Emergence of a Debate: AGPs and Public Health

A. Bezoen
W. van Haren
J.C. Hanekamp*

Heidelberg Appeal Nederland

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A. Bezoen
W. van Haren
J. C. Hanekamp,* PhD, CEO HAN

Supervision:

J.C. Hanekamp,* PhD, CEO HAN
Prof. Dr. A.W.C.A. Cornelissen

At the request of the FEFANA, the HAN Foundation has carried out an independent study into the potential human health hazards related to the use of AGP's in livestock feed. The study is conducted under the auspices of the board of the HAN Foundation and an independent scientific supervisory committee.

Scientific Committee:

- Prof. Dr. A.W.C.A. Cornelissen (Division of Parasitology, Utrecht University)
- Prof. Dr. W. Gaastra (Division of Bacteriology, Utrecht University)
- Prof. Dr. W.P.M. Hoekstra (Department of Molecular and Cellular Biology, Utrecht University)
- Prof. Dr. J. Verhoef (Clinical Microbiology Group, Utrecht University)
- Prof. Dr. A. Bast (Human Pharmacology and Toxicology; Maastricht University)
- Prof. Dr. R.H. Meloen (Molecular Recognition, Utrecht University)

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HAN
*hjaap@xs4all.nl +31(0)79 346 03 04/+31(0)79 346 06 43 (fax)
www.stichting–han.nl

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Executive Summary

The HAN foundation

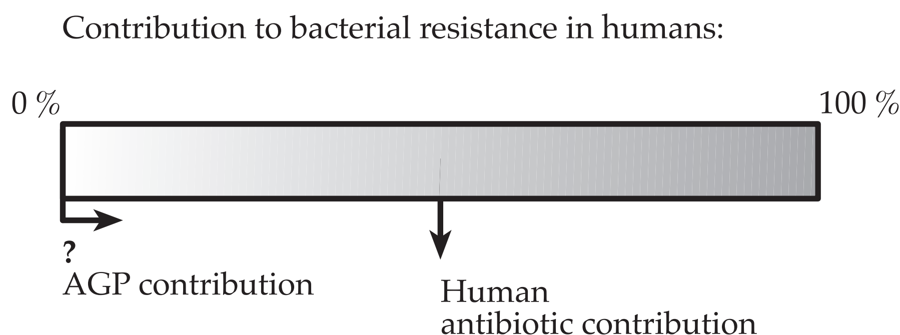
The HAN foundation (stichting Heidelberg Appeal Nederland) was established in 1993 in the Netherlands and is registered in the Chamber of Commerce in Amsterdam. HAN is an independent non-profit making alliance of scientists and science supporters whose aim is to ensure that scientific debates are properly aired, and that decisions which are taken and action that is proposed are founded on sound scientific principles. Members are accepted from all walks of life and all branches of science. HAN has at present over 800 donors, including almost 200 professors. HAN will be particularly concerned to address issues where it appears that the public and their representatives, and those in the media are being given misleading or one-sided information. Our primary role is to contribute to the scientific debate itself. Our second role is to provide an independent voice to the media, the general public and the educators, and by doing so, HAN aims to provide a balance on scientific issues. One of the activities of the HAN Foundation is to conduct scientific research at the request of third parties. Such research is performed by the HAN foundation only, supported by an independent scientific supervisory committee. To ensure that the study is executed in an independent fashion the HAN foundation has the right to publication regardless of the outcome of the research. The content of this particular report is approved by the HAN board of directors and the independent scientific supervisory committee only.

The issue

The question has been raised whether the use of antimicrobial growth promoters (AGPs) in animals can result in resistance within human bacteria. Transfer of resistance to antibiotics from livestock to humans is the point of concern here. The question is whether or not this implies a threat to human health. FEFANA (Fédération Européenne des Fabricants d'Adjuvants pour la Nutrition Animale; European federation of feed additive producers) asked the HAN foundation to re-evaluate the risk associated with the use of antimicrobial (antibiotic) growth promoting agents in livestock feed in relation to public health.

In a simplified manner, the risk issue concerning AGP use and human health can be depicted as follows, keeping in mind that any type of use ('presence') of antibiotics will result in the rise of resistant bacteria, in the species in which it is being used:

Figure 1 Possible sources of human bacterial antibiotics resistance



The risk assessment thus revolves around the question to what extent, if at all, the use of AGPs in animal rearing contributes to bacterial antibiotic resistance already present in humans.

The data

A prerequisite in this hazard scheme is the transfer of animal bacterial antibiotic resistance from animals to humans. A risk assessment thus in part requires data concerning this resistance transfer. Unfortunately, these data are in essence non-existent. Van den Bogaard *et al.* (1997b) claimed that a turkey and a farmer had the same strain of vancomycin-resistant *E. faecium*. Until now this letter is the only one that describes indistinguishable strains in animals and humans suggesting a possible transfer of bacteria. However, it was not proved that this strain really colonised the human gut. Furthermore, since other reports describing similar cases are not available, reproducibility is absent. Generalisation from this particular observation is scientifically unsound and without foundation as transfer mechanisms of DNA are manifold taking into account the different bacteria species and genera and the several resistant traits of interest.

Resistance transfer -although crucial- is, however, only part of the total risk assessment process. The acquiring of resistance by micro-organisms under selective antibiotics pressure is far from uniform and in many cases not fully resolved. Furthermore, the epidemiological consequences of resistance transfer from animals to humans, once established in a reproducible manner, need to be taken into account. Epidemiological data to this date do not show that use of AGPs in animal rearing compromised the use of related antibiotics in human medicine. Therefore, past experiences do not reveal that AGPs are a major source of resistance within human bacteria even after 30 years of use. Moreover, there are no indications that human infectious diseases are on the increase as a result of the use of AGPs. Risk analysis also requires the positive (health) effects to be taken into account such as improved animal welfare and the reduction of the shedding of pathogenic zoonotic micro-organisms.

It is clear that reproducible and documented data concerning antibiotic resistance transfer from animals to humans is lacking. This makes a formal risk assessment of this issue not possible. By definition risk assessment can not be based only on the possibility (the hazard identification) that antibiotic resistance could in theory be transferred from animals to humans. A quantitative scientific basis is needed for that. Risk analysis guarantees that sound scientific data are applied in weighing both the positive- against the negative health effects.

In conclusion

- The human health risk concerning the use of AGPs cannot be properly assessed for lack of data.
- The contribution to human bacterial antibiotic resistance from animal bacterial resistance cannot be fully assessed for lack of data.

- Sofar, AGP use did not compromise the human therapeutic use of related antibiotics.
- Sofar, epidemiological data do not show an increase of infectious diseases as a result of the use of AGPs.

- Thorough documented *in vivo* cases showing the spread of antimicrobial resistant Gram-positive bacteria from livestock to humans are in essence non-existent.

- Resistance transfer from animals to humans is only part of the entire risk chain. The major parts of this chain of events comprise of a micro-biological/ genetic part, an animal-human transfer part and an epidemiological part.

- Assessing the human health risk in relation to AGPs involves making a full scientific inventory. Beneficial aspects such as animal welfare in relation to the use of AGPs and the influence of AGPs on the spread of pathogenic zoonotic organisms also need to be taken into consideration.
- A comprehensive multidisciplinary research effort is needed to properly assess all aspects of the use of AGPs in animal husbandry.

1 The Issue

1.1 Introduction

1.1.1 General

Resistance of bacteria against antibiotics, meaning that antibiotics do not have a bactericidal or bacteriostatic effect due to the rise or inherent capability to withstand the antibiotics in question, used in human medical treatment can be a serious public health risk. It is known that the use of antibiotics can lead to the origination/emergence of antibiotics resistant bacteria (Levy, **1997**). Examples of bacteria that have become resistant to human antibiotics are:

- Methicillin–resistant *Staphylococcus aureus* (MRSA)
- Penicillin–resistant pneumococci
- Vancomycin–resistant enterococci (VRE)

It seems that there is hitherto no well–founded consistent scientific basis for the suggestion that these resistances –in part– originate from the several decades of animal feed additives use (the so–called Antimicrobial Growth Promoters (AGPs)), with a possible exception of the VREs. There is, however, extensive evidence and clinical experience that links these bacterial resistances with the human use of medicinal antibiotics both in hospitals and the local community. In spite of all this, bacterial resistance originating from animal use of antibiotics has become a subject of extensive political and scientific debate within the European Community.

Antibiotics, when added to the feed, decrease the time and the amount of feed needed to reach slaughter weight (Nefato, **1997**). It has been shown that the use of antibiotics for this goal selects for resistant bacteria in animals (Hummel *et al*., **1986**; Bager *et al*., **1997**; Klare *et al*., **1995**; Van den Bogaard *et al*., **1996**, **1997b**; Aarestrup *et al*., **1997**, **1998**). Some of the growth promoters used in feed are structurally related to antibiotics used in human medicine. Their mode of action on bacterial cells can then be identical (or highly comparable). Resistant bacteria found in animals might in this way be resistant to antibiotics used in human medicine. This is called cross–resistance. The concern now is that resistance, as found in animals, might spread to humans. This spread might add to the already widespread existence of bacterial resistance within humans resulting from human use of antibiotics. The reasoning behind this is simple and straightforward, albeit tentative:

Scheme 1.1.1.1 Risk scheme concerning AGPs and human health

> Bacteria in the animal gut and faeces contain resistant bacteria, caused by the use of antibiotics as growth promoters in livestock feed, which might be transferred to humans in one way or the other. Those resistant bacteria might themselves be a human health threat or they might transfer their resistance to other bacteria capable of colonising the human gut. Virulent resistant strains might cause illness not easily treated by known antibiotics.

In other words the human gut might be colonised by resistant bacteria previously present in animals. The second possibility is the transfer of resistance determinants from bacteria previously present in animals to human bacteria commonly present in the gut or to human pathogens. If resistance in the animal is due to the use of antibiotics in the feed, mixing antimicrobials with feed could in theory contribute to the emergence of serious infections in man.

It should be noted that most AGPs are active against Gram–positive bacteria and not against Gram–negative bacteria.[1] Antibiotics that are active against Gram–negative bacteria are usually not active against Gram–positive bacteria and vice versa. Examples of Gram–negative bacteria are *Escherichia coli* and *Salmonella typhimurium*. An example of a Gram–positive bacterium is *Staphylococcus aureus*. The antibiotics discussed in this report are active against the Gram–positive bacteria group. The bacteria considered in this report belong to the Gram–positive group. The antibiotics resistance transfer issue is thus limited to the Gram–positive bacteria group when discussing the relevant AGPs.

1.1.2 Overview

Vancomycin (an antibiotic) resistant enterococci (VRE) were first detected in hospital patients in Europe in the late 1980s. Since then these bacteria have been isolated frequently in all parts of the world (Bates, **1997**). VRE can be a problem for immuno–compromised patients, who have a severe disease or have been surgically operated. Also people who are wounded by an accident or carry medical devices like catheters have shown to suffer infections caused by VRE (Weinstein, **1998**; Bogle and Bogle, **1997**). In hospitals, the majority of VRE are isolated from patients in intensive care units and other specialised wards (Bates, **1997**).

Later it appeared that not only patients with clear symptoms of infection carried VRE, but also other patients in the hospital and people on admission to the hospital (Jordens *et al.*, **1994**; Gordts *et al.*, **1995**; Klare *et al.*, **1995**). This indicated that the problem was not solely a hospital matter.

It was found that within community people VRE was also quite widespread. These bacteria were also detected in sewage, waste water, animals and meat. Where these VRE originated is not always clear.

It is necessary to elucidate how VRE emerge and to find the source of VRE in community and hospitalised people. Do these bacteria arise in humans or are bacteria or resistance genes transferred from other sources to humans adding to the resistance of human bacteria?

Glycopeptide antibiotics, like avoparcin, vancomycin and teicoplanin, can cause emergence/selection of resistant bacteria. This has been show in humans who received vancomycin or teicoplanin (Van der Auwera *et al.*, **1997**), as well as in animals which received avoparcin as growth promoter (Bager *et al.*, **1997**; Klare *et al.*, **1995**, Van den Bogaard *et al.*, **1996**). As glycopeptide antibiotics are rarely used to treat patients in Europe, the use of avoparcin as growth promoter in feed was suggested as source for resistant bacteria present in humans (due to cross–resistance).

Avoparcin has been used in Europe in animal feed until 1997. At the moment up to ten other antimicrobials are allowed as growth promoter in animal feed. So avoparcin is not the only feed additive that may have an effect on the prevalence of resistant bacteria in humans.

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[1] The plasma membrane of *Gram-positive bacteria* is surrounded by a thick cell wall, typically 250 Å wide, composed of peptidoglycan and teichoic acid. *Gram-negative bacteria* on the other hand have a more complex membrane system. Their plasma membrane is surrounded by a 30 Å wide peptidoglycan wall, which in turn is covered by an 80 Å outer membrane comprising of protein, lipid and lipopolysaccharide. Because of the different layered cell-wall structure of the Gram-negative bacteria in comparison to the Gram-positive bacteria, antibiotics against Gram-positive bacteria are mostly inactive against Gram-negative bacteria.

In the USA avoparcin is not used as a growth promoter in animal feed. When comparing European and USA data about the prevalence and relatedness of VRE a better insight in the epidemiology (emergence and spread) of VRE might be obtained.

1.2 Objectives and methods

1.2.1 Objectives

The main objective of this report is to reassess the risk to human health caused by antimicrobial growth promoters (AGP) used as feed additives. To be able to do this, several sub–questions have to be answered.

- Does the use of antimicrobial growth promoters (antibiotics) lead to the spread of AGP resistance beyond the sphere of livestock production? There is strong evidence for the presence and emergence of bacteria in animals resistant to antibiotics present in the feed. This is not a point of controversy at the moment. It is useful to know which antibiotics are used to promote animal growth. Then it will be made clear which of them possibly form a threat to human health. The prevalence of resistance to these antibiotics (and their structural analogues used in human medicine) will be listed.
- Are there documented cases that show the spread of antimicrobial resistant bacteria from livestock to humans? Resistance to avoparcin–vancomycin (used in feed and to treat humans respectively) is quite wide spread among pigs and poultry (less in cows). Articles that describe the spread of VRE or other resistant bacteria from livestock to humans, if present, will be evaluated.
- What is the risk of the use of antibiotics in feed to human beings and how does this relate to other risk factors? Risk factors for humans concerning antibiotic resistance will be discussed. The use of antibiotics in feed as a risk factor will be evaluated.
- Can these data be generalised to all AGPs? Not only avoparcin and its relation with resistance to vancomycin will be studied, also other antibiotics used in feed and showing cross–resistance with antibiotics used in human health care will be included.

1.2.2 Methods

Literature of the last decade containing data about resistant bacteria in animals and humans will be analysed. First, factors leading to the emergence of resistant bacteria will be studied. It is important to know whether resistant bacteria are a threat for all humans or whether certain risk groups can be distinguished. Subsequently, we will focus on the resistant bacteria originated in animals due to the use of antibiotics in feed. Do they cause a threat to human health? Especially claims describing the transfer of resistant bacteria or resistance genes from animals to humans will be studied thoroughly. To be able to compare data obtained in different research groups, laboratory methods to isolate, identify and compare resistant bacteria will be reviewed.

Quite a few hurdles concerning scientific studies into resistance transfer from animals to humans have to be taken before unambiguous answers can be given. Proper comparison of data is difficult. Can resistance percentages found in animals, humans and water samples be compared or be related to each other? The following should be noted:

- Data collection and comparison should contain a thorough description of the history of the samples taken.
- Relating the use of antibiotics to the prevalence of antibiotic resistance, the history of antibiotics used in the feed and as therapy (humans and animals) has to be known.
- Different methods are used to isolate and identify resistant bacteria making data analysis and comparison complicated.

- Testing resistance to multiple antibiotics is useful in comparing strains and resistance plasmids. However, when it concerns e.g. VRE, usually only resistance to vancomycin is tested. Phenotypic and genotypic methods have to be combined when strains are compared (antibiotic susceptibility, PCR of resistance genes, PFGE of the genome).[2]

Interviews with people in the field–feed producing organisations, laboratory scientists, clinical microbiologists– will be arranged. These interviews will contribute to a good overview of amounts of antibiotics used, important literature, laboratory methods and problems occurring in hospitals.

We will not discuss the precise action of antimicrobial growth promoters on feed conversion and growth of the animal nor will we discuss environmental issues related to the use of AGPs. Also economic aspects of continued or decreased use or even a total ban of AGPs will be excluded from this study. Moreover, it is imperative that the economic, commercial and ethical aspects surrounding this issue be separated from the human health risks in relation to the use of AGPs in animal rearing. Finally, the burden of proof within the scientific arena requires a tremendous experimental effort, a thorough scientific and philosophical rigour, and a transparent presentation of results contributing to a more lucid scientific discussion.

[2] PCR: Polymerase Chain Reaction; method to amplify specific pieces of DNA.
PFGE: Pulsed-Field Gel Electrophoresis; method to make a fingerprint of a (bacterial) genome

2 Assessing the Risk

2.1 Introduction

The risk assessment procedure concerning this issue is a process, which covers a considerable number of steps. In this chapter we will look at the different phases and will see whether relevant scientific data are available. The data needed for this procedure will be discussed in the chapters following this chapter. In general *risk* is defined as follows:

> Risk is the probability that an adverse effect due to an agent or activity will occur.

In contrast, a *hazard* is an agent or activity with the potency (a possibility) to cause an adverse effect. Although the use of antibiotics as growth promoters in animal feed formally represents a health hazard to humans, it remains to be determined whether the use of antibiotic growth promoters poses a real human health risk. In principle, two aspects define risk:

- Unwanted consequences (loss, harm, death, damage)
- Probability

In order to assess the risk of a certain activity –in this case the use of antibiotics as growth promoters in relation to human health– the following three aspects need to be defined (Kaplan and Garrick, **1981**):

- Defining the unwanted consequences (***scenario***):
  'What are the negative consequences of a certain activity?'
- Defining the extent of these consequences (***scenario range***):
  'What is the extent of the unwanted event both in terms of space and time?'
- Assessing the probability of the scenario and the scenario range (***probability***):
  'What is the probability of the unwanted event?'

Kaplan and Garrick define risk as follows (Kaplan and Garrick, **1981**):

> 'It is a subjective thing –it depends upon who is looking. ... risk depends upon what you do and what you know and what you do not know.'

The subjective aspect is primarily related to the fact all answers concerning risky issues lie in the future. The mythical 'crystal ball' in which the future is depicted indeed remains mythical and thus unobtainable. Choices are always made on the basis of a limited amount of knowledge available to us at a certain point in time. The AGP issue is thus described using the three questions Kaplan and Garrick defined:

- **Scenario:**
  Bacteria in the animal gut and faeces contain resistant bacteria, caused by the use of antibiotics as growth promoters in livestock feed, which might be transferred to humans one way or the other. Those resistant bacteria might themselves be a human health threat or they might transfer their resistance to other bacteria capable of colonising the human gut. Virulent resistant strains might cause illness not easily treated by known antibiotics. (It should be noted that by definition, this tentative scenario might contribute only in part to the total resistance already present within human bacteria as a result of human antibiotic use.)
- **Extent:**
  As bacteria are capable of multiplying at tremendous rates, untreatable infections might be a global threat. Which people are at high risk for possessing or acquiring bacteria resistant to antibiotics used as growth promoter and their analogues in human health?

- **Probability:**
The probability that humans die due of an infection caused by resistant bacteria originating in animals fed with animal feed containing AGPs is the prime question in need of an answer. For this event to occur, many different events must have taken place. For all these events the probability needs to be determined. This aspect forms the heart of the assessment procedure. Are AGPs a real risk to our health? This risk must be seen in the light of the total use of antibiotics both in animals and humans.

Other effects as a result of the use of AGPs will not be included in the assessment described in this report. Those effects are, however, not irrelevant in relation to human health and are part of a total risk assessment including all facets of the use of AGPs in livestock rearing. There are e.g. clear indications that –apart from improved animal welfare due to the use of AGPs– use of AGPs decreases the shedding of pathogenic zoonotic organisms such as *Salmonella* by competitive exclusion (TNO, **1998**).

2.2 The risk chain

The risk assessment of the use of AGPs in relation to human health comprises of a large number of steps, which need to be taken into account. The chain of events can be –in a limited way– described as follows in the form of a number of questions:

Does the use of antibiotics as growth promoters give rise to resistant animal bacteria? Possible sub–questions are:

- Has acquired bacterial antibiotic resistance as a result of the use of AGPs been observed and established in the relevant Gram–positive bacteria present in the animal gut?
- If so, has the bacterial resistance mechanism (or mechanisms) been elucidated?
- What type of bacterial antibiotic resistance mechanisms exists?
- Are resistance mechanisms against antibiotics comparable?
- Is it possible to generalise specific bacterial antibiotic resistance data to other types of bacterial antibiotic resistance?
- In what manner is the acquired genetic antibiotic resistance information stored?
- Can the acquired antibiotic resistance genes be spread to other microorganisms?
- Is the acquired resistance in question of a permanent or transient nature?

Does *in vivo* transfer of resistant bacteria or animal bacterial resistance traits to humans or bacteria residing in the human gut respectively, if at all possible, pose a human health hazard? This question needs to be divided in the following sub–questions:

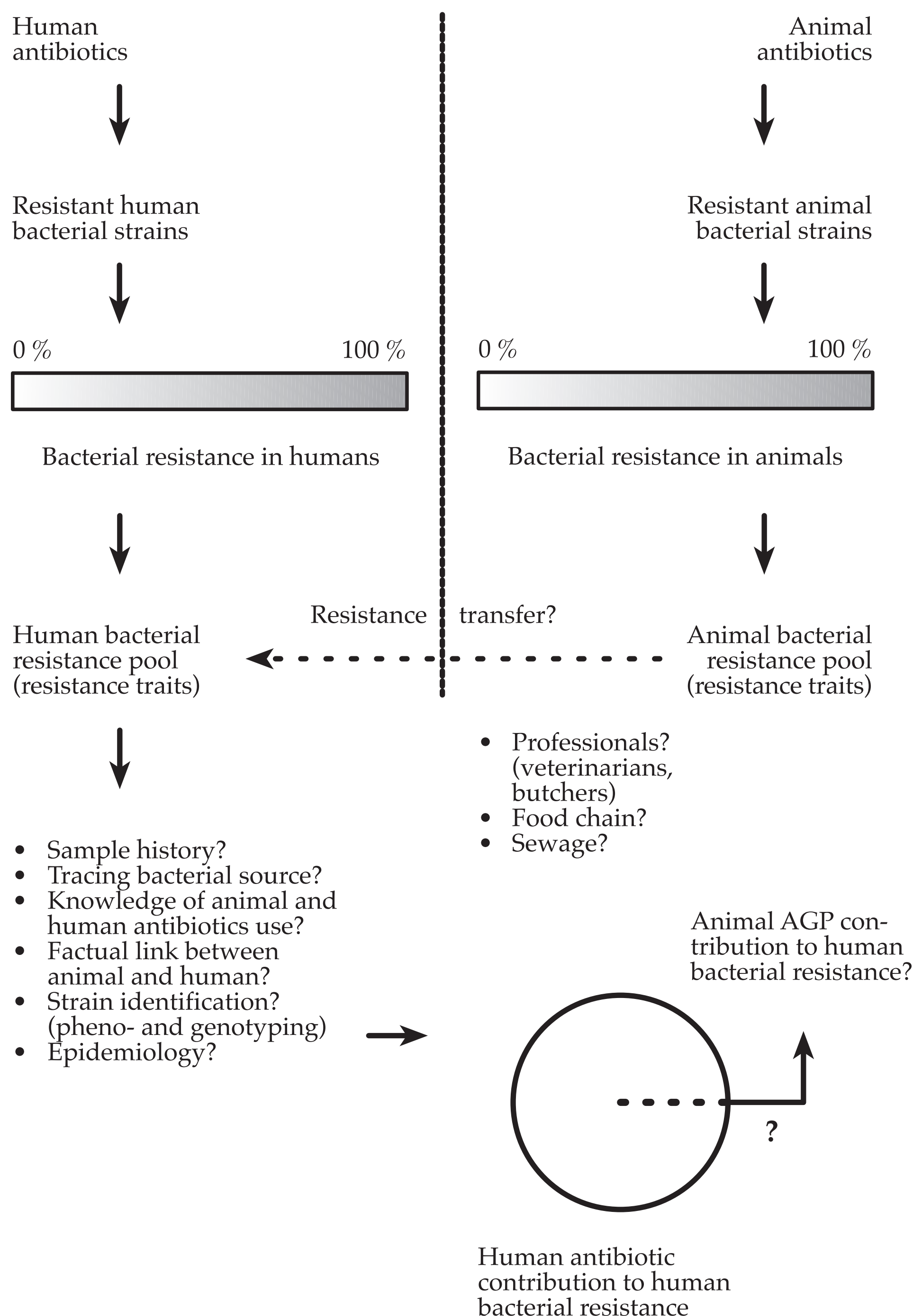
- Is transfer of bacterial resistance from animals to humans at all possible?
- What kind of transfer routes is there? (The plausibility of each route needs to be determined.)
- Are specific strains capable of colonising humans or are they to be regarded transient passengers?
- Are permanent colonising bacteria or transient passengers capable of transferring their resistance traits to other bacteria?
- What is the influence of the already present gut flora on the appearance of exogenic bacteria?
- Is the strain itself transferred to humans or does it concern the transfer of resistance traits to other strains already present in the human gut or known to be capable of colonising the human gut?
- Has bacterial antibiotic resistance transfer from animals to humans been observed and established in a reproducible manner?
- In what way can resistance transfer from animals to humans be established in a reproducible manner?

What are the epidemiological implications once transfer of animal resistant bacteria (or their resistance traits) has been observed and established in a reproducible manner? This question, again, needs to be split up in the following sub–questions:

- Has bacterial antibiotic resistance transfer from animals to humans been observed and established in a reproducible and a statistical relevant manner?
- What is the transfer frequency between animals and humans?
- Are there data to show that animal bacterial antibiotic resistance already contributed to the total human bacterial antibiotic resistance?
- Is there epidemiological data available showing an increase in human infectious diseases in relation to the use of AGPs?
- Did the use of AGPs already compromise the use of analogues human antibiotic therapeutics?
- In general are bacterial strains with antibiotics resistance more dangerous to humans than bacterial strains without antibiotics resistance?
- Are the animal bacteria themselves a hazard to human health?
- Are animal bacteria with antibiotics resistance capable of transferring their resistance traits to known human infectious organisms like MRSA?
- Does human colonisation necessarily result in disease?
- Can humans themselves, once infected with animal bacteria with resistance traits, act as a bacterial source towards other humans or animals?

These questions are in need of answering before any conclusions concerning the human health implications in relation to the use of AGPs can be drawn in a consistent manner. A formal risk assessment will at least include all these above–mentioned questions. The risk chain can, in a simplified manner, be depicted as follows:

Figure 2.2.1 The risk chain



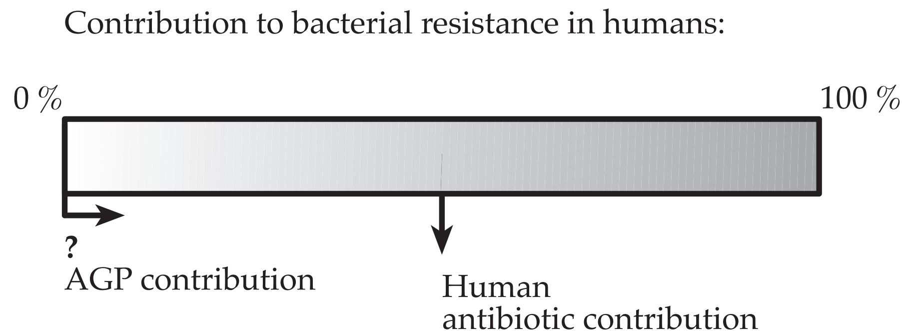
The question marks represent the research question in need of answers if the entire risk assessment concerning the use of AGPs and human health is to be made. A probability assessment of all those individual events in the 'risk chain' requires a tremendous amount of scientific data.

It has been shown that the use of antibiotics as growth promoters selects for resistant bacteria in animals. (So does any other type of use of antibiotics.) So, step 1 of the risk chain has been clarified beyond reasonable doubt. However, this does not necessarily answer the other

questions in the risk chain. Every event needs to be scrutinised in order to assess its probability. All answers taken together results in an overall probability assessment concerning the human health risk in relation to the use of AGPs.

In a simplified manner, the risk issue concerning AGP use and human health can be depicted as follows, keeping in mind that any type of use ('presence') of antibiotics will result in the rise of resistant bacteria, whether in man or animal:

Figure 2.2.2 Possible sources of human bacterial antibiotics resistance



The risk assessment thus revolves around the question to what extent, if at all, the use of AGPs in animal rearing contributes to bacterial antibiotic resistance already present in humans. Below we shall go through the risk chain in a step–wise manner.

2.3 Questions and answers

In a series of questions and answers we will make an effort to pinpoint the AGP issue. We will start off with the basics and work to the central themes. The answers mapped in this fashion will give some clues about the human health risks involved in the AGP use.

Table 2.3.1 Questions and answers part 1

Question	Answer
Which antibiotics are of interest in the AGP–debate?	Those antibiotics showing cross–resistance towards 'human' antibiotics namely avoparcin, tylosin, virginiamycin, spiramycin and Zn–bacitracin, avilamycin.
Against which bacteria are those antibiotics active?	Primarily Gram–positive bacteria: enterococci, streptococci, staphylococci.
Is the emergence of bacterial resistance in animals against those antibiotics documented?	**Avoparcin**: yes, namely enterococci; **tylosin**: yes, namely enterococci, staphylococci, *Campylobacter*; **virginiamycin**: yes, namely enterococci, staphylococci, streptococci; **Zn–bacitracin**: questionable.
How is resistance in general accomplished?	**Avoparcin/vancomycin**: Transferable acquired resistance: the *vanA* gene cassette (Tn*1546*) and the *vanB* gene cassette (Tn*1547*) both observed in *E. faecium*, *E. faecalis*, *S. bovis*. Non–transferrable intrinsic resistance: *vanC1*, *vanC2*, *vanC3* genes observed in *E. gallinarum* and *E. casseliflavus*. **MLS_B antibiotics** (tylosin, spiramycin, virginiamycin, erythromycin, Synercid®, lincosamide). Some examples: Staphylococcal *vat* (plasmid) against streptogramin A; staphylococcal *vgb* (plasmid) against streptogramin B; staphylococcal *ermA* (Tn, chromosome) against all MLS_B antibiotics; enterococcal *satA*, against streptogramin A. **Zn–bacitracin**: No transferable resistance genes known.

Table 2.3.2 Questions and answers part 2

Question	Answer
What is the host range of the bacteria in question?	Some species of enterococci, streptococci and staphylococci.
Are bacteria in animals that have become resistant to the AGPs, in principle capable of colonising the human gut?	Bacteria present in animals are in theory capable of transferring to humans in case of close human–animal contact e.g. through faeces or intestines, meat consumption, or vegetables manured with animal faeces.
Are there documented cases, showing *in vivo* transfer of resistant bacteria from livestock to humans?	In essence non–existent. Van den Bogaard (**1997b**) reported to have characterised indistinguishable strains of vancomycin–resistant enterococci present in turkeys and a farmer. However, the presented results were not reported to have been reproduced. Furthermore, it was not made clear whether it concerned permanent colonisation or one of a more transient nature. Generalisation from this observation is by definition not possible.
How frequent does colonisation of the human gut with animal bacteria occur?	No data available.
Is transfer of plasmids/transposons possible in the human gastro–intestinal system?	Bacteria of the same species, related species or other genera do exchange DNA. Laboratory data are available showing that transfer is possible. No in vivo data are available, however.
How frequent does resistance transfer between animal and human bacteria in the human gut occur?	No data available. The link between similar bacterial strains of human and animal origin is difficult to establish. A thorough analysis of strains is a requirement, both phenotypically and genotypically. Sample history and documented antibiotics use is essential.
Which plasmids/transposons have been detected in human and animal bacterial samples?	The *vanA* gene cassette Tn*1546*. The presence of other resistance traits in samples has hardly been studied.
Are there documented cases, showing human infections being caused by resistant bacteria originating from animals?	No data available. Similarly, data concerning the frequency of infections due to transfer of animal bacteria to humans is not available. AGP–use did sofar not show any increase in human infection rates.
Is it possible to follow the flow of resistance genes?	In theory, yes. It requires a tremendous interdisciplinary research effort, however.
To what extent has resistance transfer from animals to humans has contributed to the total bacterial resistance in humans within the AGP context?	No data available. It is clear, however, that antibiotics used in the course of treatment cause the development of resistant bacteria in e.g. hospital patients. An example is the rise of MRSA.

2.4 Reassessing the risk

Presently the AGP issue is hotly debated within the European Community. Resistance in human bacteria is in part thought to arise due to the use of AGPs in animal rearing. This, however, remains to be seen. Use of an antibiotic in any fashion will give rise to resistance in bacteria. History shows that human use of antibiotics has generated widespread resistance in bacteria capable of causing infectious diseases within man. The rise of the MRSA bacteria is a classic example showing that widespread use of human antibiotics gives rise to multiresistant infectious bacteria. The question whether animal uses of antibiotics –in this case, as growth promotion– will add to this resistance is at the centre of this study. This report hopefully will contribute to this debate in a consistent manner.

The questions stated in chapter 1 are as follows:

- Does the use of antimicrobial growth promoters (antibiotics) lead to the spread of AGP resistance beyond the sphere of livestock production?
- Are there documented cases that show the spread of antimicrobial resistant bacteria from livestock to humans?
- What is the risk of the use of antibiotics in feed to human beings and how does this relate to other risk factors?
- Can these data be generalised to all AGPs?

The two tables depicted above show that data needed for a complete risk assessment, which encompasses the whole of the risk chain, is grossly lacking. Data concerning transfer of Gram–positive bacteria resistant to AGPs from animals to humans is in essence non–existent. Van den Bogaard *et al*. (**1997b**) claimed that a turkey and a farmer had the same strain of vancomycin–resistant *E. faecium*. Until now this letter is the only one that describes indistinguishable strains in animals and humans. Moreover, it was not shown that this strain really colonised the human intestine and was not a transient passenger. Furthermore, reproducibility is lacking making this observation in effect open for debate and in want of thorough scientific scrutiny. Apart from these comments, extrapolation from this observation to other organisms or antimicrobial resistance traits is not possible.

This lack of essential data complicates matters substantially. Although resistance transfer is the crux of the risk assessment it is only one of the many steps in the risk chain depicted above. Analysing resistance transfer already encompasses the following:

- The bacterial strain and/or the resistance trait present in the human should be identical to a bacterial strain and/or resistance trait present in the meat consumed. In many reports meat samples and human samples are compared that might be totally unrelated insofar that the meat of the animal that is consumed is not traced, so the relation between resistant bacteria and consumption of meat is not evident or even non–existent.
- Preferable, the exact source of the resistant bacteria has to be elucidated. If the source is probably an animal, the usage of antibiotics in its feed should be known. Otherwise a possible relationship between antibiotic usage in animal feed and resistant bacteria in humans cannot be confirmed.
- When meat samples are examined, one needs to be sure that resistant bacteria found are not the result of contamination during processing, preparing or transport of meat.
- Typing methods for identifying bacteria have to be specific enough to detect small differences between bacterial strains and their resistance traits.
- On farms it is easier to trace the animal, which is causing the presence of resistant bacteria in the intestines of the farmer than from people in a town consuming meat. When a farmer does not eat meat produced on its own farm, the direct transfer of resistant bacteria from animals or animal faeces to the farmer could be detected. Reproducibility is essential in this case.

When discussing human bacteria resistance against antibiotics in relation to animal bacterial resistance as a result of AGPs transfer of resistance from animals to humans is a highly complicating factor, which at present is not solved for lack of quality data. The question is whether this is a solvable problem. Hitherto, the use of AGPs in animal rearing did not show deteriorating human health as a result of infectious diseases caused by resistant bacteria. Use of human antibiotics díd result in the rise of resistant human bacteria. The following table serves to illustrate this point (Kirst *et al*., **1998**):

Table 2.4.1 VRE infections in relation to vancomycin use

	USA	UK	Denmark
VRE infections in humans	++++	+	0
Avoparcin (AGP)	0	+++	+++
Vancomycin (kg in 1996)	11,279	320	60

Solving the AGP issue as a possible contributor to human bacterial resistance is hampered by lack (or even absence) of data, methodological inadequacies, experimental difficulties, lack of reproducibility and etceteras.

The risk assessment might be considerably simplified choosing for a ‘human’ approach. Resistance data of the human therapeutic use of antibiotics is probably much more available. Human bacterial resistance will primarily come from the human therapeutic use of antibiotics. Bacterial antibiotic resistance in animals might contribute to human bacterial resistance if –and only if– antibiotic resistance is transferred to humans. A formal risk assessment of the human use of antibiotics in relation to the rise of resistant human bacteria might –in an indirect manner– elucidate the possible animal contribution. This approach circumvents a number of fundamental difficulties such as the bacterial resistance transfer issue. In conclusion the following can be stated:

- The human health risk concerning the use of AGPs cannot be properly assessed for lack of data.
- The contribution to human bacterial antibiotic resistance from animal bacterial resistance cannot be fully assessed for lack of data.

- Sofar, AGP use did not compromise the human therapeutic use of related antibiotics.
- Sofar, epidemiological data do not show an increase of infectious diseases as a result of the use of AGPs.

- Thorough documented *in vivo* cases showing the spread of antimicrobial resistant Gram-positive bacteria from livestock to humans are in essence non-existent.
- Resistance transfer from animals to humans is only part of the entire risk chain. The major parts of this chain of events comprise of a micro-biological/ genetic part, an animal-human transfer part and an epidemiological part.

- Assessing the human health risk in relation to AGPs involves making a full scientific inventory. Beneficial aspects such as animal welfare in relation to the use of AGPs and the influence of AGPs on the spread of pathogenic zoonotic organisms also need to be taken into consideration.
- A comprehensive multidisciplinary research effort is needed to properly assess all aspects of the use of AGPs in animal husbandry.

3 Antibiotics: Use and Resistance Mechanisms

3.1 Summary

Below we shall summarise point by point the issues described in this chapter.

- Antibiotics are chemical compounds, produced by living organisms (such as fungi or bacteria), that are detrimental to other competing organisms. Usually these compounds kill or inhibit growth of bacteria or other microorganisms.
- Antibiotics are used both in human and animal medicine and as growth promoters (AGPs) in animal feed.

- AGPs discussed in this report are primarily active against Gram–positive bacteria (with a limited overlap towards Gram–negative bacteria) thus resistant Gram–positive bacteria are of our main concern.

- Bacteria can either have an intrinsic or an acquired resistance against antibiotics. Intrinsic resistance can only be passed on through cellular multiplication (bacterial offspring). Acquired resistance against antibiotics is in principle transferable to other organisms. This is the point of concern in the AGP discussion in relation to human health.

- Bacterial antibiotic resistance can be acquired in basically the following ways:
- *through chromosomal mutations (without selective antibiotic pressure)*
- *through DNA transfer (with selective antibiotic pressure)*

- In general transfer of resistance traits can be achieved by:
- *transformation (DNA uptake from the environment)*
- *transduction (DNA transfer with the aid of a bacteriophage (a virus))*
- *conjugation (DNA transfer by direct cell to cell contact)*

- A number of biochemical resistance mechanisms against antibiotics are:
- *enzymatic breakdown or modification of the antibiotic (β–lactamases)*
- *overproduction of target*
- *two versions of antibiotic target; one sensitive, one resistant*
- *change of target site so that antibiotic does not bind*
- *eliminate entry ports of the cell (decreased uptake)*
- *produce pumps that export antibiotics out of the cell (decreased uptake)*
- *missing of target enzyme or metabolic pathway (intrinsic)*

- The most important risk factor for the emergence of resistant bacteria is contact with antibiotics. Every use of antibiotics selects for bacteria that are less susceptible for that antibiotic (and related antibiotics).
- The continued use of small amounts of antibiotics as AGPs in animal feed will promote bacterial resistance to this antibiotic within livestock.
- The prolonged presence of antibiotics in animal feed increases the risk of resistance transfer within livestock.

In this chapter it will be shown that the use of antibiotics as AGPs results in the rise of resistant strains of Gram–positive bacteria within livestock. This is not a point of discussion. However, to what extent (if at all) the existence of resistant strains of bacteria in livestock is a human health threat is still an open question that needs answering. In the next chapter we shall look at this issue more closely.

3.2 Antibiotics: categories

3.2.1 General

Antibiotics are chemicals produced by specific types of bacteria or fungi. They can be used to treat bacterial infections because they stop the growth of bacteria or are able to kill them (respectively bacteriostatic and bactericidal activity). In this way the infection can be stopped and the immune system of the animal or human infected is capable of dealing with the (remaining of) the bacteria (Bryan, **1982**).

Some antibiotics are active towards many bacterial species, while others are more specific (broad/wide and narrow spectrum antibiotics respectively). Antibiotics with a broad spectrum are aminoglycosides, tetracycline and imipenem. Structural unrelated antibiotics are able to act on the same place in/at the bacterial cell. For instance the antibiotics D–cycloserin, fosfomycin, bacitracin, glycopeptides all act on cell wall synthesis.

3.2.2 Categories of antibiotics

Antibiotics can be divided in different groups, according to their structure or their target site in the cell. Some of these, like the β–lactam antibiotics and the tetracyclines have been used in human medicine since the 1940s (Levy, **1998**). In the early days of twentieth century of medicine the antibiotic as formed by the producing organism was used. To increase the performance and specificity of antibiotics the 'basic' antibiotic can be chemically modified. For instance, ampicillin and methicillin are semi–synthetic penicillins derived of penicillin G (Schlegel, **1992**).

Table 3.2.2.1 Structural subdivision of antibiotics (Bryan, 1982; Leclerq and Courvalin, 1991; Lambert et al., 1992; Schlegel, 1992; Allignet et al., 1996; SCAN 1998a; Murray, 1998)

Antibiotic Groups	Antibiotics
ß–lactam antibiotics:	
Penicillins	benzylpenicillin, ampicillin, ureidopenicillin, amoxycillin, piperacillin, methicillin
Cephalosporines	first, second and third generations cephalosporin, cephalothin, cephalordin, cephaglycin
Carbapenems	imipenem
Aminoglycosides	streptomycin, kanamycin, gentamicin
Glycopeptides	vancomycin, avoparcin, teicoplanin
Macrolides:	
14–membered rings	erythromycin, roxithromycin, oleandomycin
15–membered rings	azithromycin
16–membered rings	spiramycin, tylosin, carbomycin, clarithromycin
Lincosamides	lincomycin, clindamycin
Streptogramins:	
Streptogramins A	streptogramin A, pristinamycin IIA, virginiamycin M, mikamycin A, synergistin A
Streptogramins B	streptogramin B, virginiamycin S, pristinamycin IB, mikamycin B, synergistin B
Combinations	dalfopristin/quinupristin (Synercid®), virginia–mycin
Tetracyclines	minocyclin, tetracycline, chlortetracycline
Folic acid synthesis inhibitors	sulfamethoxazol, trimethoprim
Quinolones	nalidixic acid, ciprofloxacin, enrofloxacin
Others	nitrofurantoin, sulfonamide, 2,2–diamino–pyrimidine; Zn–bacitracin

Another way antibiotics can be subdivided is their mode of action. Antibiotics act specifically on bacterial cells or on processes in these cells. For the scope of this report it is not necessary to understand the mode of action of all antibiotics towards the cell or cellular processes. What will be described is the mode of action for the specific antibiotics important for this report. These are antibiotics that might cause a problem for human health. The classes of antibiotics that will be considered are the glycopeptides, macrolides and streptogramins. These antibiotics interfere with cell wall production (glycopeptides) and the synthesis of proteins (macrolides, streptogramins).

Table 3.2.2.2 Modes of action of antibiotics (Bryan, 1982; Russell and Chopra, 1990; Levy, 1998)

Point of interference	Examples
Cell wall production	- Inhibition of cross–linking of peptidoglycan - Interference with pentapeptide formation - Inhibition of transport of peptidoglycan precursors through the membrane
Protein production	- Prohibition of initiation of protein synthesis due to binding to the 30S subunit prior to formation of 70S subunit - Preventing elongation due to interference with linking of mRNA to tRNA - Inhibition of elongation by binding to the 50S ribosomal subunit or elongation factors
Nucleic acid production	- interference with nucleotide metabolism (e.g. dihydrofolate synthesis) - inhibition template function DNA - inhibition polymerases and other enzymes involved with DNA and RNA synthesis
Folic acid production	–

3.3 Antibiotic usage

3.3.1 General

Humans mainly receive antibiotics to treat bacterial infections. Physicians or dentists prescribe antibiotics in hospitals or within the community. In hospitals antibiotics can also be provided prophylactically, preventing infections (for example previous to and during operations; Gopal Rao, **1998**).

Antibiotics are given to farm animals for a number of purposes. The prophylactic use is more common in farm animals then it is in humans. When one animal in a herd or pig–house has an infectious disease, often the whole herd is treated. Besides the therapeutic or prophylactic use most of the animals reared on farms receive antibiotics in their daily feed. This is done because of the positive effect these antimicrobials have on animal growth and the amount of feed needed to reach slaughter weight.

The table presented below gives an impression of antibiotic use. On a national or local scale the amounts and the fields of use might differ substantially:

Table 3.3.1.1 Indication of use of antibiotics in different fields (Harrison and Lederberg, 1998)

Targets	Fields of use	Percentage of total
Humans	Hospital	20%
	Community	80%
Animals	Therapeutic	20%
	Prophylactic/growth promotion	80%

Amounts of antibiotics used cannot be compared easily between humans and animals. Below the most important factors that complicate matters are listed (Mudd, **1998**; Van den Bogaard, **1997a**; Levy, **1997**).

- Dosages applied are different for each antibiotic and each application
- A difference in potency of antibiotics affects the total weight used
- The potency of the antibiotic preparations used in animal feed might vary
- The time–scale on which antibiotics are used influences the impact the antibiotic has on the animal/human and its environment

Usually rough figures are given about the yearly antibiotic use on animal farms. For comparison between different antibiotics and different applications the most accurate way is to compare the defined daily dosages (of the active compound) that are given to an animal or human. When a human or animal is treated for an infection, the dosage of antibiotics is usually recorded. Also the amounts of antibiotics present in the feed obtained from feed companies that mix antibiotics with the product is traceable.

Some attempts have been made to compare amounts used by humans and animals. According to Van den Bogaard (**1997a**) the total amount animals receive in one year is in the same order of magnitude as the amount humans receive (430 mg/kg body weight for poultry, 125 mg/kg for pigs, 55 mg/kg for cattle and 100 mg/kg for humans). In the UK, according to FRANA/Nefato, animals use less antibiotics than humans, 57 million people uses three times more antibiotics than the 198 million animals do.

However, by only comparing total amounts of antibiotics the situation is presented in an oversimplified manner. To be able to assess the risk of a certain use of antibiotics, not only amounts are important. The impact the antibiotic has on the flora in the intestines of the individual/animal treated is of paramount importance. This is not only related to the amount of an antibiotic administered, but also on the type of antibiotic and the time–scale of treatment. In general, the impact on the environment will be larger when treatment is prolonged and when more individuals/animals are treated per geographic area (Levy, **1997**; Nord, **1993**).

3.3.2 Antibiotics used in animal husbandry

Below is out–lined why antibiotics are used in feed, which antibiotics are used, in what amounts and on which scale.

The positive effect of antibiotics on growth was discovered incidentally. Stokstad and Jukes (**1949**) used the remaining of a fermenter culture of *Streptomyces aureofaciens* as a cheap source of vitamin B_{12}. Cultures of this actinomycete were used to produce chlortetracycline. The chickens receiving the remaining material grew better than could be expected from the vitamin B_{12} alone and it seemed that the presence of chlortetracycline was responsible. Soon other antibiotics showed to have similar effects. Since the 1950s it became a routine to add low levels of antibiotics to animal feed (Van den Bogaard, **1997b**). In many countries turkeys, chickens, pigs and calves receive feed that contains several antibiotics in low amounts. In this report principally the use of antimicrobials in Europe will be discussed.

According to studies in the Netherlands, the use of AGPs leads to an increase in growth of 1 to 8 % compared to animals that do not receive AGPs (Jongbloed, **1998**; Westerhuis, **1998**). The profit depends on the age of the animal and the animal species. Little pigs show a growth improvement of 3 – 8 %, while for broilers the effect is 2 – 4 %. The effect growth

promoters have on older pigs, sows, laying hens and cows is less clear and lies between 0 – 3 %.

Growth promoters enhance digestion of the feed, the feed conversion (amount of feed (kg) needed to obtain 1 kg increase in weight) is improved. For pigs (22 up to 103 kg) it has been shown that the feed conversion decreased from 2.84 to 2.74 (Nefato, **1997**).

In Sweden, where antimicrobials were forbidden in the feed in 1986, it was shown that it took 3 to 5 days more before pigs reached 25 kg of weight without growth promoters (Viane, **1997**). So more feed is needed in the absence of growth promoters. This leads to an increase in the amount of faeces that is produced till the time the animals reach their final weight (Nefato, **1997**).

A wide range of antimicrobial additives is used or has been used in animal husbandry to promote growth. Often the drug used as growth promoter is not used therapeutically for animals. The amount of an individual growth promoter animals receive lies in the range of 5 – 100 parts per million (ppm = mg/kg feed). The most common dosage is 20 – 50 ppm. The amount depends on the antimicrobial given, animal species and its age. The amounts that are applied usually are the maximum allowed dosages or amounts close to this maximum (see Feed Additive Directive 70/524 EC). AGPs in feed are as follows:

Table 3.3.2.1 Kinds of antimicrobial growth promoters added to the feed (provided by Op den Kamp, 1998; Gezondheidsraad, 1998)

Animals	Type of AGP in feed
Turkey	virginiamycin, Zn–bacitracin
Chicken	avilamycin, flavomycin, spiramycin, virginiamycin, Zn–bacitracin
Pigs (up to 25 kg)	avilamycin, olaquindox, salinomycin, tylosin, virginiamycin
Pigs (25 kg up to 4 months)	as above plus Zn–bacitracin
Pigs (4 months until death)	avilamycin, salinomycin, tylosin, virginiamycin, Zn–bacitracin
Sows and breeding sows	virginiamycin
Calves	virginiamycin, Zn–bacitracin
White meat cows	flavomyin, virginiamycin, Zn–bacitracin
Red meat cows	monensin, flavomycin, virginiamycin

The amount of feed animals consumes and the percentage of the feed that contains antibiotics in the Netherlands is given below.

Table 3.3.2.2 Amounts of feed consumed by different animal species and percentage of feed with added AGPs in the Netherlands in 1996–1997 (Gezondheidsraad, 1998; PDV, 1997; IKC, 1998)

Animals	Amounts of feed (tons)	Percentage of feed containing AGPs
Turkeys	120,000	98
Broilers	1,150,000 – 1,175,000	95 – 99
Laying/breeding hens	1,900,000	10
Piglets	1,930,000	100
Pigs up to 16 weeks	1,500,000	100
Pigs up to 6 months	2,700,000	95
Sows and breeding sows	1,525,000 – 1,650,000	15 – 20
Calves	300,000	20
White meat cows	400,000	100
Red meat cows	360,000	90

In total, approximately 250 – 300 ton of antibiotics are mixed yearly with the feed in the Netherlands (Nefato, **1998**; Piron (FEFANA–Alpharma), **1998**).

3.3.3 Regulations for the use of antimicrobial agents

Already in the Swann report (**1969**) it was stated that antibiotics showing cross–resistance with antibiotics used in human health care should not be used as growth promoters. The ban of tetracycline and penicillins as growth promoters was recommended because these antibiotics are also used as a human medicine. In the 1970s the use of tetracycline and penicillin as growth promoter was banned in the European Community (Witte, **1997**; Gezondheidsraad, **1998**). In 1970, a European directive was published (70/524 EEC) that contained prerequisites for the use of antimicrobial growth promoters (Butaye *et al.*, **1998c**). Only growth promoters should be used that:

- have a proven growth promoting effect
- are active towards Gram–positive bacteria
- should not be used as growth promoter and as medicine (for animals or humans)
- should not be related to antibiotics used as a human medicine
- should not be resorbed from the intestine (to prevent the presence of residues in the meat)

In 1997 more items have been added to directive 70/524 EEC (Nefato, **1997**):

- Every product is checked on the basis of a dossier containing safety, quality and effectivity measurements
- For every product one company is responsible
- Every 10 year the component has to be re–evaluated, with the latest scientific knowledge as guideline

Before an antibiotic is accepted for usage in animal feed some important features needs to be analysed: chemical structure, mechanism responsible for drug resistance and cross–resistance with antibiotics used in human healthcare. Once approved, new registration–files are made every few years, containing the latest facts about the antibiotics involved (safety of use).

Not all the growth promoters currently used meet the prerequisites mentioned in directive 70/524 EEC. Especially the point that antibiotics used as growth promoter should not be related to antibiotics in medicine or should not be used in medicine is often not complied

with. As can be seen in the table below many growth promoters have structurally related relatives that are used in human health care.

Table 3.3.3.1 Antimicrobials that are used or have been used in animal husbandry in Europe as AGPs and their analogues used in human health care (Bryan, 1982; Witte, 1997; Butaye et al., 1998c; Op den Kamp, 1998; Aarestrup et al., 1997, MAFF, 1998)

Animal AGP use	Country	Class	Human health care	Resistant
Avilamycin	NL, UK, BEL	Orthosomycins	Everninomycin	–
Avoparcin[3]	Europe	Glycopeptide	Vancomycin Teicoplanin Daptomycin	enterococci
Zn–bacitracin	Europe	Polypeptide	Zn–bacitracin	*clostridia,* enterococci
Carbadox	Europe	Quinoxaline	Unknown	Gram–negative
Flavomycin	Europe	Phosphoglycolipid	Unknown	
Monensin	Europe	Ionophore	Unknown	
Nourseothricin[4]	East–GER		Streptothricin	enterobact.
Tylosin/ Spiramycin	Europe	Macrolide	MLS_b antibiotics: Erythromycin	enterococci staphylococci streptococci
Virginiamycin	BEL, NL, DK, UK	Streptogramins	Synercid®	enterococci staphylococci streptococci

The antibiotics causing most (political) concern are avoparcin (not used anymore in Europe), virginiamycin, tylosin and Zn–bacitracin. The human health risk these antibiotics might cause will be discussed and evaluated in this report.

3.3.4 Antibiotics in animal feed

Avoparcin

Avoparcin is a glycopeptide antibiotic which has been widely used as feed additive since 1975 (Witte, **1997**). This antibiotic is not metabolised when ingested by pigs and chickens, so it leaves the body in the active form (Bager, **1997**). In humans, vancomycin and to a lesser extent teicoplanin are important tools, albeit limited, in treatment of bacterial infection caused by multiple resistant bacteria (mainly multiresistant staphylococci, enterococci and pneumococci). Bacteria resistant to avoparcin have been shown to be cross–resistant to vancomycin and teicoplanin (Cormican *et al.*, **1997**).

Avoparcin was banned in the EC in January 1997. Denmark decided to ban avoparcin already in 1995 as a result of a report of the Danish Veterinary Laboratory (**1995**). In January 1996 Germany was the second country to ban avoparcin. The decision of the German Federal Institute for Consumer Health Protection and Veterinary Medicine to ban avoparcin was partly based on the Danish study mentioned above.

[3] Not used anymore.
[4] Idem.

In enterococci the vanA gene cluster mediates the high level of resistance to vancomycin. The possibility that the *vanA* gene may be transferred to other bacteria like methicillin–resistant *Staphylococcus aureus* (MRSA) was important to choose for a preventive protection of human health.

In October 1995 a question (no. 82) concerning the continued use of avoparcin as a feed additive was addressed to the SCAN. At the European level, the SCAN is the advisory board about the use of antibiotics as feed additives. The SCAN evaluated the report of the Danish Veterinary Laboratory (**1995**), especially on the point of relevant scientific data that supported the need for banning avoparcin. According to the SCAN the Danish demonstrated the presence of glycopeptide–resistant enterococci in isolates from the majority of pig and poultry farms that used avoparcin. Also it was made clear that the transfer of resistance genes from *E. faecium* to *E. faecalis* could be achieved in the lab (Noble *et al*., **1992**). A third point was that resistance to avoparcin leads to cross–resistance to vancomycin and teicoplanin. The DVL report, however, did not present evidence that the use of avoparcin as a growth–promoting agent caused disease in man or that existing diseases in animals or man increased or worsened notably. *So, SCAN concluded that there was no direct evidence that the use of avoparcin in animal feed presented a risk for human health.*

The European Community, however, decided to ban the use of avoparcin by January 1997 as a precautionary measure, partly based on the argument that the risk for human health could not be ruled out. SCAN namely also concluded that:

> '... [it] cannot be ruled out with sufficient certainty that the use of avoparcin in feed may lead to the spread of glycopeptide–resistance beyond the sphere of livestock production.' (SCAN, **1996**)

Such a conclusion is by definition derived from the fact that no amount of scientific experiments will be sufficient to exclude with absolute certainty a certain risk related to the use of AGPs.

Macrolides and streptogramins

The antibiotics tylosin, spiramycin and virginiamycin belong to the macrolide–lincosamide/streptogramin B (MLS_B) group of antibiotics. Tylosin and spiramycin belong to the macrolides, while virginiamycin is a member of the streptogramin group. It is known that within the group of MLS_B antibiotics cross–resistance can occur. Antibiotics of the MLS_B class are used as medicines in humans and animals. Macrolides are used to treat respiratory tract infections (caused by Gram–positive bacteria) outside the hospital, but are also applied to treat infections with (Gram–negative) *Campylobacter* spp. (Aarestrup *et al*., **1998**). Resistance mechanisms are known that lower the susceptibility of multiple compounds belonging to this antibiotic class (Allignet *et al*., **1996**).

Tylosin and spiramycin were approved for use as feed additives in the EEC in 1970. Tylosin is allowed for use in pigs and piglets, while spiramycin can also be used for poultry, calves, lambs and fur animals (SCAN, **1998a**).

In Denmark (**1995**) 52.3 tonnes of tylosin were used as additives in pig feed, while 0.5 tonnes of spiramycin was added to the feed of broilers. Also 9.5 tonnes of macrolides were used in therapy of animals. In Finland 0.74 tonnes of macrolides were solely used to treat diseased animals (mainly *Serpulina* infections).

Denmark banned the streptogramin virginiamycin as a feed additive in 1998 under a safeguard clause. SCAN was asked to review the scientific material on which the Danish government based its ban. SCAN is highly critical in its comments on the scientific evidence presented to them (SCAN, **1998b**). SCAN concludes the following:

> '… 1. no new evidence has been provided to substantiate the transfer of a streptogramins or vancomycin resistance from organisms of animal origin to those resident in the human digestive tract and so compromise the future use of therapeutics in human medicine
>
> 2. the development of vancomycin resistance amongst *E. faecium* and methicillin–resistant strains of Staphylococcus aureus, …, are evidently a cause for concern. However, the data provided in the Danish report does not justify the immediate action taken by Denmark to preserve streptogramins as therapeutic agents of last resort in humans.
>
> 3. as survey data … failed to detect a single case of VRE, as Denmark has amongst the lowest incidence of MRSA in Europe and North America, and as coagulase–negative staphylococci remain sensitive to vancomycin, there are no clinical reasons to require the introduction of streptogramins as human therapeutics in Denmark now or in the immediate future. …'

In countries that permit the use of streptogramins in both animal production and human medicine, notably France and the USA, the use of pristinamycin (a human therapeutic antibiotic) has not been compromised by the use of virginiamycin as a growth promoter.

Zn–bacitracin
To date, as a human curative, bacitracin is only used topically to cure infections of the skin or mucous membranes. Lately also patients with VRE infections are treated (Chia *et al.*, **1995**). In the future bacitracin might be used to treat MRSA infections as well.

3.3.5 Antibiotics in human health care

As antibiotics kill or inhibit growth of bacteria, they can have a serious impact on the human intestinal flora. In the human intestine many bacteria are present. The predominant genera are the anaerobic *Bacteroides*, *Fusobacterium*, *Bifidobacterium*, *Clostridium*, *Propionibacterium*, *Eubacterium* and the facultatively anaerobic Enterobacteriacea (*E. coli*, *Enterobacter*), *Lactobacillus*, *Enterococcus* and *Streptococcus* (Drasar and Barrow, **1985**). A precise composition of 'normal human intestinal flora' (quantitatively and qualitatively) can not be given, partly because not all bacteria can be cultured easily.

If a patient suffers from an infection caused by Gram–negative bacteria, this usually is treated with broad–spectrum antibiotics like cephalosporines and fluoroquinolones. The Gram–negative bacteria are killed and Gram–positive bacteria, like enterococci can then cause overgrowth (Murray, **1990**). As a consequence, these bacteria can cause severe damaging health effects.

There are three groups of antibiotics that can cause severe effects on the intestinal flora:

- orally administered antibiotics; these are not well absorbed from the gastrointestinal tract
- antibiotics that are absorbed but subsequently excreted in the bile
- parenterally given antibiotics that are subsequently excreted in the intestinal tract

A group of antibiotics that causes strong suppression of the intestinal flora are the MLS_B antibiotics. This leads to overgrowth (or sometimes new colonisation) of streptococci, staphylococci, clostridia and enterococci (Nord, **1993**). Also glycopeptides, like vancomycin and teicoplanin, lead to serious effects when administered orally. These antibiotics act against staphylococci, enterococci and pneumococci. It has been used clinically since the 1950s, but frequent use started in the late 1970s and the early 1980s (Murray, **1998**). Teicoplanin is also used in human medicine, but to a lesser extent.

Kirst *et al.* (**1998**) have collected data about the vancomycin usage in the United States and several European countries. From the beginning of the 1980s the use in the Unites States increases rapidly until 1992. It now seems that vancomycin use is stabilising around 10,000 kg a year. In France the use is also more or less constant the last years, around 1,100 kg a year. The same holds true for the Netherlands, where around 60 kg is used. In Germany, Italy and the United Kingdom vancomycin consumption is still increasing (1996: 629 kg, 538 kg and 349 kg respectively). Not only the total use is important, but also the use per inhabitant. This is listed in the table below.

Table 3.3.5.1 Use of vancomycin in the USA and in Europe (Kirst *et al.*, 1998)

Country	Population (1995; million)	Consumption (kg/year; 1995)	Consumption/capita (mg/year; 1995)
USA	260.5	11,279	42.5
France	59.4	1,125	19.3
UK	58.9	320	5.4
Germany	81.4	509	6.2
Italy	59.7	511	8.9
The Netherlands	15.7	60	3.8

In the United States the use of vancomycin is clearly higher than in Europe. For developing resistance (impact of the antibiotic) the amount used, the number of treated individuals as well as the population density (for AGPs: farm animal density) (Levy, **1997**). In the USA, being a large country, the amount of antibiotic prescribed is high, as well as the number of individuals treated. This, combined with the effect vancomycin has on the intestinal flora, may cause resistant strains to emerge and spread easily.

Proper use of antibiotics can decrease the risk of selecting for resistant bacteria. Antibiotics should only be given when necessary, this means in the case of (serious) infections caused by bacteria. They should not be used to treat common colds and other infections caused by viruses (Levy, **1998**; Gopal Rao, **1998**; Huovinen, **1997**). Often antibiotics are provided without knowing which organism is causing the infection. Tests to determine the microorganism causing the infection are not carried out routinely partly because most tests are time–consuming and thus costly. This also holds true for the testing of susceptibility of the infectious bacteria. Another point to be considered is the way antibiotics are administered. It is important to finish the whole treatment. Another point of concern is that in many countries antibiotics can easily be bought without a medical receipt. Moreover, hygienic measures taken in hospitals reduce the spread of resistant bacteria and will keep the rise of resistant bacteria more or less in check.

3.4 Cellular processes and antibiotics

3.4.1 Cell wall synthesis

An important difference between mammalian and bacterial cells is the presence of cell walls in the latter, positioned outside the cytoplasma membrane. The basic structure of the cell wall is a polymeric peptidoglycan, called murein. Gram–positive bacteria contain larger amounts of peptidoglycan in their cell wall compared to the Gram–negative bacteria (Schlegel, **1992**). N–acetylglucosamine (N–Gluc) and N–acetylmuramic acid (N–Mur) building blocks form the backbone of murein. Muramic acid contains a peptide chain of four or five amino acids.

Polymer strands can be connected by peptide bonds formed between peptide chains of the muramic acid. A whole layer or even a net work (Gram–positive cell walls) of peptidoglycan can be composed in this way (Russell and Chopra, **1990**; Schlegel, **1992**).

In the cytoplasma the cell wall precursors are formed. In enterococci and in *S. aureus* a pentapeptide is linked to the muramic acid. First a tripeptide consisting of L–Ala, D–Glu and L–Lys is attached to the muramic acid, after which the dipeptide D–Ala–D–Ala is added. N–Gluc and N–Mur are coupled and together form disaccharides. Subsequently, the completed disaccharide N–Gluc–N–Mur is transported through the cytoplasma membrane by the aid of a lipid carrier (Arthur *et al.*, **1996**; Reynolds *et al.*, **1998**). Then these subunits are incorporated into a growing peptidoglycan chain, which after some modifications will form part of the cell wall.

Antibiotics have been developed that interfere with bacterial cell wall synthesis. Known antibiotics interfere with pentapeptide/disaccharide formation, transport of peptidoglycan precursors through the membrane (Zn–bacitracin) and cross–linking of peptidoglycan (vancomycin). The advantage of these antibiotics is that they act specifically on bacteria and are (in principal) not toxic to humans.

Vancomycin (avoparcin, teicoplanin): mode of action

Vancomycin binds to the D–Ala–D–Ala side of the pentapeptide in N–Gluc–N–Mur disaccharides, inhibiting the incorporation of these dimers into the growing peptidoglycan (Baptista *et al.*, **1996**). The two D–Ala molecules are present in pentapeptides of muramic acid in enterococci (Baptista *et al.*, **1996**), as well in *S. aureus* (Schlegel, **1992**).

Zn–bacitracin: mode of action

Bacitracin indirectly inhibits the transport of peptidoglycan building blocks (N–acetyl glucosamide – N–acetylmuramic acid dimers) through the cytoplasma membrane. A monophosphate lipid carrier transports this disaccharide. After the dimer is released at the site of the cell wall the lipid molecule remains in the membrane in its pyrophosphate (PP) form. Bacitracin binds to the PP–lipid and inhibits its dephosphorylation. In this way the lipid carrier is not able to transport new disaccharides through the membrane (Russell and Chopra, **1990**). Zn–bacitracin is active against Gram–positive bacteria. The antibiotic is very active towards *Clostridium perfringens* (Alpharma, **1998**).

3.4.2 Bacterial protein synthesis

Proteins are constituted of amino acids coupled together. The mRNA (formed by transcription of DNA) possesses the code for the sequence of these amino acids. Before protein synthesis starts, amino acids that need to be incorporated in the protein are coupled to specific tRNAs, leading to aminocacyl–tRNA molecules.

In bacteria protein synthesis is mediated by 70 S ribosomes. These ribosomes exists of two subunits, of 50 S and 30 S. Both subunits contain rRNA and proteins (Watson *et al.*, **1987**). There are three stages in protein synthesis involving ribosomes: the initiation, the elongation and the termination phase (Cocito *et al.*, **1997**).

In the initiation phase the mRNA is bound to the 30S ribosome. After the first amino acid (bound to tRNA) in the protein code is found, the two ribosomal units are joined into the 70S ribosome (Russel and Chopra, **1990**; Cocito *et al.*, **1997**). In the elongation phase amino acids are added to the growing protein chain. The 50S subunit of the ribosome consists of two important sites: the A (acceptor) site, which binds the next tRNA–amino acid molecule and the P site, which binds the growing peptide chain (peptidyl–tRNA). The addition of the next amino acid to the peptide chain is catalysed by the peptidyl transferase centre (PTC) (Watson *et al.*, **1987**; Russel and Chopra, **1990**; Cocito *et al.*, **1997**). When termination sequences in the mRNA are reached the protein synthesis stops. The completed polypeptide is removed from the ribosome. The mRNA also leaves the ribosome, followed by separation of the two ribosomal subunits (Russel and Chopra, **1990**; Cocito *et al.*, **1997**).

The following antibiotics discussed in this report interfere with bacterial protein synthesis. Antibiotics can interfere with different processes in protein synthesis. They can bind to 30 S or 50 S ribosomal subunits or to the mRNA. When they bind to the 30 S ribosomal subunit before the 70 S ribosome is formed, initiation of protein synthesis is prevented. Some antibiotics interfere with the linking of the mRNA codon to the tRNA anticodon, preventing elongation of protein synthesis (Cocito *et al.*, **1997**). Antibiotics that bind to the 50 S ribosomal subunit or to elongation factors, that are connected to the ribosome for short periods, inhibit elongation of protein synthesis.

The macrolides, streptogramines B and lincosamines together form the MLS group of antibiotics. These antibiotics (mainly) disturb functioning of the ribosome during protein elongation. The AGPs tylosin, spiramycin and virginiamycin belong to this class of antibiotics.

Macrolides: mode of action
Macrolides contain a ring constituted of C en O–atoms (lactone ring), which is substituted with one or two (amino) sugar moieties (Russel and Chopra, **1990**). The lactone ring can be 14–, 15– or 16–membered. This group of antibiotics binds to the 50 S ribosomal subunit. Probably they act on the release of peptidyl–tRNA from ribosomes when translocation from the P to the A site takes place.

Lincosamides: mode of action
Lincosamides consist of 14, 15 of 16–membered lactone rings. These antibiotics are inhibiting the peptidyl transferase function of the 50 S ribosomal subunit. This means that the growing peptide chain can not be transferred from the peptidyl to the acceptor site.

Streptogramins: mode of action
Streptogramins can be divided into two groups, group A and B. Both types are macrocyclic lactone rings. The A–group streptogramins contain a large unsaturated non–peptide ring. The B–group consists of cyclic hexadepsipeptides which contain unusual amino acids (Russel and Chopra, **1990**; Cocito *et al.*, **1997**).

Streptogramins of the A group can bind to 50S subunits or 70S ribosomes when they are not in the elongation phase. Most likely streptogramins A bind to the free peptidyl trans-

ferase catalytic centre (Chinali *et al.*, **1987**; Russell and Chopra, **1990**). In this way protein synthesis cannot enter the elongation phase. To the streptogramin A group belong virginiamycin M and virginiamycin S (Cocito *et al.*, **1997**).

The B group streptogramins belong to the MLS_B group of antibiotics. They are acting on the elongation step of protein synthesis. Binding of aa–tRNA to the A site and peptidyl transfer from the P site is prevented. Translocation of the growing polypeptide chain is not inhibited (Cocito *et al.*, **1974**; Ennis and Duffy, **1972**).

The two groups of streptogramins are acting synergistic towards Gram–positive bacteria. When an antibiotic of the A type binds to the ribosome, conformational changes in the 50 S ribosome occur. This leads to an increase affinity for the B group streptogramins towards this ribosome subunit (Moureau *et al.*, **1983**). When streptogramin A and B are acting individually, they are only bacteriostatic which means that growth is stopped, but can be resumed when cells are transferred to antibiotic free medium. When administered together the effect is bactericidal (Chinali *et al.*, **1987**).

3.5 Bacterial resistance and its transfer: basics

3.5.1 Location of resistance genes

Bacteria can obtain antibiotic resistance in a few distinct ways (Van Egeraat, **1991a**; Bryan, **1984**; Russel and Chopra, **1990**). Resistance traits can be present on different parts or pieces of DNA: the chromosome, plasmids and/or transposons. To be able to discuss transfer of resistance, it is necessary to gather insight in how resistance against antibiotics in bacteria is accomplished and where resistance genes are located. The location is highly influential on the possibilities of transfer.

Plasmids

Besides the large chromosome, bacteria often possess small circular pieces of DNA called plasmids. In general, plasmids contain genes that are not necessarily needed for the host bacterium. Multiple copies are usually present in the bacterial cell. A plasmid can replicate (multiply) independent of the DNA of the chromosome. Plasmids can be transferred to bacteria of the same species or bacteria that are less related. Resistance genes are often present on plasmids. The presence of more than one resistance gene on one plasmid is not uncommon.
There are conjugative plasmids and nonconjugative plasmids. The conjugative plasmids are capable of moving to another cell. These plasmids are usually larger than the nonconjugative plasmids (Bryan, **1982**).

Transposons: insertion sequences and complex transposons

Transposons are pieces of DNA that can migrate through the genome of an organism (Saedler and Gierl (eds.), **1996**). They can be part of plasmids and bacteriophages but also occur on the bacterial chromosome.

Insertion sequences (simple transposons) are mobile DNA elements present in bacteria. They usually contain only the transposase gene. They can transpose themselves, this means they are cut out of their location in the DNA and are residing somewhere else. In doing this, the IS cause genome rearrangements, such as deletions, inversions, duplications and replicon fusions (Ohtsubo and Sekine, in Saedler and Gierl (eds.), **1996**). Insertion sequences usually consist of 800 – 2500 base pairs and have a few to a few hundred copies per genome. The

sequence codes for a transposase enzyme and often resistance genes are also present. The left and right ends of an IS contain inverted repeats of 10 – 40 bp. These repeats play a role in the transposition of the sequence. This transposition is different from the homology dependent recombinations that can occur in cells.

Complex tranposons can be part of plasmids but also occur on the bacterial genome. A transposon is a piece of DNA of 750 up to 40.000 base pairs. The transposon consists of genes coding for enzymes that cut themselves out of a larger piece of DNA and incorporate the transposon somewhere else. Complex transposons contain one or more genes with different functions. These can be genes for antibiotic resistance.

When a transposon containing resistance genes inserts itself in a plasmid it can be transferred to another cell. When the plasmid is able to replicate itself in the new host, or if the transposon moves to another replicable plasmid or inserts in the chromosome, this cell becomes resistant to the antibiotic (Summers, **1996**).

3.5.2 Intrinsic and acquired resistance

Bacteria acquiring resistance against an antibiotic is a form of adaptation under biochemical stress. The information thus generated is stored and passed on to other bacterial organisms in several ways.

First, the two types causing resistance will be discussed namely:

- Intrinsic resistance
- Acquired resistance

Subsequently, the possible mechanisms of resistance in a bacterial cell are described. Then the resistance mechanisms towards the growth promoters and antibiotics of importance for this report will be discussed. The last step is to explain the possibilities of transfer of genes from one bacterium to another.

Intrinsic

Already before antibiotics were used throughout the world, bacteria resistant to some antibiotics existed. This resistance called *intrinsic resistance*, is due to properties of the cell and mediated by chromosomal genes (Russell and Chopra, **1990**). The antibiotic is prevented from entering the cell or reaching its target, or the target is not sensitive to the antibiotic. The intrinsic type of resistance cannot be transferred to other bacteria, only to the offspring of the cell.

The predominant form of intrinsic resistance is resistance mediated by the shape and constituents of the cell wall. This barrier prevents some antibiotics from entering the cell. In Gram–negative bacteria this type of resistant often has been noticed. The outer membrane of Gram–negative bacteria can prevent the entrance of some β–lactams into the cell. Also large antibiotics like bacitracin, vancomycin and teicoplanin cannot pass the porins of the Gram–negative outer membrane.

Enterococci can be intrinsically resistant to penicillins, cephalosporins, aminoglycosides, clindamycin and Zn–bacitracin (Murray, **1998**; Baquero, **1997**; De Neeling *et al.*, **1997**; Alpharma, **1998**). *E. faecium* can also be intrinsic resistant to sulphonamides and trimethoprim. This bacterium is able to take up folic acid derivatives and is not dependent

on the production of tetrahydrofolic acid which can be inhibited by the above mentioned antibiotics (Russell and Chopra, **1990**).

Acquired
There are basically two routes bacteria can acquire resistance towards antibiotics.

- Chromosomal mutations
- DNA transfer

Chromosomal mutations can arise at any time; the presence of antibiotics does not influence the mutation frequency (Russell and Chopra, **1990**; Levy, **1998**; Bryan, **1984**). Single nucleotides can be changed in the DNA leading to replacement of an amino acid in the translated protein. Also larger base rearrangements can take place in the form of deletions, duplications, translocations and inversions (Russell and Chopra, **1990**).

In this way, without selective pressure, it is possible that a bacterium gains resistance to an antibiotic. An example is when a gene coding for a penicillin–binding protein is altered in one or more bases. This can be sufficient for preventing binding of penicillin to the protein, which makes the cell resistant (Russell and Chopra, **1990**).

The second way bacteria can acquire resistance genes is by taking up functional DNA from other bacteria by *transformation*, *conjugation* or *transduction* (Bryan, **1982**; Van Egeraat, **1991**; Russell and Chopra, **1990**; Summers, **1996**; Levy, **1998**). This can be favoured when antibiotics are present.

Below we shall describe the three manners in which acquired resistance can be transferred from one bacterium to another.

Transformation
When bacteria die, the soluble DNA can remain in the surroundings. Some bacterial cells that are related (competent) can pick up part of this DNA. It is possible that a piece of DNA containing resistance genes is integrated in the chromosome or plasmid (Levy, **1998**). The uptake of DNA from the environment has been observed in many Gram–positive and Gram–negative bacteria, like *Streptococcus pneumonia*, *Bacillus* spp., *E. coli*, *Haemophilus* spp. and *Pseudomonas* spp. (Schlegel, **1992**; Watson *et al*., **1987**).

In the laboratory it has been shown that cells are less competent when growing exponentially. In nature, where bacteria often spend their time in stationary or low–growth phase, transformation could occur frequently (Summers, **1996**). Factors concerning the microenvironment of bacteria are thought to play an important role in transformation (presence of competent factor for some Gram–positive bacteria, shielding from DNA degrading enzymes, pH). No precise estimation of the transformation frequency in nature can be obtained by performing laboratory experiments.

Transduction
In this case DNA is transferred with the aid of a bacteriophage (a virus that infects bacteria). The bacteriophage is able to infect a bacterial cell and subsequently new particles are produced in this cell. When DNA is packed inside a new bacteriophage particle a piece of DNA from the bacteria can be incorporated. The bacteriophage is released from the cell and capable of infecting bacteria related to the one that just released the phage. The phage attaches to the cell wall of the bacteria and injects its DNA, including the piece of DNA ob-

tained from its previous host. This piece of DNA can be maintained in the host cell and e.g. in the case of a plasmid replicate independently in the cell. For bacterial genera like *Eschericia*, *Salmonella*, *Shigella*, *Pseudomonas*, *Vibrio*, *Staphylococcus* and *Bacillus* transduction has been reported (Schlegel, **1992**; Summers, **1996**). Transduction most often occurs within genera or species, because bacteriophages usually do not have a wide range of hosts.

Conjugation

Conjugation is the transfer of DNA by direct cell–to–cell contact (Watson, *et al.*, **1987**; Summers, **1996**). This usually occurs between bacterial strains that are related. This active transfer of DNA can occur between related or less related bacterial strains. Resistance to antibiotics can be acquired by transfer of a plasmid (with or without transposons), a conjugative transposon or another piece of DNA that is able to integrate into the chromosome or into a plasmid or gene cassette (Scott, **1991**; Summers, **1996**).

In the conjugation process the donor and recipient strains make contact, after which a channel between both cells emerges, through which a plasmid or other pieces of DNA can be transported (Dunny *et al.*, **1991**). When a plasmid containing a resistance gene (or gene cassette) is transferred and it is able to replicate and be transcribed, the recipient cell will have gained resistance to that antibiotic. This also holds true when a piece of DNA containing a resistance gene has been transferred and is incorporated into the chromosome or into a plasmid.

3.6 Biochemical defence mechanisms against antibiotics

3.6.1 General

It is important to understand that a wide variety of resistance mechanisms exist, dependent on the bacterial species and the specific antibiotic. Bacteria are capable of dealing with antibiotics in one (or more) of the following ways (Bryan, **1982**; Russel and Chopra, **1990**; Levy, **1998**; Hawkey, **1998**):

- Enzymatic breakdown or modification of the antibiotic (b–lactamases)
- Overproduction of target
- Two versions of antibiotic target; one sensitive, one resistant
- Change of target site so that antibiotic does not bind
- Eliminate entry ports of the cell (decreased uptake)
- Produce pumps that export antibiotics (decreased uptake)
- Missing of the target enzyme or metabolic pathway (intrinsic)

In the next paragraph the resistance mechanisms important in the context of this report are summarised. These mechanisms lead to the antibiotics dysfunction.

Also the genes or gene cassettes that give rise to resistance are described. Their location (chromosomal, on transposons or on plasmids), combined with a characterisation of the gene and its environment, can provide insight about the spread of these resistance treats. It also shows that bacterial resistance comes in many variations making comparison and extrapolation a difficult enterprise. This complicates the final risk assessment further.

3.6.2 Bacterial cell wall defences

Glycopeptides: target modification

Resistance to the glycopeptides vancomycin and teicoplanin (both are used in human medicine, however on a limited scale) can be observed in different variations. Four phenotypes of glycopeptide resistance can be distinguished (Baptosta et al., 1996; Perichon and Courvalin, 1997; Murray, 1998). These resistance variations differ in the minimum concentrations of vancomycin and/or teicoplanin needed to inhibit bacterial growth (MIC: minimal inhibitory concentration):

A : high resistant to vancomycin (MIC 64 to > 1000 μg/ml)
intermediate to high resistant to teicoplanin
(MIC 16–512 μg /ml)
B : intermediate to high resistant to vancomycin
(MIC 4 to > 1000 μg /ml); susceptible to teicoplanin
(MIC 0,5 – 1 μg /ml)
C : low resistant to vancomycin, susceptible to teicoplanin
D : constitutively intermediate resistant to vancomycin,
low–resistant to teicoplanin

The A and B types are acquired types of resistance, while the C and D types are intrinsically present. The resistance of the A and B type is inducible, while resistance of the C and D type is constitutive.

Resistance of the VanA type

A gene cluster that is present on transposon Tn1546 is responsible for high–level resistance to vancomycin. This transposon is also present on multiple plasmids. Tn1546 was first isolated from E. faecium BM4147 and contains seven genes that regulate and cause resistance to vancomycin and teicoplanin resistance (Arthur et al., 1993). Resistance to vancomycin and teicoplanin is induced when these antibiotics (or other inducers that inhibit polymerising of the cell wall precursors) are present (Baptista et al., 1996). On the transposon two regulatory genes are present: vanR and vanS. These genes encode proteins that probably sense the presence of vancomycin and subsequently activate the promoter for the vanH, vanA and vanX genes (Baptista et al., 1996).

Resistance to vancomycin is achieved by the co–operation of different actions in the cell. In short: the D–Ala–D–Ala end of the pentapeptide in N–Gluc–N–Mur disaccharides is changed in D–Ala–D–Lac. This prevents vancomycin from binding to N–Gluc–N–Mur and cell wall polymerisation can proceed with slightly different building blocks.

Resistance of the VanB type

The vanB gene cluster consists of homologues of the vanA cluster, the difference being that instead of the vanZ gene the vanW gene is present. It has been shown that Tn1547 contains the vanB cluster (Quintilliani and Courvalin, 1996) usually present on the chromosome. Just as the vanA gene, the vanB gene encodes a ligase that catalyses the production of D–Ala–D–Lac. The sequence identity of the two genes is 73 % (Evers et al., 1996).

The vanB cluster is induced by vancomycin but not by teicoplanin. If only a single copy of the vanB operon is present (chromosomal) it is important that VanYb is formed, so that the pentapeptide chain of the disaccharides can be converted to the tetrapeptide (cleavage on

D–Ala). Then cell wall synthesis can proceed with precursors ending on D–Ala–D–Lac (Reynolds, 1998).

Resistance of the VanC type

The intrinsic type of resistance mediated by *vanC* is found in *E. gallinarum* and *E. casseliflavus*. Resistance is not transferable to other bacteria (only to the progeny). *VanC1* is specific for *E. gallinarum*, while *vanC2* is found in *E. casseliflavus*. A comparable gene has been detected in *E. flavescens* and was called *vanC3* (Dutka–Malen *et al.*, **1991**; Navarro and Courvalin, **1994**; Clark *et al.*, **1998**).

In these weakly resistant strains two precursors are formed that can be incorporated in the cell wall: disaccharides containing peptides ending in D–Ala–D–Ala and in D–Ala–D–Ser (Reynolds, **1998**). A normal ligase is present that catalyses the production of D–Ala–D–Ala. The gene product of *vanC* is a second ligase, involved in the production of D–Ala–D–Ser.

Vancomycin has a 5 times stronger affinity for peptides ending in D–Ala–D–Ala then for acyl–D–Ala–D–Ser. This causes a low–level resistance to vancomycin. Teicoplanin binds more or less equally to both peptidoglycan precursors, explaining why cells remain susceptible to this antibiotic.

Zn–bacitracin: cellular export (?)

When bacteria like streptococci and staphylococci are cultured on media containing subsequent higher concentrations of bacitracin, resistance can develop. This type of resistance is transient, because in the majority of the cases resistance disappears when bacteria are transferred to media without antibiotics (Alpharma, **1998**). Resistance genes to bacitracin have not been found on extrachromosomal elements (Threlfall, **1985**).

In *Bacillus licheniformis*, the bacterium that produces bacitracin, genes leading to resistance to bacitracin are found. Three genes, *bcrA*, *bcrB* and *bcrC* are responsible. The proteins that are expressed form an ABC–transporter, which exports bacitracin out of the cell. The bcr genes have not been isolated from other bacteria resistant to bacitracin (Podlesek *et al.*, **1995** and **1997**). However, the mechanism of transporting unwanted compounds out of the cell by an efflux pump is quite common. It is not known if the ABC–transporter in *B. licheniformis* is specific for bacitracin.

3.6.3 Bacterial protein synthesis defences

The group of macrolides, streptogramins B and lincomycins usually is regarded as one class of antibiotics, the MLS_B class. Resistance to MLS_B antibiotics is found in staphylococci like *S. aureus* and in enterococci and can be induced or is constitutively present. The resistance genes can be present on plasmids or transposons. The biochemical mechanisms against antibiotics are discussed below.

Target modification

The major type of resistance found against the MLS_B class of antibiotics is modification of the target of the antibiotic. This is accomplished by methylation of an adenine residue in the 23S RNA of the 50S subunit of the ribosome (Russell and Chopra, **1990**; Leclercq and Courvalin, **1991a**). The gene responsible is the *erm* gene, of which different variations are present in many bacteria (MMM, **1997**; SCAN, **1998a**; **1998b**; Leclercq and Courvalin, **1991a**). In *S. aureus ermA* and *ermC* can be present, while in *Streptococcus sanguis ermAM* has been found (Murphy, **1985**; Horinouchi *et al.*, **1983**). The methylation of the 23S RNA prevents binding of the MLS_B group of antibiotics to the 50S ribosomal subunit. In this way

the streptogramins A still can bind to the 50S ribosome. This type of resistance can be inducible or constitutive.

When erythromycin or other macrolides with 14 or 15 ring atoms are present, resistance to these macrolide antibiotics in staphylococci and group A streptococci can be induced. The bacteria will remain susceptible to macrolides that contain 16 ring atoms, lincosamides and streptogramins (Leclercq and Courvalin, **1991**; Seppälä *et al.*, **1993**). This inducible resistance pattern is regulated at the translation level. When no inducers are present, the mRNA containing the methylase sequence is formed, but its secondary structure prevents the methylase from being translated. When erythromycin is present it binds to ribosomes upstream the methylase sequence. This probably changes the secondary structure of the mRNA, allowing the methylase to be translated (ermC, Leclerq and Courvalin, **1991**). In *E. faecalis* and *E. faecium* of animal and human origin the transposons Tn*917* and Tn*1545* have been detected that contain genes encoding for erythromycin resistance. The transposon Tn*1545* encodes for erythromycin, kanamycin and tetracycline resistance.

In streptococci another type of induced resistance has been observed. In this case macrolides and lincosamides can be inducers. Not only resistance to 14 and 15–ring macrolides is induced, but resistance to all the MLS_B antibiotics.

Under selection of MLS_B antibiotics which are not inducers, mutants can be formed that are constitutively (permanently) resistant to the MLS_B antibiotics. In the DNA upstream the methylase gene point mutations, deletion or repeats can disturb the secondary structure of the mRNA. Then no inducing antibiotic is needed to cause a structural change, and the enzyme is constitutively formed. Constitutive resistance to MLS_B antibiotics is a wide–spread phenomenon and has been found in amongst others *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp. *Bacteroides* spp., *Campylobacter*, *Bacillus*, and *Lactobacillus* (Leclercq and Courvalin, **1991a**).

Decreased uptake

Resistance to streptogramins can be achieved by two different mechanisms. Both mechanisms prevent the antibiotic reaching its target:

- Permeability impairment
- Inactivation

The first mechanism results in a decreased uptake of the antibiotic by the bacterium. The impairment of the permeability is seen in the case of streptogramin A–like compounds. First the vga gene was identified on *S. aureus* plasmids. This gene codes for an ATP–binding protein that presumably is involved with the active efflux of the antibiotic (Allignet *et al.*, **1992**). Later Allignet and El Solh (1997) isolated a comparable gene, vgaB, encoding a protein with the same function. In *Staphylococcus epidermis* the same mechanism was detected. The efflux mechanism in this bacterium is inducible for 14 membered ring macrolides and streptogramin B antibiotics (Ross *et al.*, **1990**).

Inactivation of the antibiotic is the second mechanism of resistance to streptogramin antibiotics. Streptogramin A antibiotics can be inactivated by acetyltransferases. There are three staphylococcal genes that code for an acetyltransferase (Allignet *et al.*, **1998**). The mechanism of inactivating streptogramin A is also found in *E. faecium*; they are encoded by the satA gene (Rende–Fournier *et al.*, **1993**).

Streptogramin B antibiotics can be inactivated by lactonase or hydrolases. In staphylococci genes can be found that code for enzymes cleaving the macrocyclic lactone ring of streptogramins B. An example is the lactonase encoded by the *vgb* gene (Allignet *et al.*, **1988**). Recently a comparable lactonase has been found in *Staphylococcus cohnii subsp. cohnii* (Allignet *et al.*, 1998). These proteins have 67% amino acid identical.

As shown above bacteria are capable of developing a wide–range of defence mechanisms against antibiotics. These cellular processes have a specific genetic structure and biochemical make–up.

3.7 Selective pressure and resistance

3.7.1 The costs and benefits of resistance: a bacterial viewpoint

The most important risk factor for the emergence of resistant bacteria is contact with antibiotics (Gopal Rao, **1998**). Every use of antibiotics can select for bacteria that are less susceptible for that antibiotic and related antibiotics.

The time–scale at which the antibiotics are present is important. The longer bacteria are in contact with antibiotics, the higher the chance that they mutate into less sensitive strains. Also the chance of acquiring resistance genes from other bacteria increases.

As long as an antibiotic is present it is profitable for a bacterium to possess resistance genes to withstand this antibiotic. When the antibiotic is no longer present, resistant genotypes can show lower growth rates than the susceptible ones. The carriage of a gene or genes that is/are not necessary costs extra energy, the so–called maintenance energy (Schlegel, **1992**). Also normal processes in the cell can be changed, which can also be a burden to the cell (Lenski, **1997**; Lenski and Nguyen, **1988**).

3.7.2 Reversal of resistance

A possible strategy to eliminate cells carrying resistance traits is to remove the antibiotic or ban the use of the antibiotic for a while. Studies to find out whether resistance genes are lost and on what time–scale are needed. Lenski (**1997**) found that if some susceptible bacteria are still present in the organism or in the close environment, they reduce the persistence of antibiotic–resistant bacteria. The higher the relative growth rate of the susceptible cells compared to the resistant cells the faster the resistant population will decline.

If this would be the only principle (applicable on every bacteria/antibiotic combination), the solution to the problem of antibiotic resistance would be easy. However, the negative effect that resistance genes can have on the growth rate is subject to evolutionary change. If the energy costs of the resistance gene are not that high the resistant bacteria will survive for longer periods.

It is important to have an indication of how long resistance persists in a bacterial cell after the antibiotic has been removed. Levy (**1986**) studied the effect of taking tetracycline for five days on his own faecal flora. The maximum amount of tetracycline–resistant bacteria was detected after two days. When the administration of tetracycline stopped, it took 15 days to go back to the situation before treatment. The time it takes to return to the situation before antibiotics were present will depend on the (amount of) antibiotic used, the bacteria that gain resistance and the susceptible population. If the susceptible population in the individual, group of animals or humans has diminished and there is no contact with other popula-

tions the resistant strain can survive for longer periods. Levy (**1986**) showed that chickens in a closed environment with multiresistant *E. coli* in their faeces, kept these bacteria for months. On the other hand, when 4 chickens with resistant bacteria were housed together with 10 chickens with susceptible bacteria the resistant bacteria were lost.

In the oral cavity of almost all people tetracycline–resistant α–streptococci can be detected. This does seem related to (recent) tetracycline use. These streptococci all have acquired *tet* genes. The normal flora has not been able to overgrow the resistant bacteria, so now the resistant flora has become the dominant flora. Also women with urogenital infections not receiving tetracycline were all positive for *tet* resistant streptococci and peptostreptococci (Roberts and Hillier, **1990**).

The rise and decline of antibiotics resistance in bacterial populations is a complex process that is not solely dependent on the presence or absence of antibiotics. If resistance has developed in animals the question still remains if and in what way humans are susceptible for these organisms. Are resistant bacteria of animal origin capable of colonising the human intestine and/or are they capable of transferring resistance genes?

4 Bacterial Antibiotic Resistance and Human Health

4.1 Summary

To compare antibiotic resistant bacterial strains it is important that strains are thoroughly characterised. It is recommended to use multiple phenotypic and genotypic characterisation methods. Many articles presenting antibiotic resistance data just contain prevalence data and can only be used to compose a view of the presence of resistant bacteria in animals, humans or meat. Data about resistance (MICs) cannot always be compared, because different methods of isolation and testing of bacteria are being used. The given percentages of resistant bacteria are not always very accurate. Only articles that use multiple genetic methods to examine strains are reliable when claims of transfer are being put forward. In summary, the following points put forward in this chapter are:

- The acquiring of resistant bacteria by humans in general consists of two distinct routes (the human therapeutic antibiotic use being of dominant importance):
- *Use of antibiotics by humans can cause resistant bacteria – in the intestines– to emerge*
- *Resistant bacteria or resistant genes can be acquired by contact with:*
- *exogenic sources containing resistant bacteria. (This route incorporates the*
- *dissemination route from animals harbouring antibiotic resistant bacterial*
- *strains)*

- The animal–human link might comprise of several dissemination routes:
- *direct contact with an animal or animal faeces*
- *the consumption of meat or fish*
- *the consumption of vegetables or fruit*
- *human to human spread*
- *contact with water containing faeces*

- Two scenario's can be drawn when the spread of bacterial resistance from animals to humans is discussed:
- *When a resistant bacteria of animal origin is able to colonise the human gut the resistance in effect has been transferred from the animal to the human. However, a bacterium has to survive the stomach. When it enters the intestines it has to be able to multiply in sufficient amounts before it truly colonises the human. The time the specific bacterial strain is able to stay in the intestines determines if it is a transient passenger or a permanent resident. Resistance genes present on the bacterial chromosome, on plasmids or on transposons can be expressed during transient or permanent colonisation. Chances increase, however, when the stay is prolonged.*

- *The second possibility is that resistance genes are being transferred from bacteria present in meat or animals to bacteria that are commonly found in human intestines. Transfer of genes can take place in the gut or prior to ingestion, after which the resistant bacteria may be able to colonise the human gut. Resistance traits present on plasmids or on transposons have a chance of being transferred to another bacterium. Genes present on the bacterial chromosome, but not on a transposon, have a much lower chance of being transferred.*

- The categories of people at higher risk of being infected and circumstances that increase risk are as follows:
- *immunocompromised persons (elderly patients, ill neonates, etc.)*
- *patients subjected to surgical operations, people with burns*
- *patients with breathing devices, catheters and drains*
- *type of ward: Intensive Care, renal units, hematology ward, surgical ward*
  transfer of patients between wards or between hospitals

- *use of antibiotics (cephalosporines)*
- *prolonged stay in the hospital*
- *hygienic measures taken (or not taken) in the hospital*

- The following criteria in the elucidation of possible bacterial antibiotic resistance transfer from animals to humans are essential:
- *The bacterial strain and/or the resistance trait present in the human should be identical to a bacterial strain and/or resistance trait present in the meat consumed. (In many reports meat samples and human samples are compared that might be totally unrelated.)*
- *The exact source of the resistant bacteria needs to be elucidated. Otherwise a possible relationship between antibiotic usage in animal feed and resistant bacteria in humans cannot be confirmed.*
- *Recent use of antibiotics by the people concerned needs to be established and documented.*
- *When meat samples are examined, one needs to be sure that resistant bacteria found are not the result of contamination during processing, preparing or transport of the meat.*
- *Typing methods for identifying bacteria have to be specific enough to detect small differences between bacterial strains and their resistance traits.*

- The elucidation of factual bacterial resistance transfer from animals to humans (and from humans to humans) requires fenotypic and genotypic methods that are the most discriminatory, so that even small differences between strains and resistance traits can be distinguished.
- For a complete analysis also the presence of plasmids and/or other resistance traits present should be determined.
- Below the techniques for isolation and characterisation of resistant bacterial strains are listed:
- Isolation of resistant strains and phenotypic characterisation:
- *Enrichment cultures (different concentrations of the antibiotic used in selection)*
- *Determination of species and sub–species: biochemical methods an ready–to–use kits*
- *Determination of Minimal Inhibitory Concentrations (MIC; different methods and media)*
- *Determination of MICs of single or multiple antibiotics*

- Genotypic characterisation:
- *Pulsed Field Gel Electrophoresis (PFGE) of chromosomal DNA (digested by SmaI)*
- *Region amplification within a (resistance) gene by PCR; regions within In: intergenic amplifiction by PCR*
- *Ribotyping*
- *Long PCRs of transposons*
- *Conjugational studies*

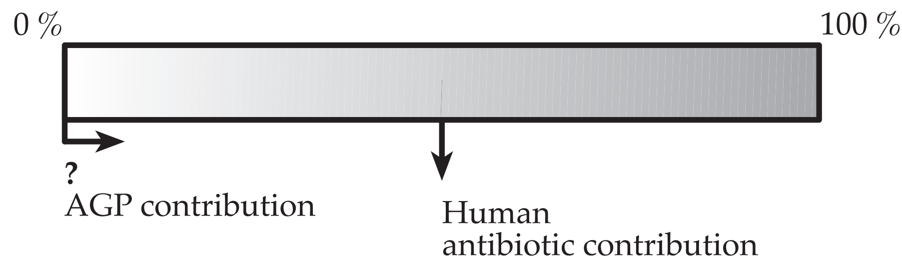
4.2 Introduction

The acquiring of resistant bacteria by humans in general consists of two distinct routes:

- Use of antibiotics by humans can cause resistant bacteria to emerge present in the intestines
- Resistant bacteria or resistant genes can be acquired by contact with sources containing resistant bacteria (either from animal or human origin)

In essence the different proportions (if there are more than one) adding to the total human bacterial resistance needs to be determined which can be depicted as follows:

Figure 4.2.1 Possible sources of human bacterial antibiotics resistance

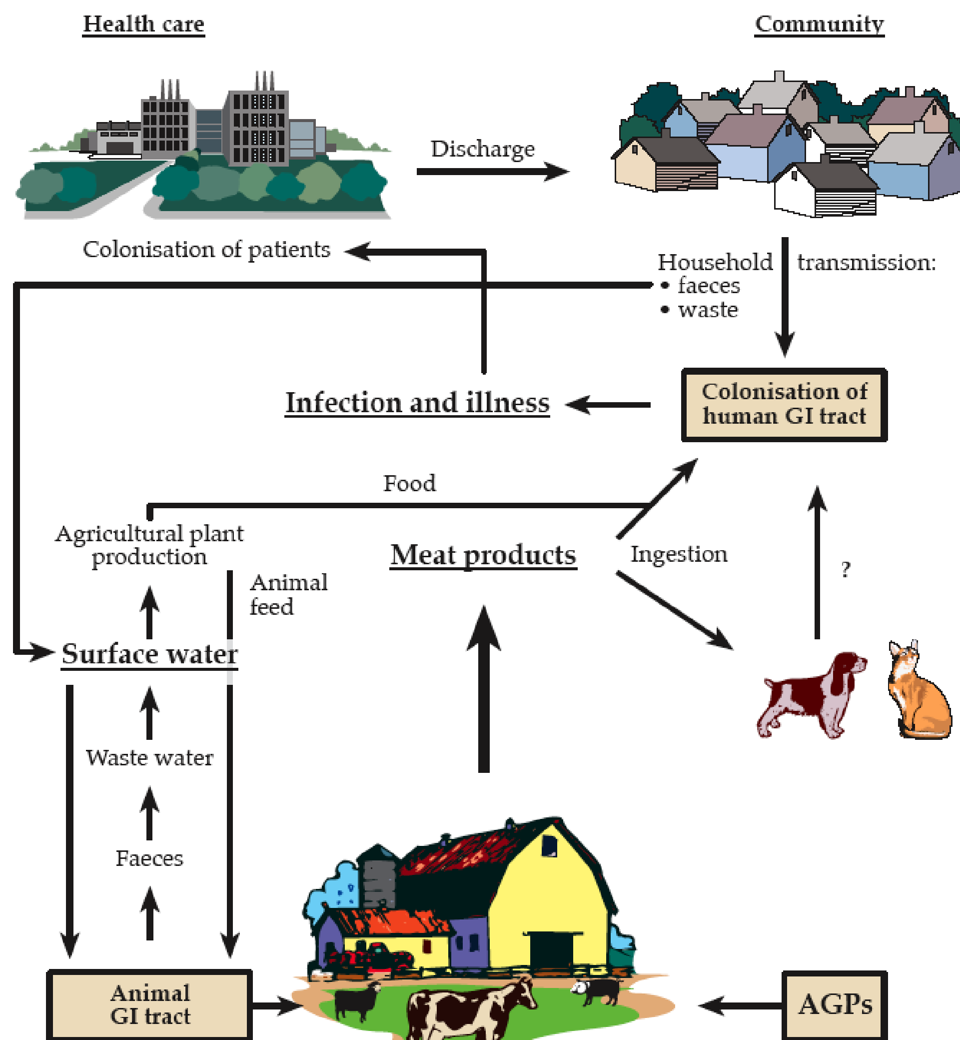


It is important to know which sources cause a risk for human health and to compare the risks of these different sources. A pool of resistant bacteria in animals might in theory be a risk when these bacteria or their resistance traits are transferred to humans or human bacteria. The question that now rises is whether resistance genes easily spread to other hosts in the same environment or to populations in other environments.

4.3 'Spreading the disease'

Below a tentative scheme is presented with possible dissemination routes of resistant bacteria or their resistance traits from animals to humans:

Figure 4.3.1 Possible reservoirs of antibiotic resistant Gram–positive bacteria and possible transfer routes (modified from Witte (1997, 1998); McDonald et al. (1997))



It is by no means clear that such routes are in fact a reality or will actually contribute to the total antibiotics resistance in human bacteria. This scheme is on all levels heavily debated. Until now little evidence is presented to substantiate this scheme. We will however for clarity discuss the presented dissemination routes.

Direct contact with an animal or animal faeces

The people most intensively in contact with animals or animal faeces are farmers, slaughter-house workers and veterinarians. Farmers and their families are frequently in contact with animal faeces, e.g. when they are cleaning a cowshed or a sty. Also direct contact with animals might be a source of resistant bacteria transfer. Another group are slaughterhouse workers. These people can be contacted with animal faeces and intestinal contents. Veterinarians can also come in contact with animal faeces or intestinal contents.

Not only the faeces of husbandry animals can contain resistant bacteria. Also faeces from pets fed with feed containing resistant bacteria can be a risk for humans.

Another risk forms the contact with antimicrobials themselves possibly generating resistant bacteria present in the human bowl. Farmers are exposed when they administer the drug, fill the feeding trays or when antimicrobial dust is present (MAFF, **1998**).

The consumption of meat or fish

Meat can be contaminated with faeces or the intestinal contents during slaughter. If the meat is not sufficiently cooked some bacteria can survive and enter the human intestine. Also when the used utensils (knife, cutting board) are used for preparing uncooked food this food can be contaminated (Kruse and Sorum, **1994**).

Fish cultured in an aquaculture, like salmon, is sometimes treated or fed with antibiotics. The consumption of fish containing resistant bacteria might lead to the presence of resistant bacteria in the human intestine.

The consumption of vegetables or fruit

One way in which food can be contaminated with bacteria originating from animals is that the environment gets contaminated. If vegetables are fertilised with faeces from animals containing bacteria resistant to antibiotics, the bacteria can survive on the vegetable. Vegetables are also treated with antibiotics, so residues can be present or resistant bacteria can evolve on the plant. Bacteria that show intrinsic resistance to antibiotics, like *E. casseliflavus* and *E. gallinarum* which show low resistance to vancomycin can be present on vegetables (Van den Braak *et al.*, **1997**). Raw vegetables and salads also can contain resistant enterobacteria (Levy, **1998**; Feinman, **1998**).

In a number of experiments people were given sterile food (no living bacteria present) to investigate the effect on resistant bacteria (Corpet, **1988**). People that ate normal food carried up to one million times more lac+ enteric bacilli resistant to tetracycline in their faeces then the group that ate sterile food.

Contact with water that contains faeces

People drinking contaminated water or swimming in contaminated water can be exposed to resistant bacteria.

Human to human spread

Human to human spread involves not only the tentative scheme of animal to human transfer with subsequent human to human spread but more importantly the rise of resistant human bacteria as a result of therapeutic antibiotic use with a subsequent spread of human resistant bacteria. People in hospitals often are treated with antibiotics. Especially people who are treated for a long time with antibiotics have a high risk of the emergence of resistant bacteria in their intestines. They are susceptible for overgrowth of their own minor intestinal flora (e.g. enterococci). Resistant bacteria are propagated when antibiotics are given for extended periods. Resistant bacteria like VRE have been shown to spread from patient to patient in, but also between hospitals (Clark *et al.*, **1993**; Sader *et al.*, **1994**). The Dutch Health Council pointed out that (Gezondheidsraad, **1998**):

> '… The development of resistant bacteria in hospital patients is mainly caused by antibiotics used in the course of treatment. However, the cause of resistance development among the general public is less clear. …'

The reason for a 'division' between the hospital environment and the community is not substantiated in the Dutch Health Council report. Patients treated and cured enter the community possibly carrying small numbers of resistant bacteria, which might easily spread within the community. Resistant bacteria can be transferred (transmit) indirectly through the personnel, when hand washing and disinfecting of equipment is not carried out properly (Gopal Roa, **1998**). This might also be a dissemination route towards the community. The hygienic measures taken in different hospitals vary highly, as well as the prevalence of resistant strains (Verhoef, **1998**, personal communication). A less frequent way of transmission is by the presence of airborne droplets. The moving of patients between wards in one hospital or between different hospitals increases the risk of spread of resistance.

Outside hospitals bacteria can be transferred from one person to another. Increased international travelling contributes to a global spread of resistant bacteria and resistance gene cassettes. The hospital might therefore in general be a source of resistance within human bacteria.

Two different events that in theory might lead to animal to human transfer of resistance can be distinguished:

- When a resistant bacteria of animal origin is able to colonise the human gut the resistance in effect has been transferred from the animal to the human. However, a bacterium has to survive the stomach. When it enters the intestines it has to be able to multiply in sufficient amounts before it truly colonises the human. The time the specific bacterial strain is able to stay in the intestines determines if it is a transient passenger or a permanent resident. Bacteria like enterococci and enterobacteria are capable of permanently colonising the large intestine (Drasar and Barrow, **1985**). Resistance genes present on the bacterial chromosome, on plasmids or on transposons can be expressed during transient or permanent colonisation. Chances increase, however, when the stay is prolonged. In short:

  animal bacteria → human + 'animal' bacteria

- The second possibility is that resistance genes are being transferred from bacteria present in meat or animals to bacteria that are commonly found in human intestines. Transfer of genes can take place in the gut or prior to ingestion, after which the resistant bacteria may be able to colonise the human gut. Resistance traits present on plasmids or on transposons have a chance of being transferred to another bacterium. Genes present on the bacterial chromosome, but not on a transposon, have a much lower chance of being transferred. In short:

  animal bacteria → human bacteria + DNA animal bacteria → human + human bacteria + DNA animal bacteria

Data concerning transfer of Gram–positive bacteria resistant to AGPs from animals to humans is in essence non–existent. Van den Bogaard *et al.* (**1997b**) claimed that a turkey and a farmer had the same strain of vancomycin–resistant *E. faecium.* Until now this letter is the only one that describes indistinguishable strains in animals and humans. Moreover, it was not shown that this strain really colonised the human intestine and was not a transient passenger. Furthermore, reproducibility is lacking making this observation in effect open for debate and in want of thorough scientific scrutiny. Apart from these comments, extrapolation from this observation to other organisms or antimicrobial resistance traits is scientifically unsound and without foundation. The following hampers proving a transfer case:

- The bacterial strain and/or the resistance trait present in the human should be identical to a bacterial strain and/or resistance trait present in the meat consumed. In many reports meat samples and human samples are compared that might be totally unrelated insofar that the meat of the animal consumed is not traced, so the relation between resistant bacteria and consumption of meat is not evident.
- Preferable, the exact source of the resistant bacteria has to be elucidated. If the source is probably an animal, the usage of antibiotics in its feed should be known. Otherwise a possible relationship between antibiotic usage in animal feed and resistant bacteria in humans cannot be confirmed.
- When meat samples are examined, one needs to be sure that resistant bacteria found are not the result of contamination during processing, preparing or transport of the meat (Klein *et al.*, **1998**).
- Typing methods for identifying bacteria have to be specific enough to detect small differences between bacterial strains and their resistance traits.
- On farms it is easier to trace the animal which is causing the presence of resistant bacteria in the intestines of the farmer compared to people in a town consuming meat. When a farmer does not eat meat produced on its own farm, the direct transfer of resistant bacteria from animals or animal faeces to the farmer could be detected.

4.4 Infectious bacteria

Bacteria can cause several severe infections in humans. To be able to control the occurrence and spread of nosocomial infections (hospital acquired infections) it is useful to be aware of the risk factors for developing such an infection. In general, the condition of the patient, the use of antibiotics and the impact of hygienic measures in the hospital are important risk factors. The categories of people at higher risk and circumstances that increase risk are listed below (Bates *et al.*, 1993; Gordts *et al.*, 1995; Bogle and Bogle, 1997; Jones, 1996; Weinstein, 1998; Murray, 1990; 1998):

- immunocompromised persons: (elderly patients, ill neonates, patients with underlying disease)
- patients subjected to surgical operations, people with burns
- patients with breathing devices, catheters and drains
- type of ward: Intensive Care, renal units, hematology ward, surgical ward
- transfer of patients between wards or between hospitals
- use of antibiotics
- prolonged stay in the hospital
- the impact of hygienic measures taken (or not taken) in the hospital

The use of high amounts of antibiotics in hospitals contributes to the emergence of nosocomial infections. An example of this is the use of cephalosporins to treat infections caused by Gram–negative bacteria. The Gram–negative bacteria are killed, but resistant Gram–positive bacteria already present, like enterococci, can substantially increase in numbers and subsequently cause a superinfection. Bacteria containing multiple resistance genes are the most dangerous to humans, because of the higher change of resistance to the antibiotic(s) used for treatment. Some of the resistance traits present probably emerged and are maintained because of (high) prescription of antibiotics to humans in hospitals and the community.

The Gram–positive bacteria usually involved in (nosocomial) infections are described below. The genera *Enterococcus*, *Staphylococcus*, *Streptococcus* and *Clostridium* often cause nosocomial infections. For example, 34 % of hospital acquired infections are caused by enterococci, *S. aureus* and coagulase–negative staphylococci (Weinstein, **1998**). The infections usually associated with the above–mentioned bacteria are:

Table 4.4.1 Some infectious diseases caused by bacteria

Type of Infection	Description
Sinusitis	Inflammation of hollow in a bone or blood channel
Bacteraemia	Presence of bacteria in the blood
Endocarditis	Inflammation of the membrane between the two heart valves
Meningitis	Inflammation of the three membranes enclosing the brain and spinal cord
Osteomyelitis	Inflammation of the bone
Otitis media	Inflammation of the middle ear
Pneumonia	Inflammation of the lungs

Enterococci

Enterococci can play a role in urinary tract infections and infective endocarditis (Murray, **1990**). Other nosocomial infections caused by enterococci are surgical wound infections and bacteraemia. Around 11 % of the bacteraemias are caused by VRE. When blood infection occurs often more then one pathogen is present (Jumaa *et al.*, **1997**).

Enterococci can also be found in infections located at the intra–abdomen and pelvis. Also ill neonates (premature babies) can be infected. Patients, who have undergone surgery, like a liver transplant or surgery of the central nervous system have a higher risk of becoming infected (Murray, **1990**, **1998**). Other factors that increase the chance that a patient develops a VRE infection are the treatment with (third generation) cephalosporins or aminoglycosides for a long time as well as a long stay in the hospital (Bogle and Bogle, **1997**).

In the majority of people, however, enterococci are part of the microflora. Van den Bogaard (**1997a**) reported that 91 % of human faecal samples (province of Limburg, NL) contained enterococci (106/117). Schouten *et al.* (**1997**) found lower percentages, around 78 % in elderly people in the proximity of Nijmegen (NL). Whether these findings portray a 'normal' community situation remains questionable. More data are needed from comprehensive epidemiological studies.

Normally, the amount of enterococci found in the human microflora is quite low compared to other bacteria; 0.5 % of all bacteria are enterococci. When antibiotics kill part of the intestinal flora, resistant enterococci can cause overgrowth, which might lead to infection. In hospitals mainly *E. faecalis* is isolated; *E. faecium* accounts for 10–15 % of the isolates. *E. faecium* is more often carrying resistance genes (Murray, **1990**).

Many enterococci are intrinsically resistant to cephalosporins, penicillins, aminoglycosides, clindamycin and Zn–bacitracin. Also resistance caused by acquired genes has been found. This concerns resistance against tetracycline, erythromycin, clindamycin, chloramphenicol, vancomycin and trimethoprim (Baquero, **1997**; De Neeling *et al.*, **1997a, c**; Hoeffler and Zimmerman, **1997**; Alpharma, **1998**).

Vancomycin–resistant enterococci usually contain the mobile Tn*1546* or Tn*1547*, containing the 7 genes responsible for vancomycin and teicoplanin resistance (Arthur *et al.*, **1993**, Quintiliani and Courvalin, **1996**). The fear exists that VRE may not only transfer their resistance genes to other enterococci, but also to (methicillin resistant) Staphylococcus aureus strains. MRSA can still be treated with vancomycin. When multiple resistant MRSA is also resistant to this antibiotic, very few or no antibiotics are available for treatment. This tentative scenario is a powerful motive to ban antibiotics within livestock rearing.

Staphylococcus aureus

Staphylococcus aureus is involved in infections in hospitals (Baquero, **1997**); 12 % of the bacteriaemias is caused by *S. aureus*, while this organism is responsible for surgical wound infections and skin infections in 28 and 21 % of the cases. Also osteomyelitis and endocarditis can be caused by *S. aureus*. The mortality rate can increase when resistant strains are involved, as shown by Romero–Vivas *et al.* (**1995**) who compared the fatality of bacteraemias caused by MRSA and MSSA (methicillin susceptible SA).

Between 1946 and 1950 penicillins, tetracyclines and macrolides were used to treat infections caused by staphylococci. As early as in 1950, penicillin was not successful in 80 % of hospital–acquired *S. aureus* infections because these strains produced β–lactamases. This high resistance percentage is still valid for the *S. aureus* strains isolated in (Dutch) hospitals.

In the 1960s methicillin was used instead of penicillin to treat *S. aureus* infections (Jones, **1996**). This antibiotic is a semi–synthetic penicillinase–resistant penicillin (De Neeling *et al.*, **1997c**). Later, methicillin resistant strains were isolated, as well as cephalosporin resistant strains. Gentamicin was subsequently used to treat MRSA. However, during the 1970s MRSA strains resistant to gentamin arose.

MRSA can be found globally. Resistance to this nosocomial pathogen is especially high in Japan, where around 60 % of the *S. aureus* strains are methicillin–resistant. In Australia and Southern Europe this percentage is approximately 15, and in the USA hospitals 29 % of the strains are resistant (Voss and Doebbeling, **1995**; Panlilio *et al.*, **1992**).

In the Netherlands this percentage is much lower, around 0.3 % between 1989 to 1995. (De Neeling *et al.*, **1997**). In nursing homes the prevalence of MRSA was also very low, 0.15 % in 1992 – 1993 (Frenay *et al.*, **1993**). In Denmark and the United Kingdom comparable prevalence levels of MRSA are found, 0.5 and 0.6 % (Jones, **1996**).

Vancomycin is one of the few antibiotics that can be used to treat infections caused by MRSA. For some MRSA strains (especially in the USA) this is the only antibiotic that is still effective. In the lab the conjugational transfer of vancomycin resistance from *E. faecium* to S. *aureus* has been observed (Noble *et al.*, **1992**). The arising organism carrying this resistance trait might not be treatable *in vivo*.

In 1997 three patients geographically separated *S aureus* resistant to vancomycin was detected (Levy, **1998**). Antibiotics were still effective to treat these infections. A strain isolated in Japan was intermediately resistant to vancomycin (MIC= 8 μg/ml). No genes seemed to be transferred from *Enterococcus*. Cell wall synthesis was increased making vancomycin less effective (Bogle and Bogle, **1997**).

Streptococcus pneumoniae

Streptococcus species often are present in the mouth and intestines of animals and humans. Infections caused by *S. pneumoniae* can be dangerous to young children, elderly and people being immunosuppressed (De Neeling, **1996**).

S. pneumonia is the major cause for pneumonia and is also involved in a large number of cases of meningitis, bacteraemia, sinusitis and otitis media (Baquero, **1997**; De Neeling, **1996**). The number of sick people (morbidity) and the mortality (amounts of deaths) caused by these diseases is relatively high.

Penicillin used to be the antibiotic of choice for treatment of above mentioned infections. Since the end of the 1960s *S. pneumonia* strains resistant to penicillin have been isolated. Especially in Spain, Hungary and Iceland the emergence of resistance is probably related to the high use of β–lactam antibiotics (De Neeling, **1996**).

Streptococcus bovis
This is a D group *Streptococcus*, frequently present in the intestines of humans and animals. This bacterium can cause endocarditis, bacteraemia, neonatal infections and meningitis (Horaud and Bouguénec; **1987**). Strains resistant to erythromycin, vancomycin, kanamycin, streptomycin and tetracycline, although not predominant, have been isolated (Poyart *et al.*, **1997**).

Coagulase negative staphylococci
Infections by coagulase–negative staphylococci increasingly occur in intensive care patients, as well as in people being immunosuppressed or having prosthetic devices.

Staphylococcus epidermis and *S. haemolyticus* often are resistant to methicillin, oxacillin, aminoglycosides, macrolides, lincosamides, teicoplanin and exceptionally to vancomycin (Swartz, **1994**; Baquero, **1997**). Usually, coagulase–negative staphylococci can still be treated with vancomycin (De Neeling *et al.*, **1997c**).

4.5 Resistance selection through antibiotics

4.5.1 Glycopeptides as human medicine and AGP

Glycopeptides used in humans select for vancomycin–resistant enterococci (VRE)
Van der Auwera *et al.* (**1996**) tested the effect of the glycopeptides teicoplanin and vancomycin on human enterococcal flora. Twenty–two volunteers that were not positive for VRE were administered vancomycin or teicoplanin. After three weeks of antibiotics exposure, 64 % of the faecal samples contained VRE. The use of glycopeptides was probably responsible for the emergence of VRE in the human intestine. Another possibility is that VRE were already present in very small numbers (that is below the detection level) before treatment and that they have been selected for as a result of the use of glycopeptides.

Other, less likely possibilities are that the people were exposed to contaminated food or that their stay in a hospital (during the experiment) favoured colonisation with VRE.

Glycopeptides (avoparcin) used as feed additive select for VRE
In Denmark avoparcin (an AGP) was used in feed consumed by poultry and pigs, but has not been used for calves (Aarestrup *et al.*, **1996**). Faecal samples from poultry flocks, pig herds and calves were checked for the presence of VRE before the ban on avoparcin. It was shown that the *E. faecium* and *E. faecalis* present in calves were susceptible to avoparcin. On the other hand, 72 % of the poultry flocks and 20 % of the pig herds contained vancomycin–resistant *E. faecium.*

Bager *et al.* (**1997**) also determined the prevalence of VRE in faecal samples taken from 12 pig farms where avoparcin was used and from 10 pig farms where avoparcin was not used recently. At the farms using avoparcin 8 of the 12 herds contained VREfm, while these bacteria were isolated only from 2 out of 10 herds on the farms where no avoparcin was

used. Data concerning the use of avoparcin in pig herds were obtained from feed–mills that were the exclusive suppliers of feed.

Klare *et al.* (**1995**) showed the presence of vancomycin resistant enterococci in manure of a pig and a poultry farm in the German county Saxony–Anhalt where avoparcin was used. In the manure of an egg–harvesting hen farm in the same region where no avoparcin was mixed with the feed, no glycopeptide resistant enterococci were found. In isolates obtained from poultry the number of vancomycin–resistant enterococci out of total enterococci was higher than in isolates from pigs.

Van den Bogaard *et al.* (**1996**; **1997a**) determined the prevalence of vancomycin–resistant enterococci in Dutch turkey flocks having avoparcin mixed in their food and in flocks not receiving this antibiotic. Of the 12 turkey flocks that were fed without avoparcin, 8 % of faecal samples contained vancomycin–resistant enterococci, while 60 % of flocks fed with avoparcin contained these resistant bacteria.

The above mentioned examples both in human medicine and in animal farming show that the use of glycopeptides such as vancomycin and avoparcin selects for resistant strains of enterococci. It remains to be seen if this resistance is of a permanent nature.

4.5.2 Virginiamycin used as AGP

In many (European) countries virginiamycin (a streptogramin) is added to the feed of broilers and pigs (Witte, **1998**; Aarestrup *et al.*, **1998**). In Denmark the prevalence of resistant bacteria amongst enterococcal isolates of pigs, broilers (Aarestrup *et al.*, **1998**; DVL, **1998**) and cattle (Aarestrup *et al.*, **1998**) was studied. It was found that 40 – 68 % of the *E. faecium* isolated from Danish pigs and broilers (probably) fed with virginiamycin show resistance against this antibiotic. In *E. faecium* from cattle (which not receive virginiamycin) just 8 % was resistant to virginiamycin. Some years after the Finnish ban of virginiamycin in 1990, resistance amongst broiler and pig isolates was much lower than in Denmark (20 % respectively 2 % contained virginiamycin *E. faecium*; DVL, **1998**). In staphylococci isolated from diseased animals (pigs, cattle from Denmark), very low resistance percentages were found (0–1 %).

SCAN noted that the causal relationship between the use of virginiamycin and the development of resistance to this antibiotic is not as clear cut as presented by the Danish studies mentioned above (SCAN, **1998b**). SCAN's criticism is mainly of a methodological nature leaving the observations done in Denmark open for debate.

4.5.3 AGPs also acting against Gram–negative bacteria

Tylosin

In Denmark, pigs and broilers consume feed containing tylosin, but cattle do not. Aarestrup *et al.* (**1997**) analysed faecal samples of swine, cattle and broilers on the presence of antibiotic resistant *Campylobacter* species (a Gram–negative bacterial species). The activity of 16 antibiotics including tylosin, spiramycin (growth promoters) and erythromycin (used in human and animal medicine) was determined. The *Campylobacter* species most isolated from pigs was *C. coli*. Out of 99 *C. coli* strains derived from pigs, 73 and 74 % was resistant to tylosin and erythromycin respectively. In broilers the prevalence of resistance was lower: 18 % out of 17 strains was resistant to tylosin/erythromycin. In cattle and broilers *C. jejuni* was the most isolated strain. Out of 29 *C. jenuni* strains isolated from cattle, 3 % was resis-

tant to tylosin/erythromycin. Of broiler derived *C. jejuni* strains, 6 % was resistant to tylosin/erythromycin.

Nourseothricin
Hummel *et al*. (**1986**) studied the effect of the usage of nourseothricin as a swine feed additive in former East–Germany. No structural analogues are used as medicine for animals and humans. Resistance to this antibiotic was found in *E. coli* strains isolated from pigs, as well as with farm related people, healthy people and people with urinary tract infections. The emergence of resistance in animals was clearly due to the use of nourseothricin. Also it was shown that resistance (or resistant bacteria) to this antibiotic was spread from animals to humans. This example is frequently referred to as an example of resistance transfer from animals to humans disregarding the fact that *E. coli* is a zoonotic Gram–negative organism.[5] This example therefore bears no relation to the AGP issue described in this report.

4.6 Research efforts and data compatibility

4.6.1 General

To be able to answer the question whether resistance transfer occurs, data should be compared and analysed thoroughly. In the box below some questions are listed:

- Animals : Are the animals under investigation fed with feed containing antibiotics?
- Humans : Did the human population/individual in question receive antibiotics? If so, what kind of antibiotics?

- Is sample history well documented?

- Which method is used to determine resistance?
- Are the results presented in a comparable way?

- Are the isolates well characterised?
- Thorough phenotypic and genotypic characterisation, using multiple techniques, is needed to show a close relationship between strains.
- Not only the chromosome of the bacterial strain should be examined well. Also plasmids, when present, have to be characterised.

To be able to show a relation between bacteria found in animals and in humans and to be able to compare resistance data between different research groups these questions need to be kept in mind. Useful evidence for gene transfer or bacterial colonisation is only provided when these criteria are met.

4.6.2 Research methods, data compatibility and reproducibility

Source of samples
In general, for comparison of data, it is essential that the source of the samples be known. When trying to find out whether a relation exists between the use of antibiotics and the prevalence of antibiotic resistant bacteria, the origin of samples is imperative. For samples taken from animals it is essential to know if this flock or group of animals has received feed containing antibiotics. Moreover, it should be known which type of antibiotics was admin-

[5] A zoonotic organism is communicable between animals and humans under normal conditions.

istered to the animals. In studies where meat (pork, beef) is examined for the presence of resistant bacteria, the sampled meat cannot always be traced (back) to the farm of origin. The same holds true for chickens bought in shops. Usually their growing conditions are not known.

The source of resistant bacteria found in meat or poultry is not always evident. These bacteria could have been selected by the use of antibiotics in the feed of the animal or the meat could be contaminated during transport, slaughter or in the butcher shop. Data found in the scientific literature can roughly be divided into two groups:

- Data directly related to the use of antibiotics in feed
- Other data that give an impression of the prevalence of resistant bacteria in the environment

Isolation and phenotypic/genotypic characterisation of strains
In the articles that provide characterisation data, a wide variation in methods to isolate and characterise strains and to determine resistance patterns can be found.

The use of multiple techniques by different research groups can be a problem in comparing data. For example, the use of different isolation techniques (media, enrichment procedures) can lead to different numbers of resistant bacteria. Another problem is the criteria used to state if strains or resistance traits in animals and humans are related. Which techniques are suitable to detect relationships and when is a resistance trait in animal ánd human bacteria considered identical?

Below the techniques for isolation and characterisation of resistant bacterial strains are listed. This is basically a step–wise procedure resulting in information about the specific micro–organism studied and its resistance profile.

- Isolation of resistant strains and phenotypic characterisation:
- *enrichment cultures (different concentrations of the antibiotic used in selection)*
- *determination of species and sub–species: biochemical methods an ready–to–use kits*
- *determination of Minimal Inhibitory Concentrations (MIC; different methods and media)*
- *determination of MICs of single or multiple antibiotics*

- Genotypic characterisation:
- *Pulsed Field Gel Electrophoresis (PFGE) of chromosomal DNA (digested by SmaI)*
- *region amplification within a (resistance) gene by PCR; regions within In: intergenic amplifcation by PCR*
- *ribotyping*
- *long PCRs of transposons*
- *conjugational studies*

4.6.3 Isolation of resistant strains and phenotypic characterisation

Vancomycin–resistant enterococci
A problem is that resistant enterococci/streptococci are only present in small numbers in the large intestine (compared with *Bacteroides* and *E. coli*). In general, when enterococci are present in human faeces (90 % of the people), their total number is around $1 \bullet 10^7$ per gram. When VRE are present they form around 0.5 % of the total number of enterococci (Drasar and Barrow, **1985**; Van den Bogaard *et al.*, **1997a**).

To be able to detect vancomycin–resistant enterococci, enrichment cultures are often needed. This means samples are streaked on (agar) plates supporting growth of the desired organism (Schlegel, **1992**). For isolation of enterococci a few media are suitable:

- Enterococcosel selective agar plates (Klein *et al.*, **1998**; Van den Braak *et al.*, **1997**)
- Enterococcosel broth combined with kanamycin esculin azide agar (Van den Braak *et al.*, **1998**)
- Bile esculin azide agar (Sahm *et al.*, **1997**)
- Iso–sensitest broth followed by blood agar (Kirk *et al.*, **1997a**)
- *Streptococcus* agar plates (Van den Bogaard *et al.*, **1997a**)

It is evident that not all research groups use the same media for enrichment, which makes resistance percentages found difficult or even impossible to compare. An extra enrichment procedure for vancomycin resistant enterococcal strains can be achieved by supplementing plates with vancomycin. The concentration of vancomycin used may vary between different research groups. For example, bile esculin azide agar containing 6 mg/ml vancomycin is used (Sahm *et al.*, **1997**) as well as Enterococcosel agar containing 32 mg/ml vancomycin (Klein *et al.*, **1998**).

Klein *et al.* (**1998**) showed the effect different isolation procedures can have on the resistance percentages found. They examined meat samples for the presence of VRE. Two different methods for culturing VRE were used:

- the use of Enterococcosel selective agar plates supplemented with vancomycin (32 μg/ml)
- an overnight pre–enrichment method with buffered peptone water followed by inoculation of Enterococcosel selective agar plates supplemented with vancomycin.

A much higher resistant percentage was found with the second, double enrichment method, 8 % VRE (out of 555) as compared with the direct method where 0.5 % VRE (out of 555) was detected. Resistance percentages mentioned in the literature need to be looked with caution as methods of determination vary widely.

Vancomycin resistance is predominantly found in enterococci. Enterococci can be subdivided in different species groups and species. Of these different species, only *E. faecium* and *E. faecalis* have been shown to carry the clinical relevant *vanA* or *vanB* gene complex (VRE). These species are the major enterococcal species in poultry, pigs, cattle, dogs and humans. Enterococci like *E. gallinarum* and *E. casseliflavus* that contain *vanC* genes are also found. This C type of resistance is not transferable and the level of resistance obtained is much lower than resistance of the VanA and VanB types.

To be able to determine the amount of VRE in a sample, it is important that enterococci can be identified to the species level (Devriese *et al.,* **1991; 1993a**). Some common characteristics for enterococci are growth at 45 °C, 10 °C, and at pH 9.6. These parameters can be used to distinguish enterococci from other bacteria. If the bacteria involved have been shown to be enterococci, then distinct features of the species can be checked. *E. faecalis* for example, can be distinguished by its tolerance to 0.04 % tellurit. Likewise, *E. gallinarum* can be distinguished from *E. faecium*, *E. casseliflavus* and *E. flavescens* because of its motility and lack of pigmentation. Also 'ready–to–use' kits are applied for the identification of enterococci. The API20 strep and the RAPID ID 32 strep are two of these kits (Kirk *et al.*, **1997a**; Hill *et al.*, **1997**). The API strep 20 combined with detection of species specific genes is very reliable (Dutka–Malen *et al.*, **1995**), while the RAPID ID 32 strep misidentifies many *E. faecium* strains for *E. galinarum*.

Staphylococci
Often staphylococci normally colonising animals or humans have different properties. *Staphylococcus aureus* can be divided in six biotypes (A–F; Hajek and Marsalek, **1971**). In humans biotype A is the major biotype, while biotype B is typical for poultry and pigs. Biotype C is typical for cattle, sheep and goats. *S. intermedius* is the dominant species in dogs and cats (Cox *et al.*, **1985**) and is also present in horses.

In pigs the *S. aureus* biotypes A and C can also be present (Devriese, **1984**), but no strains common for pigs were detected in man. A biotype closely resembling biotype B can be present in humans frequently contacted with meat or animals (Isigidi *et al.*, **1990**).

The different biotypes can be identified in culturing tests (biochemical differences). In addition to biotyping, phagetyping can be done. Different biotypes can be lysed by different phages, but also by the same phage (Shimizu, **1977**; Isigidi *et al.*, **1990**).

Streptococci
Like the staphylococci, streptococci show host–adaptation. This adaptation can be species related or present at the biotype level. A method often used to classify streptococci is based on the presence of cell wall carbohydrate antigens (serogrouping according to Lancefield). This method can be combined with biotyping based on biochemical/culturing tests (Devriese, **1991**). In the same serogroup however, strains that colonise different hosts can be determined (e.g. *Streptococcus agalactiae* has a human and bovine biotype). *S. pneumoniae* and *S. pyogenes* are strains typical in primates. *S. equisimilis* occurs in humans and pigs, but the strains can be distinguished by plasminogen activator tests. Strains present in human or animal, only show activity for the plasminogen in that specific host (McCoy *et al.*, **1991**).

4.6.4 Determining resistance data: phenotypic characterisation

General
Resistance of bacterial strains can be detected in vitro by determination of the MIC (minimal inhibitory concentration) of a specific antibiotic. Estimation of the MIC is an important tool for distinguishing between resistant and susceptible strains. It should be noted that MIC–values do not always closely correlate with the effect *in vivo*. A reliable MIC estimation in this case means that the effect of the antibiotic *in vivo* (e.g. the effect of the antibiotic as a therapeutic agent) closely resembles the *in vitro* data.

Bacteria are grown in the presence of (different concentrations or a gradient) antibiotic. The lowest concentration where no growth occurs is defined as the MIC. In the table below the different methods to determine the MIC of an antibiotic are shown.

Table 4.6.4.1 Methods to determine antimicrobial susceptibility. After inoculating with bacteria, the broth or agar plates are inoculated for 16 – 20 hours (Devriese et al., 1993a; Woods and Washington, 1995; NCCLS, 1997; Jorgensen and Ferraro, 1998)

Method	Number of bacteria
1a. Broth macrodilution (two–fold dilution; 1 – 2 ml broth)	$5 \cdot 10^5$ cfu/ml
1b. Broth microdilution (two–fold dilution; microdilution trays, 100 μl	$5 \cdot 10^5$ cfu/ml
2. Agar dilution test (two–fold dilution; agar plates)	$1 \cdot 10^4$ bact./drop
3. Etest® (AB BIODISK; gradient in agar; agar plates and strips)	$1 – 2 \cdot 10^8$ cfu/ml
4. Disk diffusion test (gradient in agar; agar plates and disks)	$1 – 2 \cdot 10^8$ cfu/ml

A test often used to determine antimicrobial susceptibility (and a MIC–value) is the agar dilution test (method 2). Agar plates containing increasing concentrations of the antibiotic are inoculated with the same amount of bacteria and the growth is monitored. A comparable test is method 1, where the bacteria are grown in broth containing different concentrations of antibiotics. This can be done in small tubes or in microtiter plates containing even smaller amounts of liquid. Another method where a MIC–value can immediately be derived from the results is the Etest® (method 3). Here strips with antibiotic are placed in the agar, after which the MIC can be read from the strip (border growth/no growth). In method 4 also a gradient is formed in the agar, but by placing an antibiotic disk on the agar plate. In this way an inhibition zone is observed around the disk. The size of this zone (in mm) indicates the susceptibility of the bacteria for this antibiotic. Categories of zone sizes (susceptible, resistant) have been determined for some antibiotic–bacterium combinations.

Tests to determine the MICs are *in vitro* tests, so the effect of an antibiotic on the bacteria *in vivo* can not be guaranteed. The concentration of the antibiotic at the site of infection is important and the time period the antibiotic is present (Bryan, **1982**).

For each antibiotic used in human health MICs have been documented prescribing when a certain strain is considered resistant or susceptible. Some antibiotics are characterised as intermediate resistant at a specific concentration range; in this case the effect of the antibiotic is not that/always clear. For antibiotics only used as growth promoter criteria and methods for testing and evaluating resistant and susceptible strains are less well documented. In this way it is more difficult to provide reliable/reproducible prevalence data for growth promoters then it is for antibiotics used in human medicine. When resistance criteria are poorly documented, all MIC data of the strains examined should be presented. When showing the MIC–range it is possible to compare data obtained in other laboratories.

Influence of media and incubation conditions

Comparability of MIC data is hampered by the use of different media and incubation conditions by different research groups. Not all enterococcal strains show the same susceptibility to an antibiotic when the test is repeated under different conditions. It is essential that the circumstances, which provide the most reliable MIC is determined and reproducibility can be guaranteed.

In most countries recommendations have been formulated on how to perform antimicrobial susceptibility testing (National Committee for Clinical Laboratory Standards (NCCLS, **1997**), Dutch Working Group for Antimicrobial Susceptibility Testing, British Society of Antimicrobial Chemotherapy). These recommendations are updated regularly (every few years). The methods and the media to be preferably used are described. Also susceptibility

and resistance criteria are provided. The use of control strains, with known MICs under the applied test conditions, is also recommended as a quality control for the performance of a test (Gordts *et al*., **1995**).

The recommendations regarding susceptibility testing are no strict rules. Variations in testing methods, susceptibility criteria and the media used, occur between different research groups and different countries. Also old testing criteria are sometimes used. Therefore, it is important that world–wide recommendations are developed and fulfilled.

Influence of media and incubation conditions: examples

An important quality item for MIC determinations is the number of very major errors made. A very major error is defined as a resistant strain misinterpreted as susceptible. (A major error is defined as a susceptible strain being misinterpreted as resistant.)

According to Kohner *et al*. (**1997**) determination of the MIC on a Mueller–Hinton medium in a broth microdilution or agar dilution test is a good method to detect resistance due the *vanA* or *vanB* gene system in enterococci. On the other hand, when it concerns vancomycin resistance due to the *vanC* gene the Mueller–Hinton medium is far less applicable; a high percentage (65 – 90 %) of very major errors is found. The Mueller–Hinton medium is recommended by the NCCLS and therefore regarded as a standardised procedure. Kohner *et al*. (**1997**) however, recommend the use of the brain heart infusion medium (BHI) in testing vancomycin resistance (VanA, B and C phenotype) with the broth dilution and agar dilution method. A disadvantage of this medium is its complexity and the lack of standardisation in testing methods (Butaye *et al*., **1998a**).

Especially when regarding susceptibility testing of growth promoters it is not known which testing conditions give the best results. Butaye *et al*. (**1998a**) examined the effect of different conditions in Mueller–Hinton II agar dilution tests on the MICs of growth promoters acting against enterococci. Enterococci of *E. faecalis*, *E. faecium*, *E. avium*, *E. gallinarum* and *E. cecorum* species groups were tested. The effect of adding sheep blood or CO_2 as well as differences between aerobic and anaerobic incubation was studied.

Important differences could be observed between the examined strain–growth promoter–condition combinations. Nevertheless it was possible to distract general conditions which give satisfactory results in most of the cases.

When testing *E. faecium* and *E. faecalis* only, Mueller–Hinton II medium without blood and aerobically incubated is suitable. When testing more enterococcal species the recommended conditions are Mueller–Hinton II medium containing blood, while incubation should take place under a CO_2–enriched atmosphere.

Susceptibility testing as part of phenotypic characterisation of resistant strains

When comparing antimicrobial susceptibility patterns the strains containing the *vanA* or *vanB* gene can be separated from the strains containing *vanC*, because the *vanA* and *vanB* genes give rise to higher MICs. *VanA* containing strains are highly resistant to vancomycin, *vanB* strains show intermediate resistance. Enterococci containing *vanC* and *vanB* are not resistant to teicoplanin. When a strain is of the VanC type, it is probably not *E. faecium* or *E. faecalis*, but belongs to the *E. gallinarum* species group. However, these phenotypic tests only lead to predictions of resistance genes present. To be sure these tests should be combined with genetic characterisation.

Testing of resistance to multiple antibiotics can be a useful tool to distinguish between different populations of resistant strains. For example, when VREs are involved, some researchers just detect vancomycin resistance, while others check the susceptibility to many other antibiotics. When determining more extensive resistance profiles of strains a better insight in resistance traits often present together, can be obtained. Supplementing this phenotypic method with genotypic data (e.g. plasmid isolation combined with PCR), a more thorough insight in the spread of resistance genes from whatever source can be obtained.

4.6.5 Genotypic characterisation

Many genetic methods can be used to characterise strains and their resistance traits. Important in transfer of resistance is to find out whether strains or resistance genes are transferred from animals to humans (and from humans to humans). It is necessary to use the methods that are the most discriminatory, so that even small differences between strains can be distinguished. For a complete analysis also the presence of plasmids and/or resistance traits present should be determined.

Pulsed–Field Gel Electrophoresis (PFGE)

A method widely applied to identify relationships between enterococcal strains is Pulsed–Field Gel Electrophoresis (PFGE). The bacterial genome is digested with a restriction enzyme (e.g. SmaI),[6] giving rise to up to 25 fragments. This procedure can be carried out with different isolates, and the large fragments obtained (10 kb to 10 Mb) can be separated on agarose gels with a pulsed electric field. Comparison of patterns of bands obtained from different isolates provides information about the relation/evolution of these isolates (Miranda *et al*., **1991**; Kaufmann, **1998**). If the PFGE patterns of two strains are indistinguishable, the strains contain identical genomes. When isolated during outbreak of infection, the presence of identical strains indicates the spread of one strain. If isolates differ in one or two bands, the strains are related and when three or more bands are different, the strains most likely are not related.

Klare *et al*. (**1995**), Van Belkum *et al*. (**1996**) and others have used PFGE as a method of characterising strains in the case of VRE. This method seems to be applicable for *E. faecium* and *E. faecalis*, but not for *E. casseliflavus* and *E. flavescens* (Clark *et al*., **1998**). Patterns of *E. casseliflavus* strains could be as different as patterns of *E. casseliflavus* and *E. casseliflavus–E. flavescens*.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a genetic typing method, where the presence or absence of specific (e.g. resistance) genes can be determined. To do this, the sequence of a prototype gene must be known. From the ends of the target sequence, two oligonucleotides of about 20 bases, called primers, are synthesised (Saunders and Clewley, **1998**). These are used to search for the gene of interest. Subsequently, several rounds of reactions multiply this specific sequence. When sufficient DNA is produced, it can be analysed. In the case of resistant enterococci, primers are available to amplify the *vanA*, *vanB*, *vanC1*, *vanC2* and *vanC3* gene (Dutka–Malen *et al*., **1995**; Clark *et al*., **1998**). Below the genes identified in specific enterococcal strains, which can be used for PCR are listed:

[6] Enzyme that recognises a specific nucleotide sequence in the DNA and recognising this sequence subsequently cleaves the double stranded DNA at this position.

Table 4.6.5.1. Genes used to identify strains and/or resistance traits by PCR (Dutka–Malen et al., 1995; Clark et al., 1993; 1998; Poyart et al., 1997; Gordts et al., 1995).

Gene	Strain
vanA	*E. faecium, E. faecalis*
vanB	*E. faecium, E. faecalis*
$ddl_{E.\ faecium}$	*E. faecium BM4147*
$ddl_{E.\ faecalis}$	*E. faecalis*
vanC1	*E. galinarum*
vanC2	*E. casseliflavus*
vanC3	*E. flavescens*

VanA and *vanB* genes can be present in both *E. faecium* and *E. faecalis*. In order to discriminate between *E. faecium* and *E. faecalis*, specific chromosomal genes can be amplified, like the *ddl* genes, encoding D–Ala:D–Ala ligases. The $ddl_{E.\ faecium}$ gene is isolated from prototype *E. faecium* BM4147. A comparable gene is present in *E. faecalis*, then called $ddl_{E.\ faecalis}$ (Dutka–Malen and Courvalin, **1990**).

Enterococci of the *E. gallinarum* species group can be distinguished by the detection of one of the *vanC* genes. These genes encode a D–Ala–D–Ser ligase (Dutka–Malen *et al.*, **1991**; Navarro and Courvalin, **1994**). The *vanC1* gene is specific for *E. gallinarum* and is not present in other enterococci. Because the *vanC2* and *vanC3* genes are very similar, it is not always possible to distinguish between *E. casseliflavus* and *E. flavescens* (Clark *et al.*, **1998**; Dutka–Malen *et al.*, **1995**).

To control whether the PCR procedure has been carried out well, control strains can be included. From these strains it is known that a certain gene is present or lacking.

In many articles describing the prevalence of vancomycin resistance, only the presence of the *vanA* gene is checked by PCR. The detection of this gene can be used to show the amount of *vanA* resistant bacteria in a population, e.g. human isolates.

Sequencing of resistance genes can be carried out to detect how closely related these genes are. Casewell and Beighton (**1996**) stated that resistance genes present in different organisms often have near identical sequences (conserved sequences). In the case of the *vanA* gene cassette, in the regions between the genes more variations can be found then in the genes themselves. Comparison of intergenic regions may be used to determine relationships between animal and human strains.

The *vanA* gene is a part of the Tn*1546* transposon (Arthur *et al.*, **1993**). This transposon contains 7 genes giving rise to the regulation of vancomycin resistance. Also two genes are present coding for a transposase and a resolvase needed for transposition of the DNA fragment. Differences in the transposon mediating vancomycin resistance can be shown by amplification of several other parts of Tn*1546*. Not only the genes present (*vanA*, *vanX*, *vanY*, *vanZ*, *vanS*, *vanH*), but also the intergenic regions *vanS–vanH*, *vanX–vanY* and *vanY–vanZ* are amplified (Casewell and Beighton, **1996**). Strains can be distinguished by comparing the presence and the lengths of the fragments. Sometimes insertion sequences are present in intergenic regions. If all fragments are identical in size (and in sequence, which is rarely determined) the resistance trait is identical and probably will have the same origin. (It still has to determined at this point where these sequences come from and how the resistance

genes can be transferred to another bacterium.) Is the complete Tn*1546* transferred, or are the new acquired IS capable of moving by the aid of the transposase gene coded by orf1?). The method of amplifying intergenic regions by PCR is used by different research groups (Casewell and Beighton, **1996**, Woodford *et al.*, **1998** and Van den Braak *et al.*, **1998**).

Random amplified polymorphic DNA analysis (RAPD)

With this technique primers for PCR are not chosen out of a known sequence of a gene. Oligonucleotides of arbitrary sequences are composed, which match at multiple sites within (for example) genomic DNA (Ralph and McClelland, **1998**). The fragments of amplified DNA can be separated by gel electrophoresis. In this way, a 'fingerprint' of the DNA examined is obtained. Differences in fingerprints of different isolates can be used to distinguish between those isolates and equalities may reveal relatedness.

Southern blotting (e.g. ribotyping)

Genes related to each other could be detected by Southern hybridisation. Genes being different in up to 30 % of their DNA can be detected with this technique. As with PFGE, first the genomic DNA of bacteria is isolated and digested with a suitable restriction enzyme. A pattern of bands is obtained, which is separated by agarose gel electrophoresis. The DNA is transferred to a nylon membrane (Southern blotting). A (large) part of the prototype of the gene of interest is used as probe to identify corresponding genes on the membrane. The (radiolabelled) probe only binds to pieces of DNA that contain this gene.

An example of Southern hybridisation is ribotyping. This technique is used to identify differences in coding regions for ribosomal RNA of the strains to be characterised. Related strains will contain DNA coding for rRNA on identical fragments on the nylon membrane. The ribosomal RNA genes are detected using a specific probe, usually 16S and 23S rRNA from *E. coli* (Jordens, **1998**). When using appropriate enzymes for restriction and controls are included, this method can be used to contribute to resolve such issues as whether an outbreak of infection is due to spread of a single strain. Bates *et al.* (**1993**) used this technique to type vancomycin resistant *E. faecium* isolated from hospital patients and general practice patients.

DNA sequencing

To detect recent transfer of resistance genes between bacteria (a few nucleotide differences), the DNA can be sequenced (Nikolich *et al.*, **1994**). Sequences of different DNA preparations can be compared with each other (genes from prototype strain and new samples). DNA sequencing of pieces of DNA amplified by PCR can be used to detect changes within the gene. If nucleotides are the same there is a close relationship between the two genes. Sometimes nucleotides have changed, but the amino acids they code for are conserved. Then the genes have diverged earlier in history (Casewell and Beighton, **1996**). However, when dealing with human samples, the question whether resistance is due to resistance genes coming from animals or humans cannot be easily answered.

When dealing with antibiotic resistant strains, it is imperative to determine the location of the resistance genes. Southern blotting of chromosomal DNA and plasmid DNA is useful for this goal. Also the plasmid(s) present can be analysed (Johnson and Woodford, **1998**). Enzymes restrict plasmid DNA can be separated from chromosomal DNA and subsequently. The size of the plasmid and the size of restriction fragments can be detected by gel electrophoresis. A coefficient of similarity can be used to compare the restriction patterns (Dice, **1945**; Zervos *et al.*, **1998**; and Platt *et al.*, **1988**):

$$S_D\ (\%) = (2m/(a+b)) \cdot 100$$

S_D = Dice coefficient of similarity
M = amount of restriction fragment that two plasmids (A and B) have in common
a + b: total amount of fragments obtained after digestion of plasmids A and B

The fragments obtained in restriction can also be used to detect the presence of specific resistance genes. After Southern blotting, probes of the genes of interest can be added. When combining the determination of similarity and the detection of resistance genes, plasmids can be thoroughly compared.

Another interesting point to study is the transfer of resistance traits to other cells by conjugation. More insight can be formed about the spreading of resistance genes and which genes are transferred together.

Many studies are known to test the prevalence of resistance in samples from animals, humans, meat or the environment. Some of these studies try to link the presence of resistance genes in different samples. Difficulties arise because the origin and history of many isolates is not clear or not known. Bacterial strains containing identical resistance patterns, genes or gene cassettes, could be related. However, this constitutes no hard evidence, because the actual transfer of genetic material is not witnessed.

Information about the probability of spread of resistance genes between different strains can be obtained from laboratory studies. In many laboratories where antibiotic resistance is tested, also conjugational studies are carried out in order to study the conjugation frequency within species and between different species. These studies also can give an indication of the frequency of conjugation in nature.

Another important aspect is that data about which antibiotic traits are transferred together can be obtained. Isolating and characterisation of plasmids and transposons can achieve this. If multiple plasmids are present in one strain their compatibility can be observed. Susceptibility studies can be done in strains containing single plasmids or in strains that contain combined single plasmids after conjugation.

Conjugation experiments in the laboratory
Mating experiments can be carried out on filters lying on agar plates or in broth containing the antibiotic of choice (Poyart *et al.*, **1997**). Usually the recipient strains contain a selection marker, which can be a resistance gene against an antibiotic being active against the donor strain. An example is the use of streptomycin–resistant recipient strains with donor strains resistant to vancomycin (Poyart *et al.*, **1997**). For detection of transconjugants agar plates containing streptomycin and vancomycin are used. The frequency of transfer was calculated by dividing the number of transconjugants/ml by the number of recipients/ml.

Restriction fragment length polymorphism (RFLP) of transposons containing resistance genes
Another method to detect differences in the genetic structure of resistance genes (or gene cassettes) was reported by Haaheim *et al.* (1998). Primers of the left and right end of the transposons containing *vanA* resistance and *vanB* resistance were composed, resulting in long PCR fragments. Material of standard strains containing Tn*1546* result in an amplified product of 10,414 bp, while the fragment amplified within Tn*1547* was 5,959 bp. The amplified products are nearly as long as the original transposon (10,8 kb and 7,1 kb; Arthur *et*

al., **1993**; Quintiliani and Courvalin, **1996**). The amplified products can be cleaved with restriction enzymes, and the pattern of fragments obtained from different isolates can be compared. This method is called restriction fragment length polymorphism (RFLP).

5 Prevalence of Bacteria Resistant to Antibiotics

5.1 Introduction

To get an indication of the spread of resistance (genes) it is useful to obtain data about the prevalence of resistance from a wide variety of samples. Below data are presented concerning resistance against antibiotics used as growth promoter and/or their analogues used to treat humans. Gram–positive bacteria resistant to avoparcin/vancomycin, MLS_B antibiotics (virginiamycin, tylosin, streptogramins, erythromycin) and Zn–bacitracin are of the highest interest. The growth promoters in animal feed select for resistant Gram–positive bacteria in animals.

To date, bacteria resistant to these specific growth promoters and related antibiotics used as human medicine, have been found in humans, animals, meat and waste water. Most of these data are derived from point prevalence studies and can only be used to give an impression of prevalence of resistance.

In most articles no information is given about the origin of the samples and bacteria are not thoroughly characterised, phenotypically or genotypically. Only when an article includes these items, the prerequisites are met that allow proper comparison of data. Only then the article can contribute to the discussion about possible reservoirs (animals, humans, sewage) of resistance genes and transfer of resistance genes between these different reservoirs. In the following sections articles that contain useful data are described in more detail.

5.2 Resistance prevalence

5.2.1 Prevalence of vancomycin–resistant enterococci

When considering vancomycin–resistant enterococci (VRE), this only includes enterococci with *acquired* resistance genes. The gene clusters that can be involved are the *vanA* and *vanB* clusters, of which *vanA* is the most wide–spread. These gene clusters can be present in the clinically important *E. faecium* and *E. faecalis*, from which *E. faecium* more often contains resistance genes.

In many articles it is described that vancomycin–resistant enterococci were found, but no further characterisation is done. Relevant articles should at least contain data identifying the strains to the species level and detecting the gene (cluster) causing resistance.

From these relevant articles, information can be obtained concerning emergence and spread of resistance. Questions that need to be answered are mentioned below. It goes without saying that these questions are relevant for all types of antibiotics used in human and animal medicine and as AGPs.

- Does the specified use of antibiotics select for resistance?
- What is the prevalence of VRE in different samples (animal, human, and sewage)?
- What is the origin of VRE infections? Are they principally community acquired or nosocomial?
- Are strains and/or transposons present in different sources genetically related?
- Are examples present showing the transfer of vancomycin resistance from animals to humans? In what way is this transfer documented?

In pigs and poultry prevalence is high; from 5 up to 50 % of the faeces samples contained VRE. In calves no VRE are found, probably because avoparcin has never been used as growth promoter in calves. Also in domestic animals VRE can be found. In meat there is a big difference shown between chicken meat and pork/beef meat. In almost all chicken samples VRE are found, while in other meat just up to 8 % of the samples is positive.

Human samples can in principle be divided in samples from hospitalised and non–hospitalised people. The prevalence of VRE in samples is highly dependent on the geographic region and –when hospitalised– whether there is an outbreak of VRE. In hospital samples when no outbreak takes place 3.5 up to 6 % of the samples contain VRE, while during outbreaks this can increase to 14 %. In non–hospitalised people percentages from 1.5 – 4 up to 10 – 30 % have been reported.

5.2.2 Origin, transfer and spread of VRE

An infection caused by resistant bacteria is considered nosocomial when resistant bacteria emerge in the hospital and are being transferred from one person to another. Hospital used antibiotics can select for resistance either through bacterial resistance acquirement or selection from the already present resistant bacteria in the patient (Bogle and Bogle, **1997**).

In the first variant VRE strains should be closely related due to person to person spread, while in the second case patients might carry non–related strains, selected from their own flora. In outbreaks of enterococcal infections sources of resistant strains can either be endogenous or exogenous.

Bingen *et al*. (**1991**) reported that in a French hospital isolates from 15 children (present in the hospital in an overlapping time–scale) were all different. Hall *et al*. (**1992**) came to the same conclusion when analysing *E. faecium* and *E. faecalis* strains isolated from UK hospital patients. It was also reported that some patients carried multiple, distinguishable strains of VRE (at the same time or some time later). Also 23 isolates from 14 patients which had been given vancomycin prophylactically were all different according to their PFGE pattern (France, Plessis *et al*., **1995**). In the Netherlands comparable observations were made. VRE isolated from hospitalised patients and community patients, characterised by PFGE and PCR were reported to be heterogeneous (Endtz *et al*., **1997**).

Bates *et al*. (**1993**) found that out of 8 clinical *E. faecium* isolates resistant to vancomycin 4 were from renal patients (UK). These 8 strains were clonally related (shown by SalI restriction enzyme analysis and ribotyping of ribosomal RNA genes with EcoRI cuts). Hill *et al*. (**1997**) examined 51 *E. faecium* strains isolated in a London hospital. By PCR the *vanA* gene was detected in all strains. Multiple different strains were present when PFGE patterns were studied. Some strains were shown to have spread through the hospital, while others were isolated in single cases.

Miranda *et al*. (**1991**) checked the relatedness of enterococcal hospital isolates in the USA by PFGE. The 34 samples analysed were from 5 different hospitals/medical colleges and from the Centre for Disease Control (CDC) in Atlanta. The hospitals were located at the North East Coast (Boston, New York and Richmond), Wisconsin and California. PFGE results showed 10 to 19 visible bands in each of the isolates. The 34 isolates showed 27 different restriction patterns. Five restriction patterns were obtained from more than one isolate. Isolates with identical patterns were only present within one location. Samples from the CDC and from Wisconsin were all different. The isolates tested within one region often had just a few bands different from each other. Two of the isolates from Philadelphia with the same

pattern showed a high similarity with one of the isolates from Richmond. The conclusion is that within hospitals strains can be spread. The observation that isolates from the same region can be related (few bands different) might indicate a spread between hospitals.

Clark *et al.* (**1993**) examined 105 glycopeptide resistant human enterococcal isolates obtained from 1988 till 1992 in 31 USA hospitals. MICs of multiple antibiotics were determined by the broth microdilution method. In total, 66 % of the strains had a VanA phenotype, 25 % VanB, 5 % VanC, while 4 % of the strains could not be classified. The presence of the vancomycin resistance genes was determined by PCR. A thorough characterisation including susceptibility patterns, PFGE and PCR of genes encoding vancomycin resistance was carried out for part of the strains with a VanA or VanB phenotype. All these strains contained one or more plasmids (1 up to 60 kb). Hybridisation with *vanA* probe showed the *vanA* gene was present on plasmids of 34 or 60 kb. Some plasmid profiles were identical in strains from different hospitals. Also multiple plasmid patterns could be obtained from some hospitals.

In total, 21 different PFGE patterns were found. PFGE patterns were shared by seven strains isolated from three different hospitals in the state New York and by six strains isolated from two hospitals in the state Missouri (the only pattern found in these two hospitals). When combining PFGE and plasmid patterns more different strains could be observed. Strains with the same PFGE pattern were shown to contain different plasmids. The conclusion is that strains with identical chromosome can be isolated from patients in the same and in different hospitals. Plasmid patterns cause variation within strains with an identical PFGE pattern. This probably means that the plasmids themselves and the plasmid content of cells are not very stable. When bacteria are growing and multiplying they can loose some plasmids, and by conjugation some new ones can enter the cell or transposons can jump into existing plasmids.

Schoonmaker *et al.* (**1998**) monitored the presence of vancomycin–resistant enterococci (*E. faecium*) in two patients during and after their stay in an USA hospital. They could isolate 13 strains from the same patient in five months time. Up to 6 different strains could be isolated from the same sample (faeces or urine). The bacterial isolates were characterised using PFGE, determination of plasmid profiles, PCR of the *vanA* gene and MIC determination. Both patients stayed in the hospital for long times (more than two months). They also had more than one surgical operation and were treated with multiple antibiotics (vancomycin, metronidazole, ciprofloxacin and others). These three factors contribute to the emergence of resistant bacteria. During treatment with vancomycin VRE emerged.

In the United States the prevalence of VRE in the community has not been examined to a large extent (McDonald *et al.*, **1997**). Bonten *et al.* (**1996**) checked the presence of VRE in faeces op patients on entering the hospital; 4 % of 301 patients carried VRE in their faeces. In two other studies no VRE were detected (Coque *et al.*, **1996**; Bais *et al.*, **1996**). On the other hand it has been shown in the USA that the use of vancomycin in hospitals did result in the rise of human VRE. The following table serves to illustrate this point (Kirst *et al.*, **1998**):

Table 5.2.2.1 VRE infections in relation to vancomycin use

	USA	UK	Denmark
VRE infections in humans	++++	+	0
Vancomycin (kg in 1996)	11,279	320	60
Avoparcin (AGP)	0	+++	+++

For the sake of argument we added the avoparcin use in the three countries mentioned. (Avoparcin has never been used in the USA.) The avoparcin use seemingly had no measurable effect on the rise of VRE.

5.2.3 Meat as a possible source of resistant bacteria in humans?

VRE in humans

VRE might be present within humans. Very little, however, is known about the presence of VRE in the bowel of the non–hospitalised community. Humans might acquire VRE through the consumption of meat. When this transmission route is valid, a first minimal prerequisite is the presence of VRE in meat. As a consequence of consuming meat, the prevalence of VRE (*E. faecium*, *E. faecalis*) might be higher in people who eat meat than in vegetarians providing the livestock receives avoparcin in their feed and providing VRE are capable of colonising the human gut permanently. Transient presence of VRE is also a possibility but is more difficult to detect and if detected might be mistaken for a permanent colonisation. In this context reproducibility is a prerequisite.

When isolating VRE out of meat and human faeces, the bacteria have to be identified to the species level as intrinsically vancomycin resistant *E. gallinarum* and *E. casseliflavus* can be present in fruit and vegetables, consumed by vegetarians as well as meat–eaters (Murray, **1998**). Few articles are published where the prevalence of VRE in faeces of both consumer groups have been examined (Schouten *et al.*, **1997**; Van den Braak *et al.*, **1997**).

Schouten *et al.* (**1997**) investigated the presence of vancomycin–resistant enterococci in faeces of elderly vegetarians (meat–free for 3 – 86 years) and elderly people eating meat in the Netherlands. The elderly vegetarians and the meat–eaters lived in two separated homes for elderly people. By using a selective enrichment procedure for enterococci, no vancomycin–resistant enterococci were found in samples from vegetarians. In contrast, 10 % of the samples from meat–eaters contained vancomycin–resistant *E. faecium* (MIC > 256 μg/ml). However, it is not noted which concentration of vancomycin was used in the selective enrichment procedure. The concentration of antibiotic used in selection effects the number and kind of bacteria selected making these observations difficult to interpret.

In another Dutch study (Van den Braak *et al.*, **1997**) rectal swabs of 318 vegetarians and 276 meateaters were examined for the presence of VRE. The vegetarians were meat–free for ±30 years. The samples were collected from all over the country. In contrast to the study of Schouten *et al.* (**1997**), vancomycin–resistant enterococci were found in higher percentages in the faeces of vegetarians compared to faeces of meat–eaters. However, all strains isolated from vegetarians were *E. casseliflavus* (21 strains) or *E. gallinarum* (12 strains), containing the *vanC2 vanC1* gene respectively. From meat–eaters, 14 VRE were isolated, of which only one *E. faecium* containing the *vanA* gene. The other strains were *E. gallinarum (7)* and *E. casseliflavus (6)*, containing the *vanC1 vanC2* gene respectively.

VRE in meat

Chickens bought in shops often contain VRE (Bates *et al.*, **1993**; Klare *et al.*, **1995**; Thal *et al.*, **1995**; Van den Braak *et al.*, **1997**; **1998**; Woodford *et al.*, **1997**). Van den Braak *et al.* (**1998**) detected VRE in 79 % of poultry product bought in Dutch shops and markets. Usually the origin of these chicken carcasses is not traced and neither is their feed regime. Whether or not antibiotics were used is therefore not known. In this way it is not possible to relate the prevalence of VRE with the use of avoparcin as growth promoter.

Klein *et al.* (**1998**) started a study concerning the prevalence of VRE in meat a few months after avoparcin was banned in Germany (ban since January 1996). The banning of this antibiotic might decrease the prevalence of VRE in meat.

Minced pork and beef meat, originated from different counties in Germany, were examined in a meat–processing plant in Berlin (EU–admitted). In total 555 meat samples were collected from different slaughter houses, in 55 days between May 1996 and April 1997. The cutting surface was thoroughly disinfected. The total number of enterococci found in the meat ranged from 5 to 710 colony–forming units (CFU) per gram. Three out of 555 samples were positive for VRE (around 10 CFU/g). The samples were plated directly on Enterococcosel selective agar plates supplemented with vancomycin. A variation on this method, with prior overnight enrichment, resulted in 46 samples with VRE out of 555 samples (1 to 9 CFU/g). The conclusion Klein *et al.* (**1998**) drew was that VRE does not seem to be widespread in pork and beef and when present, their numbers are low.

Interesting in the context of risk analysis is to find out which enterococcal species are found in meat and to which antibiotics they show resistance. Klein *et al.* (**1998**) identified 209 enterococcal strains to the species level. *E. faecalis* was predominant (182 strains), while *E. faecium*, *E. casseliflavus*, *E. gallinarum*, *E. durans*, *E. hirae* and *E. avium* were present from 8 down to 1 strain. Out of the 34 VRE found 12 were *E. faecalis* and 13 were *E. faecium*. *E. durans*, *E. hirae* and *E. gallinarum* were also present (3, 1 and 5 samples). Identification was done with the Rapid ID 32 Strep identification kit, which is known to misidentify some *E. faecium* strains as *E. gallinarum* (Kirk *et al.*, **1997a**; Hill *et al.*, **1997**). When the total number of *E. faecium* and *E. faecalis* amongst meat enterococci is compared it is clear that *E. faecium* more often contains vancomycin resistance than *E. faecalis*. In all the VRE samples (*E. faecium* and *E. faecalis*), the *vanA* gene was present in the genomic DNA. The '*E. gallinarum*' containing this gene, probably were *E. faecium*.

To further type the strains, resistance patterns of vancomycin–susceptible and vancomycin–resistant strains towards 16 (groups of) antibiotics were determined. The majority of VRE were also described to be resistant to avoparcin, virginiamycin and tylosin, three feed additives widely used. Resistance to imipenem, streptomycin, erythromycin, tetracycline, chloramphenicol and trimethoprim–sulfa–methoxazol is much more common in VRE than in non–VRE. Resistance to methicillin, cephalosporins (ceftriaxone, cephalothin) and clindamycin is found in more or less equal percentages in both groups.

The study described above by Klein *et al.* (**1998**) started a few months after avoparcin was banned. It is therefore possible that the extent of vancomycin resistance has already declined. When no antibiotics are fed to the animal, the normal, not–resistant intestinal flora might be able to overgrow the vancomycin–resistant enterococci. Klein *et al.*, (**1998**) suggest that VRE can be present in the community, without a link to the use of avoparcin in feed. Larrocke and Bulte (**1997**) also found very low or no VRE in 225 samples that were taken before the ban on avoparcin. Because of the low prevalence of VRE in meat (in par-

ticular after the ban) it is not likely sufficient bacteria will survive the human stomach and reach the human intestines (apart from colonisation difficulties). It is therefore not convincing that meat could contribute significantly (if at all) to the spread of VRE in humans. The hypothesis that meat is a source of VRE in humans remains unproven.

5.2.4 Genetic identification of similarities and differences between VRE in animals, meat, sewage and humans

In many articles resistant isolates from animals and humans are described. Often only the (major) gene responsible for resistance is searched for. In the case of phenotypically or presumed vancomycin–resistant enterococci, PCR is used to verify the presence of the *vanA* gene. This gene has been detected in (mainly) *E. faecium* isolates from hospital patients, community patients, animals from farms, pets, meat and waste water (Aarestrup *et al.*, **1996**; Gordts *et al.*, **1995**; Van den Braak *et al.*, **1997**; Klare *et al.*, **1995**; Devriese *et al.*, **1996**; Van den Bogaard *et al.*, **1997a,b**; Van Belkum *et al.*, **1996**).

Indeed, *E. faecium* containing the *vanA* gene as part of Tn*1546* is the most frequently found resistant *Enterococcus*. To answer the question whether avoparcin used in feed contributes to the presence of VRE in human isolates, resistant strains of different animal species and humans have to be thoroughly compared. However, sole detection of the *vanA* gene is not sufficient when trying to reveal whether strains are related. Also, vancomycin resistance due to the *vanA* gene is not the only genotype. Other genes (or gene systems) giving rise to vancomycin resistance are the *vanB*, *vanC1* and *vanC2* genes, which can be detected by PCR (Dutka–Malen *et al.*, **1995**).

Detection of which type of vancomycin resistance is involved is therefore the first step in characterising strains. Subsequently, it has to be elucidated whether isolates are related. The detection and characterisation of one single resistance gene is not sufficient. Vancomycin resistance genes (VanA and VanB type) are present on transposons, which contain multiple genes involved in resistance. The transposon in each strain needs to be characterised in order to reveal differences between resistance traits in bacteria. To find out whether strains are identical or highly related their chromosomes have to be compared. A combination of PFGE and PCR (within transposons) is accurate in distinguishing and relating VRE, while ribotyping is much less discriminatory.

In pets VRE have been detected (Van Belkum *et al.*, **1996**; Devriese and Haesebrouck, **1996**). Van Belkum *et al.* (**1996**) showed that two *E. faecium* strains (*vanA* genotype) isolated from a dog and a cat showed the same PFGE pattern. Only PFGE was used to determine whether strains were related. The use of multiple techniques is preferred when claiming indistinguishable strains. It is possible PCR amplification of multiple fragments within Tn*1546* reveals different fragments in the two tested strains. Below articles are described that use this technique to characterise strains.

Bates *et al.* (**1993**) examined vancomycin–resistant *E. faecium* isolated from faecal samples of hospitalised people, visitors of general practitioners, farm animals, sewage and dead chickens. Of renal patients 14 % had VRE in their faeces, while 2 % of general practice patients contained these bacteria. Within the hospital 8 strains with identical ribotypes (16S rDNA) were found: 4 within the renal unit where VRE were first detected and 4 in other hospital wards. These strains were also analysed with PFGE (DNA digested with SalI) and the patterns obtained were identical. To explain this, spreading from one human to the other must have occurred. In other human derived samples 9 different ribotypes could be distinguished. Ribotyping was also used to group samples from farm animals, sewage and dead

chickens. Fourteen ribotypes out of 48 VRE isolates could be distinguished. These ribotypes were compared with the ribotypes found in the human samples. In some cases the ribotypes of two or more samples matched.

Taking these results into account, Bates *et al.* (**1993**) suggested that the feeding of antibiotics to animals could be a source for the presence of resistant organisms in bacteria isolated from humans. However, only ribotypes of isolates from different sources are compared in this article. It has been noted that ribotyping is not an adequate method in comparing VRE (Bogle and Bogle, **1997**). The data obtained by Bates *et al.* (**1993**) do not include PFGE patterns and/or *vanA* gene cassette examination of strains to be categorised identically by ribotyping. In this way no valid conclusions about relations between strains can be made.

Kirk *et al.* (**1997c**) compared 20 human VRE isolates, obtained from a hospital in London with 20 VRE isolated from poultry carcasses bought in supermarkets. All VRE harboured the *vanA* gene, as detected by PCR. All strains had distinguishable PFGE patterns. Besides these genotypic tests, also phenotypic methods were used. Strains could be distinguished when looking at the production of enzymes capable of hydrolysing a range of substrates. Also MIC values to glycopeptides, MLS_B and other antibiotics were determined. Resistance profiles of animal and human strains were different. It was concluded human and poultry VRE strains were not related.

5.2.5 Comparison of genes and intergenic sequences in Tn*1546*

As resistance due to the presence of Tn*1546* is widespread and has clinical importance, the structure of this transposon will be discussed in more detail. The vanA gene cluster present on Tn*1546* (Arthur *et al.*, **1993**) mediates high level resistance to vancomycin and intermediate resistance to teicoplanin in *Enterococcus faecium.*

In the articles discussed below, the presence and length of genes and intergenic regions in Tn*1546* are determined and compared. Casewell and Beighton (**1996**) remarked that structural genes (for example in Tn*1546*) often show a high degree of similarity, also if they have not diverged recently. They suggested analysis of the intergenic regions of the vancomycin resistance operon as more suitable than sequencing structural genes to detect close relationships.

The *vanS–vanH*, *vanX–vanY* and *vanY–vanZ* intergenic regions of chicken and human samples were amplified and compared. Also by PCR it was checked whether the *vanX*, *vanY* and *vanZ* genes were present. Two differences between human and chicken samples were observed:

- In all human samples the *vanY* gene was missing
- In human isolates only the *vanS–vanH* intergenic region was present, while the chicken strains contained all three intergenic regions (Casewell and Beighton, **1996**; Kirk *et al.*, **1997b**).

The *vanX–vanY* and *vanY–vanZ* regions of the chickens were similar to the previously reported sequence in strain BM4147 (Arthur *et al.*, **1993**). Also some sequences were present that were larger than the known *vanX–vanY* intergenic region. So clear differences between the gene clusters of humans and chickens were observed. Besides PCR amplification of regions within Tn*1546*, PFGE was used to characterise strains. No PFGE patterns of human and chicken strains were found to be identical. The conclusion Kirk *et al.* (**1997b**) draw was that clear differences between the *vanA* gene cluster present in humans and chickens exist.

The human samples, however, were isolated from one hospital ward. It might be possible that the human samples represent an epidemic spread of one single transposon.

In an English study carried out by Woodford *et al*. (**1998**) 10 primer pairs to be used in PCR were constructed to detect differences in the *vanA* gene cluster of 107 isolates derived from different sources.

Table 5.2.5.1 Isolates examined by Woodford *et al.* (1998)

Source	Specification
Humans	64 clinical isolates
Animals	1 chicken, 3 pigs, 1 turkey, 1 duck, 1 pony
Raw meat	19 chicken, 4 pork, 4 beef
Sewage	8 isolates

These isolates could be divided in 24 groups (named A to X) based on the presence, absence or differing size of the amplicons derived after PCR. The genes shared by isolates of all groups were the *vanS*, *vanH* and *vanA* genes. This part resides in the middle of Tn*1546*. From the 24 groups, A, B, H, T, U and W contained human as well as non–human isolates. The other groups contained only human or only non–human isolates. Twenty of the 107 isolates (group A) contained a transposon identical to Tn*1546* of *E. faecium* BM4147 (Arthur *et al*., **1993**; isolated from a hospital patient). In the non–human samples the A type was the most prevalent. The *vanA* gene cassettes derived from raw chicken meat could be placed in the groups A, B, H, T, U and V. Only the V group was not identified in human samples.

One third of the glycopeptide resistant enterococci of human origin belonged to the H group. This *vanA* cluster was also detected in two raw chicken samples. An example of an insertion is the presence of the IS*1542*, 1300 bp in length, probably located at the ORF2–*vanR* intergenic region. This sequence appeared to be present in 37 isolates.

Three *vanA* gene clusters (A, B, C) contained all ten expected amplified gene products. The gene products in the *vanX*–*vanY* and/or the *vanY*–*vanZ* region of the groups B and C are longer than the amplified product of BM4147.

In the remaining 21 groups one or more amplicons were not present and quite often some amplicons were larger or smaller compared to the reference strain. In the genes coding for transposition, but also in the genes or intergenic regions downstream of *vanA* regions were often missing.

Van den Braak *et al*. (**1998**) used a similar method of mapping Tn*1546* by PCR. From butchers, supermarkets and poulterers throughout the Netherlands, 305 poultry products were obtained from June until September 1996. The samples containing VRE were compared with 20 human clinical VRE (19 *E. faecium* and 1 *E. faecalis*) and 4 vancomycin–resistant *E. faecium* from non–hospitalised patients. Of the poultry products, 79 % contained vancomycin–resistant enterococci. Of 242 VRE strains, 59 % were *E. faecium*, while also *E. durans*, *E. hirae* and *E. gallinarum* were found. All these strains possessed the *vanA* gene, except the *E. gallinarum*, which contained *vanC1*.

To get an indication about possible differences in the first place, randomly 5 human and 5 poultry strains were selected. PCR was carried out with primers (developed by Miele *et al*.,

1995) derived from structural Tn*1546* genes (*vanR*, *vanS*, *vanH*, *vanA*, *vanY*, ORF1 and ORF2 genes). The fragments obtained were identical in size for the poultry and the human strains. Also when using primers for amplifying the intergenic *vanS–vanH* and *vanY–vanZ* regions the products were identical in size. However, when the *vanX–vanY* region was amplified in both sample groups, in two out of 5 poultry strains this region was 1300 bp instead of 540 bp as observed in the other samples.

Subsequently, the *vanX–vanY* intergenic regions of 142 VRE (*E. faecium*) isolated from poultry and the 19 human clinical strains were amplified. All human strains and 58 % of the poultry strains showed a 540 bp *vanX–vanY* intergenic region. The remaining poultry isolates contained a 1300 bp fragment, including the insertion sequence IS*1216V*.

Also PFGE was applied on DNA of the poultry samples. Out of 100 genotypes, two PFGE types were more abundant than others. These two PFGE patterns and patterns of other poultry strains were not found in faecal samples of patients in the Netherlands (Endtz *et al.*, **1997**).

Jensen *et al.* (**1997**, **1998**) examined the *vanA* cluster in 40 *E. faecium* isolates obtained from humans, pigs and poultry (Denmark, UK and USA). Six regions in the gene cassette were amplified by PCR. Sequencing of the *vanA*, *vanX* and *vanR* regions revealed almost 100 % similarity. In the intergenic regions between ORF2–*vanR*, *vanX–vanY* and *vanS–vanH* insertions could be detected. Two insertion sequences were identified: IS*1251*, IS*1216V*. Also sequences resembling insertion sequences were detected.

According to the differences observed (in the intergenic regions) the isolates were grouped into 13 types. The first, being identical to the transposon in BM4147, was found in 10 human and animal isolates. The second type transposon was also found amongst human and animal isolates (11). Isolates could not be placed into groups according to their origin (animal, human or geographic).

This type of research is seriously hampered by the fact that the comparison of human and animal strains is done without knowing whether there is a link between the chicken in the shops and the people with VRE. It is not known whether the people carrying VRE ate the actual chicken containing the VRE under scrutiny in the researchprogram. Although chicken meat was bought in different places, not all chicken to be sold may have the same genetic pattern found in the samples examined. A last remark is when the use of avoparcin in feed is thought to be a primary cause for prevalence of resistance it has to be known whether the poultry flocks were fed with avoparcin.

At farms, animals and their keepers live close to each other. When antibiotics are used and resistant bacteria in the animals emerge, they might be transferred to members of the farmer family. Transfer of resistant bacteria from animals or meat to slaughterhouse workers might also happen. Unfortunately, limited research work has been done on bacterial isolates from farmers and their animals.

Van den Bogaard *et al.* (**1997b**) examined the prevalence of vancomycin–resistant *E. faecium* in faeces from turkeys, turkey farmers, turkey slaughterers and healthy persons in the same region. The isolated strains were reported to have been analysed using both phenotypic (MIC determination) and genotypic methods. The genetic analysis consisted of PFGE, PCR amplification of genes and intergenic regions in Tn*1546* and hybridisation with specific probes. At one of the 47 turkey farms involved the bacterial isolates from the turkeys

were indistinguishable to the isolate of the farmer. PFGE resulted in an identical pattern of 17 bands. PCR of *vanA*, *vanR*, *vanX* and intergenic regions *vanXY* and *vanYZ* showed DNA fragments of the same lengths in both strains. These two identical strains contained a Tn*1546* different than the one in the vancomycin reference strain *E. faecium* BM4147. In this specific *E. faecium* the *vanZ* gene is present, while in both strains isolated from the turkey and the farmer this gene was missing. A second difference noticed was the *vanXY* fragment being 600 bp longer in both the turkey and farmer strain. It was not shown, however, that this strain really colonised the human intestine and was not a transient passenger. Furthermore, reproducibility is lacking making this observation in effect open for debate and in want of more thorough scientific scrutiny. Moreover, it is not clear whether the VRE are capable of dispersing resistant traits to already present bacteria in the human gut. (Apart from these comments, extrapolation from this observation to other organisms or antimicrobial resistance traits is scientifically unsound and without foundation.)

Lately, research groups are focussing on the structure of the *vanA* gene cassette when comparing isolates. The transposon Tn*1546* is analysed by PCR, using primers to amplify genes and intergenic regions within the transposon. Because different groups use different primers and different numbers of primers for amplification, it is not easy to tell how many variations of Tn*1546* are reported. When studying articles, it becomes clear Tn*1546* is a heterogeneous transposon (present in many variations). According to Woodford *et al.* (**1995**), who showed 24 variations of Tn*1546*–like elements to be present in enterococci, even more diversity is likely.

At present, most studies describe human isolates and isolates from dead chicken. From these chicken it is not reported from which farm they came and also information about antibiotic use is lacking. Woodford *et al.* (**1998**) showed a high variation in human clinical isolates, chicken, pork and beef meat, sewage and animal faeces. However, in this study, no information about the raising conditions of the animals and the antibiotic use of patients was given. In this way, no direct relation between use of avoparcin as growth promoter and the prevalence and spread of resistance can be found. To study variations in Tn*1546*–like elements with the goal to reveal dissemination routes, it is necessary to compare samples of well–known origin. Moreover, they remarked that there are still more possible variations of this transposon, containing or missing other elements (Handwerger *et al.*, **1995**; Arthur *et al.*, **1993** respectively). Also the results of Van den Bogaard *et al.* (**1997b**), Braak *et al.*, **1998**, Casewell and Beighton (**1996**) and Kirk *et al.* (**1997**) show the heterogeneity of Tn*1546*–like elements. Different research groups use different primers for amplification of regions within Tn*1546*. Also the number of primer pairs used and thus the number of amplified sequences differs. This complicates the comparison of results. (Which isolates contain equal transposons?) Resistant enterococci of human and poultry origin can be distinguished by PFGE; in most cases they have different PFGE patterns.

5.3 Resistance to MLS_B antibiotics

5.3.1 Prevalence

The MLS_B antibiotics used as growth promoters are tylosin, spiramycin and virginiamycin. Their structural analogues used in human medicine are erythromycin, pristinamycin and Synercid®.

The prevalence of MLS_B resistant enterococci, staphylococci, streptococci will be described in the section below. Resistant enterococci have been isolated from animals, humans and

meat. Staphylococci have been isolated from animals and humans, while MLS_B resistant streptococci only have been isolated from humans.

In this section an overview is given of prevalence and spread of MLS_B resistance. As for VRE certain questions need to be answered:

- Does the use of tylosin, spiramycin or virginiamycin select for resistance?
- What resistance genes are detected in MLS_B resistant strains and are they present on transposons/plasmids?
- Are strains and/or genes present in different sources (human/animal) genetically related?
- Are examples present showing the transfer of MLS_B resistance from animals to humans?

Below articles providing information about emergence of resistance and prevalence of resistant bacteria in animals and humans will be described. The possible relation between use of AGPs in feed and clinical resistant strains will be discussed.

Different types of resistance to macrolides can be observed in *S. aureus*, enterococci and streptococci isolated from different sources. Bacteria can be inducible resistant to erythromycin alone (also non–inducible?) or to all MLS_B group antibiotics. Also a constitutive resistance mechanism is described, where strains are continuously resistant to the whole MLS_B group of antibiotics, including erythromycin, spiramycin, tylosin, lincomycin and streptogramin B antibiotics (Lacey, **1988**; Leclercq and Courvalin, **1991a**; Seppälä *et al.*, **1993**). Clinical MLS_B resistance can be arranged by multiple, not related genes. For every mechanism and in different bacteria other genes are responsible for resistance. Because different bacterial genera and multiple resistance genes are involved the emergence and spread of MLS_B resistance is a very complicated issue. The evidence for a causal relationship between the use of e.g. virginiamycin and the development of resistance to this antibiotic is not a clearcut issue (SCAN, **1998b**).

Virginiamycin

In many (European) countries virginiamycin (a streptogramin) is added to the feed of broilers and pigs (Witte, **1998**; Aarestrup *et al.*, **1998**). In Finland and Norway the use of virginiamycin was banned in 1990 and 1991 respectively. This offers a situation in which the prevalence of resistance might in theory result in a clear–cut comparison between countries with a differing AGP regime. Also useful is the fact that in cattle virginiamycin is not used as a growth promoter. When comparing data from cattle to data from pigs/broilers the influence of virginiamycin on the prevalence of resistance can be studied.

It was reported that 40 – 68 % of the *E. faecium* isolated from Danish pigs and broilers (probably) fed with virginiamycin show resistance towards this antibiotic. In *E. faecium* isolated from cattle (not receiving virginiamycin) only 8 % was resistant to virginiamycin. A study carried out after the Finnish ban of virginiamycin showed resistance percentages were lowered: 20 % and 2 % of *E. faecium* isolated from broilers and pigs, was resistant to virginiamycin (DVL, **1998**). In staphylococci isolated from diseased animals (pigs, cattle from Denmark), even lower resistance percentages were found: 0 – 1 % (Aarestrup *et al.*, **1998**).

SCAN, however, criticised these data in their report dealing with the Danish ban on virginiamycin (SCAN, **1998b**). SCAN noted that the overall number of strains examined was small and the method of antibiotic testing used, unique to Denmark, is difficult to relate to definitions of resistance used elsewhere. Methodological limitations are made explicit by the fact that a far higher proportion of *E. faecium* isolated demonstrated resistance to macrol-

ides than to virginiamycin, which makes any conclusion about the origins or mechanisms of resistance difficult to elucidate.

Tylosin
Aarestrup *et al.* (**1998**) studied the prevalence of tylosin and erythromycin resistance in pigs, broilers and cattle. Resistance was determined by a tablet diffusion test on Mueller–Hinton agar. In Denmark 90 – 91 % of the enterococci isolates from swine are resistant to tylosin and erythromycin. Of isolates obtained from broilers 59 % contained resistant strains. In Finland, a country using less tylosin than Denmark, the percentage of pig strains resistant to tylosin was around 15 %, while 9 % out of 542 enterococci isolated from broilers were resistant (MMM, **1997**). For spiramycin (as tylosin a macrolide) comparable figures were presented: in Denmark 88 – 89 % of the enterococci isolated from pigs are resistant, while in Finland 14 % of the pig and 9 % of the broiler isolates contained resistant enterococci (SCAN report, **1998a**; MMM, **1997**).

As stated earlier, results usually are not comparable in a straightforward manner, as different methods are often used to isolate and characterise bacteria. This also holds true for the Danish and Finnish data. In determining susceptibility, different methods are used and no MIC distribution patterns and zone diameters are provided, enabling data comparison (SCAN, **1998a**). Aarestrup *et al.* (**1998**) used a tablet diffusion test (inhibition zones for the antibiotics are determined), while in the study of MMM (**1997**) a dilution method was used. Here MICs could be determined directly. The second difference between the two studies is the method used to isolate bacteria from the samples taken. Different amounts of resistant bacteria are obtained when enrichment methods are used or when samples are plated directly. In Norway (with a virginiamycin ban) isolates were grown on vancomycin–containing plates and no resistant strains were found, in contrast to the Finnish results.

5.3.2 Human antibiotics and resistance prevalence

General
In the Netherlands the majority of macrolides, streptogramins and glycopeptides is used as antimicrobial growth promoter. In humans four tons of macrolides are being used for treatment, while animals receive in total (therapy and growth promoter) approximately 56 tons (Gezondheidsraad, **1998**). Resistance to glycopeptides, macrolides and streptogramins has been observed in animal and human isolates. A thorough characterisation of resistant strains may reveal insight in the dissemination route of resistance traits.

Van den Bogaard *et al.* (**1997a**) examined the prevalence of resistance to glycopeptides, macrolides and streptogramin antibiotics in enterococcal isolates in the northern part of the province Limburg, the Netherlands. The samples were obtained from an area where, at that time, avoparcin, virginiamycin and tylosin were used as animal growth promoters.

Table 5.3.2.1. Enterococci resistant to vancomycin, Synercid® and erythromycin (Van den Bogaard *et al.*, 1997a).

Source	Enterococci/ sample	Resistant to: Vancomycin	Synercid® (?)	Erythromycin
Human	106/117	12%	30%	50%
Pigs	242/282	34%	75%	84%

Resistance to erythromycin appear to be high in both animal and human isolates. In pigs, resistance percentages to glycopeptides, Synercid® and erythromycin are higher than in humans.

In the Netherlands, vancomycin is sparingly used to treat humans (60 kg a year). It is possible that humans treated with vancomycin in the hospital, carry resistant bacteria with them when the hospital stay is ended. They may transfer resistant bacteria to other people in the community. Because much more avoparcin has been used in animal husbandry, resistance transfer from animals to humans does not seem a viable route as in the case of regular transfer from animals to humans, human bacterial resistance should be a regular occurring measurable phenomenon.

Erythromycin was used to treat animals to a lesser extent than to treat humans. Erythromycin has been used for a long time and in high amounts to treat humans. Therefore, it may well be possible that erythromycin resistance in humans is simply due to use of this antibiotic by humans.

The data on Synercid® are uninterpretable. The paper does not distinguish between *E. faecium* and *E. faecalis*. The latter is intrinsically resistant while the former is the only organism against which Synercid® would be expected to be effective.

The MLS_B antibiotics group: erythromycin and tylosin

In the 1980s erythromycin was much more used in human medicine in the UK than lincomycin and clindamycin, which show toxic effects. In animal husbandry tylosin was used in larger quantities in feed then spiramycin and lincomycin.

A large study was carried out by Lacey (**1988**) in the UK, who tested human staphylococcal, streptococcal isolates on the presence of MLS_B resistance (in total 3812 strains). Of the *S. aureus* strains, 13 % (out of 3300) showed resistance to macrolides. The majority of these strains (87 %) were inducible resistant to erythromycin alone. The other strains contained a constitutive system, which gave rise to resistant to all MLS_B antibiotics. Of *Streptococcus pyogenes* (172 strains) only isolates were detected with the inducible erythromycin resistance pattern (7 %).

Cormican *et al.* (**1997**) determined MIC–ranges for glycopeptides, erythromycin and clindamycin (a lincosamide) towards human clinical samples obtained in the USA. In the USA avoparcin has not been used in feed, contrary to tylosin. The results were reported as MIC–ranges and also the concentrations needed to obtain 50 % and 90 % growth inhibition were shown.

In this study all enterococci possessing the *vanA* gene also are resistant to erythromycin. This may indicate that the *vanA* gene and *erm* genes are transferred simultaneously. More research, like conjugation studies where transfer of resistance traits present on one plasmid can be monitored, is needed to confirm or deny this possibility. Also the origin of resistance traits in human strains has to be traced. Resistance to erythromycin in human bacterial strains is probably of human origin due to the use of erythromycin in human medicine.

In Finland the majority of antibiotics to treat humans (88 %) is given to non–hospitalised people. To be able to control emergence and spread of resistance, it is important to know in which sectors of use (animal feed, treatment, and human treatment in hospital or in community) emergence is high. Some studies have been carried out to determine the emergence of

macrolide resistant bacteria due to the use of antibiotics in the community (Huovinen *et al.*, **1997**).

Seppälä *et al.* (**1992**) monitored the prevalence of resistance to erythromycin in group A streptococci from humans in Finland from 1988 to 1990. Streptococci were isolated on Mueller–Hinton agar containing 5 % sheep's blood and 2 μg erythromycin/ml, incubated with 5 % CO_2. As well in throat (272), blood (3087) and pus (1349) samples a large increase in resistance was found. Percentages increased from 5 % in 1988–89 to 13 % in 1990 for throat and pus isolates, while in blood samples a shift was observed from 4 % in 1988 to 24 % in 1990. An interesting question is whether the rise in erythromycin use (almost 300 %) was responsible for the higher prevalence of resistance. A difference in resistance percentages could be observed between the six regions that were examined. In Kuopi the highest resistance percentage was found: 29 and 54 % for throat and pus samples in 1990. The link with erythromycin use was not very clear as the regions Helsinki and Turku used comparable amounts, but resistance percentages were lower. A complexing factor is that resistance levels are already rather high (Huovinen *et al.*, **1997**). Resistance percentages can increase in two ways, which are hard to discriminate: new strains may emerge due to antibiotic use or strains already present may disseminate. However, a study carried out by Seppälä *et al.* (**1995**) concerning > 10,000 samples isolated in 1991 showed resistance to erythromycin was significantly (P= 0.006) related to the consumption of erythromycin by non–hospitalised people.

In trying to prevent a further increase of erythromycin resistance in Finland, from 1992 onwards the consumption of macrolides was decreased. This was achieved by a campaign where the pharmaceutical industry as well as physicians and scientists were adequately informed about the resistance problem (Huovinen *et al.*, **1997**).

In a few years time (1991 – 1994) a new non–inducible phenotype of erythromycin resistance emerged and spread. Many of these predominant T4 serotype strains were nearly identical, as revealed by identification methods as restriction analysis and random amplified polymorphic DNA analysis. Also a predominant inducible phenotype was detected (T28 serotype). Multiplication and survival of a few different strains may therefore cause the increase of erythromycin resistance.

Streptococcus pyogenes is mainly found in humans and cats. In farm animals this *Streptococcus* is not common (Huovinen *et al.*, **1997**). On the other hand, enterococci resistant to tylosin and erythromycin have be found in Finnish animals (MMM, **1997**). The transfer of genes from these enterococci to streptococci present in humans cannot be ruled out.

In Finland the use of tylosin was banned in 1990. For the Finnish the presented figures about prevalence of resistance and cross–reactivity within the MLS_B group of antibiotics is sufficient for continuing the ban on tylosin (MMM, **1997**). However, convincing evidence that macrolides used as feed additives cause a significant risk to human health is not available. Additional research is needed to characterise resistant strains more completely and provide methods to compare resistant strains present in humans and animals (SCAN, **1998a**).

The MLS_B antibiotics group: Synercid®

A promising new antibiotic combination for treatment of Gram–positive cocci is Synercid®. Synercid® is composed of 30 % quinopristin (peptide macrolactone, streptogramin B) and 70 % dalfopristin (polyunsaturated macrolactone, streptogramin A) and was in phase III of

clinical trials in 1997 (Fantin *et al.*, **1997**). These two antibiotics are structurally unrelated, but they act synergistically when injected simultaneously. Adding the antibiotics to susceptible *E. faecium* cells revealed the synergistic effect. Two µg/ml of dalfopristin or quinupristin separately are sufficient to impede growth. When Synercid® is added the MIC is lowered from 2 to 0.5 µg /ml (Bouanchaud, **1997**). Because two components are involved it is expected that the selection of resistant bacteria will be reduced (Bouanchaud, **1997**).

In North America and Canada Jones *et al.* (**1998**) carried out a large study concerning the action of Synercid®. The majority (95 – 97 %) of 1011 E. faecium strains were sensitive to Synercid®. The MIC that killed 90 % of the *S. aureus* strains (>10,000) was 0.5 µg /ml. The MIC range for Synercid® towards Gram–positive bacteria can be derived from Bouanchaud, (**1997**). The presence of other resistance traits (vancomycin, erythromycin) does not seem to influence resistance levels towards quinupristin/dalfopristin. In France, Germany, North America and Canada resistance percentages of staphylococci towards Synercid® are comparable. Synercid® was also tested in Germany, where streptogramins have not been used before. MRSA isolates were highly sensitive to this antibiotic preparation. *E. faecium* isolates from blood cultures also showed a low percentage of resistance against Synercid®: 4.2 % (Witte *et al.*, **1998**; Elsner *et al.*, **1998**). *E. faecalis*, however, is intrinsically resistant to Synercid®.

In most countries streptogramins have never been used to treat humans. In France, however, a synergistic mixture of a streptogramin A and B, called pristinamycin has been used for more than 20 years in human medicine. During a comparable period, virginiamycin, a structural analogue, has been used in Europe as an animal feed additive (Pfizer, **1998**). Virginiamycin can cause selection of resistant bacteria in animal livestock. The question is whether this poses a threat to human health. Therefore studies monitoring resistance to streptogramins in France can be useful. SCAN noted that in countries that permit the use of streptogramins in both animal production and human therapeutic medicine (France and the USA) the use of pristinamycin has not been compromised by the use of virginiamycin as growth promoter (SCAN, **1998b**). The use of virginiamycin in animal feed (France) probably does not contribute to the prevalence of resistance strains in humans.

5.3.3 Genetic analysis of MLS_B resistance

Enterococci (animals and humans)

The Danish virginiamycin resistant *E. faecium* isolates obtained from pigs and poultry were evaluated on the presence of resistance genes. As shown by PCR, the *satA* gene was present in 25 % of these isolates (DVL, Technical Report 1, **1998**). *E. faecium* isolates from healthy people, farmers, chickens and pigs obtained in the Netherlands were also checked for the presence of the *satA* gene. Of the animal samples, approximately 18 % contained this gene, while the human isolates were positive in 58 % of the cases (Danish Veterinary Laboratory, Technical report 2, **1998**).

Staphylococci

The Danish Veterinary Laboratory (Technical Report 3, **1998**) examined the presence of the *vatB* gene in clinical staphylococcal samples from broilers. Unfortunately, the history concerning use of antibiotics in feed was not known. Out of 52 isolates, two *Staphylococcus xylosus* strains carried the *vatB* gene. The presence of this gene caused the bacteria to be resistant to pristinamycin and virginiamycin. In *S. aureus* strains, most abundant in the isolates examined, the *vatB* gene, nor the *vat* or *vga* gene was found.

Allignet *et al.* (**1996**) studied the resistance patterns of staphylococci isolated from humans, resistant to or intermediate resistant to streptogramin A. The resistance to MLS_B antibiotics and the presence of *vga*, *vat*, *vatB* and *vgb* genes in these staphylococcal isolates was determined. When staphylococci are resistant to streptogramin A and B mixtures (e.g. pristinamycin: pristinamycin PIIA and PIB) they are also resistant to the A component, but not always to the B component.

The presence of above mentioned genes in phenotypically resistant *S. aureus* (36), *S. epidermidis* (14), *S. haemolyticus* (4), one *S. cohnii* subsp. *urealyticum* and one *S. simulans* was determined. The *vatB* gene was more prevalent in *S. aureus* then in CNS. When the *vatB* gene was present, none of the other genes could be detected. The *vga* gene was sometimes detected as the only resistance gene, but more often combined with *vat* and *vgb*. This combination was found in *S. aureus*, *S. haemolyticus*, *S. cohnii* subsp. *urealyticum* and *S. simulans* strains. The resistance genes were detected on plasmids of sizes ranging from 6 to 95 kb.

Streptococci

In *S. pneumoniae* (pneumonococci) two types of resistance to antibiotics of the MLS_B group can be distinguished. One type is due to target modification by the product of *ermAB*, giving rise to resistance to all MLS_B antibiotics. The second type is an efflux mechanism, specific for macrolides (Johnston *et al.*, **1998**; McDougal *et al.*, **1998**).

Clinical *S. pneumoniae* isolates (5,029) from Canadian hospitals and laboratories were first checked by broth microdilution on the presence of erythromycin resistance. Resistant strains were subsequently screened for the presence of resistance genes.

Out of 5,029 isolates 147 were shown to be erythromycin resistant (2.9 %). The MLS_B and the M phenotype were dominant. The MLS_B phenotype corresponded in most bacteria with the presence of the *ermB* gene, while the M phenotype contained the *mefE* gene.

In pneumococci plasmids are rare and genes giving rise to resistance to MLS_B antibiotics have been found on conjugative transposons present on the genome. MLS_B type of resistance in *S. pneumonia* has been found on different (related) transposons: Tn*1545*, Tn*1545* with some deletions, and Tn*917*–like elements, as a part of Tn*3872* (composite transposon; McDougal *et al.*, **1998**).

Twelve strains were examined by PFGE (determination of clonal relationship), serotyping, antimicrobial susceptibility testing, PCR of *ermA*, *tet(M)* and *aphA–3* and blotting by probes for the same genes. PCR–analysis showed that the *ermAM* gene and the *tet(M)* gene were present in all isolates. One strain also contained the *aphA–3* gene (kanamycin resistance). All strains contained one or more copies of Tn*917* on their genome, as was shown by Southern blotting with a probe. This transposon was inserted in a Tn*916*–like transposon.

5.3.4 Prevalence of resistance against Zn–bacitracin

Zinc–bacitracin is added to the feed of laying hens, chickens, turkeys, pigs, calves and lambs. A large part of *Enterococcus faecalis* and *E. faecium* strains is resistant to zinc–bacitracin (Alpharma, **1998**). Bacteria are resistant to Zn–bacitracin when the MIC is > 10 μg/ml. Enterococci contain a specific compound in their cell walls, a lipoteichoic acid. It is possible that this acid is responsible for resistance (Krogstad and Parquette, **1980**) It seems that during the last 40 years no increase in resistance is traceable (Alpharma, **1998**).

However, the data described are derived from multiple studies in different countries. The methods used to determine prevalence of resistance differ substantially.

Resistance to bacitracin has been detected phenotypically. The next step is to find genes that cause resistance to this antibiotic. Some enterococci are intrinsic resistant to bacitracin (Alpharma, **1998**). As described earlier in this report, chromosomal genes have been found in bacitracin producing organisms, coding for proteins responsible for transport of the antibiotic out of the cell. Other bacteria resistant to bacitracin, do not seem to contain these genes (Podlesek *et al.*, **1997**). Also no resistance genes to bacitracin have not been found on plasmids (Threlfall, **1985**).

When bacteria like streptococci and staphylococci are grown on media containing increasing concentrations of bacitracin resistance can develop. This type of resistance is transient, because in the majority of the cases resistance disappears when bacteria are transferred several times to media without antibiotics (Alpharma, **1998**).

5.4 Conjugational transfer of genetic material

Estimates of the frequency of horizontal transfer of resistance genes can in principle be obtained by laboratory studies. Donor and acceptor cells are cultured together and DNA may be transferred. The transconjugants (acceptor cells containing DNA from donor) are being selected for. In the laboratory high numbers of both bacterial species are present and the conditions (growth medium) are manipulated in a way conjugation is promoted. However, the preferred conditions for conjugation between specific strains are not exactly known. In nature (for instance in manure or inside the gut) some unknown factors may be important. Also, numbers of bacteria and/or the ratio between donors and acceptors may be substantially different from laboratory conditions.

Laboratory studies only give a rough indication of conjugation frequencies, and useful information about how specific transposons or plasmids are being transferred can be obtained.

A common thought is that bacteria of the same species can exchange DNA more easily than non–related bacteria. However, when wide–range conjugative plasmids or conjugative transposons are involved, genes can be transferred to bacteria of the same species, other species and in some cases even other genera (Summers, **1996**). The transfer of DNA between bacteria used to live in the same environment is thought to be more common. To gain more insight in the likelihood of gene transfer between specific bacteria, it is important to study data describing the exchange of DNA between bacteria, within and between different groups.

Transfer of vancomycin resistance

A particular *E. faecium* strain, isolated by Heaton and Handwerger (**1995**) in the USA, contains two plasmids involved in transfer of vancomycin resistance. The first (no. 702, 41 kb, non–conjugative plasmid) contains a fragment closely related to transposon Tn*1546*, which is part of the larger Tn*5506*, which also contains the *ermB* gene, responsible for erythromycin resistance. The other plasmid (no. 703, 55 kb, conjugative) resembles a sex pheromone–response plasmid common in *E. faecalis.*

The *E. faecium* strain was mated with plasmid free *E. faecalis* strains, resulting in two types of transconjugants (Heaton and Handwerger, **1995**; Heaton *et al.*, **1996**):

- Transconjugants with both plasmids; transconjugation frequency < 1 • 10^{-5}.
- Transconjugants containing one plasmid (no. 701; 92 kb), that is composed of material from plasmid 702 and 703. Plasmid 701 showed to be highly conjugative, causing cells to aggregate and reaching a frequency of transconjugation (transfer of vancomycin resistance) of 1.3 • 10^{-3}. This new plasmid also contained Tn*5506* (first present in plasmid 702) with the *ermB* gene and the *vanA* gene cassette.

Tn*5506*, a 39 kb composite transposon contains insertion sequences (IS) at both ends: on the left end IS*1216V2* was detected, while on the right end IS*1216*V1 and IS*1252* were found. IS*1216*V–like elements may play a role in transposition of resistance genes or gene complexes in enterococci. They were also found on transposon Tn*5482* harbouring vancomycin resistance in another *E. faecium* strain (Handwerger and Skoble, **1995**). Also an *E. faecalis* strain had IS*1216*V–like elements downstream a chromosomal β–lactamase gene (Rice and Marshall, **1994**), while a penicillin–resistant *E. hirae* strain contained a similar IS (Piras *et al*., **1990**; Raze *et al*., **1994**).

Poyart *et al*. (**1997**) isolated a vancomycin, erythromycin and tetracycline *S. bovis* strain from an ill child. A *vanB*–like gene (inducible vancomycin resistance) was present on the chromosome. This gene previously has been detected on conjugative pieces of DNA, 90 to 250 kb long (Quintiliani and Courvalin, **1994**). A gene for tetracycline resistance (*tet* (*M*)) was present on the *S. bovis* chromosome, as part of a sequence related to the conjugative transposon Tn*916*. The *ermB* gene, resulting in erythromycin resistance, was detected on the chromosome and on a plasmid (29.5 kb).

Mating experiments were done to test the transferability of resistance markers within species and between species. Transfer was positive as the transfer frequency was > 1 • 10^{-8}. Eighteen different donor–recipient combinations were tested using *S. bovis*, *E. faecalis* and *E. faecium* strains.

Transfer of tetracycline and erythromycin resistance genes was never detected when using *S. bovis* NEM760 as a donor strain. On the other hand, *vanB* was shown to be transferred to *E. faecalis* and *E. faecium* strains. The *vanB* gene was transported on a 100 kb element, fitting in the size range of 90 – 250 kb fragments observed by Quintiliani and Courvalin (**1994**).

The transconjugants obtained in this way, (*E. faecalis* NEM820, 824 and 825), were shown to act as donors, as the *vanB* gene was transferred in some cases. Together with the *vanB* fragment, a plasmid was shown to be co–transferred.

Transfer of MLS_B resistance

MLS_B resistance can be due to the presence of a composite transposon consisting of Tn*917* in Tn*916*–like elements. Filter mating studies of *Streptococcus pneumoniae* possessing this composite transposon, were carried out by McDougal *et al*. (**1998**). Four different donor strains were used, but no transfer could be detected. Under the same conditions, control strain *S. pneumoniae* BM4200, containing Tn*1545*, was able to transfer its transposon (8 • 10^{-8} per donor cell). Also when using an *E. faecalis* containing Tn*916* as donor 5 • 10^{-7} transconjugants per donor cell were found.

Woodford *et al*. (**1997**) showed that vancomycin–resistant *E. faecium* strains isolated from raw chicken (3) and a patient (1) in the UK were also resistant to quinupristin/dalfopristin. One of the chicken strains resistant to Synercid®, was able to transfer erythromycin and

clindamycin resistance to a sensitive *E. faecium* in the laboratory. The resistance mechanism (genes responsible) was not studied in this *E. faecium* transconjugant. It was suggested that the use of virginiamycin in chicken feed is responsible for the presence of MLS_B–resistant enterococci in meat. It seems that transferable MLS_B resistance is already present in *E. faecium*.

Conjugational studies have been carried out between virginiamycin resistant *E. faecium* strains and susceptible *E. faecium* BM4105. In these filter–mating tests resistance arose in 12 of the 15 recipient strains. The transfer of the *satA* gene was reported in 7 cases, while the resistance trait causing resistance in the other 5 cases was not known (DVL, Technical report 4, **1998**). The transfer frequency was determined in matings between the transconjugant BM4105 strain (*satA* gene) and the same strain without resistance. However, it should be noted that obtained data concerning transfer do not predict transfer frequencies in natural environments or between less related strains (SCAN, **1998b**). At best, transfer frequency observed in vitro is an indication of the maximum rate possible as the experiment used a single strain both as donor and recipient (SCAN, **1998b**). Moreover, enterococci are known to be promiscuous and exchange genetic information between similar strains is a common phenomenon (Clewell, *et al.*, **1995**).

5.5 The animal–human link?

5.5.1 General

As explained earlier resistance traits can in theory be transferred from animals to humans in two ways:

- The complete bacterial strain is transmitted
- Movable elements present in animal bacteria are transposed into bacteria able to reside in the human gut.

In the first case strains are thought to be transmitted through the food chain or during animal–human contact. In the second case two alternatives can take place: transfer of plasmids or transposons outside or inside the human gut (McDonald *et al.*, **1997**).

In order to prove that bacteria or resistance genes have been transferred from animals to humans isolates should be characterised thoroughly. The problem whenever strains are compared to study possible transfer routes, many research groups do not trace the origin of the isolates. Especially when it concerns resistance to growth promoters that have been used for a long time (avoparcin, tylosin/virginiamycin, Zn–bacitracin). Not only animals contain resistance genes to these antibiotics but also humans in contact with animals or eating meat may already contain resistant bacteria acquired on previous occasions. Resistance to these antibiotics (especially avoparcin) is widely spread among animal bacteria. The use of structural analogues in human medicine (vancomycin) or the same antibiotic (Zn–bacitracin) is complicating the monitoring of spread of resistance even further. All together, it is very difficult to prove a direct transfer of resistance genes or resistant bacteria from animals to humans.

The studies unequivocally detecting the emergence and spread of resistance due to the use of antibiotics in animal feed, are studies monitoring resistance immediately from the start of use. Unfortunately, only one of such a study, concerning the introduction of nourseothricin and the emergence and spread of resistance genes, is known. This example is frequently

referred to as an example of resistance transfer from animals to humans disregarding the fact that *E. coli* is a zoonotic Gram–negative organism. This example therefore bears no relation to the AGP issue described in this report.

5.5.2 Cases providing evidence?

Enterococci

Van den Bogaard *et al.* (**1997b**) examined the prevalence of vancomycin–resistant *E. faecium* in faeces of turkeys, turkey farmers, turkey slaughterers and healthy persons from the same region. Resistance percentages were reported to be 50 %, 39 %, 20 % and 14 % respectively. The strains isolated were analysed using both phenotypic (MIC determination) and genotypic methods. The genetic analysis consisted of PFGE, PCR amplification of genes and intergenic regions in Tn*1546* and hybridisation with specific probes.

At one of the 47 turkey farms the bacterial isolates from the turkeys were identical to the isolate of the farmer. In the PFGE pattern all 17 bands were identical. Genes and intergenic sequences within Tn*1546*, the transposon responsible for vancomycin and teicoplanin resistance, were amplified and were indistinguishable. These two identical strains differed from the vancomycin–resistant reference strain *E. faecium* BM4147. The strain isolated from the turkey and the farmer did not contain the *vanZ* gene that is present in *E. faecium* BM4147. The second difference was found in the *vanXY* fragment, which was 600 bp larger in the turkey/farmer strain.

Another example of transfer of resistance bacteria from (dead) animals to humans was published by Das *et al.* (**1997b**). A truck driver who had an accident (right femur fracture) within a factory packaging chicken developed an infected wound. From a wound swab vancomycin resistant *E. faecalis* was isolated. Also swabs were taken from chicken carcasses and surfaces in the factory. Eight out of 21 samples contained VRE with the *vanA* gene, with a similar result for the patient (2 *E. durans*, 2 *E. gallinarum*, 2 *E. faecalis*, 2 *E. hirae* and 1 *E. casseliflavus*). It was most likely the patient became infected in the factory, while he did not carry VRE in his faeces, had not taken antibiotics recently and had not been hospitalised previously.

Staphylococci

Isigidi *et al.* (**1990**) collected samples from working people, veterinary students and people working in meat processing plants. Also samples from meat products, shrimps, slaughter waste and live poultry were obtained. The *Staphylococcus aureus* strains isolated were characterised by biotyping and phage typing.

Different isolates were typed as the poultry biotype, poultry like biotype, human biotype, ovine biotype and bovine biotype. Some strains were not typeable. Only the strains isolated from live poultry all showed the poultry biotype. Also from chicken neck skins the majority (97.4 %) of the strains were of this biotype.

In human isolates and in meat products the amount of poultry like (positive for protein A as only difference from normal poultry) biotypes was quite high. Especially in samples from people who worked with meat this biotype formed a large part of the population (30.4 % up to 61.3 %). In people frequent in contact with meat also the real poultry biotype could be identified (poultry abattoir, pork butcher's meat plants, turkey meat plants). Also veterinary students contained poultry like (8.3 %) and poultry biotypes (3.9 %).

5.5.3 In conclusion

In essence, evidence of acquired antibiotic resistance transfer from animals to humans is non–existent. Establishing such evidence in a scientifically consistent manner is hampered by multiple factors such as methodological inadequacies and differences, absence of sample history, use of analogues antibiotics in both animals and humans, lack of reproducibility of experiments, limited data comparability, absence of monitoring programs, etc. Therefore the proportion of antibiotic resistance within human bacteria resulting from the use of antibiotics as animal growth promoters can not be established. Epidemiology, however, sofar does not show an alarming rise of antibiotic resistance within human bacteria as a result of the introduction of antibiotics as growth promoters. The use of virginiamycin as a growth promoter for instance did not compromise the use of the related human antibiotic therapeutic pristinamycin.

It is exceedingly difficult to prove a resistance transfer case. The routes of possible transmission of resistant bacteria (or their resistance traits) from animals to humans are intricate and manifold. Routes comprise among others the food chain, direct contact, sewage etc. The food chain e.g. might be described as a chain of events (phases, steps) from farmer to the kitchen with each step having a barriers with lesser or greater efficiency. Some steps in this food chain might on the other hand augment the number of bacteria present. The end stage of the food chain is the total sum of all the relevant steps.

Humans have been exposed to antibiotics in hospitals and the community for many decades now. History shows that the use of antibiotics as human medicine will give rise to resistant bacteria. The MRSA is a prime example. It goes without saying that the (ab)use of human antibiotics in the past resulted in the loss of many human antibiotic therapeutics. The lack of hygienic measurements within hospitals will add to the spread of resistant bacteria within the hospital and the community and will result in an increase of resistance within human bacteria.

The ‘animal link’ in the scheme of things is far from clear for different reasons described in this report. This issue therefore deserves a more thorough and consistent scientific approach than sofar has been the case. A global monitoring system parallel to the introduction of new antibiotics both in the animal and human world will most certainly contribute to the gathering of relevant data so desperately needed.

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Appendix I: Definitions

Acquired resistance: resistance obtained to an antimicrobial agent by a micro–organism that used to be susceptible; the resistance genes have been transferred from another bacterium.
Antibiotic: antimicrobial agent produced by bacteria or fungi; is used to treat an infectious disease that stops the growth (bacteriostatic) or kills (bactericidal) the infectious micro–organisms.
AGP: antimicrobial growth promoter: promotes conversion of the feed and growth of the animal by inhibiting growth or killing specific micro–organisms.
Amo: amoxycillin.
Antimicrobial agent: compound produced chemically or by micro–organisms that kills bacteria or inhibits their growth.
Bacteriophage: virus that infects bacteria.
Bacteriostatic: inhibits bacterial growth.
Bactericidal: causes bacterial death.
BEL: Belgium.
CAN: Canada.
CDC : Centers for Disease Control.
CFU: colony forming unit.
Chl: chloramphenicol.
CNS: coagulase–negative staphylococci.
Commensal: (of an animal or a plant): living with, on, or in the other, without doing harm to both.
Coloniser: bacterial strain that is able to enter the intestines of a human or animal and capable of settling permanently.
Co–selection: selection and spread of genes, coding for different properties together; for instance vancomycin resistance genes are co–selected with tylosin resistance genes when tylosin is used as growth promoter.
D–Ala: D–alanine, an amino acid, building block of peptides and proteins.
D–Ala–D–Ala: D–alanyl–D–alanine.
D–Glu: D–glutamic acid, an amino acid, building block of peptides and proteins.
DK: Denmark.
DNA: desoxyribonucleic acid, composed out of bases, sugars and phosphate. The sequence of the 4 bases A, T, C and G in the DNA dictates which proteins can be formed in the cell.
DVL: Danish Veterinary Laboratoria.
Ery: erythromycin.
Genome: inheritable information in the cell, all the DNA that is present (in bacteria in one large molecule).
Feed conversion: the amount of feed (kg) used per kg growth of an animal.
FEFANA: Fédération Européenne des Fabricants d’Adjuvants pour la Nutrition Animale (European federation of feed additive producers).
F–factor: fertility–factor. The F–factor is 94.10^3 bp large, about 1/3 of the genes are involved with transconjugation.
FIN: Finland.
FRA: France.
Fus: fusidic acid.
GER: Germany.
Germ free: without micro–organisms.

Intrinsic resistance: when a bacterial species is not influenced by an antimicro–bial agent; this can be due e.g. to cell wall composition.
IR: inverted repeat, present at the ends of insertion sequences, binding place for transposase.
IS: insertion sequence, simple transposon, composed of transposase gene and inverted repeats (IR).
L–Ala: L–alanine, an amino acid, building block of peptides and proteins.
L–Lys: L–lysine, an amino acid, building block of peptides and proteins.
MAFF: Ministry of Agriculture, Fisheries and Food, UK.
MIC: minimal inhibitory concentration, minimal concentration of antibiotic that inhibits bacterial growth.
MRSA: methicillin resistant Staphylococcus aureus.
NCCLS: National Committee for Clinical Laboratory Standards.
Nefato: Vereniging van Nederlandse fabrikanten van voedertoevoegingen (Organisation of Dutch producers of feed additives).
N–Gluc: N–acetylglucosamine, building block of peptidoglycan.
NL: the Netherlands.
N–Mur: N–acetylmuramic acid, building block of peptidoglycan.
Nosocomial infection: infection that arises in the hospital.
ORF: open reading frame; piece of DNA that codes for a protein.
Oxy: oxytetracyclin.
PCR: Polymerase Chain Reaction; amplification method for specific pieces of DNA.
PFGE: Pulsed Field Gel Electrophoresis. The chromosome of a bacterial strain is cut with an enzyme (SmaI) that produces up to 20 DNA fragments. These large fragments are separated on a gel using a pulsed electric field.
Prophylactic: to prevent infections (e.g. an antibiotic can be administered prophylactically).
RAPD: random amplification of polymorphic DNA.
Replication: multiplying of DNA strains.
Resistant: when a bacterial strain is not killed or growth is not inhibited by an antimicrobial agent.
Restriction enzyme: enzyme that recognises a specific sequence in DNA. An example is SmaI, which recognizes the sequence CCCGGG.
Rif: rifampicin.
Str: streptomycin.
Sul: sulphamethoxazole.
Susceptible: when a bacterial strain is killed or the growth is inhibited by an antimicrobial agent.
Tet: tetracyclin.
Tn1546: transposon that contains the vanA gene cassette.
Transconjugants: bacterial strain that has taken up DNA (e.g. resistance genes) from a donor strain.
Transient passenger: bacterial strain that is able to enter the human (or animal) intestine but is not able to stay there permanently.
Tri: trimethoprim.
UK: United Kingdom.
USA: Unites States of America.
Van: vancomycin.
VRE: vancomycin–resistant enterococci; strains that contain the vanA or vanB gene (cluster) and are intermediate to highly resistant to vancomycin (vanB respectively vanA).
WHO: World Health Organization.

Appendix II: AGP Dosages

Animal	Age	Growth Promoter	Dosage (ppm)
Turkey	0 – 4 weeks	Virginiamycin	20
		Zn–bacitracin	50
	> 4 weeks	Zn–bacitracin	20
Chicken		Avilamycin	10
		Flavomycin	5
		Spiramycin	20
		Virginiamycin	15
		Zn–bacitracin	50
Pig	Up to 25 kg	Virginiamycin	50
		Tylosin	40
		Avilamycin	40
		Salinomycin	50 – 60
		Olaquindox	50 – 100
	25 kg to 4 months	Avilamycin	40
		Olaquindox	50
		Salinomycin	30
		Tylosin	40
		Virginiamycin	40
		Zn–bacitracin	50
	4 months to slaughter	Avilamycin	20
		Flavomycin	5
		Salinomycin	20 – 60
		Tylosin	20
		Virginiamycin	20
		Zn–bacitracin	50
Sows; Breeding sows		Virginiamycin	20
Calves		Virginiamycin	40
		Zn–bacitracin	80
White meat cows		Virginiamycin	40
		Flavomycin	16
		Zn–bacitracin	80
Red meat cows		Virginiamycin	25
		Monensin	40
		Flavomycin	10

Appendix III: The Human Intestinal Flora[7]

Place	Bacteria	Amount/gram content
Stomach	Lactobacilli	10
Small intestine:		
Start	Lactobacilli	10
Before centre	Lactobacilli	100
After centre	Lactobacilli	100
Latter part	Lactobacilli	$1.6 \cdot 10^4$
	Bacteroides	$1.6 \cdot 10^5$
	E. coli	$4.0 \cdot 10^5$
Caecum (intestinal junction)	*Bacteroides*	$7.9 \cdot 10^7$
	E. coli	$3.2 \cdot 10^6$
	Clostridia	100
	Streptococci/ Entereococci	$1 \cdot 10^7$
Faeces	Lactobacilli	$2.5 \cdot 10^6$
	Bacteroides	$1 \cdot 10^{10}$
	E. coli	$4.0 \cdot 10^7$
	Clostridia	$1 \cdot 10^4$
	Streptococci/ Entereococci	$1 \cdot 10^7$

[7] Numbers of major human microflora (when living in temperate climate zones). Drasar and Barrow (**1985**).

Appendix IV: Resistance Genes against Streptogramins, Lincosamide and Macrolides

Gene	Organism	Location	Enzyme	Function	Antibiot.	Ref.
vat	Staph.	pla/chr	Acetyltransferase	Inactivation	Str. A	Sk97
vatB	Staph.	pla	Acetyltransferase	Inactivation	Str. A	A95
vatC	*S. cohnii subsp. cohnii*		Acetyltransferase	Inactivation	Str. A	A98
satA	*E. faecium*		Acetyltransferase	Inactivation	Str. A	R93
vgb	Staph.	pla	Lactonase	Inactivation	Str. B	A88
vgbB	*S. cohnii subsp. cohnii*		Lactonase	Inactivation	Str. B	A98
linA	Staph.	pla	Nulceoti-dyl–transferase	Inactivation	L	Le,Co 91b
vga	*S. aureus*	pla	ATP–binding prot. efflux		Str. A	A92
vgaB	Staph.		ATP–binding prot. efflux		Str. A	A97
mrsA	Staph.	pla		Export	MS	
mefE	Strep.			Efflux pump	M	D98/ J98
ermA	Staph.	tran/chr	Methylase	Target mod.	MLS_B	Sk,F 97/Pa 96
ermC	Staph.	pla	Methylase	Target mod.	MLS_B	Sk,F 97/Pa 96
erm–AM	Strep.		Methylase	Target mod.	MLS_B	Hor85/Le , Co91

Str. A: streptogramin A; Str. B: streptogramin B; L: lincosamides; M: macrolides; MS: macrolides plus streptogramins; MLS_B: macrolides, lincosamides and streptogramins B; pla: plasmid; chr: chromosome; tran: transposon; mod.: modification

A88: Allignet *et al*. (**1988**).
A92: Allignet *et al*. (**1992**).
R93: Rende–Fournier *et al*. (**1993**).
A95: Allignet and El Solh (**1995**).
A97: Allignet and El Solh (**1997**).
A98: Allignet *et al*. (**1998**).
Sk/F97: Skurray and Firth (**1997**).
Pa96: Paulsen *et al*. (**1996**).
Hor83: Horinouchi *et al*. (**1983**).
Le/Co91: Leclercq and Courvalin (**1991**).
D98/J98: McDougal *et al*. (**1998**); Johnston *et al*. (**1998**).
Le/Co91b: Leclercq and Courvalin (**1991b**).

Appendix V: MIC Values

Antibiotic	MIC (mg/ml)	Clasification	Species	Ref.
Ampicillin	≥ 32	Resistant		2, 6
Ampicillin	≥ 2	Resistant		14
Ampicillin	≥ 16	Resistant	Gram –	14
Apramycin	≥ 16	Resistant		7
Apramycin	≥ 32	Resistant	*E. coli*	10
Avoparcin	> 8	Resistant		3, 14
Chloramphenicol	≥ 8	Resistant	*S. pneumonia*	17
Chloramphenicol	≥ 32	Resistant		6
Chloramphenicol	≥16	Resistant		14
Clindamycin	≥ 1	Resistant	*S. pneumoniae*	17
Enrofloxacin	≥ 2	Resistant		6
Enrofloxacin	≥ 2	Resistance: low		14
Enrofloxacin	≥ 8	Resistance: high		14
Erythromycin	≥ 1	Resistant	*S. pneumoniae*	17
Erythromycin	> 2	Resistant		2
Erythromycin	≥ 8	Resistant		6
Flavomycin	≥ 32	Resistant		11
Gentamicin	≥ 2000	Resistent: high		1
Gentamicin	≥ 16	Resistant		6
Gentamicin	≥ 2	Resistance: low MIC		14
Gentamicin	≥ 8	Resistance: high MIC		14
Lincomycin	≥ 1	Resistant		14
Mupirocin	< 100	Resistance: low		5
Mupirocin	> 500	Resistance: high		5
Nalidixic acid	≥ 32	Resistant		6
Neomycin	≥ 16	Resistant		7
Penicillin	≤ 8	Susceptible		1
Penicillin	≥16	Resistant		1
Penicillin G	< 0.1	Susceptible	*S. pneumoniae*	16
Penicillin G	0.1 – 1.0	Intermediate	*S. pneumoniae*	16
Penicillin G	≥ 2.0	Resistant	*S. pneumoniae*	16, 17
Pristinamycin	≤ 0.5	Susceptible		12
Pristinamycin	1	Intermediate		12
Pristinamycin	≥ 2	Resistant		12
Pristinamycin IIA	≤ 2	Susceptible		12
Pristinamycin IIA	4	Intermediate		12
Pristinamycin IIA	≥ 8	Resistant		12
Pristinamycin IB	> 8	Resistant		12
Quinupristin/ Dalfopristin	≥ 4	Resistant (manuf.)		–
Spectinomycin	≥ 32	Resistant		–
Spiramycin	≥ 8	Resistant		7
Streptomycin	≥ 2000	Resistance: high		1
Synercid®	≥ 2	Resistant	*E. faecium*	13

Antibiotic	MIC (mg/ml)	Clasification	Species	Ref.
Teicoplanin	>4	Resistant		2
Teicoplanin	≤ 8	Susceptible		1, 8
Teicoplanin	16	Intermediate		1
Teicoplanin	≥ 32	Resistant		1, 8
Tetracycline	≥ 8	Resistant	*S. pneumoniae*	17
Tetracycline	≥16	Resistant		6
Trimetoprim	≥ 1	Resistant		14
Tylosin	>32	Resistant		3, 7
Vancomycin	≤ 4	Susceptible		1, 8
Vancomycin	8 – 16	Intermediate		1
Vancomycin	≥ 32	Resistant		1, 8
Vancomycin	>4	Resistant		2
Virginiamycin	>4	Resistant		3
Zn–bacitracin	10	Breaking point	Gram +	15

MICs determined for Gram–positive bacteria like enterococci and staphylococci, unless otherwise indicated.

1. Clark *et al*. (**1993**).
2. Dutch Working Group for Antimicrobial Susceptibility Testing
5. 3 Kaukas *et al*., **1988** (Bogaard **1997**, ref. 10)
6. 4 see article comparing MIC determining methods for regulation
7. Ramsey *et al*., **1998**
8. National Committee for Clinical Laboratory Standards, 1990, 1994, **1997** (Gordts *et al*., **1995**, ref. 16; Aarestrup *et al*., **1997**, ref 14 and 15).
9. Aarestrup *et al*., **1997**.
10. Hunter *et al*., **1993**.
11. Devriese and Haesebrouck, **1996**.
12. Allignet *et al*., **1996**
13. Jones *et al*., **1998**. Poster, Fourth International Conference on the macrolides, azalides, streptogramins and ketolides, Barcelona.
14. British Society of Antimicrobial Chemotherapy (Working Party, 1988 and 1991).
15. Alpharma, **1998**.
16
17 McDougal *et al*., **1998**

Appendix VI: Infectious Gram + Bacteria

Bacteria	Host range	References
Enterococci:	Animals, humans	Devriese *et al.*, **1997**
E. casseliflavus		
E. caecorum	Cattle	
E. durans		
E. faecalis	Humans, chicken, turkey, pigs, cats, dogs	
E. faecium	Humans, young chicken, turkey, pigs	
E. flavescens		
E. gallinarum		
E. hirae	Cattle, pigs, dogs	
Streptococci:		
Strep. agalactiae	Humans, cattle	
Strep. bovis group D	Humans, animals	Poyart *et al.*, **1997**
Strep. dysgalatiae	Bovine	
Strep. equisimilis	Humans, pigs	McCoy *et al.*, **1991**
Strep. porcines	Pigs	
Strep. hyointestinalis	Pigs	
Strep. hyovaginalis	Pigs (sows)	Devriese *et al.*, **1997**
Strep. thoralentis	Pigs (sows)	Devriese *et al.*, **1997**
Strep. pyogenes	Humans, primates, cats	Devriese, **1991**; Huovinen *et al.*, **1997**
Strep. pneomoniae	Humans, primates	Devriese, **1991**
Strep. equi	Equine	
Strep. zooepidemicus	Equine	
Staphylococci:		
***S. aureus* A**	Humans	Hajek, **1976**; Devriese, **1984**
S. aureus B	Poultry, pigs	
***S. aureus* C**	Humans (skin), cattle, sheep, goats	
S. aureus D		
S. aureus F		
S. cohnii subsp. Cohnii	Humans (skin)	Liassine *et al.*, **1997**; Allignet *et al.*, **1998**
S. cohnii subsp. urealyticum	Humans, primates	Liassine *et al.*, **1997**
S. epidermidis	Humans	
S. suis	Humans	MAFF, **1998**
S. haemolyticus		Liassine, **1997**
S. intermedius	Cats, dogs	Hajek, **1976**
S. hyicus	Pigs	Devriese, **1984**
Clostridia:		
Clostridium difficili		MAFF, **1998**
Clostridium perfringens		MAFF, **1998**

Appendix VII: Major Nosocomial Infections

Disease	Bacteria
Urinary tract infection	*E. coli*, staphylococci, streptococci, enterococci
Wound infections	*Staphylococcus*
Surgical, injuries, burns	Pseudomonas
Bacteriaemia	Staphylococci
Endocarditis	*Staphylococcus*, *Streptococcus*, enterococci
Meningitis	*S. pneumoniae*, *Neisseria*
Osteomyelitis	*S. aureus*
Otitis media	*S. pneumoniae*, *Pseudomonas aeruginosa*
Pneumonia	*Streptococcus*
Sinusitis	*S. pneumoniae*
Gastro enteritis	**Gram –**: *C. jejuni*, *E. coli*, *Salmonella typhimurium*, *Salmonella enteritidus*
Syndrom of Guillain–Barré	*C. jejuni*
Sepsis	**Gram –**: *E. coli* **Gram +**: Staphylococci, *Streptococcus*
Intra abdominal infection	Enterococci
Scarlet fever	*Streptococcus*
Respiratory tract infections	*Streptococcus*, *Enterococcus*

Information derived from Baquero (**1997**); Dutch Health Council (Gezondheidsraad, **1998**)

Appendix VIII: Zn-bacitracin Resistance in Gram-positive Bacteria over the Last 40 Years

Bacterial genus/species	Source	Resistant	Country	Reference
Streptococcus	Humans	20 % (137)	USA	Weil *et al.*, **1953**
Strept.	Humans	No increase	USA	Weil *et al.*, **1955**
Strept. group B	Humans	0 % (12)	Germany	Soedermanto *et al.*, **1996**
Strept. group B	Canine	0 % (17)	Germany	Soedermanto *et al.*, **1996**
Strept. group B	Bovine	0 % (16)	Germany	Soedermanto *et al.*, **1996**
Staphylococcus	Humans	10 % (136)	USA	Weil *et al.*, **1953**
Staph.	Humans	No increase	USA	Weil *et al.*, **1955**
Staph.	Humans	0 % (> 200)	UK	Lowbury, **1960**
Staph.	Humans	< 5 % (7633)	USA	Griffith, **1964**
Staph.	Canine	3 % (501)	Norway	Kruse *et al.*, **1996**
S. aureus	Humans	0 % (345)	Germany	Hentschel *et al.*, **1979**
S. aureus	Humans	2 % (106)	Various	Maple *et al.*, **1989**
S. aureus	Humans	< 10 % (349)	USA	Flournoy *et al.*, **1990**
S. aureus	Humans	0 % (16)	Brazil	Rodrigues *et al.*, **1993**
S. aureus	Humans	1.5 % (119)	USA	Everett *et al.*, **1995**
S. aureus	Humans	8 % (36)	Turkey	Hizel *et al.*, **1997**
S. aureus	Poultry	0 % (445)	Germany	Hentschel *et al.*, **1979**
S. aureus	Poultry	0.5 % (224)	Belgium	Devriese, **1990**
S. aureus	Bovine	0 % (246)	Belgium	Devriese, **1980**
S. aureus	Swine	0 % (124)	Belgium	Devriese, **1980**
S. aureus	Cattle	0.4 % (281)	USA	McDonald *et al.*, **1981**
S. aureus	Cattle	5 % (92)	Brazil	Cavalieri *et al.*, **1996**
S. aureus	Cattle	0 % (211)	Denmark	DANMAP, **1997**
S. hyicus	Swine	0 % (138)	Belgium	Devriese, **1980**
S. hyicus	Swine	0 % (71)	Denmark	DANMAP, **1997**
Coagulase neg. staphylococci	Humans	6 % (261)	USA	Everett *et al.*, **1995**
Coagulase neg. Staphylococci	Cattle	0 % (371)	Denmark	DANMAP, **1997**
Clostridium perfringens		0 % (90)	Canada	Mohoney, **1973**
Clostridium spp.	Cattle	13 % (68)	Belgium	Dutta and Devriese, **1983**
C. perfringens	Poultry	> 50 % (80)	Japan	Benno *et al.*, **1988**
C. perfringens	Poultry	6 % (31)	Belgium	Devriese *et al.*, **1993**
C. perfringens	Poultry	0 % (44)	India	Das *et al.*, **1997**a
C. perfringens	Swine, poultry	8 % (450)	Europe	Alpharma, **1998**
C. perfringens	Swine	0 % (32)	Belgium	Devriese *et al.*, **1993**
C. perfringens	Swine	3.8 % (79)	USA	Alpharma, **1998**
C. perfringens	Cattle	9 % (32)	Belgium	Devriese *et al.*, **1993**
Enterococcus faecalis	Poultry	2 % (60)	UK	Barnes *et al.*, **1978**
E. faecalis subsp. liquefaciens	Poultry	100 % (23)	Belgium	Dutta and Devriese, **1982**
E. faecalis	Poultry	100 % (8)	Belgium	Dutta and Devriese, **1982**
E. faecalis	Swine	3 % (225)	Denmark	DANMAP, **1997**
E. faecalis	Swine	100 % (36)	Belgium	Gezondheidsraad

Bacterial genus/species	Source	Resistant	Country	Reference
Enterococcus ***faecium***	Swine	100 % (5)	Belgium	Gezondheidsraad
E. faecium	Poultry	77 % (13)	UK	Barnes *et al.*, **1978**
E. faecium	Poultry	67 % (15)	Belgium	Dutta and Devriese, **1982**
E. faecium	Poultry	41 % (54)	Denmark	DANMAP, **1997**
E. faecium	Cattle	8 % (13)	Denmark	DANMAP, **1997**
E. faecium	Swine	31 % (58)	Denmark	DANMAP, **1997**
E. hirae	Swine	0 % (12)	Belgium	

Modified from Alpharma, **1998**.