# Annex A: Avilamycin

## A.1 Introduction

Avilamycin is a mixture of oligosaccharides of the orthosomycin group, that are produced by *Streptomyces viridochromogenes*. Other members of this group include curamycin and everninomycins (Wolf, 1973). Avilamycin is mainly active against gram-positive bacteria. The compound is used for growth promotion in swine and poultry, at dosages ranging from 5 to 40 ppm for swine and from 2.5 to 10 ppm for poultry. It has never been used for therapeutic purposes in either human or veterinary medicine but a related compund, everninomycins, has been suggested for evaluation in human therapy (Chopra *et al.*, 1997; Nicas *et al.*, 1997). In rats and swine, oral avilamycin is primarily excreted in faeces (Magnussen *et al.*, 1991).

## A.2 Mode of action and resistance mechanisms

Avilamycin acts on the bacterial ribosome, by inhibiting the binding of formylmethionyl-tRNA to the 30 S ribosomal subunit (Wolf, 1973). This blocks the formation of the 70 S initiation complex in bacterial protein synthesis. This inhibition occurs in *in vitro* ribosomal systems from both gram-positive and gram-negative bacteria (Wolf, 1973), so the difference in avilamycin susceptibility of gram-positive and gram-negative bacteria is probably due to differences in factors outside the protein synthesizing system, like cell wall composition. Experimental data indicate that avilamycin interferes with the attachment of tRNA to the ribosome by binding to the 30 S subunit (Wolf, 1973).

No data has been published on mechanisms of resistance, but it seems logical to assume that structural changes in the ribosomal 30 S subunit or ribosomal protection could confer resistance to avilamycin in naturally susceptible bacteria. In view of the ease and speed at which resistance to some other anti-ribosomal antimicrobials (e.g. aminoglycosides and tetracyclines) has emerged, it is very important to investigate possible resistance mechanisms to avilamycin and how these may interfere with the effect of other anti-ribosomal substances.

Full cross-resistance to everninomycins would be expected in avilamycin-resistant bacteria, as the two compounds are structurally very similar (see figure A.I). Cross-resistance to unrelated antimicrobials in avilamycin-resistant bacteria has not been reported, but there is no information on

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Figure A.I. Tentative sketch of avilamycin and everninomycins

possible cross-resistance to antimicrobials with a similar site of action (i.e. the binding site for formylmethionyl tRNA), such as viomycin and capreomycin, among others. Cross-resistance between structurally unrelated compounds may be seen when these compounds have the same site of action. Even when the sites of action are not identical, cross-resistance could, theoretically, appear if structural changes in the ribosome, or protecting proteins, affect both antimicrobial binding sites. In view of this, knowledge of resistance mechanisms is necessary in order to asses possible risks regarding resistance.

## A.3 Development of resistance

Unfortunately, only two publications concerning bacterial susceptibility to avilamycin have been found. This is hardly enough to illustrate the prevalence of avilamycin resistance, but the data from these studies are compiled in table A.I.

Table A.I. Reported prevalence of avilamycin resistance

| **Bacterial species** | **Source of isolates** | **No. Of isolates** | **Year(s)** | **Resistance in %** | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *Clostridium perfringens* | various | 95 | 1991 | 0 | Devriese *et al*, 1993 | Belgium |
| *Staphylococcus hyicus* | swine | 71 | 1995-96 | 0 | DANMAP, 1997 | Denmark |
| *Staphylococcus aureus* | cattle | 211 | 1995-96 | 0 | DANMAP, 1997 | Denmark |
| *Coagulase negative staphylococci* | cattle | 371 | 1995-96 | 0 | DANMAP, 1997 | Denmark |
| *Enterococcus faecalis* | swine | 225 | 1995-96 | 1 | DANMAP, 1997 | Denmark |
| *Enterococcus faecium* | swine | 58 | 1995-96 | 2 | DANMAP, 1997 | Denmark |
| *Enterococcus faecium* | poultry | 54 | 1995-96 | 69 | DANMAP, 1997 | Denmark |
| *Enterococcus faecium* | cattle | 13 | 1995-96 | 0 | DANMAP, 1997 | Denmark |

In Denmark, 84 % of the annual consumption of avilamycin in feed is used in poultry, which is reflected in the differences in resistance to this compound in *E. faecium* from cattle swine and poultry, respectively. The consumption of avilamycin increased four-fold between 1994 and 1995, coinciding with its introduction into poultry feed. Unfortunately, there is no data available on avilamycin resistance in *Enterococcus faecium* isolates collected before this increase in the consumption. As no data has been published on the development of resistance, it is not possible to evaluate the risk of this. An adequate amount of this type of data is essential in risk evaluation and should be made available as soon as possible.

## A.4 Acquisition of resistance

No published information has been found about genes conveying resistance to avilamycin, transfer of avilamycin resistance, or bacterial hosts for resistance genes. If no such investigations have been undertaken, they should be planned immediately.

## A.5 Impact of resistance on animal and human health

As avilamycin is not yet used for therapy in humans or animals, resistance would not be expected to cause clinical problems unless cross-resistance to other substances was present. However, in the future orthosomycin compounds may well be of interest for clinical use. For example, everninomycins have been suggested as a new important candidate for use in human therapy (Cormican and Jones, 1996; Urban *et al.*, 1996; Chopra *et al.*, 1997; Nicas *et al.*, 1997). The impact of avilamycin resistance on the life span of future drugs is difficult to assess, but could be substantial.

## A.6 Other effects on the microflora

### A.6.1 Salmonella

Hinton (1988) investigated the effect of in-feed avilamycin on salmonella colonisation in chickens. Groups of 10 chickens were given avilamycin in the feed at concentrations of 2.5 or 10 ppm, alone or together with monensin. Medicated birds were compared to non-medicated controls. Four replicate experiments were performed, in two study designs, where the birds were either initially infected on the day of purchase or one week later. All birds

were infected with *Salmonella* Kedougou in the feed for two weeks, at concentrations from 1.6 to 176 bacterial cells per g feed. All birds in the same replicate experiment received the same dose of organisms. Samples for bacterial culture were taken on day 7 and 14 after the introduction of infected feed. The author concluded that no evidence was obtained to suggest that avilamycin, at concentrations of 2.5 or 10 ppm in the feed, favoured colonisation of the intestinal tract in chickens with *S.* Kedougou when they were challenged with this organism in the feed.

This is the only published study on the effect of avilamycin on intestinal salmonellae. One single study, no matter how well performed, is hardly enough to form the basis for any definite conclusions, especially when this study did not result in any clear evidence as to whether avilamycin presents a risk in this aspect or not.

### A.6.2 Other enteric pathogens

No publications on the effects of avilamycin on other enteric phogens have been found.

## A.7 Effects on specific animal diseases

Avilamycin at growth promoting levels has been shown to reduce the amount of *Clostridium perfringens* in the intestinal tract of chickens (Elwinger *et al.*, 1993; Elwinger *et al.*, 1995) and may thus be used prophylactically against necrotic enteritis in poultry. Kyriakis (1989) investigated the effect of avilamycin at 40 or 80 ppm in the control of stress-induced post-weaning diarrhoea in piglets. The author stated that avilamycin at 80 ppm had a significant ($p<0.05$) effect in reducing diarrhoea and mortality in newly weaned piglets. Even at the level of 40 ppm, which may be used for growth promoting purposes in piglets, avilamycin notably reduced diarrhoea, although these figures were not statistically significant. Mortality was, however, significantly ($p<0.05$) reduced in the piglets that received 40 ppm avilamycin in the feed. At 40 ppm, avilamycin did not have a growth promoting effect in this experiment. Thus, in this study design, with piglets that were already sick, avilamycin served as a therapeutic or prophylactic agent and not as a growth promoter.

## A.8 Toxicological aspects

No information about possible toxic effects either on the target species or on humans has been found. Such effects may be totally absent or, as it is a

comparatively new compound, reports on allergy and other side effects may not yet have appeared.

One publication (Magnussen *et al.*, 1991) has been found concerning residues. The results in this study indicate that avilamycin fed to swine at a concentration of 60 ppm gives rise to small but measurable residues in tissues. At zero withdrawal time the residue levels were 0.14 ppm in muscle, 0.66 ppm in liver, 0.34 ppm in kidney and 0.55 ppm in fat. There is no available information to suggest that this would present a risk for the consumer.

## A.9 Environmental effects

Source separated municipal solid waste and agricultural waste can be utilised for biogas production. Substances with an antimicrobial effect against anaerobic bacteria could disturb this process. In studies on manure from pigs and poultry fed avilamycin, Sutton (1989) reported efficient operation of experimental and large mesophilic digesters. The presence of avilamycin appeared to alter the metabolism of the microflora, increasing the efficiency to degrade volatile solids.

No other publications on possible environmental effects of avilamycin have been found. As the compound is produced by a soil microbe, it would be expected to be microbially degraded in soil.

## A.10 Summary comments

Use of avilamycin in poultry appears to have caused an increase in avilamycin resistance in *E. faecium* from this animal species. This resistance might have appeared after only a few years of avilamycin use. Avilamycin is closely related to everninomycins, and cross-resistance could shorten the therapeutic life span of everninomycins if they were to be used in human therapy. The overall information about the possible effects of avilamycin in various aspects is much too scarce to form the basis of a risk assessment.

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# Annex B: Bacitracin

## B.1 Introduction

Bacitracin is a complex mixture of cyclic polypeptides produced by *Bacillus subtilis* and *Bacillus licheniformis*. The compound has bactericidal effect on gram-positive bacteria but little activity against gram-negative organisms (Prescott and Baggot, 1993). It is most commonly used in complex with zinc which seems to stabilise the antibiotic complex (Quinlan and Gutteridge, 1989).

Bacitracin is poorly absorbed from the gastrointestinal tract (Donoso *et al.*, 1970; Froyshov *et al.*, 1986), as well as from skin and mucosal surfaces. Absorbed bacitracin is excreted by glomerular filtration (Prescott and Baggot, 1993).

The substance is used in human therapy, mostly for topical treatment of superficial infections of the skin and mucosal surfaces. However, its effectiveness against vancomycin-resistant enterococci has led to an increase in its use for oral treatment (O'Donovan *et al.*, 1994; Chia *et al.*, 1995). In veterinary medicine, bacitracin has been suggested for the treatment and/or prevention of proliferative adenomatosis in swine (Kyriakis *et al.*, 1996), swine dysentery (Jenkins and Froe, 1985), and for clostridial infections in man (Caputo *et al.*, 1994) and various animal species (Carman and Wilkins, 1991; Prescott and Baggot, 1993). Two diseases may be of particular interest in this respect, namely proliferative adenomatosis in swine and necrotic enteritis in poultry (Prescott and Baggot, 1993).

Recommended dosages for prophylaxis and therapy in poultry are in the range between 50 and 200 ppm, and for swine around 250 ppm (Prescott and Baggot, 1993). The corresponding dosages permitted for growth promotion are between 5 and 100 ppm for poultry, and between 5 and 80 ppm for swine. Growth promoting dosages for calves, lambs and kids are in the range of 5-80 ppm and for fur animals 5-20 ppm.

Bacitracin is widely used for laboratory purposes, in selective media for bacterial culture and in cell culture media. It is also used experimentally as a protease inhibitor (Fukuda *et al.*, 1995).

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Figure B.I. Tentative sketch of bacitracin

## B.2 Mode of action and resistance mechanisms

Bacitracin inhibits the formation of bacterial cell wall peptidoglycan by complexing directly with the lipid isoprenyl pyrophosphate (IPP) carrier, inhibiting the dephosphorylation reaction that is required for its regeneration. This leads to accumulation of phospholipids inside the cell and inhibition of cell wall formation. Proposed resistance mechanisms include active efflux, increased production of IPP kinase, suppression of autolytic systems, reduced membrane permeability and suppressed exopolysaccharide secretion (see below).

No information about cross-resistance to other substances has been found. Cross-resistance might be expected in some cases, like when autolytic systems are suppressed. This would be expected to convey cross-resistance to other cell wall inhibitors. Reduced membrane permeability might convey cross-resistance to substances with similar diffusion properties or mechanisms for uptake.

It has been claimed that bacitracin "cures" resistance against other antimicrobials and that its use as a feed additive would therefore be purely beneficial from a resistance point of view (Walton, 1978; Gedek, 1981; Walton, 1984; Walton and Wheeler, 1987). However, due to weaknesses in study design in relation to the specific question to be answered, as well as

inconsistent results, the only conclusion that can be drawn from these studies is that bacitracin does not seem to induce resistance to any of the other, unrelated, antimicrobials tested. However, as resistance to bacitracin was not determined or characterised, the results provided little information about the potential for cross-resistance. Some of the studies mentioned show synergistic effects of bacitracin together with other antimicrobials, but do not investigate any effects on actual resistance genes. Undoubtedly, antimicrobials interfering with cell wall synthesis, such as bacitracin, may act synergistically with other antimicrobials that exert their effects inside the bacterial cell. One must still keep in mind that this most likely has to do with the combined mechanisms of the two antibacterials and does not mean that any resistance genes have been eliminated.

## B.3 Development of resistance

### B.3.1 Prospective studies

Only two studies that deal specifically with development of bacitracin resistance over time have been found (Linton *et al.*, 1985; Kaukas *et al.*, 1988). These include one experimental study and one field study.

In the study by Kaukas *et al.* (Kaukas *et al.*, 1988), groups of 10 chickens were fed different antibiotics, with one group serving as a non-medicated control group. The bacitracin group was given a dosage of 40 ppm bacitracin in the feed. Enterococci were isolated from cloacal swabs, identified and analysed for antimicrobial susceptibility. When comparing the incidence of bacitracin resistance in enterococci from medicated birds to that in enterococci from non-medicated birds, bacitracin exposure seems to have had little effect on the incidence of resistance in *Enterococcus faecalis* and *E. gallinarium*. *E. faecium*, however, showed an incidence of resistance to bacitracin as high as 47% in the medicated group, while in the non-medicated group the incidence was 29%. This difference was statistically significant ($p<0.005$), and indicates that bacitracin in the feed selects for resistant strains of *E. faecium.* Further, the incidence of resistance to therapeutic antibiotics, expressed as the Antibiotic Resistance Index (ARI) was significantly ($p=0.003$) higher in all groups receiving antibacterials, as compared to the control group. This increase was most likely associated with an increase in the proportion of *E.faecium* in the enterococcal population of the treated birds.

In the publication by Linton *et al.* (Linton *et al.*, 1985), the first part is a field survey comparing five commercial premises and a total of nine batches of broiler chickens. One farm, where one batch of birds was reared, used feed with bacitracin at the recommended level for growth promotion. No non-medicated controls were included and no information on whether

coccidiostats were used was provided. Cloacal swabs were taken at the beginning and at the end of the rearing period and isolated enterococci were tested for antimicrobial susceptibility. The percentage of strains resistant to bacitracin, among the strains isolated from birds fed bacitracin, increased from 87% to 100% during the rearing period. However, the enterococci were not identified to the species level and no information is provided on how many birds were sampled in each group in this experiment. This, taken together with the fact that there was no non-medicated control group, makes the results hard to evaluate.

The second part of the same publication describes an experimental study, where enterococci were also tested for their susceptibility to bacitracin. Bacitracin was not a factor of the experiment but one of the four groups of chickens was a non-medicated control. In this group, the proportion of resistant strains varied between 20% and 90% during the rearing period, which indicates that a day to day variation is not unusual and must be taken into account when evaluating this type of data.

### B.3.2 Point-prevalence studies

Data from different studies on prevalence of resistance to bacitracin have been compiled in table B.I. Unfortunately, most studies do not report MIC values and there seems to be some confusion about what should be the break-point value for different bacteria. Further, the number of strains included are often inadequate for an estimate of the prevalence of resistance. Bearing this in mind, it is noteworthy that the proportion of enterococcal isolates with presumably acquired resistance shows a range from 3-77% , varying between different animal species and different countries. To fully assess the possible importance of this, information on resistance mechanisms and transferability of resistance in enterococci is needed, as well as consumption figures for different animals and different countries.

Table B.I. Resistance to bacitracin in various bacterial species, reported in different studies

| **Bacterial species** | **Source of isolates** | **No. Of iso-lates** | **Year** | **Resis-tance in % *** | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *Clostridium perfringens* | cattle | 32 | 1991 | *9* | Devriese et al, 1993 | Belgium |
| *C. perfringens* | poultry | 31 | 1992 | *6* | Devriese et al, 1993 | Belgium |
| *C. perfringens* | poultry | 80 | 1984-86 | *>50* | Benno et al, 1988 | Japan |
| *C. perfringens* | swine | 32 | 1992 | *0* | Devriese et al, 1993 | Belgium |
| *Clostridium* spp. | cattle | * | 1979-82 | *9* | Dutta and Devriese, 1984 | Belgium |
| *Clostridium* spp. | poultry | * | 1979-82 | *6* | Dutta and Devriese, 1984 | Belgium |
| *Clostridium* spp. | swine | * | 1979-82 | *1* | Dutta and Devriese, 1984 | Belgium |
| *Enterococcus faecalis* subsp *liquefaciens* | poultry | 23 | 1980 | *21* | Dutta and Devriese, 1982 | Belgium |
| *E. faecalis* | poultry | 8 | 1980 | *62* | Dutta and Devriese, 1982 | Belgium |
| *E. faecalis* | poultry | 60 | 1977 | *17* | Barnes et al, 1978 | UK |
| *E. faecalis* | swine | 225 | 1995-96 | *3* | DANMAP, 1997 | Denmark |
| *Enterococcus faecium* | poultry | 15 | 1980 | *67* | Dutta and Devriese, 1982 | Belgium |
| *E.faecium* | cattle | 13 | 1995-96 | *8* | DANMAP, 1997 | Denmark |
| *E.faecium* | poultry | 54 | 1995-96 | *41* | DANMAP, 1997 | Denmark |
| *E.faecium* | poultry | 13 | 1977 | *77* | Barnes et al, 1978 | UK |
| *E.faecium* | swine | 58 | 1995-96 | *31* | DANMAP, 1997 | Denmark |
| *Enterococcus* spp. | humans | 9 | 1992-1993 | *11* | Everett et al, 1995 | USA |
| *Streptococcus* spp. | humans | 50 | 1992-1993 | *2* | Everett et al, 1995 | USA |
| *Staphylococcus aureus* | cattle | 211 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *S. aureus* | various | 324 | 1970-80 | *<1%* | Devriese, 1980 | Belgium |
| *S. aureus*, methicillin resistant | humans | 106 | 1989 | *2* | Maple et al, 1989 | various |
| *S. hyicus* | swine | 71 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *Coagulase positive staphylococci* | humans | 119 | 1992-93 | *17* | Everett et al, 1995 | USA |
| *Coagulase negative staphylococci* | cattle | 371 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *Coagulase negative staphylococci* | humans | 261 | 1992-93 | *6* | Everett et al, 1995 | USA |

* total number of strains for cattle, poultry and swine = 192

## B.4 Acquisition of resistance

Published information on bacitracin resistance is sparse, but some hitherto identified mechanisms of resistance to bacitracin are shown in Table B.II.

Table B.II. Mechanisms of bacitracin resistance

| Bacterial species | Gene | Mechanism | Reference |
|---|---|---|---|
| *Bacillus licheniformis* | *bcr* | active efflux | Podlesek et al, 1995 |
| plasmid pXV62, original bacterial source not given | *bac* A | production of IPP kinase | Cain et al, 1993 |
| *Enterococcus spp.* | not identified | suppressed autolytic system | Krogstad and Pargwette, 1980* |
| various gram-positive and gram-negative bacteria | not identified | reduced membrane permeability | Mukherjee et al, 1989 |
| various gram negative bacteria | not identified | suppressed exopolysaccharide secretion | Pollock et al, 1994 |

*this is the mechanism proposed although not proven in the article

Podlesek and co-workers (Podlesek *et al.*, 1995) characterised a resistance gene, *bcr*, in *Bacillus licheniformis* that codes for proteins forming an ATP-binding transport system in the cell membrane. The proposed action of this transport system is active efflux of the bacitracin molecule. Another resistance gene, located on plasmid pXV62, named *bac*A (Cain *et al.*, 1993), proposedly encodes a phosphokinase involved in IPP metabolism. A similar enzyme has been characterised in *Staphylococcus aureus* (Sandermann Jr and Strominger, 1971), although its possible effects on the bacitracin susceptibility of this bacterium was not investigated. Resistance to bacitracin due to altered cell membrane permeability has been reported (Mukherjee *et al.*, 1989). Resistance due to suppressed autolytic enzyme systems has also been suggested (Krogstad and Pargwette, 1980). Suppression of autolytic enzymes makes the bacterium resistant to substances that inhibit peptidoglycan synthesis (Tomasz *et al.*, 1970; Krogstad and Pargwette, 1980).

Other mechanisms for reduced bacitracin susceptibility that have been suggested include increased production of the carrier IPP, which may competitively overcome the inhibitory effect of bacitracin on peptidoglycan synthesis, and cessation of exopolysaccharide synthesis (Pollock *et al.*, 1994). The excretion of polysaccharides require the same carrier IPP that is needed for the synthesis of peptidoglycan and a halt in this excretion will leave more IPP available for cell wall synthesis. This will also occur in the

absence of essential components required for exopolysaccharide synthesis, e.g. in an environment depleted of certain sugars (Pollock *et al.*, 1994).

Transfer of bacitracin resistance seems to have attracted little attention from researchers. Transduction between strains of *Streptococcus pyogenes* has been shown to occur (Stuart and Ferretti, 1978). However, this study did not investigate what gene(s) and mechanisms were involved in the observed resistance. No other studies, concerning transfer or non-transfer of bacitracin resistance have been found. Bacitracin, like other substances that inhibit late stages in peptidoglycan synthesis, has been shown to induce the expression of the vancomycin resistance gene, *van*A in enterococci (Allen and Hobbs, 1995; Lai and Kirsch, 1996). The practical aspects of this are not clear but it is not likely to be of any clinical importance.

## B.5 Effects on specific animal diseases

Some reports indicate that bacitracin, even at concentrations used for growth promotion, may prevent necrotic enteritis in poultry (Wicker *et al.*, 1977; Prescott *et al.*, 1978; Stutz *et al.*, 1983). Stutz and co-workers (1983) found that supplementing a soybean protein and sucrose-based diet with levels of 5.5, 16.5, or 55 ppm of bacitracin significantly reduced the number of *Clostridium perfringens* organisms in the ileal contents of chicks ($p<0.05$). Prescott and co-workers (1978) reported that inclusion of bacitracin at 200 or 400 mg/gallon in the drinking water was effective in treating experimentally induced necrotic enteritis in chickens and incorporation of 100 mg/gallon in the drinking water prevented its occurrence. Wicker and co-workers (1977) found a significant ($p<0.01$) decrease in mortality due to necrotic enteritis in chickens given 11, 33 or 55 ppm bacitracin in the feed, as compared to non-medicated birds. These studies suggest that bacitracin at the concentrations used for growth promotion also has prophylactic and therapeutic effects on necrotic enteritis in poultry.

Some authors have investigated the possible effects of orally administered bacitracin on the immune response to certain forms of challenge (Harmon *et al.*, 1973; Wasinska, 1980). Harmon and co-workers (1973) compared non-medicated pigs to pigs fed bacitracin at a concentration of 55 ppm during various lengths of periods. After repeated intraperitoneal injections of sheep red blood cells or phenolised *Salmonella* Pullorum, serum antibody titers were tested by agglutination and hemagglutination. The authors stated that the immunological response to sheep erythrocytes was little affected by bacitracin in the feed, whereas the antibody response to *Salmonella* Pullorum was significantly enhanced by medicated feed. Various explanations for this were discussed in the paper.

Wasinska (1980) investigated the antibody response and resistance to infection after vaccinating pigs against erysipelas, colibacillosis and swine

fever. In addition, non-vaccinated controls were included in the study. Pigs were fed bacitracin at a concentration of 70 ppm for six months, and compared to pigs fed no antimicrobial. It was concluded that the supplementation of feed with bacitracin did not exert any negative effects on the antibody response in vaccinated pigs, nor did it decrease the effectiveness of immunisation, as determined by experimental infection.

## B.6 Impact of resistance on animal and human health

Increased resistance to bacitracin in clostridia and enterococci could lead to therapeutic failures when bacitracin is used for the treatment of infections with these organisms in animals and humans. Without further information about the extent of the therapeutic use of bacitracin, the impact on human and animal health of such incidents is impossible to assess.

## B.7 Other effects on the microflora

### B.7.1 Effects on salmonella colonisation

Only a few published studies investigating the association between salmonella colonisation of the gut and bacitracin in the feed have been found (Nurmi and Rantala, 1974; Smith and Tucker, 1975; Smith and Tucker, 1980; Latour and Barnum, 1981; Humbert *et al.*, 1991; Manning *et al.*, 1994). All use poultry as the experimental animal species and in-feed bacitracin at the concentrations used for growth promotion. Unfortunately, like other studies in this area, most of these suffer from weaknesses in study design and the results are often inconclusive and inconsistent. The results in the study by Manning and co-workers (1994) indicated that in chickens fed 490 ppm bacitracin, a significantly ($p<0.05$) larger proportion were infected with salmonella at the end of the experiment, as compared to non-medicated birds. All animals were kept on used litter, and challenged with $10^6$ organisms of *Salmonella* Enteritidis. The concentration of bacitracin used was considerably higher than what is used for growth promotion, and the selective culture technique used appears to be based on visual examination only. This makes the results difficult to evaluate.

In the study by Humbert and co-workers (1991) bacitracin was given at a concentration of 50 ppm to groups of chickens, with chickens receiving no antimicrobial in the feed serving as controls. Another factor in the experiment was treatment with bacterial flora from 12-week old Specific Pathogen Free-chicks (competitive exclusion, CE). All birds were challenged

with $10^4$-$10^5$ organisms of *Salmonella* Typhimurium. Half of the animals were killed on day 3 for bacteriological examination of the caeca. The rest were killed and examined on day 6. The results show a small increase in the amount of salmonella shed by medicated birds, but a slight decrease when the medicated birds were also given CE. However, in the groups not receiving CE treatment, the proportion of salmonella positive birds was 100% in both medicated and non-medicated groups. Thus, no conclusions on the influence on the prevalence of colonisation can be drawn and the study period of 6 days is too short for conclusions about differences in excretion time. The bacterial counts are presented as log means for each group, so it is not possible to determine whether the differences are caused by changes in the amount of excretion in just a few individuals or if it is an overall effect. Another minor weakness is the selective method used for salmonella isolation where the recording of salmonella was based on visual inspection and without further confirmation.

Nurmi and Rantala (1974) compared the re-isolation of *Salmonella* Infantis, after experimental infection, from the caeca of non-medicated birds and of birds fed 10 ppm or 20 ppm bacitracin with and without concurrent treatment with CE. The results indicate a decrease in the amount and prevalence of *Salmonella* shedding in birds receiving bacitracin, as compared to non-medicated birds. The feeding of bacitracin did not inhibit the positive effect of the CE treatment. The groups were very small and the percentage of salmonella shedding in the non-medicated control group varied between experiments making interpretation of the results difficult.

Smith and Tucker reported two studies including bacitracin (Smith and Tucker, 1975; Smith and Tucker, 1980). In the first (Smith and Tucker, 1975) birds were fed bacitracin at concentrations of either 10 or 100 ppm and inoculated with *S*. Typhimurium. Faecal samples were compared with samples from similarly infected non-medicated birds. It was found that the feeding of bacitracin only slightly increased the prevalence and length of salmonella shedding.

In another study by the same authors (Smith and Tucker, 1980) chickens were challenged with five different salmonella serovars (*S.* Heidelberg*, S.* Infantis*, S.* Oranienburg, *S.* Senftenberg and *S.*Typhimurium). The percentage of re-isolation of the challenge organism from faeces and caecal contents of birds fed bacitracin (10 ppm) was compared to that of non-medicated birds until 50 days of age. The study also included a comparison of salmonella infection in medicated and non-medicated birds of four different breeds fed four different feed mixes. The results indicate that bacitracin at worst slightly favours salmonella colonisation. No remarkable differences were noted between the various bacterial strains used for challenge, or between different feed mixes and different poultry breeds. The isolation procedure used in this study includes selective culturing and

identification by visual examination only. As in the study by Humbert and co-workers, it cannot be excluded that some coliforms can be mistaken for salmonella. Since one cannot be sure that this error would be the same in all groups, there is a slight risk that this may have affected the outcome of the study.

Latour and Barnum (1981) used ducks as experimental animals. Two concentrations of in-feed bacitracin were tested, 10 and 100 ppm, and medicated birds were compared to non-medicated birds. The challenge organism was *Salmonella* Typhimurium, $10^7$-$10^{10}$ organisms per bird, and cloacal swabs were taken throughout the experiment. The results did not show a definite trend. In two experiments there was little difference between the bacitracin-medicated groups and the controls and in two other the samples from medicated birds yielded significantly ($p<0.05$) more salmonellae than the untreated animals. However, the groups were small and the variability between the control groups in the various experiments regarding re-isolation of the infecting organism make the results difficult to evaluate.

Bailey and co-workers (1988) investigated resistance to salmonella infection in broilers fed various AFA. However, all groups of animals received combinations of AFA, together with competitive exclusion microflora, and therefore the effect of a single antibiotic (in this case, bacitracin) could not be determined.

No studies investigating the effect of bacitracin on the infectious dose necessary to achieve establishment of salmonella colonisation have been found, nor any studies investigating dose-response relationships or the effect on prevalence of salmonella in animal products.

### B.7.2 Other enteric pathogens

No studies on the possible effects on colonisation by other enteric pathogens have been found.

## B.8 Toxicological aspects

Bacitracin is highly nephrotoxic when administered parenterally (1993), but as it is poorly absorbed from the gut, no adverse effects would be expected after oral administration.

### B.8.1 Adverse effects in ruminants

Bacitracin is used as a feed additive in calves, lambs and kids. Adult cattle, however, react adversely to bacitracin in the feed. Sudden milk drops, in

herds fed concentrate feed contaminated with bacitracin at the feed mill, have been reported (Woodger, 1979). Higher mortality and decreased effectiveness of antimicrobial therapy was noticed in calves fed 50 ppm bacitracin in the milkreplacement in a study by Jonson and Jacobsson (1973). It is discussed whether resistant bacteria or immunosuppression in the medicated animals were the cause, but no investigations to determine the cause were included in the article. In sheep, biotransformation of plant toxins, that occurs in the rumen of some alkaloid-resistant sheep, can be impaired by oral administration of bacitracin (Wachenheim *et al.*, 1992). No conclusions can be based on these few reports, but there seems to be cause for further investigations into the possible risks for adverse effects when using bacitracin in ruminants.

### B.8.2 Allergy

Allergic reactions to bacitracin are frequently reported (Katz and Fisher, 1987; Grandinetti and Fowler, 1990; Knowles and Shear, 1995). Both anaphylaxis, eczema, urticaria and delayed reactions may be seen (Katz and Fisher, 1987). Most reports concern patients treated with bacitracin ointment. It has been stated that bacitracin is the topical agent most commonly implicated in anaphylactic reactions (Katz and Fisher, 1987). Considering this, it is somewhat surprising that no reports have been found on allergic reactions in people who come in contact with bacitracin professionally, such as farmers, hospital personnel and people working in the pharmaceutical industry.

## B.9 Environmental effects

Like for other AFA, if bacitracin reduces the amount of feed consumed per kg weight gain in the target animal, it would also be expected to reduce the amount of nitrogen output per kg weight gain.

Very little of ingested bacitracin is absorbed and most is excreted unmetabolised in the faeces (Donoso *et al.*, 1970; Froyshov *et al.*, 1986). Gavalchin and Katz (1994) studied the degradation of bacitracin in sandy loam from a non-agricultural area, mixed with chicken faeces, at different temperatures. Sterile soil-faeces mixtures were used as controls. At 4°C, inactivation of bacitracin occurred rapidly, only 23% remained after 30 days. At 20°C, however, 33% of the initial concentration was still present after 30 days of incubation. The half life for bacitracin at 4 and 20°C was calculated to 12.5 and 22 days, respectively. The inverse temperature relation of the degradation was tentatively explained by the isolation of a psycrotrophic pseudomonad capable of degrading bacitracin from the soil-faeces matrix.

Similar results have been obtained earlier by Jagnow (1978) reporting half life in soil of 22,5 days at 20 °C and 12 days at 30°C .

Vogtmann and co-workers (1978) reported that bacitracin had no adverse effects on composting but detected some depression of plant growth when fresh manure containing bacitracin was used as a fertiliser.

## B.10 Summary comments

Bacitracin has a bactericidal effect mainly on gram-positive bacteria, by inhibiting the formation of bacterial cell wall peptidoglycan. It is used, albeit not to any large extent, in both human and animal therapy. Lately it has been increasingly used for the treatment of vancomycin-resistant enterococci in humans.

In-feed bacitracin affects the antimicrobial resistance of the intestinal microflora, mainly in *E. faecium* but possibly also in other species.

Data available on colonisation by enteric pathogens in animals fed bacitracin is too inconsistent and too scarce to form the basis of any firm conclusions about the effects of bacitracin.

Bacitracin administered at growth promoting concentrations has prophylactic and therapeutic effects on necrotic enteritis in poultry.

Allergic reactions to bacitracin are documented in humans undergoing bacitracin treatment. People who are exposed to the substance on a daily basis may be at risk of being sensitised.

Bacitracin is degraded in soil. The environmental degradation appears to be inversely related to soil temperature.

In conclusion, available information is to scarce for an assessment of the possible risks of bacitracin usage to human and animal health. Bacitracin usage does not appear to represent any substantial danger to the environment.

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# Annex C: Flavomycin

## C.1 Introduction

Flavomycin, also known as flavophospholipol, bambermycins or moenomycin, is a phosphorus-containing glycolipid, mostly obtained as complexes of very similar components. It is produced by a group of *Streptomyces spp*, including *S. bambergensis*, *S. ghanaensis*, *S. geysirensis* and *S. ederensis* . At the moment it is used for growth promoting purposes only and is not included in any therapeutic drug either in human or veterinary medicine. Flavomycin inhibits cell wall synthesis, mainly in gram-positive bacteria.

Concentrations used for growth promotion are between 1 and 20 ppm for swine, 0.5-20 ppm for poultry, 2-4 ppm for fur animals, 2-16 ppm for cattle and 2-4 ppm for rabbits.

Flavomycin is not absorbed to any great extent after oral administration. When parenterally administrated it is excreted in the urine at a very slow rate and has, therefore, a prolonged activity in blood. The antimicrobial activity is reduced by serum and is optimal at pH 5.0 - 6.5. (Huber, 1979)

## C.2 Mode of action and resistance mechanisms

Flavomycin exerts its effect by inhibiting bacterial cell wall synthesis. The transglycosylation reaction necessary for peptidoglycan synthesis, that is catalysed by the penicillin-binding protein PBP 1b, is impaired in the presence of flavomycin (van Heijenoort *et al.*, 1987, Huber, 1979 #339). In this reaction the lipid-bound N-acetyl glucosaminyl-N-acetylmuramyl-pentapeptide is transferred to the peptidoglycan.

No reports on mechanisms for resistance to flavomycin have been found. This is somewhat surprising, since investigations on resistance mechanisms and resistance genes might determine whether cross-resistance to flavomycin and other antibiotics is at all possible in any bacterial species. Hudd (1983) claimed that no cross-resistance had been found in staphylococci, but without presenting any data or information on how these results had been obtained.

Flavomycin is reportedly mainly active against gram-positive bacteria. However, several publications indicate that the compound is sometimes active against gram-negative organisms, such as *Salmonella* spp. and *Escherichia coli* (Dealy and Moeller, 1976; Dealy and Moeller, 1977b; Dealy and Moeller, 1977a; Witte, 1996). Unfortunatley, pure flavomycin for

laboratory use is not commercially available. Thus, no experiments could be performed to clarify this issue.

The figures in this report is only available in the printed version

Figure C.I. Tentative sketch of flavomycin

## C.3 Development of resistance

### C.3.1 Prospective studies

Unfortunately, and somewhat surprising, most studies where antimicrobial resistance patterns are determined in faecal bacteria, before and after the supplementation of feed with flavomycin, do not include any investigations on susceptibility to flavomycin itself in these bacteria.

A study by Dealy and Moeller (1977a) shows a significant ($p<0.001$) increase in the percentage of flavomycin-resistant *E. coli* isolated from calves fed flavomycin at growth promoting levels, as compared to non-medicated calves. Since flavomycin is active mainly against gram-positive bacteria it would be interesting to see similar data on, for instance, enterococci.

Table C.I. Prevalence of resistance to flavomycin in various bacterial species

| **Bacterial species** | **Source of isolates** | **No. Of iso-lates** | **Year** | **Resistance in %** | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *Clostridium botulinum* | various | * | 1979-82 | *0* | Dutta and Devriese, 1984 | Belgium |
| *Enterococcus faecalis* subsp *liquefaciens* | poultry | 23 | 1980 | *0* | Dutta and Devriese, 1982 | Belgium |
| *E. faecalis* | poultry | 8 | 1980 | *0* | Dutta and Devriese, 1982 | Belgium |
| *E. faecalis* | poultry products | 54 | 1995-1996 | *0* | DANMAP, 1997 | Denmark |
| *E. faecalis* | swine | 38 | 1986-1995 | *0* | Devriese and Haeseb-rouck, 1996 | Belgium |
| *E. faecalis* | swine | 225 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *E. faecalis* | pork | 38 | 1995-96 | *6* | DANMAP, 1997 | Denmark |
| *E. faecalis* | beef | 21 | 1995-96 | *19* | DANMAP, 1997 | Denmark |
| *Enterococcus spp.* | swine | 21 | 1986-1995 | *71* | Devriese and Haeseb-rouck, 1996 | Belgium |
| *Coagulase negative staphylococci* | cattle | 371 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *Staphylococcus hyicus* | swine | 71 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *Staphylococcus aureus* | cattle | 211 | 1995-96 | *0* | DANMAP, 1997 | Denmark |
| *S. aureus* | various | 792 | 1970-1980 | *0* | Devriese, 1980 | Belgium |
| *Streptococcus spp.* | swine | 19 | 1986-1995 | *21* | Devriese and Haeseb-rouck, 1996 | Belgium |

* total number of strains: *C.perfringens* = 142, *C. sporogenes* = 6, *C. botulinum* = 3

### C.3.2 Point-prevalence studies

Data on prevalence of resistance to flavomycin from various studies are compiled in table C.I.

Some bacterial species are reported to be naturally resistant to flavomycin, including *Clostridium perfringens*, *Clostridium sporogenes* and *Enterococcus faecium* (Dutta and Devriese, 1984). However, susceptible strains of *E. faecium* have been reported (DANMAP, 1997). These discrepancies could be due to methodological differences regarding breakpoints etc.

## C.4 Acquisition of resistance

No published investigations on resistance genes, transfer of resistance or resistance determinants in different bacterial hosts have been found.

### C.4.1 Influence on resistance against other antimicrobials

George and Fagerberg (1984) investigated the effect of flavomycin on plasmid-mediated antimicrobial resistance in *E.coli,* and found that flavomycin decreased the transfer frequency of some R plasmids, while it increased transfer frequency of others. It also selectively inhibited growth of bacteria harbouring some R plasmids, but not others. The reason for this suppression of bacteria carrying some R plasmids is discussed in the article and the possibility of sex pili and pilin precursor proteins in the bacterial cell wall causing increased susceptibility to flavomycin in these bacteria is suggested. This theory is supported by the fact that some *E. coli* harbouring plasmids depressed for pili synthesis were not suppressed by flavomycin. If this is really the case, it would mean that flavomycin may have a suppressing effect on the spread of certain resistance plasmids. However, it would not affect transfer of resistance by transduction or transformation, nor would it decrease the spread of transposons carrying resistance genes.

A similar investigation, with similar conclusions was conducted by Sepulchre (1979). This study also includes *in vivo* experiments on effects of flavomycin in the feed on resistance in enteric microflora in pigs. However, the decrease in resistance observed in bacteria isolated from medicated pigs was also seen in the non-medicated control group, so the actual effect of flavomycin could not be determined.

Another study, by Pohl (1975), also showed decreased transfer frequency of some, but not all, R plasmids between *E.coli* strains in the presence of flavomycin.

Brophy (1988) conducted an *in vivo* study on the effect of in-feed flavomycin on prevalence of R plasmid-carrying *E.coli* in faecal samples from calves. In this experiment the introduction of flavomycin into the diet brought about a marked increase in the incidence of R plasmid-carrying *E. coli* and the number of these organisms isolated from the treated group was at any time during the trial notably higher than from the non-medicated control group. However, the results indicate that the incidence was higher in the treated group already at the beginning of the experiment. If this is correct, comparing the two groups without adjusting for the initial differences may be misleading.

Corpet (1984) used a mouse model to study changes in chlortetracycline resistance of faecal *E. coli* after supplementing the drinking water with flavomycin. The percentage of chlortetracycline-resistant isolates was lower in the flavomycin-fed group than in the control group. It is doubtful, though, whether this mouse model is practically applicable for food producing animals.

The proposed reason for flavomycin's limited action against gram-negative bacteria is that the antibiotic cannot penetrate the outer membane (Huber, 1979). If the presence of R-plasmids in some gram-negative organisms causes alteration of the cell wall surface, this may facilitate the uptake of flavomycin into the cell and thereby render these cells susceptible to flavomycin.

## C.5 Effects on specific animal diseases

The fact that all *C. perfringens* seem to be flavomycin-resistant may cause some concern regarding necrotic enteritis in poultry. Stutz and Lawton (1984) fed flavomycin at 55 ppm to chickens whithout noticing any increase of *C. perfringens* in ileal contents. Brenes et al (1989) conducted a similar experiment with similar results. However, 55 ppm is more than twice as much as the maximum dose used for growth promotion in chickens and would therefore not quite correspond to the real-life situation. No published studies evaluating the risk of increasing necrotic enteritis by feeding flavomycin at lower levels have been found.

## C.6 Impact of resistance on animal and human health

No flavomycin-related substances are used in either human or aimal therapy. Thus, at present no adverse effects would be expected from an increased resistance to flavomycin among human and animal bacteria.

Several other phosphorus-containing glycolipids with similar chemical properties and antibacterial spectra have been described (Huber and Nesemann, 1968; Meyers *et al.*, 1968; Huber, 1979). Some of these are prasinomycin, diumycin (macarbomycin), 11837 R.P, quebemycin, 19402 R.P., ensanchomycin, prenomycin and pholipomycin. As these substances are all highly active against gram-positive organisms, have extremely low toxicity and a prolonged acticity in blood after parenteral administration, it is remarkable that they have not yet been used for therapeutic purposes. In the present situation, with increasing antimicrobial resistance in both pathogenic and opportunistic bacteria, these substances would appear to be a welcome addition to the therapeutic arsenal.

## C.7 Other effects on the microflora

### C.7.1 Salmonella

A few studies on the effects of in-feed flavomycin on *Salmonella* colonisation have been published (Smith and Tucker, 1975; Dealy and Moeller, 1976; Dealy and Moeller, 1977b; George *et al.*, 1982; Humbert *et al.*, 1991).

Dealy and Moeller (1976; 1977b) investigated the shedding of *Salmonella* Typhimurium in experimentally infected calves and pigs. Animals given flavomycin at growth promoting levels were compared to non-medicated animals. The results indicate that the use of flavomycin reduced the duration and prevalence of *Salmonella* shedding in both pigs and calves. However, the experimental groups are rather small and, oddly enough, the challenge strain of *S.* Typhimurium used in the trials was susceptible to flavomycin and did not develop resistance during the experimental period. Since there are no reports on the regular susceptibility of *Salmonella* spp. to flavomycin, it cannot be determined whether this is unusual or not. If the infecting strain is sensitive to flavomycin, feeding this drug would be expected to reduce shedding. This might be regarded as therapy and not growth promotion, though.

In the study by Humbert and co-workers (1991), flavomycin was given to one of the experimental groups. In this study, chicks were challenged with *S.* Typhimurium and re-isolation of the organism was compared between medicated and non-medicated birds. The medicated groups included some feed additives, either in combination with or without competitive exclusion (CE) microflora. The results were very variable and the authors concluded that, due to interaction between the CE treatment and the feed additives, it was not possible to identify any antibiotic effect. Some objections may be

given as to the design of this study, regarding culturing methods, presentation of the results and length of study period (see annex B).

George and co-workers (1982) studied the effects of in-feed flavomycin on incidence, shedding, and antimicrobial resistance pattern of *S.* Typhimurium in experimentally infected chickens. The authors concluded that flavomycin had no effect on body weight, duration of salmonella shedding, number of salmonellae shed, tissue recoverability and total number of resistance patterns. Resistance to flavomycin was not tested.

Smith and Tucker (1975) also compared flavomycin-fed chickens to non-medicated chickens after experimental infection with *S.* Typhimurium and found a slight increase in salmonella shedding in medicated birds. This study includes the same selective technique for re-isolating the salmonellae as the study by Humbert and co-workers, where selective culture and visual examination only is used to identify salmonellae, which may lead to coliforms being mistaken for salmonellae.

### C.7.2 Other enteric pathogens

No studies regarding the effects of in-feed flavomycin on other enteric pathogens have been found.

## C.8 Toxicological aspects

The toxicity of flavomycin is very low, even after intravenous administration (Huber, 1979), and is only absorbed from the gut in small quantities (Sambeth *et al.*, 1974).Therefore, no toxic effects would be expected in either animals or humans due to the usage of flavomycin in animal feed.

### C.8.1 Allergy

Frese and Blobel (1973) studied the antigenicity of flavomycin in rabbits and found that neither oral administration nor subcutaneous injection of the substance produced any antibody responses or anaphylactic reactions. This is the only published report on flavomycin in association with allergy. It is hard to say whether data obtained from rabbit experiments are applicable to people exposed to the substance.

## C.9 Environmental effects

Like other AFA, if flavomycin reduces the amount of feed consumed per kg weight gain in the target animal, it would also be expected to reduce the amount of nitrogen output per kg weight gain.

Flavomycin shows a high degree of stability through the procedure of pelleting feed (Waals, 1973), which would suggest heat stability. The substance is degraded in soil and manure, but at a slow rate (Jagnow, 1978). According to Jagnow (1978), it takes 17 weeks for complete aerobic degradation of 10 ppm flavomycin in fresh manure, and 2 weeks in a soil mixture. Galvachin and Katz (1994) found that at 20°C or more, flavomycin, in a soil-faeces mixture, was degraded within 25 days. At 4 °C little or no degradation occured during the study period, which was 1 month. However, flavomycin does not seem to be a problem as far as environmental residues are concerned except for, possibly, areas with a constant temperature below 4°C.

## C.10 Summary comments

Flavomycin appears to be a very attractive substance for therapy, as it is fairly atoxic and has good pharmacokinetic properties. However, if its use for growth promoting purposes causes increased resistance among animal bacteria, both flavomycin and related substances may be rendered useless for therapy in animal and human medicine. In general, very little information about flavomycin is available. As the substance has been in use for more than 20 years it is remarkable that so few investigations have been published on resistance in various bacterial species, various animal species and in different geographical regions. For substances not used in therapy, such investigations are essental, since resistance will not be noticed in clinical practice.

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# Annex D: Ardacin and avoparcin

## D.1 Introduction

Ardacin and avoparcin are glycopeptide antibiotics. The glycopeptides are large molecules produced by a variety of bacterial genera including *Streptomyces*, *Actinoplanes, Nocardia* and *Kibdelosporangium*. Avoparcin and ardacin are both compounds of two or more substances with similar molecular structure.

Chemically, the glycopeptides all have a common core and differ in the four side chains (figure D.I). The basic peptide structure possesses a nucleus of seven amino acid residues and five amino acids. Sugars and amino sugars, linked to the core structure, are mainly located on the outside of the molecule. They do not markedly affect the antimicrobial activity, but give the substances different pharmacokinetic properties (Reynolds, 1990).

Glycopeptides are active against gram-positive bacteria such as staphylococci, streptococci, enterococci, corynebacteria, clostridia and *Listeria* spp. Presently known glycopeptides are not active against gram-negative bacteria because the antibiotic molecules are unable to pass through the outer membrane and hence cannot reach their target (Reynolds, 1990).

Many glycopeptides, including avoparcin, are poorly or not at all absorbed from the gastrointestinal tract (Zulalian *et al.*, 1979; Hudd *et al.*, 1983).

Avoparcin is, in accordance with Directive 97/6/EC, presently not approved in the EU. Similarly, ardacin has been approved for growth promotion in annex II of Directive 70/524/EEC according to Directive 94/77/EEC, but there are indications that the authorisation will not be prolonged.

Vancomycin and teicoplanin (formerly teichomycin) are well known substances that are used therapeutically in human medicine for the treatment of severe infections caused by gram-positive bacteria.

## D.2 Mode of action and resistance mechanisms

The bacterial cell wall is composed of a three dimensional web of cross-linked peptidoglycans. Precursors for this web are transported to the outer surface of the cell membrane on a lipid carrier. The assembly of precursors by cross linking takes place on the outer surface of the cell membrane. At this step, glycopeptides inhibit cell wall formation by binding to peptide stems of the precursor ending with D-alanine-D-alanine (D-Ala-D-Ala). The

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Figure D.I. Tentative sketch of ardacin, avoparcin, vancomycin and teicoplanin

The figures in this report is only available in the printed version

Figure D.I continued

binding is the result of five hydrogen interactions between D-Ala-D-Ala and the backbone structure of the glycopeptide. This results in the accumulation of precursors inside the cell and an arrested peptidoglycan assembly, eventually leading to the death of the bacterial cell. As the mode of action of glycopeptides derives from the core structure of the molecule, it can be assumed to be basically the same for all substances within the group (Arthur *et al.*, 1996).

Bacteria producing peptidoglycan precursors not ending in D-Ala-D-Ala will be partially or completely resistant to glycopeptides. The intrinsic resistance to high levels of vancomycin and teicoplanin seen in most *Lactobacillus* spp is due to the production of precursors ending in D-Ala-D-lactate (D-Lac) to which glycopeptides will not bind.

Enterococci belonging to the *Enterococcus gallinarium* group are resistant to low levels of vancomycin but sensitive to teicoplanin (VanC phenotype). These enterococci produce peptidoglycan precursors where D-Ala is substituted by D-serine (D-Ser). Vancomycin, but not teicoplanin, have a lower affinity for precursors ending in D-Ser (Arthur *et al.*, 1996).

In enterococci with acquired resistance, two main phenotypes have been described. Both these resistance phenotypes (VanA and VanB, see D.4) are due to the production of peptidoglycan precursors ending in D-Ala-D-Lac in stead of the normally produced D-Ala-D-Ala. As a result of this substitution, the complex between glycopeptides and the precursors will not be formed. This will allow the bacteria to grow in presence of the antibiotic. Recently, a phenotype designated VanD has been described in a strain of *E. faecium* (Perichon *et al.*, 1997). The described strain was constitutively resistant to vancomycin and to low levels of teicoplanin. Apparently, this phenotype is also the result of the production of peptidoglycan precursors ending in D-lactate.

## D.3 Development of resistance

***Experimental studies***

In a study by Walton (1978), the effects of avoparcin on resistance in faecal streptococci (enterococci) and staphylococci was investigated. Avoparcin was fed to chickens at dosages of 10 and 100 ppm and birds on a non-supplemented feed were used as control group. In all groups, there was a wide variability in the total viable count of bacteria as well as in the proportion of resistant strains. Avoparcin-resistant enterococci and staphylococci were isolated from both avoparcin-fed birds and from the control group. Similarly, vancomycin-resistant strains were detected in all groups. The author stated that there was no evidence of cross-resistance

between avoparcin and other antibiotics tested (vancomycin, among others). The results are not presented in a form that makes it possible to substantiate this claim. No species identification of resistant isolates was performed.

Kaukas and co-workers (1988), monitored the effect of several antibiotics given at growth promoting dosages. Avoparcin was fed at 20 ppm to small groups of chickens and the results were compared to those from birds given non-supplemented feed. The incidence of resistance to therapeutic antibiotics, expressed as the Antibiotic Resistance Index (ARI), was higher ($p=0.003$) in all groups receiving antibacterials, as compared to the control group. This increase could be associated with an increase in the proportion of *E. faecium* in the enterococcal population of the treated birds. The proportion of strains resistant to avoparcin (defined as having a minimum inhibitory concentration > 4µg/ml) in *E. faecium* and *E. faecalis* was higher in the control group than in the avoparcin group. The authors commented that avoparcin resistance was an overall common finding also in the other experimental groups (22-52% in birds given different antibacterial feed additives and 42% in control birds).

### *Cohort studies*

An association between the use of avoparcin and prevalence of vancomycin resistant enterococci (VRE) in animals has been reported (Aarestrup, 1995; Klare *et al.*, 1995a; Kruse, 1995; van den Bogaard *et al.*, 1996). In studies from USA where avoparcin has never been used, and from Sweden where avoparcin has not been used for 10 years, no VRE were found in samples from animals when selective techniques were used (Coque *et al.*, 1996; Greko, 1996). Thus, in the absence of avoparcin, the prevalence of VRE in animals is, at most, very low.

Bager and co-workers (1997) investigated poultry and pig farms in Denmark in a retrospective cohort study. The relative risk for occurrence of high level vancomycin resistance in *E. faecium* was 3.3 (0.9-12.3) for pig herds exposed to avoparcin. The corresponding figure for poultry flocks was 2.9 (1.4-5.9).

Taken together, there is strong evidence of a causal relationship between avoparcin use and occurrence of high level vancomycin resistance.

### *Point prevalence studies*

Shortly after the introduction of avoparcin, no glycopeptide resistance was found among 15 strains of *E. faecium* isolated on vancomycin-free media (Dutta and Devriese, 1982), nor was resistance found in other enterococcal species. In table D.I, results concerning prevalence of glycopeptide resistance from Denmark and Sweden are presented. The results in these studies were

obtained without the use of antibiotic containing media in the course of monitoring studies.

Studies using media favouring resistant isolates indicate that enterococci with high level resistance to glycopeptides are widespread among animals, including pets and horses (Bates *et al.*, 1994; Klare *et al.*, 1995b; Devriese *et al.*, 1996).

The lack of earlier data on VRE in animals precludes conclusions on whether the resistance trait was present in animal populations at the time of introduction of avoparcin in animal husbandry.

Table D.I Prevalence of glycopeptide resistance

| **Bacterial species** | **Animal source** | **No. of isolates or samples** | **Year** | **Resistance in %** | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *E. faecium* | cattle | 13 | 1995-96 | 0 | DANMAP, 1997 | Denmark |
| *E. faecium* | poultry | 54 | 1995-96 | 59 | DANMAP, 1997 | Denmark |
| *E. faecium* | swine | 58 | 1995-96 | 20 | DANMAP, 1997 | Denmark |
| *E. faecalis* | cattle | 35 | 1995-96 | 0 | DANMAP, 1997 | Denmark |
| *E. faecalis* | poultry | 225 | 1995-96 | 29 | DANMAP, 1997 | Denmark |
| *Enterococcus* spp. | swine | 46 | 1995 | 0 | Greko, 1996 | Sweden |
| *Enterococcus* spp. | poultry | 60 | 1995 | 0 | Greko, 1996 | Sweden |
| *Enterococcus* spp. | swine | 218 | 1996 | 0 | Greko, 1997 | Sweden |
| *Enterococcus* spp. | poultry | 207 | 1996 | 0 | Greko, 1997 | Sweden |

## D.4 Resistance genes and gene transfer

Resistance to high levels of vancomycin, teicoplanin, avoparcin and, presumably, ardacin (the VanA phenotype) in enterococci is mediated by a cluster of genes designated the *van*A-gene cluster (Arthur and Courvalin, 1993; Arthur *et al.*, 1996). A dissociated resistance phenotype with various levels of vancomycin resistance and sensitivity to teicoplanin characterises the VanB-type of resistance encoded for by the *van*B-gene cluster (Arthur *et*

*al.*, 1996). The recently described VanD phenotype is encoded by a gene cluster designated *van*D (Perichon *et al.*, 1997).

As shown in table D.II, there is a high degree of similarity between the main mechanisms mediating the VanA and VanB phenotypes. The explanation for the difference in teicoplanin susceptibility between these phenotypes is likely to be derived from differences between the regulatory products vanS-vanR and $vanS_B$-$vanR_B$ (Arthur *et al.*, 1996).

Table D.II. Genes encoded for by Tn*1546* (the *van*A-gene cluster) and Tn*1547* (the *van*B-gene cluster), their products and main functions (based on information from Arthur *et al.*, 1996)

| **Genes in cluster** | | **Product type of respective gene** | **Function of respective gene** | **Amino acid identity between homologous** |
|---|---|---|---|---|
| **VanA** | **VanB** | | | **products (%)** |
| *van*H | *van*$H_B$ | dehydrogenase | Formation of D-Lac from pyruvate | 67 |
| *van*A | *van*B | ligase | Binding between D-Ala and D-Lac | 76 |
| *van*X | *van*$X_B$ | dipeptidase | Hydrolysis of D-Ala-D-Ala | 71 |
| *van*Y | *van*$Y_B$ | carboxypeptidase | Hydrolysis of terminal D-Ala | 30 |
| *van*Z | - | unknown | Confers teicoplanin resistance by unknown mechanism | - |
| *van*R | *van*$R_B$ | | Initiation of transcription of vanH, vanX and vanA/B | 34 |
| *van*S | *van*$S_B$ | | Regulation of *van*R | 23 |
| - | *van*W | | Unknown function | - |
| ORF1 | - | | Open reading frame, transposase | |
| ORF2 | - | | Open reading frame, resolvase | |

Production of VanH, VanA and VanX (VanHAX), encoded for by the genes in the operon *van*HAX, is normally inducible. This means that they are

only produced in the presence of a suitable inducer such as a glycopeptide. Synthesis of VanHAX is thought to be activated (induced) by the phosphorylated form of VanR (the product of the gene *van*R). Recent evidence indicate that VanS, encoded for by the *van*S gene, controls the effect of VanR negatively by dephosphorylation in the absence of glycopeptides. VanS is likely to have a domain acting as a membrane associated sensor. Elimination of the VanS gene results in high-level constitutive activation of the *van*HAX operon (Arthur *et al.*, 1997). VanA-type resistance is induced by vancomycin, teicoplanin and apparently also by avoparcin (Klare *et al.*, 1995b). The ability of ardacin to induce VanA-type resistance has not been investigated. However, considering that the molecular structure of ardacin is partly similar to both vancomycin and teicoplanin, there is no indication that it would not be an inducer.

Conflicting data about the inducing effects of other cell wall active antibiotics such as moenomycin (flavomycin), bacitracin, daptomycin, penicillin, cephalotin have been presented (Allen and Hobbs, 1995). Recent evidence indicate that apart from glycopeptides, only flavophospholipol (moenomycin/flavomycin) has the capacity to act as an inducer (Baptista *et al.*, 1996).

The regulatory system for production of $VanH_B$, VanB and $VanX_B$ in strains with VanB-type resistance is similar to the above described. However, the regulatory system $VanR_B$- $VanS_B$ is only activated by vancomycin, explaining the phenotypic susceptibility of VanB strains to teicoplanin (Evers and Courvalin, 1996). Teicoplanin resistant derivatives of VanB type strains have been reported (Hayden *et al.*, 1993; Green *et al.*, 1995). These isolates are likely to be the result of spontaneous mutations altering the specificity of the $VanS_B$ sensor domain.

Both the *van*A and *van*B gene clusters are generally located on plasmids and/or transposons (Arthur and Courvalin, 1993). High level resistance to glycopeptides mediated by the *van*A-gene cluster has been detected in *E. faecium*, other enterococcal species (Arthur and Courvalin, 1993), *Oerskovia turbata* and *Archanobacterium haemolyticum* (Power *et al.*, 1995). The gene cluster is mostly associated with the conjugative transposon Tn*1546* and/or self-transferable plasmids (Arthur *et al.*, 1996). Transfer of the *van*A-gene cluster has been shown *in vitro* from *E. faecium* to *Listeria monocytogenes*, *Staphylococcus aureus*, and to various streptococci (Leclercq *et al.*, 1989). Transfer frequencies were $10^{-4}$ for transfer between different strains of *E. faecium* and $10^{-6}$ - $10^{-9}$ for transfer from *E. faecium* to other species.

Resistance to glycopeptides mediated by the *van*B-gene cluster has, with respect to avoparcin and ardacin, attracted less attention. The *van*B-gene cluster is transferable either directly from the chromosome by a transposon (Tn1547) or through plasmids (Quintiliani and Courvalin, 1994; Woodford *et al.*, 1995b; Quintiliani and Courvalin, 1996) at a low frequency. The *van*B-

gene cluster has been found in *E. faecalis*, *E. faecium* and, recently, in *S. bovis* (Arthur and Courvalin, 1993; Poyart *et al.*, 1997). As mentioned, the *van*B-gene cluster is induced by vancomycin but not by teicoplanin, meaning that when strains carrying the gene cluster are exposed to teicoplanin, the gene will not be activated and the strain phenotype will remain susceptible (Arthur and Courvalin, 1993). According to available information, the *van*B-gene cluster does not seem to be inducible by avoparcin. No information is available on the capacity of ardacin to induce *van*B.

Selection of mutants expressing the *van*B-gene constitutively have been reported both from *in vitro* studies as well as in clinical isolates (Hayden *et al.*, 1993; Green *et al.*, 1995). Recently, transfer experiments with strains expressing *van*B constitutively were reported (Hayden *et al.*, 1997). The resulting transconjugants were either of constitutive or of inducible type. The use of a non-inducing antibacterial such as avoparcin, and possibly ardacin, could favour strains harbouring *van*B-gene clusters with the mutation required for the gene to be constitutively expressed should the gene be present in animal populations or their environments. Further information is needed on this topic.

VRE harbouring the *van*A gene cluster, have been isolated from humans, both in hospitals and community, from swine, rabbits, dogs, cats, horses chickens, turkeys, pheasants, ducks, foods of animal origin and sewage (Bates *et al.*, 1994; Torres *et al.*, 1994; Klare *et al.*, 1995b; Chadwick *et al.*, 1996; Devriese *et al.*, 1996; DANMAP, 1997). A polyclonal nature of the VRE strains has been demonstrated (Klare *et al.*, 1995b). As shown in table D.II, the *van*A gene cluster consists of 7 gene components. It is extremely unlikely that such a complicated gene should have developed separately in so many different host populations. Its occurrence therefore suggests an interspecies spread.

Human VRE have successfully been used to colonise mice experimentally (Whitman *et al.*, 1996). This indicates that at least certain enterococcal strains can colonise, or transiently inhabit, a variety of hosts. A report on occupational exposure provides further evidence (van den Bogaard *et al.*, 1997). The prevalence of VRE in turkeys, turkey farmers, turkey slaughterers and urban residents was found to be 50%, 39%, 20% and 14% respectively. Further investigations showed that VRE isolated from one of the farmers and his turkeys could not be differentiated by phenotypic or genotypic (pulsed-field gel electrophoresis) methods. Investigations of the *van*A-gene cluster by polymerase chain reaction (PCR) and hybridisation showed the two strains to be identical in the tested areas, having an insertion in a not previously described position, between the *van*X and *van*Y gene, and a deletion in the right end of the cluster.

The question of ”identity” of genes has been a matter of debate in relation to the possible effects of the use of avoparcin in animal husbandry. The *van*A

gene cluster contains 9 genes (7 *van* and two transposition genes). Between those genes are intergenic, non-coding regions. The coding regions would be expected to be highly conserved once their sequences are optimal for function. As the intergenic regions are not essential for the function of the gene cluster, they are more likely to vary. Three recent studies have addressed the matter by amplification by polymerase chain reaction (PCR) and sequencing of the genes and/or their intergenic regions (Jensen, 1996; Haaheim *et al.*, 1997; Kirk *et al.*, 1997).

In the Norwegian study (Haaheim *et al.*, 1997), PCR for the *van*A and *van*B genes combined with restriction fragment analysis of a long PCR covering the entire gene cluster and sequencing of the intergenic *van*S-*van*H region were used to analyse the vanA gene cluster from VRE of Norwegian poultry and humans of various nationality (Swedish, Norwegian and American). In 9/12 human isolates and 7/10 poultry the results were identical, indicating horizontal transfer of the gene cluster.

Kirk and co-workers (1997), investigated 37 VRE isolates from one UK hospital and 36 VRE isolates from poultry meat bought in national supermarkets. By PCR, three intergenic regions of the *van*A-cluster were investigated (*van*S-*van*H, *van*X-*van*Y and *van*Y-*van*Z). The presence of *van*X, *van*Y and *van*Z was also determined. In the chicken isolates, all three investigated genes were amplified as well as the three intergenic regions. Evidence of a not previously described insertion sequence in various locations of the intergenic region between *van*X and *van*Y was found in some of the chicken isolates. The gene sequences of the strains from humans obviously differed from those of other described gene clusters from human strains (Arthur *et al.*, 1993; Handwerger *et al.*, 1995), as the *van*Y region as well as the intergenic regions *van*X-*van*Y and *van*Y-*van*Z failed to amplify with the primers used. Only the regions *van*S-*van*H, and the genes *van*X and *van*Z were amplified, indicating the presence of the *van*X and *van*Z genes but not the *van*Y gene. These results may be caused by an insertion or a deletion in the primer binding site for the *van*Y gene. In a recent publication by Mackinnon and co-workers (1997), a novel insertion sequence element designated IS*1476,* located within the *van*Y gene, was reported in one out of 124 clinical isolates, indicating the occasional finding of such variations. The fact that all the gene clusters from human strains in the study by Kirk and co-workers (1997) apparently had a deletion and/or insertion in the *van*Y region seems to indicate a horizontal spread of the gene within the hospital. Therefore, it is questionable whether the investigated strains can be deemed representative for human strains in general. The authors conclusion was that the results indicate that the infections with VRE in humans may not be caused by VRE from chickens. Considering the indications of a horizontal spread of the gene cluster among the human strains in this particular material and the variation between the genes of the chicken strains, indicating a

multiple origin, this study does not seem suitable for any general conclusions about the relation between human and animal strains.

Another report, cited above, of comparisons between the *van*A-clusters of different strains indicates the transmission between animal and man (van den Bogaard *et al.*, 1997). VRE isolated from a farmer and his turkeys could not be differentiated by phenotypic or genotypic methods. The *van*A gene clusters also appeared to be identical, having an insertion between the *van*X and *van*Y gene, and a deletion in the right end of the cluster.

The focus of a Danish study, reported by Jensen (1996), was slightly different. In order to investigate the degree of variation within the *van*A gene cluster, isolates from different animals and humans from a wider geographic range were investigated. Similar to Kirk and co-workers, Jensen found evidence of an insertion sequence in the *van*X-*van*Y region in 7 of 12 British human isolates. Based on sequencing of coding and non-coding regions, the remaining isolates could be divided into 3 groups, each containing isolates both from man and animals from different countries. The designation of one of the groups was based on the presence of a point mutation in *van*X and an insertion sequence (IS*1216V*) in a specific position in the transposon (Tn*1546*). The group contained isolates from humans (Denmark and USA) and pigs (Denmark and UK). Mutations within the coding regions appear to be rare. Insertion sequences are highly mobile, and frequently vary in their location. The occurrence of an insertion sequence in the same location in strains from different origins could either be interpreted as evidence of an epidemiological relationship, or as the site being a ”hot spot” for insertion of the specific sequence. However, the likelihood of both a point mutation and an insertion in a specific location occurring independently in different strains is extremely low. Therefore, the genes present in those isolates must be very closely related and their presence the result of horizontal transfer.

### *Co-transfer of genes*

As mentioned, transfer of the *van*A-gene cluster has been shown *in vitro* from *E. faecium* to *L. monocytogenes*, *S. aureus*, and various Streptococci (Leclercq *et al.*, 1989). Transfer frequencies were $10^{-4}$ for *E. faecium* to *E. faecium* and $10^{-6}$- $10^{-9}$ for transfer to other species. When resistance to MLS antibiotics was also present, the two traits were transferred *en bloc* (Leclercq *et al.*, 1989). Conjugal co-transfer of resistance to high levels of glycopeptides, erythromycin and chloramphenicol, from *E. faecalis* to *S. aureus* on the skin of hairless obese mice was demonstrated in an experiment by Noble and co-workers (1992). The mice used cannot be regarded as ”normal” mice. Nonetheless, they are a better model for the *in vivo* situation than a petri dish.

The localisation of, for instance, MLS resistance determinants on mobile gene elements together with glycopeptide resistance means that selective pressures other that glycopeptides (e.g. macrolides) can result in increased or maintained resistance levels, and vice versa.

## D.5 Effects on specific animal diseases

Glycopeptides are not used for therapy of animal diseases, although avoparcin can be used to prevent necrotic enteritis in chickens. Using a model with experimental infections, Prescott (1979) showed that inclusion of avoparcin at 20 ppm in the feed prevented necrotic enteritis, but 10 ppm was only marginally effective. Elwinger and co-workers (1996) showed that 15 ppm of in-feed avoparcin significantly lowered the caecal counts of *Clostridium perfringens* as compared to control birds given feed without additives.

## D.6 Impact of resistance on animal and human health

Glycopeptides are used in human medicine for the treatment of infections (especially nosocomial) with multiresistant enterococci and staphylococci. Glycopeptides are also used to treat certain intestinal infections. Enterococci have a remarkable capacity for acquiring resistance to antimicrobial substances (Murray, 1990; Leclerq, 1997). In many instances, vancomycin is the sole drug with activity against these infectious agents.

Infections with bacteria resistant to vancomycin and/or teicoplanin have been reported with increasing frequency in human medicine. VRE are emerging as a significant cause of hospital acquired infections (HICPAC, 1995; Woodford *et al.*, 1995a). Infections with VRE are associated with increased mortality (Linden *et al.*, 1996). A further threat is the possible spread of vancomycin resistance genes from their enterococcal hosts to multiresistant staphylococci. Although transfer of the *van*A gene cluster from enterococci to *Staphylococcus aureus* on the skin of mice in an *in vivo* model has been reported (Noble *et al.*, 1992), no such resistance has yet been reported in clinical isolates of staphylococci.

Multiple factors predispose a person to infection with VRE, but colonisation precedes most infections (Edmond *et al.*, 1995). In Europe, a community reservoir has been demonstrated. The reported rates of VRE carriage in non-hospitalised humans range from 2 to 10 % (Jordens *et al.*, 1994; Gordts *et al.*, 1995; van den Bogaard *et al.*, 1996). Whether this colonisation is transient or persistent is not known.

According to the findings of Whitman (1996), administration of glycopeptides seems to be an important factor in establishing a persistent infection. Van der Auwera (1996) studied the effects of administration of oral glycopeptides to healthy volunteers. Before exposure, no VRE were recovered from the faecal samples whereas after exposure, 64% of the volunteers gave VRE positive samples. It is not clear whether these findings were due to a pre-exposure colonisation below the detection level of the methods used or to acquisition of resistant strains following the administration of the drug. Administration of other antimicrobial drugs may also alter the intestinal microflora, thereby predisposing for colonisation.

The occurrence of VRE in food of animal origin has been demonstrated (Bates *et al.*, 1994; Aarestrup, 1995; Klare *et al.*, 1995a; Wegener *et al.*, 1997). Once VRE-containing foods have been introduced into households or hospitals, numerous occasions for transfer of the resistance genes are available. Human enterococci may acquire resistance genes from animal enterococci in the environment (i.e. on towels, cutting boards, fittings of hygiene facilities etc.). Lax enforcement of hygienic rules provide opportunities for recontamination of heat treated foods. *E. faecium*, the principal bacterial host of the *van*A-gene cluster, has a high capacity for surviving heat treatment. Panagea and Chadwick (1996) showed that several isolates of *E. faecium* had the capacity to survive exposure to +65°C for 10 minutes. Thus, food-borne transmission is possible even without recontamination if food is not heated to higher temperatures for longer times. Transmission of VRE from animals to man may also occur through direct contact, as indicated by the findings of van den Bogaard and co-workers (1997, see E.4).

Numerous reports are available on the spread of VRE between patients and hospital staff (for a review see Woodford *et al.*, 1995a). Clearly, introduction into the ward from a community reservoir or through contaminated food may lead to an increased number of colonised patients, and consequently to an increased number of infections.

## D.7 Other effects on the microflora

### D.7.1 Salmonella

Of all AFA, avoparcin is probably the best studied regarding the possible influence on salmonella colonisation. Almost half of the studies discussed in chapter 4 include avoparcin. On the other hand, no studies have been found on the effect of ardacin on salmonella colonisation. All studies use chickens as experimental animals.

Already in 1978, Smith and Tucker (1978) demonstrated an increase in the prevalence and duration of salmonella shedding among chickens fed 10 ppm avoparcin, as compared to that of nonmedicated birds, both after direct and indirect experimental infection with *Salmonella* Typhimurium. The animals were either directly infected with 03 ml of a nalidixic acid-resistant strain of *S.* Typhimurium at a concentration of $10^9$ CFU/ml, or through contact with experimentally infected chickens. Faecal samples were cultured on selective media containing nalidixic acid. However, this method also allows the growth of mutant (i.e. nalidixic acid-resistant) lactose-negative enterobacteria, and one has to bear in mind that the proportion of colonies possibly mistaken for salmonellae may not be the same in all experimental groups.

In another, similarly designed, study by the same authors (Smith and Tucker, 1980), the earlier results were confirmed. In this study, 5 different serovars were used (*S.* Typhimurium, *S.* Heidelberg, *S.* Oranienburg, *S.* Infantis and *S.* Senftenberg). Three different poultry breeds and 3 different commercial diets were also tested. All experiments gave similar results; feeding avoparcin at a concentration of 10 ppm led to a higher prevalence and a longer duration of salmonella shedding. Different inoculation dosages were also tested. It was demonstrated that, in the medicated animals, the inoculation dose required to achieve colonisation was 10-fold smaller than for the controls. This is the only study that has been found to deal with the effects on infectious dose, but it clearly demonstrates that avoparcin may increase the risk of a flock becoming infected when exposed to low doses of salmonella organisms.

In his thesis from 1981, Leuchtenberger (1981) compared the salmonella excretion of experimentally infected broilers treated with avoparcin, virginiamycin or tylosin with nonmedicated controls. In a series of experiments, 2 different concentrations of antibiotic (15 and 25 ppm avoparcin), 2 types of housing (wired cages and floor housing), 2 methods of experimental infection (direct oral inoculation or mixed in the feed) and 2 different infectious doses ($10^3$ or $10^4$ organisms of *S.* Typhimurium) were tried in various combinations. Most experiments were performed in duplicate, with groups of 20-30 animals. Sampling was performed on several occasions throughout the trials, by cloacal swabs from live birds and from the heart, liver, duodenum and caecum of killed birds at the end of the experiment. Not all birds were sampled on all occasions, though. The author concluded that feeding avoparcin, virginiamycin or tylosin, at both levels tested, can prolong the persistence of *S.* Typhimurium infection in the intestine as well as internal organs, and significantly increases the amount of salmonella found in samples from these sites. The author also stated that the duration and frequency of salmonella excretion depends on the dose and way

of infection, as well as the frequency of dosing with infectious organisms, and on the housing system.

It is rather surprising that so many of the trials yielded significant differences between treated and non-treated groups, since the sample sizes are invariably too small for anything but large differences to be detected. However it is only in the main experiments, where cloacal swabs were taken continuously, that large enough numerical differences were recorded to be interpreted as a strong tendency towards increased salmonella excretion in treated birds, compared to non-treated controls. The trials where all birds were killed and cultures made from internal organs must be regarded as inconclusive, due to too small sample sizes and too variable numerical differences in the results.

In 1981 Gustafson and co-workers published a study where chickens fed avoparcin, virginiamycin or no antimicrobial were compared after receiving *S.* Typhimurium via the drinking water (Gustafson *et al.*, 1981). The authors tried to achieve a level of infection that corresponds to the level of natural infection and the administration of the salmonellae was distributed over several days. The results indicate that the feeding of avoparcin led to a larger proportion of animals shedding salmonella as compared to controls during the first 3 weeks after infection. This proportion of positive birds then decreased to become lower than that in the control group at about 4 weeks post infection. However, in the samples taken from the caeca after slaughtering the birds at the end of the trial, the proportion of salmonella-positive birds was higher in the avoparcin-fed group than in the control group. The sample size in this study is comparatively large, 100 animals in each group (all animals were sampled on each sampling occasion). The bacteriological methods include selective culture on media containing nalidixic acid, as the experimental strain was resistant to nalidixic acid. No further identification of the presumed salmonellae isolated in this way is reported in the article. One cannot be certain that the proportion of coliforms mistakenly identified as salmonellae would be the same in all experimental groups. The major objection against the design of this study, however, is that all birds also received 100 ppm monensin. Since monensin has antibacterial effects, this must be regarded as a possible confounder. Thus, the study only investigated the differences between birds fed monensin and birds fed monensin plus avoparcin or virginiamycin.

Another study by Gustafson and co-workers (Gustafson, 1983) reported no significant difference between avoparcin-fed birds and control birds, regarding salmonella shedding after experimental infection. On the other hand the prevalence among birds raised on clean litter was significantly higher that that among birds raised on used litter.

Linton and co-workers (1985) and Hinton and co-workers (1986) reported two salmonella studies that include avoparcin. The birds were naturally

infected via contaminated food, and all animals, except for one control group in one experiment, also received monensin. This makes the results difficult to evaluate, but in the first study the authors stated that there was no statistical difference between the medicated group and the control group (Linton *et al.*, 1985) and in the other that the results were inconclusive (Hinton *et al.*, 1986).

In 1989, Barrow (1989) demonstrated a dose-response relationship between in-feed avoparcin and prevalence of salmonella shedding among experimentally infected chickens. Infections with *S.* Typhimurium, *S.* Choleraesuis, *S.* Dublin and *S.* Arizonae all gave similar results. This is the only published study on dose-response relations between AFA and salmonella shedding. It provides an explanation to why some studies yield contradictory results; the concentrations of avoparcin used in most studies (around 10 ppm) appear to correspond roughly to the breakpoint of response-no response.

Humbert and co-workers (1991) investigated the effects of various AFA, including avoparcin, on salmonella shedding among CE- (competitive exclusion) treated chickens. The authors stated that birds fed 10 ppm avoparcin had significantly more salmonella in their caeca than control birds receiving no antibiotic. However the concurrent CE-treatment, among other things, make these results hard to evaluate.

In conclusion, avoparcin increases the prevalence of salmonella shedding among experimentally infected chickens, as demonstrated by a dose-response relationship. Avoparcin also lowers the infectious dose necessary for achieving salmonella colonisation of chickens. No studies have been found regarding ardacin, but as the two substances are very similar in activity and antibacterial spectrum, there is no reason to believe that ardacin would differ from avoparcin in this respect.

### D.7.2 Other enteric pathogens

No publications have been found on the influence of either avoparcin or ardacin on the intestinal colonisation of target animals with other zoonotic pathogens.

## D.8 Toxicological aspects

Glycopeptides are poorly absorbed after oral ingestion (Prescott and Baggot, 1993).

In an experiment where radiolabelled avoparcin was fed to chickens for 7 consecutive days at the dose of 1 mg/kg body weight, virtually the entire dose was retrieved in urine-faecal samples and in gastrointestinal contents

(Zulalian *et al.*, 1979). Residues in all tissues were less than 0.05 ppm. Based on these findings, the authors concluded that essentially no avoparcin was absorbed from the gastrointestinal tract of the animals. No residues are therefore to be expected. Maximum residue levels (MRL) have not been established.

Experimental feeding of broiler chickens with 15 ppm ardacin for 30 days resulted in liver residues of up to 50 µg/kg after a 7 day withdrawal period (Gottschall *et al.*, 1995). Ardacin is not biotransformed to any large extent (Gottschall *et al.*, 1995). No MRL has been established.

No information on toxicity to target species, non-target species or humans has been found. Vancomycin is ototoxic in humans, but it is not known whether this feature is shared by ardacin or avoparcin.

Occupationally derived contact dermatitis after contact with avoparcin has been reported (Barriga *et al.*, 1992).

## D.9 Environmental effects

As for other AFA, if feeding avoparcin or ardacin leads to a reduction in the amount of feed consumed per kg weight gain, this would be expected to lead to a reduction in nitrogen output.

Like other naturally produced antibiotics, avoparcin and ardacin would be expected to be microbially degraded in soil, but no reports relating to the environmental fate of either ardacin or avoparcin have been found. Regarding avoparcin, that has been used for over 20 years in some countries, it is remarkable that such information is not publicly available.

## D.10 Summary comments

Increased glycopeptide resistance is a human health problem. Avoparcin has been shown to select for glycopeptide resistance among animal bacteria, and there is no reason to believe that ardacin would be any different in this respect. Numerous reports indicate that transfer of glycopeptide resistance between animal and human microflora can and does occur.

Avoparcin has also been shown to affect salmonella colonisation. The lowering of the infectious dose necessary for colonisation that is seen in avoparcin-treated animals indicate that avoparcin may increase the prevalence of salmonella-infected poultry flocks, especially in areas where the exposure to salmonella is low.

The available information on toxicological and environmental aspects is too scarce to form the basis of any assessment.

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# Annex E: Spiramycin, tylosin and virginiamycin

## E.1 Introduction

Spiramycin and tylosin belong to the macrolide group of antibiotics, while virginiamycin belongs to the streptogramins. Macrolides and streptogramins are not chemically related but they are often grouped together because they show similar antibacterial spectra and are functionally related in their mode of action. A third class, the lincosamides is also often included for the same reason, leading to the acronym MLS (macrolides, lincosamides and streptogramins). They all inhibit protein synthesis and bind to the 50S subunit of the bacterial ribosome. They are mainly active against gram-positive aerobic bacteria, various gram-positive and gram-negative anaerobic bacteria. Lincosamides are not used as feed additives and will therefore only be discussed where appropriate.

### E.1.1 Macrolides (tylosin and spiramycin)

Macrolides are derived from products from *Streptomyces* spp. and are characterised by a macrocyclic lactone ring attached to one or more sugar moieties and can, according to the ring structure, be divided into 14-, 15- and 16 lactone ring macrolides (fig. E.I). Erythromycin, a 14 membered lactone, and several newer derivatives are used in human medicine and are important in the treatment of common infections caused by bacteria such as staphylococci, streptococci, mycoplasmas and campylobacters. Macrolides have been suggested as the primary drugs of choice for a number of clinically significant infections in children (Adam, 1992). Among the 16-membered lactones, spiramycin and josamycin have also been evaluated for the treatment of certain protozoal diseases such as toxoplasmosis (St Georgiev, 1994).

In veterinary medicine, the 16-membered lactones tylosin and spiramycin have been widely used, both for growth promotion and for therapy. In the EU, spiramycin is approved for growth promotion in poultry, calves, lambs, kids and swine. Dosages range between 5 and 20 ppm for poultry, 5-80 ppm for calves, lambs and kids, and 5-80 ppm for swine. Tylosin is approved for growth promotion in swine, at concentrations between 5 and 40 ppm.

Important clinical applications for those and related substances are swine dysentery (*Serpulina hyodysenteriae*) and mycoplasmosis. They are also

The figures in this report is only available in the printed version

Figure E.I. Tentative sketch of erythromycin, spiramycin and tylosin

The figures in this report is only available in the printed version

Figure E.I. Continued; virginiamycin

second-choice antibiotics for a range of common infections, including mastitis caused by penicillin-resistant staphylococci. Another macrolide for therapeutic purposes, tilmicosin, has lately been introduced into the veterinary field, mainly for use against infections with *Actinobacillus pleuropneumoniae* in swine, *Pasteurella* spp. in cattle and mycoplasmosis in various animal species (Prescott and Baggot, 1993). Therapeutic dosages of in-feed tylosin for swine are usually around 100 ppm (Prescott and Baggot, 1993).

The different macrolides differ to some extent with respect to their pharmacokinetic properties. Common characteristics of the group are high intracellular concentrations and a wide distribution in tissues (Prescott and

Baggot, 1993), properties which together with their activity against important pathogens make them a valuable group for therapeutic purposes in both veterinary and human medicine.

### E.1.2 Streptogramins (virginiamycin)

Streptogramins are natural cyclic peptides including substances such as pristinamycins and virginiamycins (fig E.I). They are produced by a variety of *Streptomyces* spp. Streptogramins consist of two components; A (also called M) and B (also called S), each having bacteriostatic activity and acting synergistically.

Streptogramins have been sparingly used in human medicine, although there are geographic variations. Pristinamycin and virginiamycin are the most well known substances, but a new derivative, quinpristin-dalfopristin, has recently been launched (Pechere, 1992; Cormican and Jones, 1996; Nicas *et al.*, 1997). The role of these substances as therapeutic tools is expected to increase due to the increasing resistance to more commonly used substances. Quinpristin-dalfopristin has been suggested as a new option for the treatment of infections with glycopeptide- (vancomycin) and aminoglycoside-resistant enterococci (Cormican and Jones, 1996; Nicas *et al.*, 1997).

In animals, virginiamycin is mainly used as a feed additive for growth promotion. Examples of prophylactic or therapeutic applications are swine dysentery and clostridial infections such as necrotic enteritis in poultry.

Virginiamycin is permitted for growth promotion in poultry, calves and swine at concentrations between 5 and 50 ppm for poultry, 5-80 ppm for calves and 5-50 ppm for swine. Therapeutic dosages for swine are around 100-150 ppm and for prophylactic treatment of necrotic enteritis in poultry around 40-80 ppm (Prescott and Baggot, 1993; FASS VET., 1997).

There are few data available on the pharmacokinetics of streptogramins. Orally administered virginiamycin is not absorbed from the gut (Prescott and Baggot, 1993).

## E.2 Mode of action and resistance mechanisms

The MLS antibiotics inhibit protein synthesis by binding to the 50 S subunit of the bacterial ribosome. By binding to, or near, the peptidyl transferase centre on the ribosome, macrolides prevent the elongation of the peptide being synthesised. Intrinsic resistance to macrolides, lincosamides and streptogramin B ($MLS_B$) in gram negative bacteria is due to low permeability of the outer membrane (Leclercq and Courvalin, 1991b).

The most common mechanism of acquired resistance to MLS antibacterials is alteration of the ribosomal target. Inactivation of the drug and active efflux from the bacterial cell have also been described.

Concerning streptogramin A, the mechanisms of intrinsic resistance are unclear. Described mechanisms of acquired resistance include enzymatic inactivation and active efflux.

Depending on the exact mechanism, a variety of resistance phenotypes are found, many of which are cross-resistant to most or all members of the group (table E.I). Most studies on MLS resistance have been conducted on gram-positive cocci and clostridia. Regarding macrolide and streptogramin resistance in *Campylobacter* spp. and *Serpulina* spp., little is known about the mechanisms and the genes conferring resistance.

Further information on resistance mechanisms and corresponding genes are found in E.4.

Table E.I. Examples of phenotypic and genotypic expression of some resistance genes (R=resistant, S=sensitive)

| **Resistance gene** | **Phenotype**[1] | | | | | | **Genotype** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Er** | **Ty** | **Sp** | **SgB** | **SgA** | **SgAB** | **Er** | **Ty** | **Sp** | **SgB** | **SgA** | **SgAB** |
| *erm* | | | | | | | | | | | | |
| -constitutive | R | R | R | R | S | S | R | R | R | R | S | S |
| -inducible in: | | | | | | | | | | | | |
| staphylococci | R | S | S | S | S | S | R | R | R | R | S | S |
| streptococci[2] | R | | | R | S | S | R | R | R | R | S | S |
| *E. faecalis* | R | | | | R | | R | R | R | R | R | R |
| *ere* | R | S | S | S | S | S | R | S | S | S | S | S |
| *sbh* | S | S | S | R | S | S | S | S | S | R | S | S |
| *msr* | R | S | S | R | S | S | R | S | S | S | S | S |
| *satA* | S | S | S | S | R | S | S | S | S | S | R | S |

[1] Er=erythromycin, Ty=tylosin, Sp=spiramycin, SgB= streptogramin B component, SgA= streptogramin A component, SgAB= streptogramin components A+B

[2] *S. sanguis* and *S. pyogenes*

## E.3 Development of resistance

Most studies regarding development of resistance to macrolides and streptogramins have been investigating the association between clinical therapy and development of resistance. Thus, the dosages used are usually higher than what is permitted for growth promotion. Nonetheless, the results indicate that resistance develops in bacteria exposed to these substances. Moreover, as discussed in chapter 4, the concentrations used for growth

promotion are well above the MIC values for common intestinal bacteria. Where relevant information is lacking about the situation when growth promoting dosages are used, results from studies employing therapeutic dosages have been cited.

### E.3.1 Prospective studies

***Experimental studies***

Several studies by the research group of Linton (Linton *et al.*, 1985; Hinton *et al.*, 1986; Kaukas *et al.*, 1987; Kaukas *et al.*, 1988) illustrate the influence of macrolides and streptogramins (tylosin, virginiamycin) at growth promotion dosages on the prevalence of resistance in enterococci. In two of these studies (Hinton *et al.*, 1986; Kaukas *et al.*, 1987), the control groups were given a diet containing growth promoters (including virginiamycin) and/or a coccidiostat with antibacterial effect. A gradual increase in macrolide (tylosin) resistance in the control groups was noted in both these studies. This is in contrast to the studies where the control groups were provided feed without antibacterial agents (Linton *et al.*, 1985; Kaukas *et al.*, 1988). Considering the potential of virginiamycin to select for $MLS_B$-type resistance, it cannot be excluded that the gradual increase in macrolide resistance for the control groups in the former studies was related to the antibiotics in the diet.

In one of the studies (Linton *et al.*, 1985) the enterococci were not identified to the species level, which makes the results hard to evaluate. Moreover, part of the study was conducted on commercial farms and it is not known whether any coccidiostats or other antimicrobial substances were used during the study period. In the other studies, the isolation frequencies of different species of enterococci on different sampling occasions were compared. Again, a marked difference can be observed between the two studies where the bacterial flora of the control groups was exposed to antibacterial agents and the one where it was not. The normal changes in composition of the enterococcal flora during the first weeks of life of chickens not exposed to antimicrobial agents was further investigated in another study (Kaukas *et al.*, 1986). The results show the same trend in number of isolates of different enterococcal species as was observed in the unexposed control group in the study from 1988 (Kaukas *et al.*, 1988). Thus, the confounding effect of growth promoters and anticoccidials in the other control groups is further substantiated. All animals fed antimicrobial agents seem to have had a larger proportion of *E. faecium* in their intestinal flora than animals not receiving any antibacterials.

In the following, only the study from 1988, where the control group did not receive any antimicrobials will be discussed in detail. In this study (Kaukas *et al.*, 1988), small groups of chickens were given feed containing either avoparcin, nitrovin, virginiamycin or zinc bacitracin during the first three weeks of life. The feed of the control group, as mentioned earlier, contained no additives. Each study group consisted of ten chickens and the experiment was performed in duplicate. The incidence of resistance to therapeutic antibiotics, expressed as Antibiotic Resistance Index (ARI) was significantly higher in all groups receiving antibacterials, as compared to the control group ($p=0.003$). This increase was associated with an increase in the proportion of *E. faecium* in the enterococcal population of the treated birds. The incidence of resistance to streptogramins in bacterial isolates from the group receiving virginiamycin and from the control group differed significantly ($p<0.001$) for both *E. faecalis* (46 and 25% respectively) and *E.faecium* (88 and 26% respectively). Thus, in addition to the selection of the species *E. faecium* by virginiamycin, a specific selection for resistance traits was also observed.

In a study from 1983 (Christie *et al.*, 1983) an increase of MLS resistance over time in gram positive cocci isolated from pigs fed 100 ppm tylosin was observed. Despite weaknesses in study design, sample size and methodology, and the fact that therapeutic dosages were used, this study nevertheless implies that tylosin in pig feed affects development of resistance in both staphylococci and enterococci from these animals. This study has been much criticised. Among other things, it appears that due to a feed mixing error the control group also received some tylosin early in the study (Anonymous, 1985). However, the influence of this error on the results would have been expected to be seen as a decrease in the difference between the two groups. Thus, it ought not to have caused any overestimation of the difference.

### *Field studies*

The influence of virginiamycin on the prevalence of resistant enterococci within flocks of turkeys has been reported by Thal and co-workers (1996). Different turkeys from the same flock were found to harbour the same type of streptogramin-resistant *E. faecium* and the prevalence of resistance to both streptogramins and ampicillin increased with the age of the flock.

In a German study (Hummel and Witte, 1981) macrolide resistant staphylococci could be isolated from pigs in herds exposed to tylosin via the feed. Moreover, such resistant strains were also isolated from people who worked directly with the animals but not from their family members. All staphylococci from pigs in non-exposed herds were sensitive to macrolides. This indicates that tylosin may exert a selective pressure, not only on the microflora of animals consuming the feed with the added tylosin but also

directly on the bacteria of people handling the feed and the animals. The failure to demonstrate a spread of the resistant strains or the genes mediating resistance to non-exposed humans could indicate that a selective pressure has to be in force in order to promote further transmission. The dose of tylosin given and the precise administration principles were not detailed in the publication but from what is noted a therapeutic concentration seems most likely. In addition, all animals included in the study were fed tetracyclines, apparently on routine basis.

### E.3.2 Retrospective studies

The development of tylosin resistance in *Serpulina hyodysenteriae* was reported by Molnar (1996) in an investigation from Hungary. The proportion of strains classified as resistant to tylosin in this investigation increased from 11% during 1978-82 to 40% during 1983-87. The author assumed that the high level of resistance to tylosin was to a large extent due to the fact that since the 1980s most pig rations in Hungary have contained tylosin as an additive. However, this assumption is not substantiated in the article and no data on consumption is presented.

The prevalence of resistance to macrolides in *Clostridium perfringens* isolated from cattle, swine and poultry was investigated in two studies from Belgium (Dutta and Devriese, 1984; Devriese *et al.*, 1993). In the material included in the first study, collected between 1979 and 1982, the prevalence of resistance ranged from 1% to 21% depending on animal source. In the second study from 1991-1992, the corresponding figures were 3% to 12%. The number of animals in these studies is to small for any clear trends to be deducible, but there is no evidence of increase in the resistance rates over the years.

### E.3.3 Point-prevalence studies

#### *Enterococci*

With the exception of *E. faecium*, enterococci are naturally resistant to the A component of streptogramins (Leclercq and Courvalin, 1991b). Acquired resistance in enterococci is, according to present knowledge, of the $MLS_B$ type, encoded for by a number of *erm*-genes. In table E.II, data from several studies on resistance to macrolides in enterococci from poultry and swine have been compiled. The prevalence of resistance appears to be high in the bacterial populations studied, although somewhat lower in the material presented from Sweden.

Table E.II. Resistance in enterococci to $MLS_B$ antibacterials as reported in different studies

| **Bacterial species** | **Animal source** | **No. of isolates** | **Year** | **Resistance in %** [1] | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *Enterococcus faecalis* subsp. *Liquefaciens* | poultry | 23 | 1980 | *83* | Dutta and Devriese, 1982 | Belgium |
| *E.faecalis* | poultry | 8 | 1980 | *50* | Dutta and Devriese, 1982 | Belgium |
| *E.faecalis* | swine | 225 | 1995-96 | *91* | DANMAP, 1997 | Denmark |
| *Enterococcus faecium* | poultry | 15 | 1980 | *67* | Dutta and Devriese, 1982 | Belgium |
| *E.faecium* | poultry | 54 | 1995-96 | *65* | DANMAP, 1997 | Denmark |
| *E. faecium* | cattle | 13 | 1995-96 | *38* | DANMAP, 1997 | Denmark |
| *E. faecium* | swine | 58 | 1995-96 | *91* | DANMAP, 1997 | Denmark |
| *Enterococcus* spp. | cattle | 34 | 1979-82 | *26* | Rollins et al., 1985 | USA |
| *Enterococcus* spp. | swine | 72 | 1979-82 | *79* | Rollins et al., 1985 | USA |
| *Enterococcus* spp. | pork | 50 | 1995 | *15* | Quednau et al., 1996 | Denmark |
| *Enterococcus* spp. | pork | 50 | 1995 | *2* | Quednau et al., 1996 | Sweden |
| *Enterococcus* spp. | swine | | 1996 | *33* | Greko, 1997 | Sweden |
| *Enterococcus* spp. | poultry | 55 | 1979-82 | *50-67* | Dutta and Devriese, 1984 | Belgium |
| *Enterococcus* spp. | poultry | 93 | 1979-82 | *67* | Rollins et al., 1985 | USA |
| *Enterococcus* spp. | poultry meat | 50 | 1995 | *27* | Quednau et al., 1996 | Denmark |
| *Enterococcus* spp. | poultry meat | 50 | 1995 | *10* | Quednau et al., 1996 | Sweden |
| *Enterococcus* spp. | poultry | 207 | 1996 | *17* | Greko, 1996 | Sweden |

[1]When several macrolides have been investigated, the figures for erythromycin have been used.

### *Staphylococci*

Data on the prevalence of macrolide resistance in animal staphylococci from various studies are presented in table E.III. A majority of these strains have been isolated from pathological lesions or from skin. As can be seen in the table, there seems to be a marked difference in the prevalence of macrolide-resistance between staphylococci from different animal species. The majority of the staphylococcal strains reported as resistant in table E.III expressed the resistance constitutively.

Table E.III. Resistance in *Staphylococcus aureus* to erythromycin as reported in different studies

| **Bacterial species** | **Animal source** | **n of isolates** | **Year** | **Resistance in %** | **Reference** | **Country** |
|---|---|---|---|---|---|---|
| *S.aureus* | cattle | 517 | 1971-80 | *2-4* | Devriese, 1980 | Belgium |
| *S.aureus* | cattle | 211 | 1995-96 | *1* | DANMAP, 1997 | Denmark |
| *S.aureus* | cattle | 183 | 1995 | *1* | Nilsson, 1996 | Sweden |
| *S.aureus* | poultry | 399 | 1970-80 | *6-20* | Devriese, 1980 | Belgium |
| *S.aureus* | swine | 124 | 1973-80 | *27-53* | Devriese, 1980 | Belgium |

### *Campylobacter*

*C. jejuni* is associated mainly with poultry and *C. coli* with swine. The species most commonly isolated from humans is *C. jejuni*. The incidence of macrolide resistance in *C.coli* is much higher than in *C. jejuni*. (Wang *et al.*, 1984; Burridge *et al.*, 1986; Lacey, 1988; Jacobs-Reitma *et al.*, 1994; DANMAP, 1997). It has been implied that the widespread resistance to macrolides in *C. coli* may be linked to the use of macrolides (Davies *et al.*, 1996; Moore *et al.*, 1996). One of the few studies made on MICs of tylosin for field isolates of *C. coli* (Ryden R, cit. by Burridge *et al.*, 1986) showed that the prevalence of tylosin resistant *C. coli* among nontreated pigs, or pigs given antibacterials other than tylosin, was 55%, and in pigs receiving therapeutic doses of in-feed tylosin it was 70%. In contrast, *C. jejuni* originating from poultry are rarely resistant to macrolides. The use of macrolides is presumably higher in swine as compared to poultry as tylosin is approved for growth promoting purposes only in the former species. In view of the zoonotic character of campylobacteriosis, it is remarkable that no further investigations on the possible relation between use of macrolides as growth promoters and selection of resistant strains of campylobacters have been made.

Table E.IV. Prevalence of erythromycin resistance in campylobacter in various studies

| Bacterial species | Source | n of isolates | Year | Resistance in % | Reference | Country |
|---|---|---|---|---|---|---|
| *C. coli* | swine | 99 | 1995-96 | *55* | DANMAP, 1997 | Denmark |
| *C. coli* | poultry | 91 | 1992-93 | *12* | Jacobs-Reitma et al., 1994 | Netherlands |
| *C. coli* | sewage | 32 | 1995 | *25* | Koenraad et al., 1995 | Netherlands |
| *C. coli* | human | 58 | 1987-91 | *19* | Reina et al., 1992 | Spain |
| *C. coli* | human and swine | 80[1] | 1984 | *70* | Wang et al., 1984 | USA |
| *C. jejuni* | poultry | 55 | 1995-96 | *4* | DANMAP, 1997 | Denmark |
| *C. jejuni* | poultry | 177 | 1992-93 | *2* | Jacobs-Reitma et al., 1994 | Netherlands |
| *C. jejuni* | sewage | 121 | 1995 | *9* | Koenraad et al., 1995 | Netherlands |
| *C. jejuni* | human | 614 | 1987-91 | *2* | Reina et al., 1992 | Spain |
| *C.jejuni* | human and swine | 98[2] | 1984 | *3* | Wang et al., 1984 | USA |
| *Campylo-bacter spp.* | Poultry | 59 | 1989-90 | *5* | Cabrita et al., 1992 | Portugal |
| *Campylo-bacter spp.* | Swine | 65 | 1989-90 | *26* | Cabrita et al., 1992 | Portugal |

[1] 11 isolates from humans, 69 isolates from swine
[2] 93 isolates from humans, 5 isolates from swine

### *Serpulina*

During the 1970s, tylosin was regarded as the drug of choice in the treatment of swine dysentery. This is no longer the case, as resistance to tylosin now seems to be widespread in strains of *Serpulina hyodysenteriae* (Smith *et al.*, 1991 see also E.3.2; Prescott and Baggot, 1993; Fellström *et al.*, 1996; Molnar, 1996). A high proportion of virginiamycin resistance in this group of bacteria has also been reported from other countries in recent years (Ronne and Szancer, 1990).

## E.4 Resistance genes and acquisition of resistance

Various genes conferring the different types of resistance mentioned above (E.2) have been identified (table E.IV). These genes, often of a highly mobile type, can be transferred between bacteria, within the same species and between different species. They are often carried on ”jumping genes” (transposons) which means that they can copy themselves from, for instance,

the chromosome to a plasmid or from one plasmid to another plasmid. Further, they may be carried on self-transmissible plasmids.

Table E.IV. Examples of resistance to MLS antibacterials by different mechanisms and genes. (Data compiled from Arthur *et al.*, 1987; Brisson Noel *et al.*, 1988; Leclercq and Courvalin, 1991a; Leclercq and Courvalin, 1991b; Eady *et al.*, 1993; Mullany *et al.*, 1995; Tauch *et al.*, 1995; Weisblum, 1995a; Allignet *et al.*, 1996)

| **Phenotype** | **Mechanism** | **Gene** | **Described localisation** | **Example of species** |
|---|---|---|---|---|
| $MLS_B$ | target modification | *erm*A | Tn*554* | *S. aureus*, coagulase negative staphylococci |
| | | *erm*B, *erm*AM | Tn*551*, pAM77, Tn*917*, Tn*1545*, plus various other plasmids and transposons | *S. aureus, S. intermedius, S. hyicus, S. pneumoniae, Streptococcus spp., E. faecalis, Lactobacillus* spp. |
| | | *Erm*C | pE194, plM13, pE5, pNE131 | *S. aureus*, coagulase negative staphylococci, *S. hyicus, Bacillus subtilis* |
| | | *erm*CX | Tn*5432* | *Corynebacterium xerosis* |
| | | *erm*D | chromosome | *B. licheniformis* |
| | | *erm*BC | pIP1527 | *E. coli* |
| | | *erm*F | pBF4 | *Bacteroides spp.* |
| | | *Erm*E | chromosome | *Streptomyces erythreus* |
| | | *erm*P, *erm*Q | | *Cl. perfringens* |
| | | *erm*Z | Tn*5398* | *Cl. difficile* |
| | | *erm*J | | *Bacillus anthracis* |
| | | *erm*M | | *S. epidermidis* |
| 14-M | drug inactivation | *ere*A | pIP1100 | *E. coli* |
| | | *ere*B | pIP1527 | *E. coli* |
| L | | *lin*A | pIP855 | *S. haemolyticus* |
| | | *lin*A’ | pIP856 | *S. aureus* |
| $S_B$ | | *sbh* | pIP524 | *S. aureus* |
| | | *vgb* | pIP680 | *S. aureus* |
| $S_A$ | | *satA* | pAT424 | *E. faecium* |
| | | *vat* | pIP680, IS257 | *S. aureus* |
| | | *vatB* | pIP1156 | *S. aureus* |
| 14-M | active efflux | *erp*A | | *S. epidermidis* |
| $14\text{-}MS_B$ | | *msr*A | | *S. epidermidis* |
| $S_A$ | | *vga* | plasmids | *S. aureus, S. epidermidis* |

### E.4.1 Target modification

Cross-resistance to all $MLS_B$ antibiotics due to target modification is widespread although the prevalence differs regionally (Duval, 1985; Arthur *et al.*, 1987). The bacteria equipped with this property produce rRNA methylases which are enzymes capable of altering the ribosomal binding site of $MLS_B$-antibiotics. The alteration inhibits binding of the antibiotic and hence allows the bacterial protein synthesis to continue. The genes coding for this property in bacteria are designated *erm* (erythromycin resistance rRNA methylase). Various different *erm*-genes have been identified. They present a substantial sequence diversity but studies on their evolutionary relationship suggest that they are of ancient presence in at least *Streptomyces* spp, gram-positive cocci and *Bacillus licheniformis* (Arthur *et al.*, 1987). However, transfer of *erm* genes occurs under natural conditions to bacteria which are phylogenetically remote from the above mentioned bacteria, such as *Escherichia coli* (Brisson Noel *et al.*, 1988). This transfer is believed to be a recent event although the genes are already disseminated in enterobacteria.

Transfer of *erm*-genes has been demonstrated experimentally in numerous studies. Poyart-Salmeron and co-workers (1990) showed by way of *in vitro* mating experiments the conjugative transfer of *erm*B, carried on plasmid pIP811 together with other resistance determinants, between among others *Listeria monocytogenes* and *E. faecalis*. Doucet-Populaire and co-workers (1991) demonstrated transfer *in vitro* and in an *in vivo* model of transposon *Tn*1545, harbouring *erm*B, *aph*A3' (kanamycin resistance) and *tet*M (tetracycline resistance) between *E. faecalis* and *L. monocytogenes.* McConnell and co-workers (1991) demonstrated transfer of plasmid pAMβ1, carrying *erm*B, from *Lactobacillus reuteri* to *E. faecalis in vivo*.

#### *Inducible or constitutive expression*

Expression of *erm*-genes can be inducible or constitutive. When constitutively expressed, the methylase encoded for by the *erm*-gene will always be produced. On the other hand, when inducibly expressed, the methylase is only produced when the gene is activated by an inducer. Various macrolides may function as inducers, depending on the class of *erm* gene and bacterial host.

The type of expression is related to the class of *erm* gene but depends on a regulatory gene sequence upstream from the methylase gene sequence itself (the messenger). The mechanisms of resistance by target modification and induction of this resistance has been described by Weisblum (Weisblum, 1985; Weisblum, 1995a; Weisblum, 1995b; see figures).

The figures in this report is only available in the printed version

Figure E.II. Induction of the *erm*C gene (Adapted from Weisblum, 1995a)

The entire gene consists of a leader peptide and a series of inverted complementary repeat sequences that can redistribute and assume alternative double stranded conformations, followed by the methylase gene sequence. In the presence of a macrolide the ribosomes are inhibited ("stalled"). Activation of the messenger depends on the degree to which ribosomes, during translation of the leader peptide, are inhibited by the macrolide and thereby disrupt the secondary structure in a certain part of the control region. The result of inverted repeat sequence redistribution is the unmasking of a ribosome binding site for synthesis of the methylase. The inductive capability of different macrolides is dependent on their capability of stalling the ribosome at the right step in the translation of the leader peptide. This varies between different *erm* genes and between different bacterial species, but 14-membered macrolides are generally the best inducers. Inducibly resistant strains can convert to constitutively resistant ones by deletions or single nucleotide changes in the regulatory gene, either in the leader peptide or in the inverted repeat sequences.

### *Enterococci and streptococci*

In enterococci and streptococci (formerly group D streptococci), $MLS_B$ resistance can be expressed either inducibly or constitutively. However, in some cases all $MLS_B$ antibiotics can act as inducers which explains the diversity of resistance phenotypes coded for by similar genes. Thus, in the case of $MLS_B$ resistance, phenotypic characteristics do not necessarily provide a basis for conclusions on genotype. Reports from Japan and France have shown a trend from predominantly inducible resistance to predominantly constitutive resistance in staphylococci whereas in British and German reports inducible type still dominate (Jenssen *et al.*, 1987). A non-inducing macrolide or streptogramin would be expected to select for constitutively expressed resistance, since mutation to the constitutive form would be the only way bacteria harbouring inducible *erm*-genes could survive exposure to a non-inducer.

Investigations of Swedish isolates from humans of *Streptococcus pyogenes* revealed that the resistance determinant was present on an *erm*B-carrying conjugative plasmid, possibly originating from plasmid pAMb1 of *E. faecalis* (Schalen *et al.*, 1995). In an Italian study on the same species, in addition to the $MLS_B$ type mediated by *erm*-genes, a second phenotype, possibly due to an efflux-mechanism, was reported (Coranaglia *et al.*, 1996).

*Streptococcus suis,* originally described as a cause of outbreaks of arthritis and meningitis in piglets is today more commonly associated with bronchopneumonia in weaners and fatteners. In a study from USA (Stuart *et al.*, 1992) a substantial increase in resistance to the MLS antibacterials in *S. suis* was demonstrated when compared to a similar study undertaken previously. Further, the study showed that resistant isolates were capable of transmitting resistance by conjugation possibly mediated by a transposon similar to *Tn*916. Wasteson and co-workers (1994) investigated a collection of Norwegian isolates and demonstrated MLS-resistance in *S. suis* as determined by *erm*C and/or *erm*B genes and furthermore, that the resistance genes could be transmitted from *S. suis* to *E. faecalis.*

Enterococci are known to frequently exchange resistance genes, not only with other enterococci but also with other bacterial genera. Transfer of resistance genes between enterococci and staphylococci appears to take place in nature, maybe mediated by broad host range plasmids (Bonafede *et al.*, 1997).

### *Staphylococci*

In staphylococci harbouring *erm*C or *erm*A genes, inducible resistance is triggered by 14- and 15-membered macrolides only. *In vitro*, those strains are resistant to 14- and 15- membered macrolides. As long as the induction persists (presence of, for instance, erythromycin) the methylase will be produced and the bacterial cell will be resistant to all $MLS_B$ antibiotics. The 16-membered macrolides, lincosamides and streptogramin B cannot induce expression of the gene and will, when tested separately, remain active *in vitro*. When expressed constitutively in staphylococci, the *erm*-genes confer cross-resistance to all $MLS_B$. No information has been found on whether staphylococci constitutively expressing $MLS_B$ resistance due to mutations are likely to mutate back to the inducibly expressed form of resistance in the absence of a selective pressure for the constitutive variety, i.e. the presence of a 16-membered macrolide. Experimental data indicate that the inactive structure of the inducibly expressed resistance gene is energetically favoured (Horinouchi and Weisblum, 1981), so a mutation back to this state would not be totally unexpected. Moreover, it is not known whether constitutively expressed *erm* genes would still be constitutively expressed after transfer into a new host bacterium. As a comparison, transfer experiments with enterococcal strains expressing *van*B constitutively have shown that the resulting transconjugants can be either of constitutive or of inducible type (Hayden *et al.*, 1997).

Eady and co-workers (1993) investigated the distribution of *erm*A, *erm*B and *erm*C in staphylococci from human and animal sources. In addition, the presence of *msr*A was studied. One hundred and seventy-two human isolates of coagulase-negative staphylococci from dialysis patients, blood cultures and untreated acne patients were analysed, along with 33 isolates of coagulase-negative staphylococci from pigs and 16 isolates of *Staphylococcus intermedius* from dogs.

Table E.II. Number of staphylococcal strains from various sources harbouring macrolide-streptogramin-resistance genes. Data from Eady and co-workers (1993)

| Gene | Swine | Dogs | Humans |
|---|---|---|---|
| *erm*A | 0 | 1 | 14 |
| *erm*B | 7 | 11 | 0 |
| *erm*C | 21 | 1 | 89 |
| *msr*A | 2 | 1 | 70 |

Fourteen of the human isolates, but none of the isolates from pigs, and only one isolate from a dog harboured *erm*A. The *erm*B gene was only found in animal isolates. The *erm*C gene was the most common among human and pig isolates, while this gene was only found in one of the dog isolates. The

*msr*A gene was only found in 2 pig isolates and one dog isolate, but in 70 human isolates. Several isolates included in the study harboured more than one of the resistance determinants studied. All of the pig strains, half of the dog strains and 13 of the human strains expressed $MLS_B$ resistance constitutively. This illustrates the fact that expression of resistance genes cannot be used to predict type of resistance determinant, and that some *erm* genes circulate in both human and animal populations. Since pigs, as opposed to dogs, are not exposed to 14-membered macrolides, neither for therapy nor growth promotion, only 16-membered macrolides, the constitutive expression of *erm* genes in isolates from these animals is not surprising. Dogs, like people, may be given 14-membered macrolides for therapeutic purposes and would therefore be expected to harbour a much higher proportion of isolates with inducibly expressed *erm*-genes.

Westh and co-workers (1996) investigated the presence of *erm*A and *erm*C in human isolates of *Staphylococcus aureus* and coagulase negative staphylococci from Denmark. They found that the *erm*A gene was solely responsible for erythromycin resistance in strains isolated before 1971, but had since then gradually disappeared. Today the *erm*C determinant is responsible for 72% of erythromycin resistance in Danish *S.aureus*, according to the authors. Both constitutive and inducible expression was noticed, with the inducible form being the most common. No *erm*A genes were found in coagulase-negative staphylococci and in paired isolates, i.e. isolates of *S.aureus* and coagulase-negative staphylococci originating from the same sample, the same *erm* gene was only found in both species in 4 out of 15 pairs. This study illustrates the variability over time in the presence of different *erm* genes, a fact that emphasises the importance of identifying the genes involved in the resistance patterns studied.

### *Clostridia*

The hitherto described genes encoding for macrolide resistance in clostridia belong to the *erm* family. The most common resistance determinant seems to be *erm*Q which has been found in isolates from pigs and humans from a wide geographical range (Berryman *et al.*, 1994). A second, apparently less prevalent gene, the *erm*B-*erm*AM (also called *erm*BP) gene, has also been described in *C. perfringens* (Daube *et al.*, 1992; Devriese *et al.*, 1993; Berryman and Rood, 1995). Genes belonging to this subgroup have earlier been described in numerous bacterial genera, both gram-positive and gram-negative, indicating that they are readily transferred. Sequencing of the *C. perfringens erm*B determinant and its flanking regions by Berryman and Rood (1995) revealed a close similarity to the corresponding determinant on plasmids from *E. faecalis* (pAMb1) and *Streptococcus agalactiae* (pIP501) which are both of the conjugative, broad host range type. It was therefore

postulated that the *C. perfringens erm*BP determinant was derived from an enterococcal or streptococcal determinant. The gene *erm*B is carried by transposon Tn917, often residing on a self transmissible, broad host range plasmid. This gene is widespread in human and animal isolates of enterococci as well as in other bacteria (Rollins *et al.*, 1985; LeBlanc *et al.*, 1986).

### E.4.2 Enzymatic inactivation

Unlike target modification, which causes resistance to structurally distinct antibiotics, enzymatic inactivation confers resistance only to structurally related drugs.

Macrolide modifying enzymes have been described in lactobacilli of animal origin (Dutta and Devriese, 1981; Arthur *et al.*, 1987) and in *Streptomyces* spp. (Arthur *et al.*, 1987), but the genes in question have not been described (Arthur *et al.*, 1987). Inactivation of erythromycin by production of erythromycin esterases or phosphotransferases has been described in human enterobacteria isolated from patients undergoing treatment with erythromycin (Arthur *et al.*, 1987). Two types of esterases (I and II) are encoded for by the genes *ere*A and *ere*B, respectively (Arthur *et al.*, 1987; Leclercq and Courvalin, 1991b).

Resistance to streptogramin antibiotics, caused by the modification of both components, was described in the 70s, in *S.aureus* (Le Goffic *et al.*, 1977a; Le Goffic *et al.*, 1977b). This resistance is coded for by two genes, *sbh* and *saa,* located on a large plasmid. Most of the strains are also resistant to low levels of lincosamides although these antibiotics are not inactivated. Since this original description, several different enzymes and genes with similar activity have been described (Rende Fournier *et al.*, 1993; Allignet and El Solh, 1995). In staphylococci, resistance to the group A compound will always confer resistance to the mixtures of group A and B compounds. This is not the case with resistance to group B compounds where group A compounds still may remain active (Duval, 1985).

### E.4.3 Active efflux

Resistance due to active efflux has been reported in staphylococci. The resistance gene *msr*A described in *Staphylococcus epidermidis* encodes a transport-related protein that confers inducible resistance to 14- and 15-membered macrolides (Leclercq and Courvalin, 1991b). The strains with this characteristic are, for unknown reasons, cross-resistant to streptogramin B antibiotics. Further, a constitutively expressed resistance of efflux-type mediated by the gene *erp*A has been reported in *S. epidermidis* (Leclercq and

Courvalin, 1991b). In this case, the streptogramins retain their effect. Active efflux of streptogramin A is mediated by the gene *vga* coding for an ATP-binding protein probably involved in the active transport of the compound. This gene has been found in *S. aureus* and *S. epidermidis* (Allignet *et al.*, 1992; Rende Fournier *et al.*, 1993).

### E.4.4 Co-transfer of resistance genes

Co-transfer of vancomycin resistance and erythromycin resistance, from *Enterococcus faecalis* to *Staphylococcus aureus* has been shown to occur both *in vitro* and *in vivo* , in a mouse model (Noble *et al.*, 1992). Others have reached similar results. Leclercq (1989) achieved co-transfer of vancomycin resistance and *erm*AM, located on the plasmid plP819, between different enterococcal species and from enterococci to streptococci and *Listeria monocytogenes*. Further, the transfer of pheromone-responsive plasmids harbouring virulence- and resistance factors (among others *erm*B on Tn*917*) between strains of *E. faecalis* in an animal model has been demonstrated (Huycke *et al.*, 1992).

### E.4.5 Exposure to AFA and resistance

Evaluating resistance data between different countries or different regions, is difficult without information about the degree of exposure to various antibacterials in various regions. Little information is available concerning statistics on the quantities of MLS antibacterials used for different purposes in different countries. Moreover, differences between countries in climate, animal husbandry and animal health, frustrate comparisons. However, Denmark and Sweden are, in these respects, similar enough for a comparison to be attempted. Recent statistics from Denmark and Sweden are presented in table E.VI. The statistics on human consumption are only available as the sum of macrolides and lincosamides. An estimate of the proportion of macrolides in this group was made based on substance-specified figures from Sweden 1994 (Naturvårdsverket, 1996). The same figures were also used to estimate an average DDD of 1.7g.

Table E.VI. Usage of MLS antibacterials in 1995 in Sweden and Denmark expressed as kg active substance (Apoteksbolaget, 1996; DANMAP, 1997)

| Country | Tylosin | Spiramycin | Macrolides (total) | Virginiamycin |
|---|---|---|---|---|
| **Denmark** | | | | |
| animal therapy | | | 9500 | |
| feed additives | 52275 | 507 | 52782 | 2590 |
| human therapy | | | 6500[1] | |
| **Sweden** | | | | |
| animal therapy | 1238 | 565 | 1803 | 575 |
| human therapy | | | 6100[1] | |

[1] Estimated as described above

In the debate concerning the contribution of MLS antibacterials used as growth promoters to development of resistance, it has often been assumed that their therapeutic use dominate the selective pressure. The figures from Denmark show, however, that the amount of macrolides used for growth promotion exceeds the therapeutic use in animals by more than 5 times. Comparing the amounts used for therapy in Sweden and Denmark, the differences can be attributed to different sizes of animal populations. Thus, it is a reasonable assumption that the observed differences in prevalence of MLS resistance between enterococci isolated from Danish and Swedish animal sources (see table E.II) are largely explained by the use of MLS antibacterials, mainly tylosin, for growth promotion.

In a paper by Lacey (Lacey, 1988) data on macrolide consumption in humans (erythromycin) and animals (tylosin) during 1986 in the UK are presented. The quantities used in animals and man are about the same, 47 000 and 51 000 kg active substance, respectively. However, the majority of animals (pigs) would have received a low level of antibiotic for a long time, whereas the humans would have received a high level for a short time. Thus, more individuals were exposed for a longer time period in the animal population, as compared to the human population.

## E.5 Effects on specific animal diseases

Virginiamycin may be used for prevention of necrotic enteritis (NE) in poultry. In a large study, Jansson and co-workers (1992) found that virginiamycin given in feed at 20 ppm effectively prevented the experimentally induced NE. Another study by Elwinger and Teglöf (1991) showed similar results; virginiamycin at 20 ppm in feed notably reduced mortality ($p<0.001$) of chickens in necrotic enteritis, as compared to non-medicated groups.

Stutz and co-workers (1983) showed that virginiamycin, at 55 ppm in feed, reduced the amount of *C. perfringens* in the intestines of chickens compared to non-medicated controls ($p<0.05$). The numbers of clostridia were inversely correlated with performance data.

MLS antibacterials are still considered to be alternatives in the therapy and prevention of swine dysentery (Allen *et al.*, 1992). Although tiamulin is often the drug of choice for treatment of this disease, tylosin is in many areas important for maintaining the means to control the infection.

There seems to be a good correlation between MIC values of *S. hyodysenteriae* for tylosin and therapeutic effect of this substance. Williams and Shively (1978) found that tylosin at 100-110 ppm completely prevented swine dysentery induced by a tylosin-susceptible isolate of *S. hyodysenteriae*, while it was only partly effective against the disease induced by isolates with higher MIC values for tylosin. Jacks and co-workers (Jacks *et al.*, 1986) also prevented swine dysentery in pigs experimentally infected with a tylosin-susceptible strain, by feeding tylosin at 110 ppm. When a tylosin-resistant strain was used the disease was not prevented, although mortality was lower in the tylosin-medicated group as compared to the non medicated group. A new tylosin compound, 3-acetyl-4"-isovaleryl tylosin was also tested and found to be effective at 50 ppm.

Miller and co-workers (1972) studied the effect of different levels of virginiamycin and tylosin on pigs experimentally infected with *S. hyodysenteriae*. They found a good prophylactic effect of virginiamycin at about 25 ppm but only a moderate effect of tylosin even at about 100 ppm. Antimicrobial resistance pattern of the infecting strain is not presented, but presumably it was resistant to tylosin. Williams and Shively (Williams and Shively, 1978) fed experimentally infected pigs virginiamycin at 50 to 100 ppm. Virginiamycin could not control the disease, although clinical signs were a little less common in medicated pigs than in non-medicated animals. This was supported by the observations of Rønne and co-workers (1992) who investigated the effect of 20 ppm of virginiamycin.

McOrist and co-workers (1997) evaluated the efficacy of orally administered tylosin for the prevention and treatment of experimentally induced porcine proliferative enteritis (PPE). They found that tylosin at therapeutic levels could prevent PPE in challenge exposed pigs and could also be used for treatment of previously induced PPE. Even at growth promoting levels, tylosin was effective in preventing development of PPE. Moore and Zimmermann (1996) reported successful prevention of PPE by tylosin at about 100 ppm. Fleck and Jones (1994) compared 0, 40 and 100 ppm of tylosin for the treatment and prevention of PPE. The higher level of tylosin was effective as treatment of PPE, while 40 ppm not quite prevented emergence of clinical signs.

## E.6 Impact of resistance on animal and human health

### E.6.1 Consequences of MLS resistance

As stated earlier, macrolides and streptogramins are important therapeutic or prophylactic substances for animals. Macrolides also play an important role in human medical therapy (Kirst and Sides, 1989). Streptogramins are not, except in some countries, yet widely used in human medicine, but due to the development and spread of antimicrobial resistance among human pathogens these compounds are expected to become more commonly used in the future (Pechere, 1992; Pechère, 1996).

From an animal health point of view resistance to MLS-antibacterials in *S. hyodysenteriae* is perhaps most alarming. Uncontrollable outbreaks of swine dysentery can lead to substantial losses and eradication of whole herds may be the only option left (Bouwkamp, 1982). *In vitro* data on resistance of this bacterium seems to be a good predictor of clinical outcome after treatment (Williams and Shively, 1978; Jacks *et al.*, 1986). Unfortunately, such data indicate that the therapeutic potential of the MLS drugs is now limited (Ronne and Szancer, 1990; Gunnarsson *et al.*, 1991; Fellström *et al.*, 1996; Molnar, 1996). Buller and Hampson (1994) claimed that the present situation presents a potential threat to the pig industry.

Bearing in mind that both macrolides and streptogramins are attractive therapeutic options for several important animal and human diseases, a further increase in resistance prevalence would be most unfortunate

### E.6.2 Influence of AFA usage

The possible influence of the use of macrolides as growth promoters on the resistance of human pathogens was intensively discussed in relation to the recommendations of the Swann committee. The debate has, to a large extent, been concentrated on the possible influence on the resistance of staphylococci. Knothe (1977b) concludes, in a review on medical considerations of the use of macrolides in animal feeds, that transfer of staphylococcal strains from animals to humans is possible. However, he considers this to be of no significance since the likelihood of an animal strain actually causing disease in humans is small and the proportion of macrolide resistant strains in human medicine is low. The basis of this assumption is a review of investigations published from 1953-1971 and an accompanying paper describing the prevalence of resistance in Germany from 1968-1976 (Knothe, 1977b; Knothe, 1977a). In the latter study, prevalences of erythromycin resistance between 13 and 24 % were reported with a tendency

towards a reduction over time. In a more recent report from the same region of Germany, the prevalence of erythromycin resistance in *S.aureus* from community acquired infections was 8%, from general ward 13% and from intensive care 28% (Shah *et al.*, 1993). In some recent reports from other European countries, though, the prevalence of resistance to macrolides in staphylococci and enterococci approaches 50% (Turano *et al.*, 1994). At the time when Knothe (1977b) published the review, exchange of genes between gram-positive cocci was thought to be a rare event in natural environments.

The conclusion is thus based on the assumption that animal strains have to colonise and infect humans in order for resistance to have an impact on human health. This view is no longer predominant (see chapter 4).

Genes of the *erm*-family conferring resistance of the $MLS_B$ type have been demonstrated in both human and animal bacteria (Arthur *et al.*, 1987; Roberts and Brown, 1994; Wasteson *et al.*, 1994). This may be due either to common ancestry of the bacteria and/or to genetic exchange in more recent times.

The rarity of tylosin resistance in human pathogenic bacteria has been used as evidence that the flow of resistant organisms or their genes from animals to man is rare (Lacey, 1980; Lacey, 1981; Lacey, 1984; Lacey, 1988). In the most recent paper on this topic by Lacey (1988), the author presented five statements supporting the view that resistant bacteria in animals and humans are two separate entities;

Firstly, that animal staphylococci survive poorly in human environments. This may well be the case, but as long as resistance genes from these staphylococci (and other bacteria, such as enterococci) can be transferred to human strains, the survival of the bacteria themselves is not necessary. Moreover, other bacterial species have been found to be less host-specific.

Secondly, he stated that tylosin resistant strains may grow more slowly than sensitive cultures and would therefore disappear as the exposure to the antibiotic stops. Since use of tylosin as a growth promoter means an almost continuous exposure, this arguing seems to support the view that the use should be limited. Moreover, resistant bacteria may persist even in the absence of a selective pressure (see chapter 4). Relieving the selective pressure of antibiotic exposure in humans usually leads to a drop in the prevalence of resistance, but one cannot assume that the pool of resistance genes would disappear as long as a selective pressure is in force in animal husbandry.

Thirdly, the difficulties in transferring resistance between animal and human staphylococci were pointed out. This was supported by an earlier investigation by the same author (Lacey, 1980), showing that transfer between animal and human staphylococci can occur, but at a lower frequency and in fewer strains than between isolates of staphylococci from the same source. The low frequency of transfer in this study is not surprising, since

only phage dependent transfer is studied. Conjugative transfer is generally believed to be more common. Regarding what can be deemed a ”low” transfer frequency, see chapter 4.

Fourthly, it was stated that tylosin resistance requires the presence of erythromycin to occur, a statement that is true regarding phenotypic expression of erythromycin-inducible *erm* -encoded resistance, but overlooks the lack of correlation between genotype and phenotype in $MLS_B$ resistant staphylococci (see table E.I).

Fifthly the author proposed that the low level of tylosin used for growth promotion in pigs may be too low to select for resistance. The concentrations used for growth promotion are above the MICs of naturally susceptible bacteria (see chapter 4). Finally, the author also concluded that since there are no residues in meat, no selection pressure will be exerted in man. Acknowledging that this is true, the real concern about meat would be that it can contain microbes carrying transferable resistance genes. Besides meat, there are numerous direct and indirect contact areas between human and animal bacteria.

Lately, streptogramin resistance in animal and human bacteria has received increased attention as the streptogramin quinpristin-dalfopristin has recently been introduced for human therapy. Woodford and co-workers (1997) reported streptogramin resistance in vancomycin-resistant enterococci (VRE) isolated from raw chicken (3 isolates) and from a hospital patient (1 isolate) in the UK. The resistance trait included cross-resistance to macrolides and lincosamides and was transferable to other enterococci. The authors commented on the fact that no streptogramin is yet licensed for use in human therapy in UK, whereas virginiamycin is widely used for growth promotion in animals. A reservoir of streptogramin resistance may be present in animal bacteria. Since infections with VRE is one of the main indications for quinpristin-dalfopristin therapy, acquisition of streptogramin resistance by those organisms is most alarming. Similar observations have been reported from The Netherlands (van den Bogaard *et al.*, 1997). In this study, enerococci were isolated from healthy humans and pigs. The prevalence of streptogramin, macrolide and vancomycin resistance in human samples was 30%, 50% and 12%, respectively. The corresponding figures for the samples from pigs were 75%, 84% and 34%. The higher prevalences observed in the material from pigs indicate a higher selective pressure in this population. No information on consumption of macrolides and streptogramins in either humans or pigs in The Netherlands were given.

## E.7 Other effects on the microflora

### E.7.1 Salmonella

There are several studies available on the possible influence of antibacterial feed additives on colonisation of the intestines and the shedding of salmonella. At least 10 of these studies from the last decade involve virginiamycin and 3 of those also include tylosin.

The main problem with all studies in this area, however, is the variability in study design which makes them difficult to compare and evaluate. Weaknesses that are found in several of the studies undertaken include, among other things, inadequate number of animals in the experimental groups, lack of nonmedicated control and undocumented sensitivity of the bacteriological methods employed to recover the salmonella organisms. Taken together, the design of these studies only allows for extremely large differences in outcome. Some of the studies use commercial feed and do not state whether care has been taken to ascertain that this feed contains no antimicrobials. Since antimicrobial feed additives are very common ingredients in commercial feedstuffs this may otherwise influence the outcome of the experiment. Several authors claim that their experimental models are reproducible, but any well described method is reproducible. The results obtained with the method, however, have to be reproducible too.

Bearing in mind the weaknesses mentioned above there are some studies which are nevertheless worth further comment.

Abou-Youssef and co-workers studied the effect of in-feed virginiamycin on experimental infection with *Salmonella* Typhimurium in chickens and swine (1979; 1983). No significant differences between medicated animals and control groups were found, when comparing prevalence and duration of salmonella shedding. However, the experimental groups were small in both studies, between 5 and 20 animals per group, which would only allow for very large differences between groups to be detected.

In his thesis from 1981, Leuchtenberger (1981) compares the salmonella excretion of experimentally infected broilers treated with avoparcin, virginiamycin or tylosin with nonmedicated controls. In a series of experiments 2 different concentrations of antimicrobial (20 and 30 ppm virginiamycin, 50 and 100 ppm tylosin), 2 types of housing (wired cages and floor housing), 2 methods of experimental infection (direct oral inoculation and mixed in the feed) and 2 different infectious doses ($10^3$ or $10^4$ organisms of *Salmonella* Typhimurium) were tried in various combinations. Most experiments were performed in duplicate, with groups of 20-30 animals. Sampling was performed on several occasions throughout the trials, by cloacal swabs on live birds and from the heart, liver, duodenum and caecum of killed birds at the end of the experiment. Not all birds were sampled on all

occasions, though. The author concludes that feeding avoparcin, virginiamycin or tylosin, at both levels tested, can prolong the persistence of *S.* Typhimurium infection in the intestine as well as internal organs, and significantly increases the amount of salmonella found in samples from these sites. It was also found that the duration and frequency of Salmonella excretion depends on the dose and way of infection, as well as the frequency of dosing with infectious organisms and on the housing system.

It is rather surprising that so many of the trials yield significant differences, since the sample sizes are invariably too small for any small differences to be detected. However it is only in the main experiments, where cloacal swabs were taken continuously, that large enough numerical differences were recorded to be interpreted as a strong tendency towards increased salmonella excretion in treated birds. The trials where all birds were killed and cultures made from internal organs must be regarded as inconclusive, due to too small sample sizes and too variable numerical differences in the results to be statistically significant.

In 1981 Gustafson and co-workers published a study where chicks fed avoparcin or virginiamycin or no antimicrobial were compared after receiving *S.* Typhimurium via the drinking water (Gustafson *et al.*, 1981). The authors tried to achieve a level of infection that corresponds to the level of natural infection and the administration of the salmonellae was distributed over several days. The results indicate that the feeding of virginiamycin or avoparcin led to a larger proportion of animals shedding salmonella as compared to controls during the first 3 weeks after infection. This proportion of positive birds then decreased to become lower than that in the control group at about 4 weeks post infection. However, in the samples taken from the caeca after slaughtering the birds at the end of the trial, the proportion of salmonella positive birds was highest in the virginiamycin-fed group and lowest in the control group.

The sample size in this study is comparatively large, 100 animals in each group (all animals were sampled on each sampling occasion). The bacteriological method used for isolating the inoculated salmonella from the faecal samples include selective culture on media containing nalidixic acid, as the experimental strain was resistant to nalidixic acid. This isolation procedure would, however, also yield nalidixic acid-resistant mutants of other lactose negative enteric bacteria, and one cannot be certain that the proportion of coliforms mistakenly identified as salmonellae would be the same in all experimental groups. No further identification of the presumed salmonellae isolated in this way is reported in the article. The major objection against the design of this study is, however, that all birds received monensin at 100 ppm in the diet. Since monensin has antibacterial effects, this must be regarded as a possible confounder. Thus, the study only

investigated the differences between birds fed monensin and birds fed monensin plus avoparcin or virginiamycin.

Smith and Tucker have published several studies on the influence of various antimicrobial substances on the course of salmonella infection and excretion in broiler chickens (Smith and Tucker, 1975a; Smith and Tucker, 1975b; Smith and Tucker, 1978; Smith and Green, 1980; Smith and Tucker, 1980). One study from 1975 includes both tylosin and virginiamycin (Smith and Tucker, 1975b) and one from 1978 includes tylosin (Smith and Tucker, 1978). Tylosin was given at concentrations of 10 and 100 mg/kg feed in both studies and in the study from 1975 virginiamycin was also given at concentrations of 10 and 100 mg/kg. Duplicate experiments were performed on groups of 33-45 chickens receiving either antibiotic-containing feed or control feed without antimicrobial additives. In the study from 1975, the animals were experimentally infected with 0.3 ml of a nalidixic acid-resistant strain of *Salmonella* Typhimurium at a concentration of $10^9$ CFU/ml. In the study from 1978, the animals were infected through contact with experimentally infected chickens. Cloacal swabs were taken from all animals throughout the trials and caecal contents were sampled after slaughter at the end of each trial. The bacteriological methods are the same as those employed by Gustafson and co-workers (1981), i.e. culture on selective media containing nalidixic acid. The authors claim that very few faecal bacteria grow on this medium and the colonies of those that do can easily be differentiated visually from those of the infecting salmonella strain. No validation of this statement is presented.

In these two studies tylosin, at both concentrations tested, gave a higher rate and a greater amount of salmonella excretion in the birds given this diet, compared to that of the control groups. Virginiamycin, at the concentrations tested, caused only a slight increase, or no increase, in the rate and amount of salmonella excretion.

No studies addressing the issue of dose-response effects of spiramycin, tylosin or virginiamycin on salmonella colonisation have been found, nor any studies concerning effects on infectious dose.

Taken together, it appears that tylosin and virginiamycin might effect salmonella colonisation, but available studies can not provide the basis for any proper assessment. No information has been found on spiramycin in this matter.

### E.7.2 Other enteric pathogens

No data on influence of macrolides on colonisation with other enteric pathogens, such as *Campylobacter* spp. or *Yersinia enterocolitica* has been found.

### E.7.3 Other effects

Continuous administration of MLS antibacterials to animals might interfere with disease surveillance. Kempf and co-workers (1991) suggested that macrolide (spiramycin or tylosin) treatment might hinder the detection of *Mycoplasma gallisepticum* in cultures, or antibodies to this pathogen, in samples from subclinically infected chickens. Ronne (1992) reported a decrease in the isolation rate of *Serpulina hyodysenteriae* after introducing virginiamycin in the feed of pigs with clinical signs of infection.

Such effects need to be considered for disease control based on surveillance programs, as they may substantially affect the efficiency and the benefit/cost ratio of the program.

## E.8 Toxicological aspects

As spiramycin, tylosin and virginiamycin are safely used in clinical therapy, they are not expected to have any obvious toxic effects on the target species at growth promoting dosages.

### E.8.1 Residues

Spiramycin and tylosin were evaluated by the Committee of Veterinary Medical Products(CVMP) in 1994 and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) 1991 (1991). In table E.VII, provisional maximum residue limits (MRL) fixed by the Commission in 1995 for spiramycin and tylosin when used as veterinary medical products are shown. Corresponding values for spiramycin when used in cattle were not given at the time, but temporary MRLs for cattle as fixed by JECFA 1991 to 50, 300 and 200µg/kg for muscle, liver and kidney. respectively.

MRLs are based on ADI (acceptable daily intake). These limits are based on risk assessments and are set to ensure that residues in animal products do not harm the consumers of these products.

Table E.VII. Maximum residue limits of spiramycin and tylosin as veterinary medical products in foodstuffs of animal origin according to Commission regulation (EC) 1442/95

| Substance | Marker residue | Animal species | MRL, µg/kg | Target tissue |
|---|---|---|---|---|
| **Spiramycin** | spiramycin | swine | 600[1] | liver |
| | | | 300[1] | kidney, muscle |
| | | | 200[1] | fat |
| **Tylosin** | tylosin | bovine, swine, poultry | 100 | muscle, liver, kidney |
| | | bovine | 50 | milk |

[1] Provisional MRL, expired July 1, 1997.

According to a study by Green Lauridsen and co-workers (1988), usage of tylosin and virginiamycin at dosages used for growth promotion did not result in residues even if no withdrawal time is practised.

Published studies on residue aspects on spiramycin when given at growth promotion dosages have not been found. Spiramycin fed to pigs at approximately 8 times the maximum concentration permitted for growth promotion resulted in liver residues at zero withdrawal time of 10 times the MRL (FAO/WHO, 1991). Assuming a linear relationship, residues in swine liver, from spiramycin used for growth promotion, could be above MRL at zero withdrawal time (see chapter 5). The pharmacokinetics of spiramycin are not linear in swine (Sutter *et al.*, 1992), which could result in higher residue levels than what might be expected. Similar experiments in poultry, with similar assumptions of a linear relationship, indicates that in poultry the MRL would not be reached with spiramycin at growth promotion dosages(FAO/WHO, 1991). For tylosin at growth promoting dosages, MRL is not expected to be reached (see chapter 5).

Further investigations into possible residues from spiramycin as antibacterial feed additives (AFA) in target species are therefore necessary.

## E.8.2 Allergy

Allergic reactions triggered by macrolide therapy in humans are rarely reported (Descotes *et al.*, 1988; Periti *et al.*, 1993). Occupational contact dermatitis and/or asthma is, however, not unusual in farmers (especially pig farmers), feed plant workers, veterinarians and people working in the pharmaceutical industry (Hjorth and Weismann, 1973; Gollins, 1989; Lee *et al.*, 1989; Caraffini *et al.*, 1994; Danese *et al.*, 1994). Hypersensitivity to tylosin and/or spiramycin, indicating a certain but not absolute cross-reactivity, has been reported (Hjorth and Weismann, 1973). Airborne antigen is thought to be the main cause of these reactions.

There is no information available on the prevalence of hypersensitivity to virginiamycin, but there can be no doubt that both tylosin and spiramycin are capable of functioning as potent antigens and that people continuously exposed to dust containing these substances may become allergic. Frequent use of macrolides in animal feed may therefore be regarded as a professional hazard for farmers and other people in contact with this feed.

### E.8.3 Other immunological effects

An apparent reduction of the response to vaccination following administration of macrolides has been noted in two studies. In a study by Hassan (1990), chickens, vaccinated against Newcastle disease virus (NDV), were challenged with NDV and subsequently treated with spiramycin. This resulted in a twice as high mortality in treated and vaccinated birds as compared to non-treated, vaccinated birds. The spiramycin was given as a subcutaneous injection at a high dose, which must be regarded as a rather extreme challenge.

In one study by Vahl (1985), virginiamycin was given in the feed at a concentration of 20 mg/kg in a floorpen experiment. Immunological response to vaccination with NDV, in the form of antibody production, was measured. The NDV titers in the group receiving virginiamycin were depressed as compared to the non-treated group. In a corresponding experiment with caged birds the results were inconclusive. No challenge experiments were made.

No explanations of the mechanisms behind these findings have been provided by the authors. No other, similar reports have been found. In many countries the control of NDV infections depends on vaccination programs. In view of this, these observations certainly merit further investigation.

## E.9 Environmental effects

Like other AFA, if the use of spiramycin, tylosin or virginiamycin reduces the amount of feed consumed per kg weight gain in the target animal, they would also be expected to reduce nitrogen output per kg weight gain.

The effect of tylosin and tylosin fermentation wastes on microbial activity in soil has been investigated (Bewick, 1978). Tylosin from waste was detected in leachates and after 10 weeks 20-32% had been leached from the soil. Addition of pure tylosin to potting compost in concentrations expected from fertilising soil with the wastes (2, 20 or 40 tonnes/ha) resulted in a decrease in microbial respiration for 5-7 weeks after the addition of the antibiotic. In an experiment by Jagnow (1978), the degradation of spiramycin added at 10 ppm to chicken manure was investigated. After 4 weeks, 70% of

the added spiramycin had been degraded. However, when the manure was mixed with soil in the ratio 1:3, degradation of spiramycin was complete within a week. Gavalchin and Katz (1994) studied the degradation of tylosin and erythromycin in sandy loam from a non-agricultural area mixed with chicken faeces at different temperatures. Sterile soil-faeces mixtures were used as controls. At 20 and 30°C, inactivation of tylosin occurred rapidly and after 5 days the detection limit was reached. At 4°C however, 40% of the initial concentration was still present after 30 days of incubation. No degradation of either antibiotic occurred in the sterile controls. This indicates that the degradation process is due to microbial degradation of the antibiotic, probably by enzymatic inactivation. In the experiment, non-agricultural soil was used and the antibiotics were mixed in faeces from non-medicated birds. As the prevalence of bacteria carrying resistance genes coding for macrolide degrading enzymes can be expected to be higher in both faeces from animals given the substance in question and in agricultural soil previously fertilised with macrolide containing manure, the degradation process can be expected to be more efficient. However, during the degradation period the microbial respiration is expected to be lowered as observed by Bewick (Bewick, 1978), presumably due to impairment of sensitive microbiota.

## E.10 Summary comments

Spiramycin, tylosin and virginiamycin all belong to important antibacterial classes. Increased resistance to spiramycin, tylosin and virginiamycin would hamper the therapeutic use of substances from these classes in both animals and humans. Exposure of bacteria to spiramycin, virginiamycin and tylosin selects for resistant strains, usually carrying one or several transmissible resistance determinants. In order not to further diminish their therapeutic value, these substances should be restricted to therapeutic use. Spiramycin, tylosin and virginiamycin are potent allergens, and may as such represent an occupational hazard for farmers and feedmill workers.

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# Annex F: Carbadox and olaquindox

## F.1 Introduction

Carbadox and olaquindox belong to the group of quinoxaline-di-N-dioxide derivatives, or quinoxalines. This group consists of synthetically produced substances with activity against anaerobic bacteria but also against certain gram-negative facultative anaerobes such as salmonellae (Prescott and Baggot, 1993). Carbadox is also active against gram-positive bacteria such as staphylococci (Devriese, 1980). The antibacterial activity of the quinoxalines is markedly improved under anaerobic conditions (Prescott and Baggot, 1993). Quinoxalines that are presently approved as antibacterial feed additives (AFA) in the European Union are carbadox and olaquindox. Other substances of this class are quindoxin and cyadox.

After oral administration, olaquindox and carbadox are rapidly absorbed and are extensively metabolised (FAO/WHO, 1991; FAO/WHO, 1994). The concentration profiles of the active substances in the gut show a gradual decline from the stomach to the colon where the concentrations are below detectable levels (Baars *et al.*, 1988; de Graaf *et al.*, 1988; Spierenburg *et al.*, 1988c).

The quinoxalines are not used for in human medicine. Carbadox and olaquindox are approved as feed additives for swine for growth promoting purposes at dosages ranging from 20 to 50 ppm and 50 to 100 ppm respectively. Quinoxalines are also used at similar or higher dosages for prevention of intestinal infections (see F.7).

The use of quinoxalines has been questioned for toxicological reasons, mainly due to their genotoxic and/or carcinogenic potential (Anonymous, 1995), they are also known to have toxic effects on the target species (Prescott and Baggot, 1993).

## F.2 Mode of action and resistance mechanisms

As mentioned above, the activity of the quinoxalines is markedly improved under anaerobic conditions. The mode of action of the quinoxalines on bacteria was investigated by Suter and co-workers (1978). The authors showed that the antibacterial effect of quindoxin was always accompanied by a reduction of the drug. Such a reduction only occurred under anaerobic conditions, and generated free radicals. Based on a series of experiments, the authors suggested that radicals produced during the intracellular reduction of

The figures in this report is only available in the printed version

Figure F.I. Tentative sketch of carbadox, olaquindox, nalidixic acid and enrofloxacin

the parent substance or some decomposition products thereof damage DNA without being bound to this target. As a consequence, DNA synthesis ceases, breakdown of chromosome ensues and the bacterial cell dies.

The quinoxalines are somewhat structurally related to the quinolones (see figure F.1) which also exert their antibacterial activity by interfering with the bacterial DNA synthesis. The quinolones inhibit the activity of bacterial topoisomerases, i.e. enzymes controlling the supercoiling of DNA, converting relaxed covalently closed circular DNA to a superhelical form by an energy dependent strand breakage and resealing process (Maxwell, 1997). Although there are still unclarities as to the precise bactericidal mechanism of the quinolones, it is thought that when the gyrase is inhibited, resealing following breakage is prevented and DNA is exposed to and degraded by exonucleases.

Resistance to quinolones develop through alterations of the target of the drug and to some extent through altered permeability or increased efflux.

As the information on mechanisms of action of the quinoxalines predates the discovery of bacterial gyrases, possible similarities with the precise mechanism of action of the quinolones cannot be determined. No information on the molecular background of observed quinoxaline resistance, nor on cross-resistance between quinoxalines and other DNA-active drugs such as quinolones has been found. It is likely, however, that were resistance due to altered permeability or active efflux to occur, this would confer cross-resistance to at least the other quinoxalines.

## F.3 Development of resistance

An experimental study on germ-free mice fed carbadox, olaquindox, flavomycin and chlortetracycline was reported by Corpet (1984). The mice were inoculated with intestinal microflora from 4 piglets, and the drugs were given at dosages corresponding to those used for growth promotion in livestock. Little effect on coliform resistance against olaquindox and carbadox was observed, nor was there any effect of the quinoxalines on tetracycline resistance.

A prospective study to monitor the development of olaquindox resistance in coliforms following the introduction of olaquindox as a feed additive was conducted in commercial farms in Suffolk by Linton and co-workers (1988). Olaquindox resistance in coliforms from farms with and without use of the antibacterial are shown in table F.I. As is often the case in field studies, there were problems with sampling variability and difficulties to control the management on the farms. In spite of this, the overall results were consistent and showed an increasing level of resistance to olaquindox in coliforms from farms using olaquindox. Incidence and level of resistance increased on

neighbouring farms not using olaquindox as well, but to a lesser extent. The latter finding is not surprising as the herds were not isolated from the environment.

Table F.I. The average percentage of coliforms resistant to 50 µg/ml olaquindox in each year of the survey (from Linton *et al.*, 1988)

| **Year** | **Resistance (%)** | |
|---|---|---|
| | **Control farms** | **Test farms** |
| 1981[1] | 0.00 | - |
| 1982 | 0.03 | 0.04 |
| 1983 | 0.12 | 5.63 |
| 1984 | 0.68 | 6.14 |

[1] Sampled before olaquindox was used in the UK

The ecological aspects of olaquindox resistance were investigated by Hedges and Linton (1988). In a prospective study, chosen pens in four pig farms, two of which used olaquindox were sampled weekly. Coliform bacteria were isolated from the samples and biovariant along with sensitivity to olaquindox and other antibacterials was determined. The overall proportions of coliforms resistant to olaquindox (counts on selective medium divided by counts on non-selective medium) were 0 and 0.02%, respectively, for control farms and 1.3% and 6.5% for the farms using olaquindox. A transient appearance of olaquindox-resistant strains in one of the control farms coincided with a changeover in the occupancy of the pen. The weekly fluctuations in proportion of resistant strains were very marked.

An increase in carbadox resistance in salmonellae over time was noted in a study from Kansas by Mills and Kelly (1986). The study included clinical isolates from necropsied swine in Kansas during 1980-1983. A steady increase in carbadox resistance, from 37% 1980 to 61 % 1983 was noticed. The authors note that in-feed carbadox in Kansas is labelled for prevention of swine dysentery and for treatment of salmonella infections.

Ohmae and co-workers (1983) reported the incidence of carbadox resistance in *Eschericha coli* from cattle, pigs and chickens, collected during 1976-1980 in Japan. Carbadox resistance was only found in isolates from pigs. All isolates with a minimum inhibitory concentration (MIC) of carbadox under anaerobic conditions of >25µg/ml originated from 1 farm, where carbadox was used for prevention of swine dysentery. Baumgartner and co-workers (1985), investigating samples from the same animal species in Switzerland, found olaquindox-resistant coliforms in samples from pigs (24%) and calves (20%) but none in samples from chickens. Spanoghe and

Pohl (1987) reported a 5 times higher prevalence of carbadox-resistance in coliforms from pigs receiving carbadox, as compared to pigs not fed antibacterials, in a material collected in various European countries.

Several investigations report on a uniform sensitivity of *Serpulina* spp to carbadox (Molnar and Magyar, 1987; Walter and Kinyon, 1990; Molnar, 1996) with reported MICs generally considerably below 0.4µg/ml. Contrary to this, in a study from Canada, a $MIC_{90}$ (minimal inhibitory concentration for 90% of the isolates) of carbadox for *Serpulina hyodysenteriae* of >6µg/ml was reported (Messier *et al.*, 1990). This figure is considerably higher than those reported by other authors, indicating an emerging resistance. In an investigation from Sweden, carbadox had a MIC of >0.1µg/ml for 3 out of 67 (4%) of the Serpulina isolates investigated. Although MICs in this range might not cause the isolates to be classified as resistant, their sensitivity seems reduced as the modal MIC in this study was 0.012µg/ml. This might indicate a gradual increase in MICs, a phenomenon which is seen when stepwise mutations are needed for full resistance. Interestingly, carbadox was not used in Sweden at the time when the isolates were collected.

Two reports show the MICs of olaquindox and carbadox for individual strains of *Serpulina* spp (Williams and Shively, 1978; Fellström *et al.*, 1996). No clear relation between MICs of carbadox and olaquindox can be noted in the data presented. In the study by Williams (1978), a good correlation between MICs obtained *in vitro* and therapeutic efficacy of respective drug in experimental infections was noted. Infection with the three strains with MICs for olaquindox of 2.5µg/ml or more resulted in considerable morbidity in spite of olaquindox treatment, while the treated pigs infected with a strain with a MIC of olaquindox of 0.3µg/ml remained healthy. The same 4 strains were uniformely sensitive to carbadox both *in vitro* and *in vivo.* Taken together, these studies indicate that strains resistant to olaquindox are not cross-resistant to carbadox. The mechanisms for the observed resistance were not investigated.

Concerning the activity of carbadox against gram-positive bacteria, few publications are available. Devriese (1980) reported on the carbadox-sensitivity of 950 isolates of *Staphylococcus aureus* from cattle, poultry and pigs. All the isolates had MIC values <1.6µg/ml and no indications of acquired resistance were found. Similarly, in an investigation of MIC of carbadox for clostridia from the same animal species, all 68 isolates had MIC values below 0.25µg/ml (Dutta *et al.*, 1983).

Indications of a simultaneous increase in minimum inhibitory concentrations for olaquindox and the quinolone enrofloxacin was recently detected in a Swedish survey (Greko, 1997). The investigated strains originated from three different cathergories of piglet producing herds; 10 herds were using olaquindox as medicated feed (160 ppm), 10 herds were using zinc oxide (2500 ppm) and 10 herds used no medication. The observed

phenotypes with respect to olaquindox and enrofloxacin sensitivity are shown in table F.II. 16 out of 61 isolates with resistance to olaquindox according to the break-points assigned had MIC values of 0.25 for enrofloxacin. This intermediary MIC value of enrofloxacin was not found in isolates sensitive to carbadox. Only two isolates had higher MIC values (0.5 µg/ml).

Table F.II. Phenotypes with respect to olaquindox and enrofloxacin sensitivity in *E.coli* isolated from piglets in herds using different regimes (Greko, 1997)

| **Phenotype**[1] | **Proportion of isolates with phenotype from sampling group (%):**[1] | | | |
|---|---|---|---|---|
| | **Olaquin-dox** (n=60)[2] | **Zinc-oxide** (n=73) | **No medi-cation** (n=85) | **All groups** (n=218) |
| $Olaquindox^{R}$-$Enrofloxacin^{R}$ | 0 | 0 | 1 | <1 |
| $Olaquindox^{R}$-$Enrofloxacin^{I}$ | 15 | 9 | 0 | 7 |
| $Olaquindox^{R}$-$Enrofloxacin^{S}$ | 20 | 9 | 30 | 20 |
| $Olaquindox^{S}$-$Enrofloxacin^{R}$ | 0 | 1 | 0 | <1 |
| $Olaquindox^{S}$-$Enrofloxacin^{I}$ | 0 | 0 | 0 | 0 |
| $Olaquindox^{S}$-$Enrofloxacin^{S}$ | 65 | 80 | 68 | 71 |

[1] Determined according to MICs (µg/ml); $Olaquindox^{R}$ >32, $Olaquindox^{S}$<64, $Enrofloxacin^{R}$ >0.25, $Enrofloxacin^{I}$=0.25, $Enrofloxacin^{S}$<0.25

[2] n= number of isolates

## F.4 Acquisition of resistance

Omahae and co-workers (1983) investigated the transferability of carbadox resistance from resistant *E.coli* originating from one farm. The carbadox-resistance was transferable by conjugation to other strains of *E.coli.* Transfer was invariably linked to transfer of resistance to spectinomycin, streptomycin and ampicillin. The resistance determinants were shown to reside on a conjugative plasmid. No transfer to *Serpulina* spp was observed. Spanoghe and Pohl (1987) tested 61 *E.coli* strains with anaerobic MIC values for carbadox of >8µg/ml for their ability to transfer resistance (including carbadox). The experiments were successful in 55 and 69% of the attempts when two *E.coli* strains were used as recipients. Transfer of carbadox resistance to salmonellae was achieved only in 10% of the experiments. Transfer of carbadox resistance was constantly associated with co-transfer of

resistance to at least one other antibacterial. The most frequently linked transfer was carbadox-streptomycin-tetracycline resistance. In contrast, in similar experiments reported by Baumgartner and co-workers (1985), transfer was successful from only 3 out of 64 strains tested and no co-transfer was observed.

Linton and co-workers (1988) demonstrated transfer of olaquindox-resistance from 6 out of 12 *E. coli* strains to a laboratory strain of *E. coli*. Transfer of resistance did not correlate to the appearence of a plasmid nor was it linked to transfer or other resistance traits. The evidence therefore suggests that the gene(s) conferring olaquindox resistance were located on the chromosome.

An apparent suppression of coliforms carrying R-plasmids has been reported for carbadox and olaquindox (Gedek, 1979) but no mechanism explaining this was provided. Quinolones and novobiocin, inhibitors of DNA gyrase, have been shown to eliminate plasmids *in vitro* both by inhibiting their replication (Uhlin and Nordström, 1985) and their transfer (Weisser and Wiedemann, 1987).

No information on the precise genetic nature of either carbadox or olaquindox resistance has been found. Plasmid mediated resistance to nalidixic acid, a quinolone, was reported from Bangladesh in 1987 (Munshi *et al.*, 1987). This was later found to be due to a mutation of the recipient strain. Possibly, the transferred plasmid induced mutations leading to nalidixic acid resistance (Ahmed, cit. by Courvalin, 1990). Although speculative, a similar phenomenon could explain why the transconjugant strains in the experiments cited above had markedly lower MICs than those of the donor strains (Ohmae *et al.*, 1983; Baumgartner *et al.*, 1985). If stepwise mutations are necessary to reach higher MICs, single or two-step mutations induced by a transfered gene element might result in the comparatively lower MICs observed in the recipients.

## F.5 Effects on specific animal diseases

The preventive effect of the quinoxalines, especially carbadox, against certain intestinal diseases in animals, especially swine dysentery, is well documented.

Studies on the pharmacokinetics of the quinoxalines have shown that the intestinal concentrations gradually decline from the stomach to the colon (Baars *et al.*, 1988; de Graaf *et al.*, 1988; Spierenburg *et al.*, 1988c). As swine dysentery is primarily a disease of the lower intestine, a preventive rather than a therapeutic effect is to be expected.

Williams and Babcock (1978) investigated presence and development of carbadox-resistance *in vitro* in *S. hyodysenteriae,* and prevention of experimentally induced infection by carbadox. No carbadox-resistant strains

were found and carbadox at 5 ppm above maximum growth promoting level was found to be effective in preventing swine dysentery. Similar results were obtained by Williams and Shively (1978), who found no carbadox-resistance in isolates of *S. hyodysenteriae* and managed to prevent swine dysentery by carbadox at, or slightly above, growth promoting levels. Jenkins and Froe (1985) prevented experimentally induced swine dysentery by feeding carbadox at growth promoting levels to pigs. In a study by Jacks and co-workers (1986), carbadox was fed at 5 ppm above growth promoting level and was found to successfully prevent swine dysentery.

Raynaud and co-workers (1980a; 1980b) developed a swine dysentery model for evaluation of drug prophylaxis. Using this model carbadox was found to effectively prevent the outbreak of swine dysentery. Olaquindox only prevented the disease during the medication period, but not post-medication. It is not clear what dosages were used in this experiment.

Taylor and Davey (1980) found that carbadox at maximum growth promoting levels prevented the onset of swine dysentery and eliminated *S. hyodysenteriae* infection in experimentally infected pigs. Rainier and co-workers (1980b) investigated therapeutic effects and prevention of the carrier state in pigs experimentally infected with *S. hyodysenteriae*. Carbadox at 5 ppm above permitted growth promoting levels effectively eliminated the infectious agent from the host, thereby curing the disease without leaving any asymptomatic carriers. Similar results were shown in a similar study by the same authors (Rainier *et al.*, 1980a). Biehl and co-workers (1984) used the same dosage of carbadox and were able to successfully treat pigs experimentally infected with *S. hyodysenteriae*.

In a large scale trial in commercial pig herds in France (Raynaud and Bretheau, 1973), 50 ppm carbadox was found to be sufficient for both prophylaxis and treatment of swine dysentery.

As mentioned under F.3, olaquindox at growth promoting levels completely prevented swine dysentery induced by an olaquindox-susceptible strain (Williams and Shively, 1978). When olaquindox-resistant strains were used as challenge, the disease was not prevented, but morbidity was lower than in the non-medicated control group. Davis and Libke (1976) prevented clinical signs of swine dysentery in experimentally infected pigs by dosages of olaquindox higher than what is permitted for growth promotion.

These experimental results have been confirmed by various field studies. In a study from Pfizer Technical Information Service (Anonymous, 1980), carbadox at the maximum growth promoting dosage totally prevented occurrence of swine dysentery. Hunneman (1980) reported a notable decrease in outbreaks of swine dysentery in an area when carbadox was introduced as a feed additive in local swine herds. Molnar (1987) reported an attempt to eradicate swine dysentery from swine herds by the aid of carbadox at growth promoting levels. The eradication program failed, but on an

individual basis, medication was successful both as a preventive and therapeutic measure. No carbadox-resistant *Serpulina* spp. were isolated. Colibacillosis and salmonellosis were also successfully prevented in the medicated herds. Olson (1986) reported successful eradication of swine dysentery with in-feed carbadox at 5 ppm above the maximum growth promoting level. Wood (1987) also reported successful eradication of swine dysentery on a farm with in-feed carbadox. The dosage used in this case was not reported.

Some information is also available concerning the preventive effect of quinoxalines for other diarrhoeal conditions of pigs. Bertschinger (1976) found that olaquindox at low dosages (50 ppm) in feed was effective for prevention of experimentally induced *E. coli*-diarrhoea in piglets. Holmgren (1994) found that prophylactic treatment with 122 ppm or 173 ppm olaquindox in commercial swine herds significantly ($p<0.01$) reduced the incidence of post-weaning diarrhoea in all but one of the herds investigated. From the herd where olaquindox had no prophylactic effect, *E. coli* resistant to olaquindox were isolated. Troutt and co-workers (1974) found that carbadox at the maximum growth promoting concentration reduced clinical signs and intestinal lesions in pigs experimentally infected with *Salmonella* Cholerasuis. Winkelman and Hawkins (1996) evaluated carbadox for the control of proliferative enteropathy in swine. Carbadox at growth promoting levels was found to reduce clinical signs and pathological lesions in pigs experimentally infected with *Lawsonia intracellularis* (the causative agent of porcine proliferative enteritis).

Stutz and co-workers (1984) showed that carbadox at 55 ppm significantly ($p<0.05$) reduced the amount of *C. perfringens* (associated with necrotic enteritis) in the intestines of chickens. Carbadox is not used in poultry, so this may be of limited interest, but it is clear that carbadox is effective against clostridia, both *in vivo* and in *vitro*.

## F.6 Impact of resistance on animal and human health

Carbadox, and to some extent olaquindox, are used for prevention or therapy in veterinary medicine. Neither of those, nor related substances, are used in human medicine. As no information on possible cross-resistance to the modern quinolones, which are valuable drugs in human and animal medicine, is available, the impact of an emergence of resistance cannot be fully assessed.

Quinoxalines are valuable for prevention of swine dysentery and weaning diarrhoea. Development of resistance in *Serpulina* spp. to carbadox or olaquindox would entail reduced effectiveness of strategies employing

quinoxalines for prevention of this disease. Few drugs are available for this purpose. However, available data indicate that the development of resistance to quinoxalines is slow.

## F.7 Other effects on the microflora

The influence of carbadox treatment on salmonellosis was investigated by Troutt (1974). When fed at 55 ppm to pigs experimentally infected with *Salmonella* Choleraesuis, carbadox diminished the clinical signs and reduced the extent and severity of lesions. Development of resistance in salmonella towards carbadox has been documented (Mills and Kelly, 1986). No investigations concerning possible effects of quinoxalines on infections with resistant strains of *Salmonella* spp. have been found.

No information on the influence of quinoxalines on other food-borne pathogens such as *Campylobacter* spp. or *Yersinia enterocolitica* has been found.

## F.8 Toxicological aspects

### F.8.1 Target species

A series of investigations concerning adrenal toxicity of quinoxalines, particularly carbadox, has been published. Field observations of intoxications in pigs fed high doses of carbadox (up to 150 ppm) were confirmed by an experimental study where 150 ppm carbadox was given to weaned pigs for up to 10 weeks (van der Molen *et al.*, 1985). Clinical signs of dehydration and impaired growth were observed from 3 weeks and onwards. Histopathological examination revealed atrophy of the glomerular zone of the adrenal glands.

In a subsequent experiment, pigs fed carbadox at doses between 25 and 200 ppm were investigated (van der Molen *et al.*, 1986). The results confirmed the observation of adrenal damage as lowered plasma-aldosterone concentrations and corresponding changes in sodium and potassium in blood samples were observed for all doses. The magnitude of the effects were dose dependent.

Further investigations into the pathomorphological changes showed that after 10 weeks of carbadox at 25 ppm or more, damage to the cells of the zona glomerulosa of the adrenal gland could be observed histologically (van der Molen, 1988). Again, the effects were dose and time dependent. Only two animals per dosage group were examined histologically.

The hormonal changes induced by carbadox were further investigated (van der Molen *et al.*, 1989). After 9 weeks, the levels of plasma renin were higher in all groups fed carbadox than in the control group.

Taken together, these experiments show that carbadox supresses the mineral-corticoid secretion from the adrenal gland, inducing secondary hormonal changes in the renin-angiotensin system. These observations explain the clinical picture observed. The dose-dependent inhibition of the aldosterone production of porcine adrenal cells was confirmed by *in vitro* experiments (Spierenburg *et al.*, 1988a; Spierenburg *et al.*, 1988b; Jager *et al.*, 1994).

In a study comparing the effects of carbadox, cyadox and olaquindox similar toxic effects were noted. For olaquindox, adrenal toxcicity was observed, although less pronounced, at dosages of 100 ppm or more (Nabuurs *et al.*, 1990). Again, the effects were dose- and time dependent. The clinical signs observed were also dose-related, ranging from mild to severe. At 50 ppm of carbadox or 100 ppm of olaquindox, mild effects in the form of increased faecal dryness were observed. Other signs included dehydration (urine drinking), decreased abdominal volume and lowered haematocrit values. Further, changes in hair quality, with hair becoming longer and withered, irritable behaviour and a decrease in feed intake and weight gain could sometimes be observed. It should be noted that also in other respects, there is a difference in activity between the substances, and carbadox is permitted in feed at a concentration of 50 ppm, whereas olaquindox is used at 100 ppm.

In the *in vivo* studies cited above, the growth-rate of the medicated animals was either equal to or reduced compared to the control group. This is in conflict with the reported growth promoting effect of these substances. It is possible that in a field situation, the preventive effects of the quinoxalines against common enteric diseases act as confounders, i.e. if the (sub)clinical diseases negatively affect the growth-rate.

Accidental overdosing of olaquindox (Köfer *et al.*, 1990; Stockhofe-Zurwieden *et al.*, 1991) and carbadox (Power *et al.*, 1989) has been reported to cause death and severe adrenal damage in piglets.

As the main early sign of intoxication with quinoxalines, i.e. dry faeces, may be mistaken for recovery from enteric disease, mild intoxications are expected to be overlooked by farmers and farm workers. Further, as the symptoms are diffuse, a definite diagnosis might be difficult to reach.

## F.8.2 Adverse effects in humans

***Carbadox***

In a study where radiolabelled carbadox was fed to swine at 55 ppm for 5 consecutive days, total residues 30 days after withdrawal were around 5, 74 and 15 µg/kg in muscle, liver and kidney, respectively. After 70 days, the measured concentrations in liver were 13µg/kg (FAO/WHO, 1990).

Carbadox is rapidly metabolised to, among other metabolites, quinoxaline-2-carboxylic acid (QCA) and desoxycarbadox. In a pig study on residues of carbadox and its metabolites, feeding of 50 ppm carbadox did not result in any detectable residues after a withdrawal time of one day (Baars *et al.*, 1991). Desoxycarbadox was measurable in liver for 14 days (detection limit 1µg/kg). Liver samples yielded residues of QCA above 30 µg/kg in liver and kidney for 4 to 5 weeks. In an experiment by Rutalj (1996), QCA was still present at around 10 µg/kg at 62 days after cessation of feeding of carbadox at 50 ppm.

Detection of QCA is dependent on the extraction method used, and it has been suggested that this is due to other unknown intermediate metabolites in the pathway of carbadox to QCA (Baars *et al.*, 1991). QCA, being the major residual metabolite, has been suggested as a marker substance for residue studies (FAO/WHO, 1990).

Long term toxcicity studies on carbadox in rats have shown dose-related increases of benign and malignant liver tumors.

Positive results were reported in 14 out of 15 mammalian and non-mammalian genotoxicity studies (FAO/WHO, 1990), clearly indicating a genotoxic potential of carbadox.

Chronic administration of the meatabolite desoxycarbadox to rats has resulted in an increase of liver tumour incidence in all dosage groups (FAO/WHO, 1990; FAO/WHO, 1991). Most tests for genotoxicity have produced negative results, but positive findings were recorded in the cell transformation test and in Ames test using liver cells pre-treated with polychlorinated biphenyls.

With respect to QCA, no studies indicate reasons to suspect carcinogenic or genotoxic properties (FAO/WHO, 1990; FAO/WHO, 1991)

In the 36th report of JECFA (FAO/WHO, 1990) it was concluded that an ADI could not be established, due to the carcinogenic and genotoxic nature of carbadox and some of its metabolites. MRLs set in 1990 (FAO/WHO, 1990) for QCA as marker substance were apparently based on the detection limit of the analytical method.

### *Olaquindox*

Olaquindox is rapidly absorbed from the gut and mainly excreted via urine. No bound residues appear to be present in tissue (FAO/WHO, 1995). In a study where radiolabelled olaquindox was given to pigs as a single oral dose of 2 mg/kg body weight, <1, 2 and 1 µg/kg of total residues were detected in muscle, liver and kidney, respectively after 28 days. (FAO/WHO, 1990). At doses corresponding to recommended feed inclusion doses (2.5 mg/kg body weight) and a 28 days withdrawal period, olaquindox residues were below 5µg/kg in muscle and below 10µg/kg in kidney (FAO/WHO, 1995). The substance is extensively metabolised the animal. The metabolites found vary between tissues and between animal species (FAO/WHO, 1995). In pigs given 60 ppm olaquindox in the feed up to 16 weeks of age, and with a withdrawal period of 28 days, olaquindox residues were below 0.005 ppm in muscle and below 0.01 ppm in kidney (FAO/WHO, 1995). One of the metabolites, 3-methylquinoxaline-2-carboxylic acid (MQCA) has been chosen as a marker compound (FAO/WHO, 1995). Radiolabel studies indicate that the MQCA accounts for approximately 25% of the total residues.

In long-term toxicity studies in mice, an increase in the incidence of benign adrenal cortical adenomas and benign proliferative lesions in the lungs were noted at the highest dose level (54 mg/kg). There was no effect on the incidence of malignant tumours (FAO/WHO, 1990). In another long term study in rats, no increase in the incidence of tumours was noted.

The genotoxicity of olaquindox has been investigated in a range of *in vitro* and *in vivo* studies. Both positive and negative findings were reported in assays using various mammal and non-mammal systems (Nunoshiba and Nishioka, 1989; FAO/WHO, 1990).

The marker substance, MQCA, is known to be responsible for the bacterial mutagenecity of other quinoxaline derivatives (FAO/WHO, 1995). No studies specific for olaquindox are available.

An ADI could not be allocated by JECFA 1995, because of the genotoxic potential of the parent compound and the absence of specific toxicity studies on the metabolites. No MRL has been set.

### *Photoallergy*

Carbadox, olaquindox and other quinoxalines induce both photoxicic and photoallergic mechanisms through formation of a reactive oxaziridine reacting with proteins upon exposure to light (de Vries *et al.*, 1990b).

The photoallergic properties of olaquindox has been confirmed in studies on rats (de Vries *et al.*, 1990a). Olaquindox-induced photoallergy has been described in pig breeders following airborne exposure (Schauder, 1989;

Hochsattel *et al.*, 1991; Schauder *et al.*, 1996). In a report by Schauder (1996), twelve out of 15 patients habitually mixed mineral feed containing 1000 ppm olaquindox on the farm, two of the patients only handled pellets. The average interval of exposure at onset of clinical signs for the patients who mixed feed was 2.5 years.

The clinical symptoms are characterised by eczema worsened by sunny weather. At worst, the affected persons have to stay indoors during daytime. The reaction may be persistent and can be severely disabling (Schauder *et al.*, 1996). In spite of the experimental findings indicating similar properties of carbadox, no clinical reports have been found. Nonetheless, carbadox should be regarded as a potential photoallergen (de Vries *et al.*, 1990b).

***Some comments on toxicological aspects***

The quinoxalines are suspected of having carcinogenic and genotoxic properties (Cihák and Srb, 1983; Nunoshiba and Nishioka, 1989). Carcinogenic and genotoxic effects are not acceptable since the effect could occur at very low intake levels, especially if the substance in question is ingested regularly over a number of years. Farm and feedmill workers are a special risk group, frequently exposed to AFA when handling animal feed. If appropriate protection cannot be ensured, the handling of animal feed containing quinoxalines and other AFA with potentially genotoxic effects must be regarded as an occupational hazard both due to potential genotoxicity and to photoallergenicity. Althogh the precise exposure cannot be determined, a conservative approach is often recommended for genotoxic substances in order to prevent underestimation of the risks.

## F.9 Environmental effects

No information on the environmental fate of quinoxalines has been found. The quinoxalines are mainly excreted via urine (FAO/WHO, 1990; FAO/WHO, 1995). They are sensitive to photodegradation. Considering the potential genotoxicity of some of these substances, not only the fate of the parent substance but also of relevant metabolites should be investigated.

## F.10 Summary comments

Although transferable resistance to carbadox and olaquindox has been reported, the mechanisms and molecular biology of this or other resistance phenomena are still unclear. The precise target in the bacterium of the quinoxalines or their metabolites is still unknown. The development of resistance in important animal pathogens such as *Serpulina* spp. seems slow.

No information is available concerning cross-resistance to the most modern class of antibacterials, the quinolones. Therefore, the impact of a possible emergence of resistance in enteric bacteria cannot be fully assessed.

The preventive effects of carbadox and olaquindox against enteric diseases of pigs is well documented.

Olaquindox and carbadox have several unwanted properties related to toxicology. Both substances have toxic effects on the adrenal glands of exposed animals at the dosages used for growth promotion. Profound disturbances in the stereoid balance of the animals, resulting in clinical signs of dehydration has been reported.

The quinoxalines and some of their metabolites are, or are suspected to be, genotoxic. Olaquindox is a well known photoallergen and according to *in vitro* data, carbadox shares this property. Therefore, exposure to quinoxalines must be regarded as an occupational hazard.

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# Annex G: Buget sheets to 3.5

## G.1 Budget sheets for piglet production

(SLU INFO - OMRÅDESKALKYLER)

***Basic assumptions***

Herd size 50 sows, a sow produces 20 piglets per year, pigs are 100 % Scan-H piglets, 5 weeks to weaning, piglets sold at 25 kg (at 85 days of age), purchased feed, the feed for gilts until farrowing included, new buildings and interest rate 7%, results per sow

Assumptions on the effect from the use of antibiotics as additives in animal feed on growth 6.8%, feed conversion 4.6%, and reduction of mortality 0.6%.

***Gross margins (from table G.I)***

| | **Without AFA** | **With AFA** | **Gain** |
|---|---|---|---|
| Gross margin (TB 1) = Income minus variable costs | 3092 | 3281 | 189 |
| Gross margin (TB 2) = income minus variable and capital costs | 1376 | 1581 | 205 |
| Gross margin (TB 3) = income minus variable, capital and fixed costs | -7345 | -7140 | 205 |

Table G.I Budget sheet for piglet production

| **Income** | **Unit** | **Quan-tity** | **Price/ unit** | | **Added quan-tity** | **Price SEK** | **Added benefit** | **Sum add. be-nefit** |
|---|---|---|---|---|---|---|---|---|
| Piglets | n. | 20 | 454 | 9080 | 0.12 | 454 | 54 | |
| Premium | SEK | 20 | 19.00 | 380 | 0.12 | 19.00 | 2 | |
| Meat from culled sows | n | 0.50 | 1076 | 538 | | | | |
| Total income | | | | 9998 | | | 10054 | 56 |
| **Variable costs** | | | | | | | | |
| Replacement | n | 0.5 | 2000 | 1000 | | | | |
| Sow feed | kg | 1289 | 1.70 | 2191 | | | | |
| Piglet feed | kg | 938 | 2.16 | 2026 | 55 | 2.16 | 93 | |
| Straw, bedding | kg | 400 | 0.35 | 140 | | | | |
| Electricity | kWh | 710 | 0.45 | 320 | | | | |
| Health control fee | SEK | | | 58 | | | | |
| Sow production control fee | SEK | | | 150 | | | | |
| Cost of boar | SEK | | | 351 | | | | |
| Insurance | n | | | 70 | | | | |
| Miscellaneous | SEK | | | 600 | | | | |
| Additional vet costs if AB prohibited | | 20 | 2.00 | | | | 40 | |
| Sum variable costs | | | | 6906 | | | 6773 | 189 |
| Buildings, maintenance | SEK | 73200 | 2.1% | 1537 | | | | |
| Costs of animal capital | SEK | 1538 | 7% | 108 | | | | |
| Costs of operational capital | SEK | 1009 | 7% | 71 | 14 | 7% | 1 | |
| Savings due to shorter period to 25 kg | | | | | 14 | 1.06 | 15 | |
| **Sum of variable and capital costs** | | | | 8622 | | | 8473 | 205 |
| Buildings, maintenance and depreciation | SEK | 73200 | 8.3% | 6076 | | | | |
| Labour | hours | 23 | 115.00 | 2645 | | | | |
| **Sum of variable, capital and fixed costs** | | | | 17343 | | | 17194 | 205 |

## G.2 Budget sheets for pig meat production

(SLU INFOS OMRÅDES KALKYLER)

### *Assumptions*

Herd size 288 pigs per batch, purchased feed, dry feeding, live weight at slaughter 107 kg, slaughter weight 73% of live weight, new buildings, interest rate 7%, 2.7 batches per year, and feed conversion 2.8 kg feed per kg weight gain, results per slaughter hog

Assumptions on the effect of antibiotics used as additives in animal feed on growth 1.86%, and feed conversion 1.65%.

### *Gross margin per pig (from table G.II)*

| | **Without AFA** | **With AFA** | **Gain** |
|---|---|---|---|
| Gross margin (TB 1) = income minus variable costs | 155 | 162 | 7 |
| Gross margin (TB 2) = Income minus capital and variable costs | 62 | 71 | 9 |
| Gross margin (TB 3) = Income minus variable, capital and fixed costs | -267 | -253 | 14 |

### *Gains in a farm with 500 pigs per batch*

| | **Without AFA** | **With AFA** | **Gain** |
|---|---|---|---|
| Pigs produced per year | 1350 | 1375 | |
| Gross margin TB1 (SEK) | 209250 | 227750 | 13500 |
| Gross margin TB2 (SEK) | 83700 | 97625 | 13925 |
| Gross margin TB3 (SEK) | -360450 | -323360 | 12575 |

Table G.II. Budget sheet for pig meat production

| Per slaughter pig | Unit | Quantity | Price | SEK | Added quantity | Price | Added bene-fit | Sum of added benefit |
|---|---|---|---|---|---|---|---|---|
| **Income** | | | | | | | | |
| Meat kg | kg | 78 | 13.80 | 1076 | | | | |
| Delivery premium | SEK | | | 26 | | | | |
| | | | | 1102 | | | 1102 | 0 |
| **Variable costs** | | | | | | | | |
| Piglets costs 25 kg | n | 1.00 | 454.00 | 454 | | | | |
| Delivery fee | SEK | | | 39 | | | | |
| Feeding stuff | kg | 229.60 | 1.76 | 404 | 3.8 | 1.76 | 7 | |
| Service fee | SEK | | | 3 | | | | |
| Energy | SEK | | | 24 | | | | |
| Mortality (2 %) | SEK | 627 | 2.0% | 13 | | | | |
| Miscellaneous | SEK | | | 10 | | | | |
| **Sum variable costs** | | | | 947 | | | 940 | 7 |
| **Capital costs** | | | | | | | | |
| Buildings maintenance | SEK | 3407 | 2.1% | 72 | 64 | 2.1% | 1.3 | |
| Cost of animal capital | SEK | 183 | 7% | 13 | 4 | 7% | 0.3 | |
| Cost of operational capital | SEK | 111 | 7% | 8 | 2 | 7% | 0.1 | |
| **Sum variable & capital costs** | | | | 1040 | | | 1031 | 9 |
| **Capital costs** | | | | | | | | |
| Buildings depreciation and interest payment | SEK | 3407 | 8.3% | 283 | 63 | 8.3% | 5 | |
| Labour | hours | 0.4 | 115.00 | 46 | | | | |
| **Sum variable, capital and fixed costs** | | | | 1369 | | | 1355 | 14 |

## G.3 Budget sheets for egg production

(SLU INFOS OMRÅDES KALKYLER)

### *Assumptions*

Batch size 10000 layers, production period 66 weeks, age at start in flock 16 weeks, and at slaughter 80 weeks, purchased feed, feed consumption 2.25 per kg eggs.

New buildings, caged birds, three story cages, annual egg production 20 kg per introduced young bird and interest rate is 7%, results per 100 hens

Assumptions on the effect of antibiotics used as additives in animal feed on egg performance 1.63%, and feed conversion 1.32%.

### *Gross margins (from table G.III)*

| | **Without AFA** | **With AFA** | **Gain** |
|---|---|---|---|
| TB1 = Income minus variable costs | 4372 | 4535 | 163 |
| TB 2 = Income minus variable and capital costs | 3570 | 3913 | 163 |
| TB 3 = Income minus variable, capital and fixed costs | -31 | 132 | 163 |

Table G.III. Budget sheet for egg production

| | Unit | Quantity | Price | SEK | Added quantity | Price | Added benefit | Sum added benefits |
|---|---|---|---|---|---|---|---|---|
| **Income** | | | | | | | | |
| Eggs | kg | 1564 | 9.30 | 14545 | 21 | 9.30 | 195 | |
| Meat for slaughter | n | 69 | 1.38 | 95 | | | | |
| Quality deductions | kg | 1564 | -0.53 | -829 | 21 | -0.53 | -11 | |
| Washing costs | kg | 126 | -0.25 | -32 | 2 | -0.25 | -1 | |
| Sum income | | | | 13779 | | | 13962 | 183 |
| **Variable costs** | | | | | | | | |
| Replacement birds | n | 79 | 33.00 | 2607 | | | | |
| Growing feed | kg | 79 | 1.75 | 138 | | | | |
| Laying feed | kg | 3519 | 1.76 | 6193 | -9 | 1.76 | -15 | |
| Misc. costs | SEK | 1564 | 0.30 | 469 | 21 | 0.30 | -6 | |
| **Sum variable costs** | | | | 9407 | | | 9427 | 163 |
| **Capital costs** | | | | | | | | |
| Buildings maintenance | SEK | 22000 | 2.1% | 462 | | | | |
| Cost of animal capital | SEK | 1351 | 7% | 95 | | | | |
| Operational capital | SEK | 922 | 7% | 65 | 1 | 7% | 0 | |
| **Sum variable and capital costs** | | | | 10029 | | | 10049 | 163 |
| **Capital costs** | | | | | | | | |
| Buildings, depreciation and interest payments | SEK | 22000 | 8.3% | 1826 | | | | |
| Labour | hours | 17 | 115 | 1955 | | | | |
| **Sum variable, capital and fixed costs** | | | | 13810 | | | 13830 | 163 |

## G.4 Budget sheets for poultry meat production

(SLU INFOS OMRÅDES KALKYLER)

***Assumptions***

Flock (batch) size 80000, weight at slaughter 1.65 kg, new buildings, purchased feed, batches per year 6.75, annual production 540000 broilers, number of day old chicks per 1000 broilers slaughtered 1050, feed conversion 2.85 kg feed per kg broiler, interest rate 7% and age at slaughter 36 days, the results in a farm producing 80000 birds per batch (expected values).

Assumptions on the effect of antibiotics used as additives in animal feed on growth 2.09%, and on feed conversion 1.47%.

***Gross margins (from table G.IV)***

| | **Without AFA** | **With AFA** | **Gain** |
|---|---|---|---|
| TB 1 = Income minus variable costs | 988616 | 1065977 | 77361 |
| TB 2 = Income minus variable and capital costs | 909608 | 988677 | 79069 |
| TB 3 = Income minus variable, capital and fixed costs | -61132 | 26890 | 88022 |

Table G.IV. Budget sheet for poultry meat production

| | Unit | Quantity | Price | SEK | Added quantity | Price | SEK | Net benefita |
|---|---|---|---|---|---|---|---|---|
| **Income** | | | | | | | | |
| Broilers | kg | 891000 | 7.42 | 6611220 | 16500 | 7.42 | 122430 | |
| Quality penalty | SEK | 6611220 | -1% | -66112 | 122430 | -1% | -1224 | |
| Sum income | | | | 6545108 | | | 6666314 | 121206 |
| **Variable costs** | | | | | | | | |
| Day old chicks | n | 567000 | 2.88 | 1632960 | -10500 | 2.88 | -30240 | |
| Chicken feed | kg | 1563300 | 2.13 | 3329829 | -5544 | 2.13 | -11809 | |
| Electricity | kWh | 194940 | 0.45 | 87723 | 39910 | 0.45 | -1796 | |
| Oil | SEK | 631 | | 34070 | | | | |
| Straw, bedding | kg | 50 | 1.00 | 27000 | | | | |
| Insurance | SEK | 157 | | 84780 | | | | |
| Misc. costs | SEK | 99 | | 53460 | | | | |
| **Sum variable costs** | | | | 5556492 | | | 5600337 | 77361 |
| **Capital costs** | | | | | | | | |
| Buildings, maintenance | SEK | 5720000 | 1.3% | 74360 | 124348 | 1.3% | 1617 | |
| Cost of operational capital | SEK | 66406 | 7% | 4638 | 1298 | 7% | 91 | |
| **Sum variable and capital costs** | | | | 5635500 | | | 5677637 | 79069 |
| **Capital costs** | | | | | | | | |
| Buildings depreciation and interest rate | SEK | 5720000 | 7.2% | 411840 | 124348 | 7.2% | 8953 | |
| Labour | hours | 4860 | 115.00 | 558900 | | | | |
| **Sum variable, capital and fixed costs** | | | | 6606240 | | | 6639424 | 88022 |