 54	n = 53
<i>E. faecalis</i>	11	20	2	3
<i>E. faecium</i>	35	26	32	45
<i>E. durans</i>	122	6	20	7
<i>E. gallinarum</i>	—	1	—	—
<i>E. casseliflavus</i>	—	2	—	—

All isolates were resistant to erythromycin (MIC > 64 mg/L). The MIC distributions of the *E. faecium*, *E. faecalis* and *E. durans* isolates are shown in the Tables 8, 9 and 10 respectively.

* number of samples.

DISCUSSION

Comparing the three groups of pigs in the study, the growth rate and feed intake tended to be less in the group of animals not receiving any AMGP than in the other two groups. This resulted in a slightly better feed conversion, but there were no statistically significant differences in performance between the three groups observed. However, it should be kept in mind that the study was primarily designed to study the resistance in the faecal flora of the pigs and not performance. The total numbers of *E. coli* and enterococci per gram faeces at the start of the study were of the same size of order as the results obtained in a former study⁶⁵. The total numbers of enterococci per gram faeces increased during the study in all three different groups, but significantly more in the avoparcin exposed group. In contrast the total numbers of *E. coli* decreased in the flavophospholipol group, increased in the negative control group and remained stable in the avoparcin group. According to the concept of colonisation resistance (CR), (part of) the endogenous obligate anaerobic flora limits the concentration of (potentially) pathogenic bacteria in the intestinal tract^{71,72}. Impairment of the CR of the intestinal tract causes an increase in the faecal concentration of facultative aerobic enterobacteriaceae as well as enterococci, if resistant (or unsusceptible) strains are present⁷⁵. At the end of the study both the total numbers of *E. coli* and enterococci per gram faeces were lower in the flavophospholipol group than in the avoparcin group. Within the flavophospholipol group the total number of *E. coli* was lower at the end than at the start of the study and of *E.*

coli more or less similar to the number at the start of the study. The total number of enterococci in the faecal flora of flavophospholipol fed pigs increased during the study less compared to both other groups of pigs. Therefore it might be concluded that the CR of flavophospholipol fed pigs was not or only slightly affected and in any case less than in both other groups of animals. The increase in the numbers of *E. coli* in the negative control group A is difficult to explain, but might have been caused by bacterial overgrowth in the small intestinal tract which was controlled in both other groups by the used AMGP. As the CR of the intestinal tract also protects against colonisation with salmonellae, these observations are in concordance with the results of others. In flavophospholipol fed pigs a reduction of the number of salmonellae per gram faeces and of the duration of excretion of salmonellae has been observed, but in avoparcin fed animals the minimal infection dose of salmonellae decreased and numbers and duration of excretion increased compared to controls^{4, 5, 26}.

E. coli

Prevalence and degree of resistance in a population is correlated with antibiotic use, but this is not a monocausal relation⁶⁵. Resistance in fattening pig increases with age, crowding and stress, caused by for instance high temperatures and transport^{10, 35,42}. Some of these factors might be interrelated such as age, transport and holding time at the abattoir. It has been shown that in pigs the degree of resistant *E. coli* is higher in the faecal contents than in the colon. Stress enhances the transit time in the intestinal tract of pigs

Table 8. *Enterococcus faecium*: MIC distribution before (compartment A+B+C) and at the end of the study (compartments A, B and C).

Antimicrobial agent	Compartment	%	Number of isolates with MIC of:													MIC ₅₀	MIC ₉₀
			resistant	<0.12	0.12	0.25	0.5	1	2	4	8	16	32	64	>64		
Flavophospholipol	A+B+C	97						1	2	9	10	8	5		16	64	
	A	100								3	23				16	16	
	B	94	1				1		2	14	14				8	16	
	C	95			1			1		39		1	1		8	8	
Vancomycin	A+B+C	0					29	3	3						0,5	1	
	A	0					23	3							0,5	0,5	
	B	16					23	4		1				4	1	>64	
	C	86					5		1					37	>64	>64	

Table 9. *Enterococcus faecalis*: MIC distribution before (compartment A+B+C) and at the end of the study (compartments A, B and C).

Antimicrobial agent	Compartment	%	Number of isolates with MIC of:													MIC ₅₀	MIC ₉₀
			resistant	<0.12	0.12	0.25	0.5	1	2	4	8	16	32	64	>64		
Flavophospholipol	A+B+C	18			4	2	1	2				1	1		0,5	32	
	A	0	1		19										0,25	0,25	
	B	0			2										0,25	0,25	
	C	0			2	1									0,25	0,5	
Vancomycin	A+B+C	0				3	8								1	1	
	A	50					5	5	10						2	4	
	B	0					2								1	1	
	C	0					2	1							1	2	

Table 10. *Enterococcus durans*: MIC distribution before (compartment A+B+C) and at the end of the study (compartments A, B and C).

Antimicrobial agent	Compartment	%	Number of isolates with MIC of:													MIC ₅₀	MIC ₉₀
			resistant	<0.12	0.12	0.25	0.5	1	2	4	8	16	32	64	>64		
Flavophospholipol	A+B+C	88					0	0	15	28	10	18	16	26	9	16	64
	A	100									5				1	8	8
	B	30			1		1		12				1	2	3	2	>64
	C	83							1		1	1		2	1	16	64
Vancomycin	A+B+C	0					61	25	36							0,5	2
	A	0					1	4	1							1	1
	B	0					2	18								1	1
	C	17					2	3						1		1	1

and thus the numbers of resistant bacteria per gram faeces, because these numbers are higher in the caecum. In this study there were neither differences in housing conditions nor other stress factors or relevant differences in therapeutic use of antibiotics among the three study groups. Therefore the observed differences must be due to the differences in the AMGP supplied. The observed high prevalence of antibiotic resistant *E. coli* was in accordance with previous studies^{47,65}. Resistance against nearly all antibiotics with the exception of chloramphenicol, neomycin and gentamicin were found in almost all pigs at the start and at the end of the study. It was interesting that in group A and C the degree of resistance for ampicillin, streptomycin, oxytetracycline, trimethoprim, and the combination of oxytetracycline and ampicillin was significantly higher at the end of the study than at the start. In the flavophospholipol group, however, the degree of resistance remained at the same size of order for almost all antibiotics and was even lower for oxytetracycline. The animals in this group excreted considerably lower numbers of resistant *E. coli* in their faeces, indicating that the reservoir of resistance genes present in the intestinal flora of these animals was much lower than in the two other groups. Flavophospholipol in a concentration of 9 mg/kg added to the feed of fattening pigs prevented either overgrowth by resistant strains and/or transmission of plasmids carrying resistance genes among intestinal *E. coli* of these animals. Both phenomena have also been observed *in vitro*^{9,23,36,57}. Consequently the chance of transfer of resistance genes to other bacteria in the intestinal tract of these animals, and the risk of transfer of resistant *E. coli* via meat(products) to humans, decreased as well.

ENTEROCOCCI

The observed high prevalence and degree of resistance of enterococci in all three groups against erythromycin was to be expected. Just before the start of the study all animals were treated with tylosin, which is known to select for erythromycin resistance in faecal enterococci of pigs^{1,11}.

At the start of the study the number of vancomycin resistant enterococci in all pigs was below the detection level of the method used. The increase in both preva-

lence and degree of resistance in the avoparcin fed group was most striking. Of all excreted enterococci per gram faeces in VRE positive animals 72% were VRE. Gentamicin resistance, however, reduced in the avoparcin group during the study and was significantly lower at the end of the study than in both other groups. This might have been due to a replacement of gentamicin resistant non-*E. faecium* enterococcal species by vancomycin resistant *E. faecium* strains. Vancomycin resistance is more common in *E. faecium* and gentamicin resistance in *E. faecalis*, as indicated in Table 11. The prevalence of flavophospholipol resistant *E. faecalis* increased in the flavophospholipol group during the study, but the numbers of flavophospholipol resistant enterococci per gram faeces (degree of resistance) even decreased.

The results of the MIC determinations of single isolates clearly indicate that mass medication with tylosin causes a selection and spread of erythromycin resistance in all enterococcal species. Avoparcin selects for vancomycin resistant *E. faecium*. Like *E. faecium*, *E. durans* is probably unsusceptible for flavophospholipol, which is not very surprising as *E. durans* is closely related to *E. faecium* and has long been considered a subtype of *E. faecium*⁴⁵. However, in this study vancomycin resistance was not observed in *E. durans*. No resistance against flavophospholipol emerged during the study in the flavophospholipol fed group B, nor in the avoparcin group, but nearly all *E. faecalis* were eradicated from the faecal flora of pigs in these two groups as shown in Table 7.

CONCLUSIONS

As was to be expected from the results of other publications^{3,34,79}, the use of avoparcin as AMGP selected strongly for vancomycin resistant enterococci in the faecal flora of exposed pigs. No influence on the resistance of the faecal *E. coli* flora was observed. In the flavophospholipol treated group the prevalence of *E. faecalis* increased but the total numbers of flavophospholipol resistant enterococci per gram faeces decreased during the study, however, less than in both other groups. Flavophospholipol addition to the feed prevented almost totally the increase in numbers of resistant *E. coli*, in the faecal flora of group B. As a result

the numbers of resistant *E. coli*, excreted with the faeces in the flavophospholipol group were significantly lower than in the other two groups. This has been proposed to be a safety mark for the protection of public health. Consequently flavophospholipol use as AMGP might decrease the public health risk of dissemination of resistant Gram negative bacteria and plasmid born resistance genes from animals to humans via the food chain. In case of a risk-benefit analysis the benefits of adding flavophospholipol to pig feeds in order to reduce resistance might be considered larger than the potential risk of selection in the intestinal tract of pigs and transfer of flavophospholipol resistant *E. faecalis* to humans. However, as this study was performed on just one farm with pigs previously colonised with only three multiresistant *E. coli* strains, the results have to be confirmed in a field study comparing pigs from farms using flavophospholipol as AMGP with others that do not. It would also be interesting to investigate whether flavophospholipol addition to pig feed prevents an increase in the prevalence and degree of antibiotic resistant *E. coli* in the intestinal tract of animals when they are treated with antibiotics, such as ampicillin, oxytetracycline and trimethoprim-sulfonamide combinations.

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Chapter VIII

The effect of banning avoparcin on VRE carriage in the Netherlands

Correspondence

The effect of banning avoparcin on VRE carriage in The Netherlands

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Sir,

Acquired resistance against antibiotics is closely related to the amount of drug used, a fact observed ever since these agents were introduced into human and veterinary medicine. However, the rate of development of resistance appears to have accelerated in the past decade. In animals, antibiotics are not only used for therapy and prevention of bacterial infections, but are also added to animal feed to act as growth promoters. In some countries, antibiotic use for growth promotion is greater than veterinary usage.¹

In countries using avoparcin, a glycopeptide antibiotic, as a growth promoter, vancomycin-resistant enterococci (VRE) are commonly found in the commensal flora of food animals, on meat from these animals and in the commensal flora of healthy humans despite very limited use of vancomycin in hospitals.¹ In The Netherlands in 1996 and 1998, approximately 1500 and 1260 kg of vancomycin, respectively, was used for human therapy, whilst an estimated 80 000 kg of avoparcin was used yearly in farming until 1997. By contrast, in those countries not allowing the use of avoparcin, no VRE have been detected in food animals, food of animal origin or healthy humans in the general population.¹ Sweden banned the use of all antibiotics as growth promoters in 1986 and no VRE have been found in the faecal flora of animals, foods of animal origin or in healthy humans from the general population¹ or in hospitalized patients. The only VRE isolated from a hospital patient contained the *VanB* gene cluster and was acquired abroad.² The Swedish example strongly suggests that removal of the selective pressure in animals can remove VRE from the human population in time. Moreover, not only has clonal spread of VRE occurred from animals to humans, but it

has also been shown that the *VanA* gene cluster can be disseminated from animal to human enterococcal strains.³

In April 1997, the European Commission suspended the use of avoparcin in animals. Denmark had already forbidden its use in 1995 and Germany in 1996. After the ban in Denmark the prevalence of VRE in poultry decreased from >80% in 1995 to <5% in 1998.⁴ By contrast, the prevalence in pigs (c. 20%) did not change during this time. Similarly, in Germany a decrease in the prevalence of VRE in poultry meat decreased from 100% in 1995 to 25% of samples tested by 1997.⁵ A decline in the prevalence of VRE was also seen in faecal samples of healthy persons, from 12% in 1994 to 3% in 1997. In Italy the prevalence of VRE in poultry meats decreased from 15% to 8%.⁶

In this report we have shown that, in The Netherlands, within 2 years of stopping the use of avoparcin, the prevalence and numbers of VRE have decreased significantly, not only in the faecal flora of food animals but also in the endogenous flora of healthy humans. Faecal samples of (sub)urban residents and of pigs and broilers from different farms were collected in 1996 and 1999. The prevalence and degree of resistance to vancomycin, erythromycin and quinupristin/dalfopristin was determined and calculated as previously described.³

The observed decreases in the prevalence of VRE and of samples with a high degree of VRE in broilers is in accordance with the results described by others^{4–6} (Table). By contrast with the situation in Denmark,⁴ the prevalence of VRE in Dutch pig faecal samples also declined. The unexpected finding was the significant decrease in prevalence of enterococci resistant against dalfopristin/quinupristin in all three populations studied. This decrease probably, however, resulted from the antimicrobial growth promoter virginiamycin (a mixture of pristinamycins like quinupristin/dalfopristin) being in short supply in The Netherlands during 1997 and 1998, because of production problems. No significant differences were noted for erythromycin resistance between 1997 and 1999.

The Swedish situation and the observed decrease in VRE in Germany, Italy, Denmark and The Netherlands after the ban on avoparcin makes it likely that discontinuation of the use of avoparcin in animals will eradicate VRE not only from animals exposed to this compound but also from the healthy human population. The observed decrease in faecal enterococci resistant to dalfopristin/quinupristin shows that the recent EU ban on the use of virginiamycin as a growth promoter may reduce the number of pristinamycin-resistant enterococci in the intestinal flora of healthy

Table. Prevalence of antibiotic-resistant enterococci and percentages of samples with a high degree (HD) of resistance in faecal samples collected in the South of The Netherlands

		1997				1999							
Antibiotic	Antibiotic concentration in agar (mg/L)	humans (<i>n</i> = 117) ^a		pigs (<i>n</i> = 282)		broilers (<i>n</i> = 50)		humans (<i>n</i> = 171)		pigs (<i>n</i> = 127)		broilers (<i>n</i> = 89)	
		prevalence	HD	prevalence	HD	prevalence	HD	prevalence	HD	prevalence	HD	prevalence	HD
Vancomycin	10	12	0	34	3	80	8	6*	0	17**	0	31**	0
Erythromycin	10	50	11	84	67	94	44	47	8	85	65	92	20
Dalfopristin/ quinupristin ^b	8	30	NT	75	NT	92*	NT	12**	NT	31**	NT	57**	NT

NT, not tested.

Number of samples tested.

Only *E. faecium*.

* Significantly different $P \leq 0.05$

** Significantly different $P \leq 0.001$.

Correspondence

humans. However, because of cross-resistance with macrolides and lincosamides, which are both commonly used in human and veterinary therapy, complete disappearance of resistance to this antibiotic is less likely to occur.

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Discussion

"So far penicillin has been under strict control, but soon it will be on sale in the chemists shops. It is to be hoped that it will not be abused, as were the sulfonamides. It is the only chemotherapeutic drug which has no toxic properties so there is no medical reason for under dosage. It is the administration of too small doses, which leads to the production of resistant strains of bacteria, so the rule in penicillin treatment should be to give enough. If more than enough is given there is no harm to the patient but merely a little waste -but that is not serious when there is a plentiful supply. But I am not giving you a discourse on penicillin. Suffice it to say that it has made medicine and surgery easier in many directions, and in the near future its merits will be provided in veterinary medicine and possibly in horticulture."

Sir Alexander Fleming (1946)¹

Although the "antibiotic era" dates from Fleming's discovery of the effects of the fungus *Penicillium notatum* in 1928, it was not until the early 1940s that penicillin could be produced in a sufficiently concentrated and pure form to treat a human. Over the following six decades the development and marketing of new classes of antibiotics increased exponentially and now more than 50 penicillins, 70 cephalosporins, 12 tetracyclines, 9 macrolides, 8 aminoglycosides and 9 fluoroquinolones (among many others) are clinically available².

Few developments in the history of medicine have had such a profound effect on human life and society as the possibility to control bacterial infections. The first advances following the discovery of micro-organisms as causes of diseases, were mainly the results of improved sanitation and hygiene, which removed some of the worst foci of infectious diseases and limited the spread of infections by insects, vermin, and contaminated water and food. The development of antisera, vaccines and the introduction of antiseptics and later aseptic techniques, which still play an important part in the prevention of infections today, achieved the earliest effective direct control of infections. The arrival of antibiotics, however, changed human and veterinary medicine completely. For the first time in history the cause of a bacterial infection could be treated and often-lethal bacterial diseases cured easily. Indeed it has been suggested that the introduction of antibiotics has added ten years to the average life expectancy of humans³.

The availability of antibiotics made the future look bright and the successes helped to foster the belief among the general public, the medical community, politicians and researchers that bacterial diseases would soon be conquered. In 1969 the Surgeon General of the United States stated that it was time "to close the book on infectious diseases" and that research and public health attentions could safely shift to other problems. For the past 60 years these life-saving drugs have been used with unrestrained enthusiasm far surpassing the needs of therapy, control and prevention of infection, despite the early warnings about the emergence of resistance in pathogenic bacteria and in the intestinal flora⁴⁻⁶. Consequently there has been steady progressive erosion of the efficacy of antibiotics by a rising tide of acquired resistance in pathogenic bacteria. Fortunately total inability to control resistant bacteria is, so far, a rarity. The trouble is that this situation may not persist and that it will ultimately jeopardise our continued ability to cure bacterial infections. Not many real new antibiotics are expected to become clinically available in the near future and the new ones being marketed will mainly be used to treat infections with multiresistant bacteria, making sure that eventually resistance will also emerge in these pathogens.

When antibiotics became available to veterinary surgeons their approach was not different to that of the medical profession. Prior to that time veterinary practice was characterised by local and systemic bacterial infections for which only palliative treatment was avail-

able. In addition for a few animal diseases antisera existed. Economic losses due to epidemics could be devastating, which caused farmers to keep only limited numbers and several species of animals on their farms to spread risks. Apart from the successful treatment of individual animals, the availability of safe and (too) cheap antibiotics made it possible to treat, control and prevent epidemics caused by pathogenic bacteria that traditionally interfered with large scale animal production. As a result herd sizes increased and farmers often specialised in keeping only one species. In this so-called intensive-farming animals are kept in large numbers closely housed together. Infection control measures are aimed at the prevention of introduction of pathogenic micro-organisms into a stable or farm, but within a stable or unit micro-organisms can spread unchecked among the animals present. As animals intended for human food in intensive farming systems have short lives and bacterial infections among young animals are common, large amounts of antibiotics are necessary to control these infections and to prevent severe economic losses. Because crowding, poor hygiene and high antibiotic use are typical features of intensive farming, resistance is commonly encountered among for animals pathogenic bacteria in such premises. This causes therapy failures with increased morbidity and mortality. Moreover it promotes the veterinary use of new antibiotics, which are "critical" or "essential" for human use (i. e. little or no alternate agents to treat serious or life-threatening infections in humans) and the use of which should be restricted both in human and in veterinary medicine.

Because of the growing national and international concern about the increasing resistance of bacteria to antibiotics, the need for more concerted action to prevent, delay and control is recognised. Antibiotics are some of the most active drugs known and their benefits for therapy and control of bacterial infections in man and other animals are obvious. However they exert not a direct effect on humans or other animals, but kill an infecting pathogen. Unfortunately they are not narrowly targeted and will try to kill any bacterium they encounter. Animals consist of some 10^{12} cells per kg bodyweight, but only 10% of these are animal cells. The remainder are micro-organisms, mainly bacteria that make up the normal endogenous flora. Each time

an antibiotic is administered, the normal flora is also exposed. These bacteria have infinitely expandable and mutable populations to adapt to the presence of antibiotics in their environment. Resistant mutants and unsusceptible species of bacteria have in the presence of antibiotics a selective advantage and will replace susceptible bacterial strains. In addition genes conferring resistance are not only passed on vertically from generation to generation, but also horizontally via plasmids and transposons to neighbouring bacteria even of different species. Furthermore, large plasmids encoding multidrug resistance can be assembled by sequential addition of other mobile resistance conferring elements (integrons and transposons) and transfer multiresistance from one bacteria to another, and from a mere commensal to a pathogen. Because this is easily accomplished in places where a high density of bacteria is present, the intestinal tract forms a reservoir in which resistant bacteria and resistance genes accumulate under antibiotic pressure. Consequently the prevalence and degree of resistance in the intestinal flora of a population can therefore be used to estimate the antibiotic use in that population and to predict resistance to be encountered in pathogens⁷. That man and animals share a common pool of resistant bacteria and resistant genes is clear. Hence it remains logical to accept that antibiotic resistance in animals contributes to antibiotic resistance in humans. Resistant animal bacteria can reach humans either directly or indirectly via food products of animal origin. Control of resistance in animals is thus not only of importance to maintain the clinical efficacy of antibiotics in veterinary medicine, but is also a public health issue.

In this thesis the prevalence and degree of resistance of *Escherichia coli* and enterococci (these are considered indicator bacteria) in faecal samples of different human and animal populations were used to detect dissemination of resistant bacteria and resistance genes from food animals to the human intestinal flora. The results indicated that the prevalence and degree of resistance observed in *E. coli* and enterococci in the faecal flora of poultry and fattening pigs strongly correlated with the amounts of antibiotics these animals were exposed to. Recent exposure to high doses or prolonged exposure not only caused an increase in the prevalence i.e. the numbers of animals colonised with resistant bacteria,

but also in the numbers excreted per gram faeces. In humans it has been found that persons with a high degree of resistance in their faecal flora also excreted resistant bacteria more persistently and over a longer period of time than people with a low degree of resistance⁸. In food animals an increase of resistant bacteria in the faecal flora means that more resistant bacteria are excreted into the environment and a heavier contamination of carcasses with resistant bacteria. Foods of animal origin are frequently contaminated with resistant bacteria and humans can acquire resistant bacteria in their intestinal flora via food⁹. Persons in direct contact with animals, with a high prevalence or degree of resistance, have also more resistant bacteria in their faecal flora than other human populations and the observed resistances correlate with those in the animals they are in direct contact with. Moreover, direct clonal transmission of resistant bacteria from animal to humans and transfer of resistance genes of animal bacteria to bacteria of the endogenous flora of humans and to human pathogens have been shown. These observations provide strong evidence that antibiotic resistant bacteria and resistance genes spread to man via the food chain. To minimise public health risks it has been proposed that a low prevalence and a low degree of resistance in the faecal flora should be considered as a safety and quality mark of food animals^{10,11}. As prevention of cross-infection is not feasible in these animals, the large volumes of antibiotics used in food animals must be reduced. Of course we cannot stop treatment of animals we are responsible for in cases of serious bacterial infections. However the dependency on antibiotics in intensive farming should be diminished. Antibiotics should no longer be considered as a mere management tool to make up for suboptimal husbandry practices, but as a non-renewable resource only to be used in case of an outbreak of a bacterial infection. On every occasion that the therapeutic use of antibiotics is indicated, measures should be taken at the same time to prevent the reappearance of this infection in the future. These preventive measures could include improved housing and animal husbandry, adaptation of feed and feeding practices, and disease eradication or vaccination. Cost should never be the prevailing factor and even if these measures are more expensive than regular medication of the whole herd or flock with antibiotics they must be

applied. Antibiotics are not intended for prevention of infections or suppression of disease symptoms. Therefore preventive usage of antibiotics in whole herds or flocks should only be allowed if the efficacy of such a practice has been proven, and no other preventive measures are available. Every time, that antibiotics are used, a risk-benefit assessment should be made. Certain public health risks are acceptable in case of therapy to prevent animals suffering and to stop an outbreak of a bacterial disease on a farm; in contrast antibiotic usage for mere economic reasons is not acceptable. The use of AMGP is responsible for nearly 50% of all antibiotics used world wide in animals and is justified on the assumption that it might facilitate production of more food more cheaply. Apart from the fact that these effects in a setting of good animal husbandry are very minimal, most AMGP are used in food rich societies, where obesity rather than hunger is the major public health problem. What are the potential benefits in this case, that justify any delay in elimination of the risk? The argument that AMGP suppress bacterial infections in food animals is not valid, as these drugs are not registered for therapy and prevention of bacterial diseases but registered only as a feed additive to enhance performance. Continuous feeding of antibiotics is not a suitable way to tackle infection problems. However, certain drugs, until recently used as AMGP in the EU, could be valuable supplements to the existing veterinary antibiotic arsenal e.g. for treatment of clostridial and *Brachyspira*-infections in poultry and pigs. The amounts used for therapy will be considerably lower than as AMGP, because only a small proportion of all food animals will need these drugs and only during a short period of time. Stopping the lavish use and misuse of antibiotics in food animals will be an important step forward in increasing the safety of foods of animal origin and help to maintain the efficacy of antibiotics in veterinary medicine in the future. Phasing out of antibiotics as AMGP is a first and major step forward and the development of consensus formularia for antibiotic therapy in animals a second. These measures alone, however, will by no means solve the resistance problem in human medicine. The major cause of resistance in human pathogens is the (over)use of antibiotics in humans. Other sectors with a high use of antibiotics are aquaculture, plant culture, and food and wood conser-

vation. It is fruitless, however, to apportion blame. A more productive route for all sectors involved is to recognise the problem and agree on strategies to slow down the loss of important drugs from our therapeutic armament. With the phasing out of AMGP use and strict adherence to an agreed upon veterinary antibiotic policy the animal production sector and the veterinary sector in the Netherlands are heading for the right direction. Decontamination of animal carcasses with organic acids such as lactic acids might help to decrease the public health risk of antibiotic use in animals and of zoonotic infections, e.g. salmonellosis and campylobacteriosis even further. However, continuous information and education of butchers and consumers about meat handling and hygiene remains necessary.

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Summary

“De roos heeft gauw gedaan, de doornen blijven staan.”

(Roses wither soon, but their thorns remain.)

Jacob Cats (1577 – 1660), Dutch statesman, poet and moralist.

Antibiotics are the most effective and safest drugs ever developed. Their efficacy as therapeutics is, however, threatened by the emergence of antimicrobial resistance in pathogenic bacteria.

In the introduction the reasons for the antimicrobial resistance problems encountered today in (potentially) pathogenic bacteria are discussed. This seems to be the result of an important unwanted side effect of antibiotics. Because these drugs are not selective they kill not only pathogenic bacteria, but all susceptible bacteria that they encounter. This results not in a decrease of bacterial numbers, because susceptible bacteria are replaced by unsusceptible species and strains with acquired resistance. This is called selection of resistance. Because resistant bacteria exchange genes conferring resistance relatively easily, the lavish use of antibiotics in human and veterinary medicine, animal husbandry and agriculture, has caused an enormous increase of resistant bacteria in the intestinal flora of man and other animals, which are excreted into the environment. These resistant bacteria form an enormous reservoir of resistance genes for (potentially) pathogenic bacteria. Resistant bacteria of food animals can reach the intestinal tract of humans either directly or via the food chain. In the case of pathogenic bacteria this is a cause of zoonotic food borne infections like salmonellosis, but non-pathogenic species might either colonise the human intestinal tract or transfer their resistance genes to bacteria of the human intestinal flora. This is called dissemination of resistance. Despite that resistance is an inevitable sequel of antibiotic use, it can be limited by decreasing the selection pressure i. e. using less antibiotics (read: use them only if necessary) and by prevention of dissemination of resistant bacteria by improving hygiene and sanitation.

In chapter I the usage of antibiotics in animals is attended to. Antibiotics are given to animals not only for therapy, control and prevention of bacterial infections on prescription of a veterinary surgeon, but also continuously added as antimicrobial growth promoter (AMGP) to feeds of food animals to enhance growth, feed digestion and performance and to decrease animal waste. In the EU and the USA approximately 50% of all antibiotics (kg active ingredient at 100% purity) are used for animals and more than 95% of these antibiotics are given to animals producing food for human consumption. However, large differences between countries exist. The selection pressure on animal bacteria by therapeutic antibiotics and AMGP is high, which causes not only resistance in animal pathogens, but also in zoonotic pathogens and in the intestinal flora of these animals. As to the extent to which antibiotic use in animals contributes to antibiotic resistance in humans is still under debate, the literature on this aspect is reviewed. Clonal spread of antibiotic resistant bacteria such as *E. coli*, *Salmonella* spp., *Campylobacter* spp. and enterococci from food animals to humans has been described as well as transfer of resistance genes of animal bacteria to the human endogenous flora and human pathogens. At this moment the only way to minimise this public health risk is to use fewer antibiotics in food animals. Stopping the mere economic use of antibiotics as AMGP would diminish the overall use in animals by 30% - 50%.

In chapter II ways to minimise the use of antibiotics in veterinary medicine without hampering optimal veterinary care are discussed. This might be done by developing a veterinary antibiotic policy or prudent use guidelines for optimising the use of antibiotics by, or under supervision of, veterinary surgeons, also taking into account prevention of selection and dissemination

of resistant bacteria. The backbone of such a policy is the establishment of antibiotic formularies or guidelines: preferred and limited lists of antibiotics for more than 90% of the indications for which antibiotic therapy is warranted and regularly encountered in veterinary practice. If several antibiotics can be used, a formulary must differentiate between first, second and third choice antibiotics. Antibiotics that are very important in human medicine or that are analogues of such antibiotics are always third choice antibiotics. They can only be used after susceptibility testing has shown that first or second choice antibiotics are not effective. Such practical clinical guidelines are not only of great help to veterinary surgeons but also prevent policies and prudent use guidelines of becoming a dead letter or only a politically intended lip service. The need for continuous surveillance of antibiotic resistance and use in animals is discussed as being essential a key factors for evaluation of the impact of formularia and a timely adaptation of the formulary recommendations, and to provide an early warning system for new resistance problems that might occur.

In the following chapters the prevalence and degree of resistance in faecal indicator bacteria: *E. coli* and enterococci were used to detect selection and dissemination of resistant bacteria and resistance genes among animals and from food animals to the human intestinal flora. The prevalence (%) of resistance against a certain antibiotic is the number of samples containing bacteria (*Escherichia coli* or enterococci) resistant against that antibiotic divided by the total number of samples and multiplied by 100. The degree of resistance (%) against an antibiotic in a faecal sample is the percentage of *E. coli* or enterococci resistant to that antibiotic relative to the total number of *E. coli* or enterococci present in a sample.

In chapter III the prevalence and degree of antibiotic resistance of the faecal indicator bacteria: *E. coli* and enterococci, were determined in faecal samples of pigs collected at five Dutch abattoirs and in samples of Swedish pigs. In the Dutch pig populations the prevalence and degree of resistance both of *E. coli* and enterococci were high for antibiotics regularly used in pig medicine or as AMGP. Differences were observed between the five abattoirs, but could not be explained as no data on antibiotic consumption of the different

pig populations could be obtained. In the Swedish samples the resistance of enterococci against antibiotics used as AMGP was significantly lower than in the Dutch populations and avoparcin resistance was even absent. This was to be expected as Sweden had already banned the use of AMGP in 1986. The overall lower prevalence and degree of resistance against therapeutically used antibiotics observed in the Swedish pig samples compared to the Dutch pig populations, clearly indicated that banning the use of antibiotics as AMGP does not inevitably cause a higher therapeutic use of antibiotics in pigs. Moreover comparison of the results of the Swedish and Dutch samples showed that monitoring the prevalence and degree of resistance in faecal samples collected at slaughterhouses can be used to detect differences in antibiotic consumption between different pig populations.

In chapter IV the prevalence and degree of resistance against antibiotics commonly used for therapy or as AMGP in poultry were determined in the faecal flora of turkeys and three human populations, each with a different level of contact with turkeys: turkey farmers, turkey slaughterers and (sub)urban residents, was described. The prevalence of vancomycin resistant enterococci (VRE) was significantly higher (62%) in turkeys fed avoparcin than in turkeys not exposed to this drug (8%). No significant differences between farmers using avoparcin (37%) and those who did not (42%) were observed. The prevalence in turkey slaughterers and (sub)urban residents was 20% and 13% respectively. The Pulsed-field gel electrophoresis (PFGE) patterns of the isolated VRE in the different populations were quite heterogeneous, but isolates with the same pattern (similar genotypes) were found among turkey and turkey farmer isolates. On two occasions *Enterococcus faecium* isolates with identical PFGE patterns were found in the faeces from the farmer and turkeys of the same farm, which in addition, contained an indistinguishable *VanA* containing element not found in human isolates before. This strongly suggested transfer of resistant enterococci from animals to humans. Moreover similar *VanA* elements were not only found in isolates with the same PFGE patterns, but also in other strains isolated from humans and turkeys, which indicated that not only did clonal spread of resistant strains occur, but also a spillover of resistance genes of

turkey enterococci to enterococci in the human intestinal flora.

In chapter VI the prevalence and degree of antibiotic resistance of enterococci in the faecal flora of two chicken populations, i.e. laying hens with a low, and broilers with a high exposure to antibiotics were determined and likewise in faecal samples of laying hen farmers, broiler farmers and poultry slaughterers. The prevalence and degree of resistance for most antibiotics tested was higher in broilers than in laying hens, and in broiler farmers and broiler slaughterers than in laying hen farmers. Hence a clear correlation was found between the prevalence and degree of resistance found in faecal enterococci and the amounts of antibiotics the chicken populations were exposed to. Among the human populations there was no difference in antibiotic consumption and the observed prevalence and degree of resistance correlated with these parameters in the animals they were in contact with. The overall prevalence of resistance of faecal enterococci was significantly higher in broilers and broiler slaughterers than in laying hen farmers. The PFGE patterns of the isolated VRE (one per VRE positive faecal sample) of the five populations were quite heterogeneous, but in two cases *Enterococcus hirae* with identical PFGE patterns were isolated from the farmer and from broilers from the same farm. Moreover, similar *VanA*-elements were not only found in isolates with the same PFGE-patterns but also in other VRE isolated from humans and chickens. These results strongly indicate transmission of resistant enterococci from poultry to man, both by clonal spread and by transposon transfer from animal strains to enterococci in the human intestinal flora.

In chapter VI the prevalence and degree of resistance of *E. coli* were determined in faecal samples of three poultry populations: broilers and turkeys commonly exposed to antibiotics and laying hens with a low usage of antibiotics. In addition faecal samples of five human populations were examined: turkey and broiler farmers, laying hen farmers and broiler slaughterers and turkey slaughterers. The antibiotics tested or analogues are commonly used in poultry medicine. Ciprofloxacin resistant isolates from these 8 populations and from poultry meat samples were genotyped by PFGE. The prevalence and degree of resistance was significantly higher in turkeys and broilers than in the

laying hen population. Also for most antibiotics the observed resistance in faecal *E. coli* of turkey and broiler farmers, and of turkey and broiler slaughterers, was higher than in laying hen farmers. The same resistance patterns were found in turkey, turkey farmer and turkey slaughterer isolates, and in broiler, broiler farmer and broiler slaughterer isolates. Multiresistant isolates were common in turkey and broiler farmers but absent in the laying hen farmer population. The PFGE patterns of the isolates from the eight populations were quite heterogeneous, but on two occasions *E. coli* strains with an identical PFGE pattern were isolated from turkeys and the farmer from the same farm, and also from a broiler and a broiler farmer but from two different farms. Moreover, three *E. coli* isolates from turkey meat were identical to faecal isolates from turkeys. The results of this study strongly suggest that transmission of resistant clones of *E. coli* and resistance plasmids from poultry strains to human *E. coli* does commonly occur.

In chapter VII another approach, different from reducing antibiotic usage, to lower the prevalence and degree of resistance in faecal *E. coli* of food animals, was investigated. The "plasmid curing effect" of an AMGP: flavophospholipol, which had been described to occur *in vitro* and *in vivo* under experimental conditions, was studied under field conditions. The prevalence and degree of resistance of the indicator bacteria *E. coli* and enterococci, were determined in faecal samples of three groups of pigs being fattened at the same farm. During the last 12 weeks before slaughtering, one group received feed without any AMGP (negative control group), another group was given the same feed with 9 mg/kg flavophospholipol (study group) and the third group the same feed with 15 mg/kg avoparcin (positive control group). Faecal samples of each pig were collected at the start and at the end of the study and assessed for the prevalence and degree of resistance against antibiotics or analogues, either commonly used for therapy in pig medicine or as AMGP. Before the start of the study all pigs were colonised with multiresistant *E. coli* by mixing three resistant pig isolates through their feed.

The overall prevalence and degree of resistance of *E. coli* in the faecal flora increased significantly during the study in the negative control group and avoparcin ex-

posed group, but in the flavophospholipol group levels remained the same as at the start of the study. Before the study, in all three groups, the numbers of VRE were below the detection limit of the used isolation methods. At the end of the study the prevalence of vancomycin resistance was 44% and 41% in the negative control and flavophospholipol group respectively but only very low numbers of VRE per gram faeces were found. In the avoparcin fed group the prevalence was 72% and in 57% of the samples the majority of all enterococci present were vancomycin resistant. It was concluded that flavophospholipol under the study conditions effectively suppresses the amplification and dissemination of multiresistant *E. coli* in the intestinal flora of fattening pigs and that avoparcin use strongly selects for VRE. After confirmation of this effect of flavophospholipol in a large field study including several different farms, the significantly lower prevalence and degree of resistance of faecal *E. coli* in flavophospholipol fed animals might in a risk-benefit analysis outweigh the small increase of the risk of transfer of flavophospholipol resistant *E. faecalis* from animals to humans via the food chain. In chapter VIII the effect of the suspension of avoparcin usage as AMGP in the Netherlands in 1997 was assessed. The prevalence and degree of enterococci against three antibiotics important for human therapy and closely related to and cross resistant with three commonly used AMGP were analysed in faecal samples of pigs and broilers collected at the same slaughterhouses in 1997 and 1999, and from healthy human (sub)urban residents from the same cities and collected in the same years. The prevalence of VRE decreased significantly in all three populations. Erythromycin resistance did not change, but dalfopristin-quinupristin resistance had declined, despite the fact that the use of the related AMGP i.e. virginiamycin, was not forbidden at that time. Virginiamycin, however, was in short supply in the Netherlands in 1998 and 1999, because of production problems. These results indicate that continuation of the ban in the Netherlands on the use of avoparcin in animal feeds will not only eradicate VRE from food animals but also from the human population. Because of cross-resistance with macrolides and lincosamides, which are both commonly used in human and veterinary medicine, complete disappearance of pristina-

mycin resistance after a ban of virginiamycin is less likely to occur. The use of vancomycin in humans had not changed between 1997 and 1999. Therefore the results of this study again show that use of antibiotics in food animals not only causes resistance in the intestinal flora of these animals but that these resistant bacteria or their resistance genes are transferred to humans via the food chain.

In the discussion it is concluded that 60 years of lavish usage of antibiotics in human and veterinary has progressively eroded the clinical efficiency of antibiotics because of a rising tide of acquired resistance in pathogenic bacteria. However resistance in pathogens is only the top of the resistance iceberg and an enormous reservoir of resistance genes has accumulated in the intestinal flora of man and other animals. Fortunately there is a growing national and international concern about the increasing resistance and the need for more concerted action to prevent, delay and control bacterial resistance against antibiotics is generally recognised. In addition to other studies in the literature, the studies in this thesis show clearly that man and animals share a common pool of resistant bacteria and resistance genes and that resistant bacteria from food animals can reach humans either directly or via the food chain. Because antibiotic use in animals selects for bacterial resistance in the endogenous flora of these animals, it is logical to accept, as has been proven, that antibiotic use in animals contributes to certain resistance problems in human medicine. This makes control of antibiotic use in food animals not only of importance for maintaining the clinical efficacy of antibiotics in veterinary medicine, but also as a public health issue. Therefore a low prevalence and degree of antibiotic resistance in the faecal flora of food animals is to be considered as a safety and quality benchmark for food animals. This goal can only be achieved by lowering the need for therapeutic antibiotics in these animals and by stopping to use them for mere economic reasons as AMGP. These measures alone, however, will by no means solve resistance problems in human medicine, as the major cause of resistance in human pathogens is the (over)use of antibiotics in humans. It is fruitless, however to apportion blame. A more productive approach for all sectors involved is to agree upon strategies to prevent the loss of these important drugs from our thera-

peutic armament. With the strict adherence to a veterinary antibiotic policy, agreed upon by consensus, and the phasing out of AMGP usage the veterinary and animal production sectors in The Netherlands have clearly shown to take their responsibility for public health seri-

ously. Additional decontamination of animal carcasses after slaughtering might decrease the risk of transfer of bacterial resistance and of zoonotic infections like salmonellosis, from animals to humans even further.

Samenvatting

“De roos heeft gauw gedaan, de doornen blijven staan.”

Jacob Cats (1577 – 1660), staatsman, dichter en moralist.

Antibiotica zijn de meest effectieve en veilige geneesmiddelen, die we kennen. Hun werkzaamheid dreigt echter af te nemen door de antimicrobiële resistentie, welke in toenemende mate bij pathogene bacteriën voorkomt.

In de inleiding wordt deze resistentie-problematiek besproken. De belangrijkste ongewenste bijwerking van antibiotica is dat zij niet alleen (potentieel) pathogene bacteriën doden of in hun groei remmen, maar iedere gevoelige bacterie. Dit heeft echter geen daling van het totaal aantal bacteriën tot gevolg, omdat de gedode gevoelige bacteriën onmiddellijk vervangen worden door resistent geworden bacteriën en door ongevoelige soorten. Dit wordt selectie genoemd.

Bacteriën kunnen bovendien onderling relatief gemakkelijk genen, die coderen voor resistentie-eigenschappen, uitwisselen. Als gevolg van het jarenlange kwistig gebruik van antibiotica in de (dier)-geneeskunde, veeteelt en landbouw zijn de aantallen resistente bacteriën in de darmflora van mens en dier en in het milieu enorm toegenomen. Deze resistente commensale bacteriën vormen een reservoir van resistentie-genen voor (potentieel) pathogene bacteriën. Resistente bacteriën uit de darmflora worden bovendien uitgescheiden met de faeces. Omdat tijdens het slachtproces vlees veelal besmet wordt met darmbacteriën van slachtdieren, kunnen mensen niet alleen door direct contact besmet worden met resistente bacteriën, afkomstig van slachtdieren, maar ook indirect via voedingsmiddelen van dierlijke oorsprong. Wanneer het pathogene bacteriën zoals salmonellae betreft, zal dit vaak een (voedsel)infectie veroorzaken. Echter in geval van andere niet-pathogene bacteriën uit de darmflora van dieren kunnen mensen ongemerkt door deze resistente bacteriën gekoloniseerd worden of kunnen deze bacteriën hun resistentie-genen aan bacteriën van de

darmflora van de mens overdragen. Dit heet verspreiding van resistentie.

Ondanks het feit dat selectie en verspreiding van resistentie onvermijdelijk gevolgen zijn van het gebruik van antibiotica, kunnen de gevolgen beperkt worden door de selectiedruk te verminderen door minder antibiotica te gebruiken (lees: antibiotica alleen te gebruiken indien noodzakelijk). Verspreiding kan men voorkomen én door vermindering van het antibioticum gebruik én door het verbeteren van de hygiëne en sanitaire maatregelen. Bij producten van dierlijke oorsprong betekent dit decontaminatie van karkassen na het slachten, een goede hygiëne in slagerijen en keukens, en verhitting van het eindproduct voor consumptie.

In hoofdstuk I wordt het gebruik van antibiotica bij dieren besproken. Antibiotica worden aan nutsdieren niet alleen toegediend voor behandeling en preventie van bacteriële infecties op voorschrift van de dierenarts, maar ook continu door het veevoer gemengd als antimicrobiële groeibevorderaar (AMGB) om de groei te bevorderen, de voederconversie te verbeteren en de mestproductie te verminderen. Ongeveer de helft van alle antibiotica (kg actieve stof) wordt in de EU en in de USA gebruikt voor dieren en meer dan 95% hiervan is bestemd voor dieren die voedsel produceren voor menselijke consumptie, zogenaamde nutsdieren. De selectiedruk door antibioticagebruik bij nutsdieren is dus hoog. Dit veroorzaakt niet alleen resistentie bij bacteriën, die pathogeen zijn voor dieren, maar ook bij zoönotische bacteriën en bij bacteriën die behoren tot de normale darmflora van nutsdieren.

Omdat het nog niet duidelijk is in welke mate deze resistentie-selectie door antibioticagebruik bij dieren bijdraagt aan de resistentie-problematiek bij de mens, zijn de bestaande gegevens uit de literatuur over dit onderwerp samengevat en geëvalueerd. Klonale verspreiding van resistente bacteriën zoals *E.coli*, *Salmo-*

nella spp., *Campylobacter* spp. en enterokokken van nutsdieren naar mensen is bewezen en blijkt regelmatig voor te komen. Echter ook overdracht van resistentie-genen van faecale bacteriën van nutsdieren naar de darmflora van mensen en voor mensen pathogene bacteriën is aangetoond. De enig haalbare manier om dit risico voor de volksgezondheid te reduceren is om minder antibiotica aan nutsdieren te geven. Stoppen met het gebruiken van antibiotica als AMGB om puur economische redenen zou het totale antibioticumgebruik bij dieren in één keer met 30%-50% verminderen.

In hoofdstuk II worden mogelijkheden besproken om het diergeneeskundig gebruik van antibiotica te verminderen zonder het welzijn van dieren te schaden. Een belangrijke stap in dit proces is het opstellen van een veterinair antibioticumbeleid of richtlijnen voor het zorgvuldig gebruik van antibiotica. Een antibioticumbeleid is erop gericht om het gebruik en voorschrijven van antibiotica door dierenartsen te optimaliseren door regels op te stellen betreffende indicaties voor antibioticagebruik. Een essentieel onderdeel is het opstellen van formularia: richtlijnen, waarin voor meer dan 90% van de indicaties in de veterinaire praktijk adviezen worden gegeven of, en zo ja, welk antibioticum bij voorkeur gebruikt moet worden. Bij de keuze dient men naast effectiviteit ook rekening te houden met de kans op resistentie-ontwikkeling met het doel om deze zoveel mogelijk te voorkomen. Het aantal geadviseerde antibiotica is zoveel mogelijk beperkt en, indien voor een indicatie meerdere antibiotica geschikt zijn, wordt een volgorde aangegeven van middelen van eerste, tweede of derde keuze. Antibiotica, die gebruikt worden voor de behandeling van levensbedreigende infecties bij mensen en waarvoor geen alternatieven (meer) bestaan, zijn in diergeneeskundige formularia altijd derde keuze middelen. Deze mogen alleen gebruikt worden als bacteriologisch onderzoek heeft uitgewezen dat de verwekker niet gevoelig is voor een eerste of tweede keuze antibioticum. Dergelijke praktische richtlijnen zijn niet alleen erg handig voor praktiserende dierenartsen, maar verhinderen ook dat het antibioticumbeleid verwatert en een dode letter wordt. Ook wordt daardoor voorkomen dat een antibioticumbeleid of richtlijnen voor zorgvuldig antibioticumgebruik alleen worden geformuleerd om politiek move-

rende redenen en zo ontaarden in een opportunistische lippendienst. Op de noodzaak van een continue surveillance van resistentie in bacteriën geïsoleerd uit zieke dieren en faecesmonsters van slachtdieren, en van het antibioticumgebruik bij dieren wordt gewezen, omdat dit essentiële voorwaarden zijn voor een goed antibioticumbeleid. Door de gegevens uit deze surveillance regelmatig te analyseren kan het effect van de formularium-adviezen gemeten worden en kunnen, indien nodig, de adviezen aangepast worden. Bovendien worden hierdoor nieuwe resistentie-problemen tijdig onderkend, zodat interventie mogelijk is.

In de volgende hoofdstukken is de prevalentie en mate van resistentie van indicatorbacteriën in de faecale flora: *E. coli* en enterokokken, gebruikt om selectie en verspreiding van resistente bacteriën en resistentie-genen van nutsdieren naar de mens te onderzoeken. De resistentie-prevalentie tegen een bepaald antibioticum is het percentage monsters in een populatie, waarin *E. coli* of enterokokken, die resistent zijn tegen dat antibioticum, gevonden zijn. De mate van resistentie tegen een bepaald antibioticum in een faecesmonster is het percentage *E. coli* of enterokokken, dat resistent is tegen dat antibioticum.

In hoofdstuk III zijn de resultaten gepresenteerd van een onderzoek naar de prevalentie en mate van resistentie van indicatorbacteriën: *Escherichia coli* en enterokokken, in de faecale flora van slachtvarkens. De monsters waren verzameld aan de slachtlijn op vijf Nederlandse abattoirs en in Zweden. In de Nederlandse monsters was de prevalentie en mate van resistentie van de onderzochte indicatorbacteriën tegen antibiotica, die als AMGB gebruikt worden of als geneesmiddel, hoog. Er bestonden significante verschillen tussen de monsters afkomstig van de verschillende slachthuizen. Deze verschillen konden echter niet verklaard worden omdat geen gegevens over het antibioticumgebruik in de verschillende populaties beschikbaar waren. In de Zweedse monsters werd nauwelijks resistentie tegen de meeste AMGB gevonden en er werden geen vancomycine-resistente enterokokken (VRE) uit geïsoleerd. Vanwege het Zweedse verbod sedert 1986 op het gebruik van AMGB in veevoeder was dit te verwachten. De lagere resistentie tegen therapeutisch gebruikte antibiotica in de Zweedse monsters toonde duidelijk aan dat een verbod op AMGB niet

hoeft te leiden tot een toename van het veterinair gebruik van antibiotica. Bovendien bleek uit de verschillen tussen de Nederlandse en Zweedse monsters dat het bepalen van de prevalentie en mate van resistentie in faecesmonsters, verzameld op slachthuizen, een geschikte methode is om verschillen in antibioticumgebruik tussen populaties op te sporen.

In hoofdstuk IV is de prevalentie en mate van vancomycine-resistente enterokokken (VRE) bepaald in de faecale flora van kalkoenen en drie groepen mensen met een verschillende mate van intensiteit in het contact met kalkoenen: kalkoenhouders, kalkoenslachten en stadsbewoners. Bij kalkoenen, waarbij avoparcine als AMGB door het voer gemengd was, bleek de VRE-prevalentie significant hoger (62%) dan bij de dieren, die niet aan dat middel blootgesteld waren (8%). Bij de kalkoenhouders werd geen verschil gevonden tussen avoparcine gebruikers (37%) en niet-gebruikers (42%). De VRE-prevalentie in kalkoenslachten en stadsbewoners was respectievelijk 20% en 13%. Genotypering met behulp van pulsed-field gel electrophoresis (PFGE) toonde aan dat de VRE-stammen, geïsoleerd uit mensen en kalkoenen, zeer heterogeen waren. In twee gevallen werd echter op dezelfde boerderij een genotypisch identieke *E. faecium* stam geïsoleerd uit de faeces van de veehouder en een van zijn kalkoenen. Bovendien bevatten beide genotypen *E. faecium* een identieke variant van het *VanA*-gen cluster, dat nog niet eerder was aangetoond in VRE geïsoleerd uit mensen. Tevens werd deze variant van het *VanA*-gen ook gevonden in een aantal andere genotypen van VRE geïsoleerd uit mensen en kalkoenen. Dit bewijst dat niet alleen klonale overdracht van VRE van kalkoen naar mens plaats vindt maar ook dat verspreiding van vancomycine resistentie-genen, afkomstig van kalkoenstammen, naar enterokokken in de darmflora van mensen voorkomt.

In hoofdstuk V is de prevalentie en mate van resistentie tegen antibiotica onderzocht van enterokokken in de faecale flora van twee kippenpopulaties: legkippen en vleeskuikens en drie groepen mensen: legkippenhouders, vleeskuikenhouders en kippenslachten. Bij legkippen worden geen ABGM en relatief weinig antibiotica gebruikt; bij vleeskuikens daarentegen worden relatief veel antibiotica gebruikt. Zowel de prevalentie als de mate van resistentie tegen de

onderzochte antibiotica waren bij de enterokokken afkomstig van vleeskuikens hoger dan bij die van legkippen. Ook bij de vleeskuikenhouders en kippenslachten was de resistente prevalentie van de meeste antibiotica hoger dan bij legkippenhouders. Er bestond een duidelijke correlatie tussen de resistentie-prevalenties voor de verschillende antibiotica bij vleeskuikens en bij vleeskuikenhouders en kippenslachten. Van ieder VRE positief faecesmonster werd een isolaat getypeerd met behulp van PFGE. De gevonden genotypen in alle groepen waren erg heterogeen. Echter op twee boerderijen werd zowel uit de ontlasting van de pluimveehouder als van een vleeskuiken een vancomycine-resistente *E. hirae* geïsoleerd, beiden met hetzelfde PFGE patroon. Moleculaire analyse van de *VanA*-genen van deze stammen toonde aan dat deze ook identiek waren en dat het varianten van het oorspronkelijke *VanA*-genencluster betrof, die voornamelijk bij pluimvee gevonden worden. Deze *VanA*-varianten werden niet alleen in isolaten met hetzelfde PFGE genotype gevonden, maar ook in andere genotypen, geïsoleerd uit mensen en kippen. Dit wijst duidelijk op resistentie-overdracht van kippen naar mensen. Dit kan klonale verspreiding van resistente stammen zijn, maar het lijkt dat overdracht van transposons van kippen enterokokken naar enterokokken uit de faecale flora van de mens meer voorkomt.

In hoofdstuk VI zijn de prevalentie en mate van resistentie van *E. coli* in de faecale flora van de populaties uit de hoofdstukken IV en V bepaald. Van monsters, waarin ciprofloxacin-resistente *E. coli* waren gevonden, werd bovendien een ciprofloxacin-resistente stam getypeerd met behulp van PFGE. De prevalentie en mate van resistentie was significant hoger bij kalkoenen en vleeskuikens, beide populaties met een relatief hoog antibioticumgebruik, dan bij legkippen. Ook bij kalkoen- en vleeskuikenhouders en pluimveeslachten was de resistentie hoger dan bij legkippenhouders. Bovendien werden dezelfde resistentie-patternen gevonden bij *E. coli* isolaten van kalkoenen, kalkoenhouders en -slachten en in die van vleeskuikens, vleeskuikenhouders en -slachten. Multiresistentie kwam veelvuldig voor bij isolaten afkomstig van kalkoenen, vleeskuikens en kalkoen- en vleeskuikenhouders, maar werd niet gevonden bij legkippen(houders). De genotypen van de ciprofloxacin-resistente *E. coli* uit de acht

populaties waren erg heterogeen. Echter op twee boerderijen werden identieke genotypen geïsoleerd uit faeces van de kalkoenhouder en zijn kalkoenen en ook uit een vleeskuikenhouder en een vleeskuiken, maar deze waren afkomstig van verschillende bedrijven. Bovendien waren drie ciprofloxacin-resistente stammen geïsoleerd uit kalkoenvlees met behulp van PFGE niet te onderscheiden van het isolaat uit de faeces van een kalkoenhouder in dit onderzoek. De resultaten van deze studie geven aan dat overdracht van resistente *E. coli* stammen van kippen naar mensen en uitwisseling van resistentie-plasmiden tussen kippen *E. coli* en *E. coli*-stammen in de darm van mensen regelmatig voorkomt.

In hoofdstuk VII is onderzocht of naast vermindering van antibioticumgebruik er ook andere mogelijkheden zijn om een lage prevalentie en mate van resistentie in de faecale flora van varkens te bewerkstelligen. Uit de literatuur was bekend dat een antibioticum, flavophospholipol, dat gebruikt wordt als AMGB, *in vitro* en *in vivo* onder experimentele condities de antibioticumresistentie bij *E. coli* bacteriën vermindert. Om vast te stellen of dit effect onder praktijkomstandigheden ook optreedt, werden op een boerderij drie groepen varkens afgemest, waarbij gedurende de laatste 12 weken voor het slachten voer werd verstrekt met en zonder AMGB. Een groep kreeg voer zonder AMGB (negatieve controle), een andere groep voer met 9 mg/kg flavophospholipol (studiegroep) en bij de derde groep (positieve controle) was avoparcine (15 mg/kg) aan het voer toegevoegd. De prevalentie en mate van resistentie tegen AMGB en antibiotica, die regelmatig therapeutisch gebruikt worden, werd bepaald bij de enterokokken en *E. coli* in faecesmonsters, individueel verzameld aan het begin en aan het einde van de studie. Vlak voor het begin van de studie waren de varkens bovendien gekoloniseerd met drie multiresistente *E. coli* stammen afkomstig van varkens door deze te mengen door het voer. Gedurende de studie nam de prevalentie en mate van resistentie van de *E. coli* van varkens in beide controlegroepen significant toe. Echter bij de varkens uit de flavophospholipol-groep bleef de prevalentie en mate van resistentie gelijk aan de waarden, gevonden in het begin van de studie. Bij het begin van de studie werden in de drie groepen geen VRE gevonden. Aan het einde van de studie was de

VRE prevalentie 44% en 41% in respectievelijk de negatieve controle- en flavophospholipol-groep, maar de mate van resistentie was in beide groepen erg laag, gemiddeld circa 1%. Bij dieren, die avoparcine kregen, was echter 72% van de varkens VRE drager en bij 57% van de dieren was de meerderheid van alle enterokokken in de faecale flora vancomycine resistent. Geconcludeerd werd dat flavophospholipol de vermeerdering en verspreiding van multiresistente *E. coli* in de darmflora van varkens voorkomt en dat avoparcine duidelijk selecteert voor VRE. Indien men in een veldproef, waarbij een groot aantal varkensbedrijven betrokken is, hetzelfde effect vindt, dient men een toekomstig verbod op het gebruik van flavophospholipol als veevoederadditief kritisch te heroverwegen. In een risico-baten-analyse zou het voordeel van een vermindering van de prevalentie en mate van resistentie in de faecale *E. coli* flora van varkens door het gebruik van flavophospholipol groter geacht kunnen worden dan het nadeel van een mogelijke selectie van resistente *E. faecalis* stammen en eventuele overdracht van dergelijke stammen naar mensen.

In hoofdstuk VIII is het effect onderzocht van het verbod op het gebruik van avoparcine in veevoerders in Nederland. Daartoe werd de prevalentie en mate van resistentie van enterokokken gemeten in faecesmonsters van slachtvarkens, slachtkuikens en gezonde vrijwilligers tegen drie voor de geneeskunst belangrijke antibiotica, nauw verwant en kruisresistent met AMGB. De faecesmonsters waren verzameld op dezelfde slachthuizen in 1997, vlak voor het verbod op het gebruik van avoparcine, en anderhalf jaar later in 1999. De faecesmonsters van de vrijwilligers waren afkomstig uit dezelfde twee regio's. De VRE prevalentie was in alle drie de populaties in 1999 significant lager dan in 1997. Bij vleeskuikens daalde de prevalentie van VRE van 80% naar 31%, bij varkens van 34% naar 17% en bij mensen van 12% naar 6%. De resistentie tegen erythromycine was gelijk gebleven, maar ook de resistentie tegen quinupristine-dalfopristine was significant gedaald, ondanks het feit dat het verwante AMGB, virginiamycine, ten tijde van het onderzoek nog niet verboden was. Het bleek echter dat in 1998 en 1999 virginiamycine in Nederland nauwelijks beschikbaar was vanwege productie-problemen bij de producent. Deze resultaten tonen duidelijk aan dat continue-

ring van het verbod op avoparcine niet alleen zal leiden tot een verdere verlaging en waarschijnlijk eradicatie van VRE uit de faecale flora van slachtdieren, maar ook van mensen. Aangezien het vancomycine-gebruik in Nederland tussen 1996 en 2000 praktisch gelijk is gebleven, is de waargenomen daling van de VRE-prevalentie bij mensen ook een bewijs dat VRE drager-schap in de faecale flora van de gezonde Nederlandse bevolking veroorzaakt wordt door avoparcine-gebruik bij dieren. De daling van de quinupristine-dalfopristine-resistentie duidt aan dat in geval van een verbod van andere AMGB een zelfde daling verwacht kan worden. Het is echter niet waarschijnlijk dat door het verbod op het gebruik van virginiamycine als AMGB deze resistentie volledig zal verdwijnen, omdat kruisresistentie bestaat met andere therapeutisch veel bij mens en dier gebruikte antibiotica: de lincosamides en macrolides.

In de conclusies wordt gesteld dat 60 jaar kwistig gebruik van antibiotica, met als gevolg een vloedgolf van resistente (potentieel) pathogene bacteriën, heeft geleid tot een erosie van de klinische effectiviteit van antibiotica. Men dient zich echter te realiseren, dat de waargenomen resistentie in (voorwaardelijk) pathogene bacteriën slechts het topje van de resistentie-ijsberg is. Er is tegenwoordig in de darmflora van mens en dier een geweldig reservoir van resistentie genen, waaruit pathogene bacteriën naar believen kunnen putten. Thans bestaat nationaal en internationaal bezorgdheid over de resistentie-toename en ziet men de noodzaak in om gezamenlijk en wereldwijd maatregelen te nemen ter beheersing van resistentie-ontwikkeling in bacteriën bij mens en dier. Uit de literatuur en ook uit het in dit proefschrift beschreven onderzoek blijkt duidelijk dat bacteriën uit de darmflora van mens en dier wat resistentie genen betreft in dezelfde vijver vissen. Resistente commensale bacteriën van nutsdieren kunnen direct, maar ook indirect, via de voedselketen, mensen besmetten, koloniseren of resistentie aan darmbacteriën van mensen en pathogene bacteriën overdragen. Daarom is het zorgvuldig gebruik van antibiotica bij nutsdieren niet alleen van

belang om de resistentie-problematiek in de diergeneeskunde te beheersen, maar ook een volksgezondheidsbelang! Een lage prevalentie en mate van resistentie in de faecale flora van nutsdieren moet daarom beschouwd worden als een veiligheidsgarantie en kwaliteitskenmerk binnen de integrale keten beheersing systemen, die de kwaliteit van producten van dierlijke oorsprong "van zaadje tot karbonaadje" pogen te waarborgen. Dit kan alleen bereikt worden door minder antibiotica te gebruiken. Daarvoor is het niet voldoende om alleen het gebruik van antibiotica op puur economische gronden als AMGB te stoppen, maar dient de dierlijke productiesector ook andere maatregelen te nemen om minder afhankelijk te worden van het gebruik van antibiotica. Echter beperking van het gebruik van antibiotica bij dieren alleen, zal de resistentie-problematiek in de medische sector niet oplossen. Het probleem in die sector wordt namelijk voornamelijk veroorzaakt door antibioticumgebruik door mensen. Het heeft echter geen zin om elkaar over en weer verwijten te maken en te beschuldigen. Een positievere benadering is samenwerking van alle betrokkenen om gezamenlijk strategieën te ontwerpen om het dreigende verlies van antibiotica uit het ons ter beschikking staande arsenaal geneesmiddelen te voorkomen. De Nederlandse veehouders en dierenartsen hebben duidelijk getoond dat zij hun verantwoordelijkheid voor de volksgezondheid serieus nemen door het op korte termijn afschaffen van het gebruik van AMGB en zich te confirmeren aan het afgesproken veterinaire antibioticumbeleid. Het toestaan van decontaminatie van karkassen van dieren na het slachten kan het risico van overdracht van bacteriële resistentie en pathogene bacteriën van dier naar mens op korte termijn zonder problemen nog verder doen dalen. Voorlichting aan de vleesverwerkende sector en de consument over de noodzaak van koeling, hygiënisch werken en een goede verhitte van vleesproducten, voordat men deze consumeert, blijft echter belangrijk ter voorkoming van overdracht van pathogene en/of resistente bacteriën van dier naar mens via voedingsmiddelen van dierlijke oorsprong.

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Allerheiligen, Maastricht, 1 november 2000

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Associate expert virologist. Proyecto de investigacion sobre la rabia paralytica (paralytische rabiës met de vleermuis: *Desmodus rotundus* als reservoir), FAO-UNDP, Mexico D.F., Mexico.

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Lid van het "Scientific Steering Committee on Antimicrobial Resistance" van de Europese Commissie, Directorate B – Scientific Health Opinions. Brussel.

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Lid van het "Committee for the Containment of Antimicrobial Resistance due to Antimicrobial Use in Animals intended for Food", WHO Consultation on Global Principles for the Containment of Antimicrobial

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List of publications

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