THE EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE MONITORING IN PIGS IN RELATION TO THE GROWTH PROMOTER AVILAMYCIN

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ABSTRACT

The objectives of this study were to monitor resistance to the antimicrobial growth promoter avilamycin on pig farms, with changes in resistance over time and in relation to avilamycin use of particular interest. The aims were to consider how best to measure resistance and to determine which organisms in the faecal flora, in particular which *Enterococcus* species, were expressing resistance. Resistance to therapeutic antimicrobials used in human medicine was also to be assessed.

In considering how best to measure resistance, standard statistical methods were used and novel epidemiological techniques were also developed. The findings suggested that standard statistical formulae should be applied to calculating sample numbers for antimicrobial resistance studies and that the organism, antimicrobial and animal population of interest should be clearly defined. Furthermore, the epidemiological models suggested that the sensitivity and specificity of the tests used must be defined and that the current practice of testing a small number of colonies from a small number of animals means that resistant organisms will be missed if the prevalence of resistance is very low, and that changes in resistance below 5 per cent prevalence cannot be monitored with accuracy. When the test used is not 100 per cent specific then the current practice of confirming the presence of resistance based on one bacterium testing positive is potentially misleading.

The relationship between resistance and antimicrobial use on farms was shown to be difficult to assess due to the many factors potentially influencing the prevalence of resistance. The use of Slanetz and Bartley medium was shown to be effective in isolating *Enterococcus* species with a prevalence of isolation ranging from 0.5 to 1 but to be poorly specific for this genus. *E. faecium, E. faecalis, E. hirae and E. durans* were shown to be capable of expressing resistance to avilamycin and the relative proportion of these species was found to be different on different farms. Minimum inhibitory concentrations (MIC) of avilamycin ranged from 1μg/ml to >128μg/ml in the enterococcal isolates tested. The large number of *Escherichia* spp. and smaller number of *Yersinia* spp. isolates tested were resistant to avilamycin with MIC >128μg/ml whilst the MIC in a small number of *Campylobacter* spp. isolates ranged from 8μg/ml to 128μg/ml. Avilamycin resistant enterococci were isolated from all four farms studied and resistance had persisted or been reintroduced on one farm where avilamycin had been withdrawn from use two years previously. This was only detected when faeces were screened on avilamycin-containing medium and not by conventional individual isolate MIC determination.

The prevalence of resistance to a panel of human therapeutic antimicrobials was assessed in enterococci and *Escherichia* spp. but few conclusions could be drawn due to the small sample numbers studied.

The molecular basis of avilamycin resistance was determined in 4 enterococcal isolates. Two *E. faecium* and one *E. faecalis* had substitutions in the gene encoding ribosomal protein L16 but one *E. hirae* and one *E. faecium* had sequences identical to the sensitive reference strain.

In summary, avilamycin resistance was detected on all 4 farms studied using conventional microbiological techniques but was difficult to quantify, and it was not possible to measure changes in prevalence over time with accuracy using these methods.

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To my family and friends and in memory of Sophie,

my beloved four-legged companion.

AUTHOR'S DECLARATION

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged. It has not been submitted in any form for another degree or professional qualification.

Susan Dick

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CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1 Historical background to antimicrobial use

The use of antimicrobials to control bacterial infection is now commonplace in human and veterinary medicine with 490 tonnes of active ingredient sold for use in animals alone in 2000 (http://www.vmd.gov.uk). However, in historical terms, the development of antibacterial agents is a relatively recent phenomenon. Prior to the twentieth century, efforts at controlling bacterial infections were restricted mainly to topical antiseptics with many of these substances developed from embalming techniques used by the ancient Egyptians to preserve flesh (Nutton, 2001). However, these agents were too toxic to be taken internally and work began in the early twentieth century on developing agents that were selectively damaging to prokaryotic but not eukaryotic cells (Greenwood, 1997; Weatherall, 2001).

In 1928, Scots physician Alexander Fleming first observed that a substance produced by *Penicillium notatum*, a mould sourced from air, could exert an inhibitory effect on bacteria and the antibiotic era began (Fleming, 1929; Prescott, 2000). The first cases of successful treatment of bacterial infection in humans were reported shortly afterwards including the case of a 43 year old man from Oxford diagnosed as having a disseminated *Staphylococcus aureus* and *Streptococcus pyogenes* infection whose condition improved following the administration of penicillin. The patient relapsed and died when penicillin supplies were exhausted but the successful treatment of a case of streptococcal meningitis was described soon after (Abraham *et al.*, 1941; Fleming, 1943). Although its efficacy had been demonstrated, it was several years following its discovery before penicillin was fully purified, its structure determined

and large-scale commercial production achieved (Greenwood, 1997). Chain and Florey were largely responsible for developing penicillin for clinical use and for these achievements they, as well as Fleming, were awarded the Nobel Prize for Medicine in 1945 (Chain *et al.*, 1940; Abraham *et al.*, 1941).

Meanwhile, synthetic antimicrobials were developed before penicillin was produced in useful quantities and German (Gerhard Domagk) received the Nobel Prize in Physiology or Medicine in 1939 for his work on the compound sulphanilamide (Weatherall, 2001). Domagk had observed that prontosil, a red dye first synthesised in 1932 by Klarer and Mietzsch, was effective in preventing disease in mice and rabbits dosed with staphylococci and streptococci and it was later discovered that the antibacterial activity of prontosil was due to the release of sulphanilamide (Domagk, 1935; Horlein, 1935; Fuller, 1937). Prontosil was subsequently successfully used in the treatment of a woman suffering from puerperal fever in a London hospital in 1936. The use of both sulphonamides and penicillins for the treatment of puerperal pyrexia was associated with a significant reduction in mortality following childbirth in the United Kingdom from the middle of the twentieth century (Colebrook and Kenny, 1936; Greenwood, 1997). Following these early advances, many other antibacterial agents were developed but in recent years the majority of antimicrobial drug development has been based on the expansion of existing drug classes and it now seems unlikely that any totally new, unrelated antimicrobial compounds remain to be discovered (Greenwood, 1997).

The widespread application of antimicrobials in the treatment of animals followed their use in human medicine and since their introduction, antimicrobials from many different chemical classes have been commonly used in the therapy of microbial disease in food animals (Miller and Flynn, 2000). Early examples of antimicrobial

use in animals include a report on diseases of farm livestock by the National Veterinary Medical Association (later the British Veterinary Association) in 1941, where it was suggested that cases of mastitis be treated with oral sulphonamides or, in less severe cases, by udder irrigation with acriflavine (HMSO, 1965). Daily intra-mammary infusion of 100,000 units of penicillin in aqueous solution was also shown to be effective in the treatment of mastitis caused by *Streptococcus agalactiae* in an experimental herd at Weybridge (HMSO, 1965). However, in farm animals, antibiotics have not only been used in the treatment of disease, but also prophylactically to treat clinically normal but infected animals, and for growth promotion (Friendship, 2000).

1.2 Antimicrobial growth promoters

Growth promoters are feed additives, other than dietary nutrients, which increase growth rate and/or improve feed efficiency in healthy animals fed a balanced diet (van den Bogaard and Stobberingh, 1999) and antimicrobials used for this purpose are given to animals continuously in feed at low levels.

The "growth promoting" properties of antibiotics were first discovered in the late 1940s when fermentation waste from tetracycline production was fed to chickens as a source of vitamin B_{12}. It was discovered that the chickens grew more rapidly than when on their normal diet and it was thought that these effects were due to some unknown substances in these fermentation products (Hill, 1948; Carlson *et al.*, 1949; Stokstad *et al.*, 1949; Jukes and Williams, 1953). However, the growth-promoting effect was later found to be due to residual tetracycline (aureomycin) (Carpenter, 1950; Jukes *et al.*, 1950; Stokstad and Jukes, 1950; Whitehill *et al.*, 1950) and further experiments in pigs and poultry confirmed that other antimicrobials including

penicillin and sulphonamides also exerted a growth-promoting effect (Moore *et al.*, 1946; Luecke *et al.*, 1950; Speer *et al.*, 1951). These findings led to the subsequent commercial development of various antimicrobial growth promoters and their use has been commonplace in the United Kingdom since 1953 (Swann, 1969).

Antimicrobial growth promoters are generally used in young animals where they are also most effective (Stahly *et al.*, 1980; Lindemann *et al.*, 1985; Jones *et al.*, 1987) with young pigs, broilers, pre-ruminant and recently-weaned calves the most common recipients. Their use in adult ruminants is less common but they can be administered to this class of animal where it has been shown that their use increases milk yield (van Heijenoort *et al.*, 1987).

1.3 Consumption of antimicrobial growth promoters

In the UK, the Veterinary Medicines Directorate collates information on the sale of antimicrobials in veterinary medicine based on data provided voluntarily by the pharmaceutical industry, and information on antimicrobial sales is available on their website (www.vmd.gov.uk). In 1993, antimicrobial growth promoter sales in the UK were initially reported to be 83 tonnes of active ingredient, and this increased to a high of 122 tonnes in 1995 before falling again to 89 tonnes in 1998 (www.vmd.gov.uk/general/publications/mayra2a.htm). However, these data were recently reviewed and the findings suggested that only 46 tonnes of antimicrobial growth promoter were sold in the UK in 1998 (VMD, 2001). Such discrepancies in the data available on antimicrobial sales make it difficult to assess trends in the quantities of antimicrobials prescribed in animals. In 1999 and 2000 sales were reported to be 28 and 24 tonnes of active ingredient, respectively, but it is acknowledged that some data are missing for these years (VMD, 2001). In 2001,

recorded sales were 43 tonnes of active ingredient, which represented 9 per cent of the total sale of antimicrobial products for food-producing animals (www.vmd.gov.uk). In addition to the inconsistency of the available data on veterinary antimicrobial sales, at present it is also impossible to relate antimicrobial growth promoter sales to species in the UK as these data are not collected. However, this information is available for therapeutic antimicrobials where sales in pig-specific products have increased from 83 tonnes active ingredient in 1998 to 109 tonnes active ingredient in 2001 (www.vmd.gov.uk). This increase in therapeutic antimicrobial use has coincided with the ban on some growth promoting antimicrobials and the introduction of post-weaning multi-systemic wasting syndrome and porcine dermatitis and nephropathy syndrome which are likely to have increased the need for antimicrobial therapy. However, there has also been a reduction in the total live weight of pigs slaughtered over the same period from 1,402,000 tonnes in 1998 to 972,000 tonnes in 2001. A large component of this reduction in pig production has been due to contraction of the industry under economic pressure and under normal circumstances this would have been expected to be reflected in a reduction in antimicrobial sales. However, the introduction of these diseases has had a significant impact on pig productivity and may also have increased the need for therapeutic antimicrobial treatments and this makes these findings difficult to interpret.

1.4 Regulation of antimicrobial growth promoters

Within the EU, the use of antimicrobial growth promoters is tightly regulated. Permitted compounds may be incorporated in feed at specified concentrations for particular species as indicated in the classified Annexes of EC Directive 70/524/EEC The Feedingstuffs (Zootechnical Products) Regulations 1999. Under the above

directive, they may only be incorporated into animal feed at registered feed mills (Bishop, 2001). Farmers can purchase antimicrobial growth promoters from agricultural merchants and feed companies, which supply them in feed at the approved inclusion rate. Article 3 of the above directive states:

Community authorisation of an additive shall be given only if:

a) when used in animal nutrition it has one of the effects referred to in Article 2 (a)[1] *;*

b) taking into account the conditions of use, it does not adversely affect human or animal health or the environment nor harm the consumer by altering the characteristics of livestock products;

c) its presence can be monitored:

- *as an additive per se*
- *in premixtures*
- *in feedingstuffs or, where appropriate, in feed materials*

d) at the level permitted, treatment or prevention of animal disease is excluded: this condition does not apply to additives belonging to the group of coccidiostats and other medicinal substances;

e) for serious reasons concerning human or animal health its use must not be restricted to medicinal or veterinary purposes.

The growth promoters currently approved by the above legislation within the EU are avilamycin, flavophospholipol, salinomycin and monensin. Monensin and salinomycin are also approved for use as anticoccidials. Avilamycin is licensed as a growth promoter in pigs and chickens; flavophospholipol in cattle, pigs, poultry and rabbits; monensin as a growth promoter in non-lactating cattle and as an anticoccidial

in poultry and salinomycin as a growth promoter in pigs and as an anticoccidial in poultry (Bishop, 2001).

1.5 Mode of action of antimicrobial growth promoters

Despite being the focus of many reports, the exact mechanisms of action of antimicrobial growth promoters are poorly described (Stockholm Commission on Antimicrobial Feed Additives, 1997; van den Bogaard and Stobberingh, 1999; Aarestrup, 2000a). Their effects, however, have been summarised as improved feed utilisation, improved growth rate and disease prevention and the proposed mechanisms by which they exert these effects include alteration of the normal microbial intestinal flora, preservation of nutrients and enzymes from degradation by microbes and alteration of villous structure and function (Stockholm Commission on Antimicrobial Feed Additives, 1997). In economic terms, their most important effect is improved feed efficiency (van den Bogaard and Stobberingh, 1999) although there is also a consequent reduction in the amount of waste products excreted in urine and faeces (Thomke and Elwinger, 1998).

Early evidence that antimicrobial growth promoters exerted their effect by inhibiting components of the intestinal microflora was that, unlike the situation in conventionally raised animals, growth in germ-free chickens was not enhanced by the administration of antimicrobial growth promoters (Lev and Forbes, 1959). Furthermore, the reduction in growth rate seen in germ-free chickens following the administration of *Enterococcus faecium* could be improved by the administration of penicillin (Lev and Forbes, 1959; Eyssen and DeSomer, 1967). It has been suggested that their inhibitory effect on microorganisms in the gastrointestinal tract reduces microbial degradation of useful nutrients and thereby increases metabolisable energy

available to the animal but they are also thought to control weakly pathogenic organisms present in the gastrointestinal tract such as *Enterococcus spp.* and *Clostridium spp.* (Stockholm Commission on Antimicrobial Feed Additives, 1997; Shryock, 2000).

Early studies described changes in the physical properties of the gastrointestinal tract associated with the administration of growth promoters, with several authors reporting a reduction in weight of the small intestine (Pepper *et al.*, 1953; Coates *et al.*, 1955). Thinning of the duodenal wall in antibiotic-fed birds was also described (Jukes *et al.*, 1956). It was suggested that the gut-thinning effect observed was due to a reduction in bacterial toxin production and that this allowed improved absorption of dietary nutrients (Bogan *et al.*, 1983). A decline in mucosal cell turnover associated with a reduction in energy expenditure was also thought to be responsible for these observations and the consequent improved feed efficiency (Visek, 1978).

Other authors have suggested that the growth promoting effect of antimicrobials is due to altered metabolism of the enteric flora leading to an increase in the availability of dietary nutrients to the animal (Bogan *et al.*, 1983). Studies on the metabolism of nutrients by the enteric flora suggested that the inhibition of certain organisms including Streptococci, Enterococci and Lactobacilli resulted in a sparing of carbohydrates (Vervaeke *et al.*, 1976; Bogan *et al.*, 1983).

However, it has generally been acknowledged that antimicrobial growth promoters also have a role in disease prevention and because therapeutic antimicrobials including tetracycline and penicillin were initially used for this purpose (Smith, 1975), it has been difficult to separate their effect on pathogenic microorganisms from their other microbiological effects. Therapeutic antimicrobials are no longer permitted for use as growth promoters in the European Union, although the situation is different

in the U.S.A where, for example, oxytetracycline is still used for this purpose (van den Bogaard and Stobberingh, 1999). Although growth promoters are given at low "subtherapeutic" concentrations, this does not necessarily mean they do not have an inhibitory effect on the growth of pathogenic organisms and there are many accounts in the literature of growth promoters reducing the severity and incidence of clinical disease (Shryock, 2000).

The compounds avoparcin, bacitracin, virginiamycin and avilamycin have all been shown to be effective against necrotic enteritis in poultry caused by *Clostridium perfringens* at levels permitted for growth promotion (Wicker *et al.*, 1977; Stutz *et al.*, 1983; Hofshagen and Kaldhusdal, 1992; Stockholm Commission on Antimicrobial Feed Additives, 1997; Bolder *et al.*, 1999). Monensin has been shown to reduce the clinical signs of swine dysentery caused by *Brachyspira hyodysenteriae* (Kyriakis, 1989) and avilamycin has been shown to reduce the severity and mortality associated with post-weaning diarrhoea in piglets, despite the fact that the most likely causal agent is *Escherichia coli*, a gram-negative organism (Kyriakis, 1989). Tylosin has been shown to be effective in preventing porcine proliferative enteropathy caused by *Lawsonia intracellularis* at growth-promoting levels (McOrist *et al.*, 1997) and flavophospholipol has been shown to have an inhibitory effect on the shedding of *Clostridium spp.* (Bolder *et al.*, 1999).

In cattle, antimicrobial growth promoters and ionophores in particular have proven efficacy in reducing the incidence of bloat, mastitis and non-infectious lameness in adult dairy cows (van Heijenoort *et al.*, 1987; Lowe *et al.*, 1991). However, the mode of action of growth promoters in ruminants is different to that in monogastrics with evidence to suggest that the main effects are a reduction in energy lost due to the production of methane gas and improved efficiency of rumen fermentation by

alteration of volatile fatty acid production to favour propionic acid (Thornton *et al.*, 1976; Nevel and Demeyer, 1977; Chen and Wolin, 1978; Bogan *et al.*, 1983). The improved energy availability to the animal could explain why antimicrobial growth promoters have been associated with a reduction in the incidence of diseases associated with negative-energy balance rather than infectious diseases of the digestive tract in this class of animal.

Further evidence of the role of growth promoters in disease prevention is that although they are said to be effective in both extensive and intensive systems (Gustafson and Bowen, 1997) studies comparing conventionally raised animals and germ-free animals have shown that only the growth response of conventional animals is improved by the administration of low concentrations of antibiotic (Stockholm Commission on Antimicrobial Feed Additives, 1997) and furthermore, that animals kept in scrupulously clean conditions respond less well to growth promoting antimicrobials (Lev and Forbes, 1959; Coates *et al.*, 1963; Eyssen and DeSomer, 1967; Shryock, 2000). A study in healthy pigs under good commercial management conditions suggested that constant improvements in live-weight gain of 30g per day could be obtained even in clinically healthy pigs kept in sanitary conditions by the administration of tylosin and this was thought to suggest that growth promotion occurred independent of disease status (Jones, 1978). However, it should be pointed out that although these animals were defined as healthy by clinical examination, there may have been underlying unidentified subclinical disease problems having an inhibitory effect on growth.

These findings suggest that antimicrobial growth promoters may be more useful when husbandry standards are poorer and that since the presence of disease undoubtedly has an inhibitory effect on growth and production, their effect in reducing disease is

inextricably linked to their growth-promoting effect. It has been suggested that targeting the administration of antimicrobial growth promoters over the period when animals are most likely to encounter pathogens or are most susceptible to disease allows the prevention of diseases and the economic losses associated with disease outbreaks and that there is less need for higher levels of therapeutic antimicrobials later (Gustafson and Bowen, 1997). In addition, the welfare of the animals concerned is protected because the stress and suffering caused by disease is prevented. However, recent experiences in countries where antimicrobial growth promoters have been withdrawn suggest that animal welfare and production is not compromised provided that husbandry standards are high and given the pressure to limit antimicrobial use in agriculture, the use of antimicrobials to compensate for poor hygiene and stocksmanship in animal production is unlikely to be tolerated in the future (Aarestrup *et al.*, 2001; SVARM, 2001).

In summary, it is likely that the action of antimicrobial growth promoters involves a complex interaction of microbiological, nutritional, physiological and disease-preventing effects and that the magnitude of their effect is modified by factors such as nutritional, environmental and health status of the animals. Although the mode of action of antimicrobial growth promoters is complex, their effect on growth is less pronounced in high-health status animals and there is little evidence to suggest that their use improves animal welfare unless husbandry standards are poor.

1.6 Structure of antimicrobial growth promoters

Avilamycin is an oligosaccharide antibiotic produced by *Streptomyces viridochromogenes* (Mertz *et al.*, 1986; Stockholm Commission on Antimicrobial Feed Additives, 1997; Bishop, 2001) and is structurally related to curamycin and the

everninomycins, which have recently been considered for use in human medicine. It exerts its inhibitory effect on bacteria by blocking protein synthesis through inhibiting the function of the 30s ribosomal subunit (Wolf, 1973) and is poorly absorbed from the gastrointestinal tract (Magnussen *et al.*, 1991).

Flavophospholipol is a phosphorus-containing glycolipid produced by several *Streptomyces* spp. comprising *S. bambergiensis*, *S. ghanaensis*, *S. geysirensis* and *S. ederensis* and is active mainly against Gram-positive bacteria (Bogan *et al.*, 1983). It is a competitive enzyme inhibitor, which interferes with the transglycolase activities of penicillin-binding-proteins, thereby interfering with cell wall synthesis in Gram-positive bacteria (van Heijenoort *et al.*, 1987). Studies in chickens and pigs have shown that after oral administration it is almost completely eliminated as the intact molecule in the faeces and that no measurable residues are found in carcasses of animals fed flavophospholipol for several months (Bogan *et al.*, 1983). There are no compounds related to flavophospholipol used for therapy in either humans or animals.

Monensin is a carboxylic ionophore produced by *Streptomyces cinnamonensis* and acts by altering membrane permeability, killing the bacterial cell by lowering intracellular pH (Prescott, 2000). It has limited antibacterial activity and is active against the six species of Eimeria known to be pathogenic in chickens (Bogan *et al.*, 1983). Studies in cattle have shown that over seventy per cent of monensin is excreted unchanged in faeces and that at the levels permitted for growth promotion and coccidial prophylaxis, no residues are detectable in carcasses or eggs 24 hours after withdrawal (Bogan *et al.*, 1983).

Salinomycin is a monocarboxylic acid polyether ionophore produced by *Streptomyces albus* (Kinashi *et al.*, 1973) with a similar mechanism of action to monensin. No ionophore antimicrobials are used in human medicine.

1.7 Spectrum of activity of antimicrobial growth promoters

Not all antimicrobial agents are effective in improving growth in animals and those that are tend to be active mainly against Gram-positive organisms (von Wasielewski *et al.*, 1965; Bunyan *et al.*, 1977; Bogan *et al.*, 1983; Aarestrup, 2000a). However, the chemical structures of these compounds differ widely and there are a few antimicrobials mainly active against Gram-negative organisms, such as streptomycin, that have also been shown to have growth-promoting effects (Bunyan *et al.*, 1977). The primary site of action of antimicrobial growth promoters currently licensed for use in the European Union is the gastrointestinal tract, from which they are usually not absorbed (Bogan *et al.*, 1983; Gustafson and Bowen, 1997) and therefore the wide variety of Gram-negative and Gram-positive organisms in the gastrointestinal microflora are exposed to their action.

Avilamycin is reported to inhibit the growth of *E. faecium, E. faecalis, C. perfringens* and *Staphylococcus spp. in vitro* (Dutta and Devriese, 1982; Butaye *et al.*, 1998) and whilst there is variation in susceptibility to flavophosholipol amongst *E. faecium* strains (Aarestrup *et al.*, 1998), *Clostridium spp., Staphylococcus spp.* and *E. faecalis* are reported to be susceptible (Devriese, 1980; Dutta and Devriese, 1982; Dutta and Devriese, 1984; Aarestrup *et al.*, 1998). Monensin and salinomycin are active against *E. faecium* and *E. faecalis* although these species are more susceptible to avilamycin *in vitro* (Aarestrup *et al.*, 1998) and monensin is also active against some *Campylobacter spp., Brachyspira hyodysenteriae* and *Toxoplasma gondii* (Prescott, 2000). In addition, monensin and salinomycin also possesses anticoccidial activity (Bogan *et al.*, 1983; Prescott, 2000). Therefore, whilst antimicrobial growth promoters are active in the main against Gram-positive organisms, the ionophores in

particular have the potential to inhibit the growth of Gram-negative organisms as well as protozoa.

1.8 Significance and development of antimicrobial resistance in human pathogens

There are numerous accounts in the literature of the development of antimicrobial resistance and since the advent of antimicrobial use, it has been recognised and documented in many organisms (Abraham and Chain, 1940; Tenover, 1991; Neu, 1992). There is also evidence to suggest that antimicrobial resistance is an increasing problem with some describing it as a worldwide epidemic (Hancock, 1997; O'Brien, 1997; Salyers and Amabile-Cuevas, 1997; Hart, 1998; MAFF, 1998). Many of these papers and articles use very emotive language to describe the current state of the resistance problem, e.g., "Superbugs are beating at the gates" (Kmietowicz, 1999) and "Resistance to Antimicrobial Drugs – a Worldwide Calamity" (Kunin, 1993). It is a subject that has a high priority on the current web pages of the World Health Organization (http://www.who.int/emc.amr.html) and the U.S Food and Drug Administration (http://www.fda.gov/oc/antimicrobial/taskforce2000.html) so there is little doubt that it is perceived to be a significant problem.

There are some differences of opinion as to the prevalence of resistance genes in the pre-antibiotic era with resistant bacteria extremely rare in patients 60 years ago (Hughes and Datta, 1983) and yet evidence suggests that antimicrobial resistance was present to some extent before antibiotics were introduced (Smith, 1967; Gould, 1999). However, there is general agreement that the further development and dissemination of resistance is inextricably linked to the use of antimicrobials (SMAC, 1998; Monroe and Polk, 2000).

Shortly after the introduction of penicillin for clinical use, resistant strains of *Staphylococcus aureus* became apparent (Kirby, 1944; North and Christie, 1946) and since then, the development and use of novel antimicrobial drugs has been followed by the development of resistance to them in bacteria (Andersson and Levin, 1999) with more than 100 resistance genes now described and resistance a recognised clinical problem in many bacterial species (O'Brien, 1997). The increase in antimicrobial drug resistance in human medicine has been associated with the misuse and over use of antimicrobials (Salyers and Amabile-Cuevas, 1997; Andersson and Levin, 1999), is of economic significance and is responsible for an increase in morbidity and mortality associated with infectious disease. However, it has also been suggested that the use of antimicrobials in veterinary medicine, and agriculture in particular, has been partly responsible for the worldwide increase in antimicrobial resistance seen in human medicine (Wegener *et al.*, 1999; Lipsitch *et al.*, 2002).

1.9 The spread of resistance from animals to man

Resistance to therapeutic antimicrobials used in veterinary medicine has been documented since shortly after their introduction for clinical use (Smith, 1954; Smith and Crabb, 1956) and although resistance is perceived to be less of a clinical problem in veterinary medicine, reports of resistant organisms continue to be published (van den Bogaard and Stobberingh, 1999). As well as presenting a problem for the therapy of animal diseases, it is widely acknowledged that animals that enter the food chain can be sources of antimicrobial resistant pathogens in humans (MAFF, 1998; ACMSF, 1999). The transfer of resistant bacteria from animals to man is best described for enteric bacteria with faecal contamination of carcasses leading to the

exposure of humans to organisms originating in the gastrointestinal tract of food animals.

Resistant Salmonellae were first documented in the 1960s with multiresistant *Salmonella enterica* first isolated from man in 1965, and *S. enterica* serovar Typhimurium DT104 the most common multi-resistant strain isolated from humans in the UK in the 1990s (Threlfall *et al.*, 1997). Multiresistant clones of *S. enterica* serovar Typhimurium have been isolated from animals in the UK and the spread of multiresistance has been attributed to the use of antimicrobials in calf-rearing units (Helmuth and Protz, 1997). There is also good evidence, including the isolation of genetically similar ceftriaxone-resistant isolates from a child and a cattle herd in the USA, to suggest that resistant *S. enterica* strains have been transferred from animals to humans (Threlfall *et al.*, 1985; Threlfall *et al.*, 1994; Wall *et al.*, 1995; Calvert *et al.*, 1998; Fey at al., 2000). Antibiotic resistance has also been shown to be transferable between human and animal strains of *Escherichia coli* (Smith, 1969) and resistant *E. coli* strains have been documented as contaminants of carcasses (Linton, 1986). These findings in conjunction with other observations, including the emergence of fluoroquinolone resistance in human *Campylobacter jejuni* isolates following the introduction of fluoroquinolones in poultry (Endtz *et al.*, 1991) and the detection of apramycin/gentamicin resistant determinants in enterobacteriaceae from humans following apramycin use in animals (Johnson *et al.*, 1994), indicate that resistant organisms and resistance determinants have been transferred from animals to man via the food chain.

1.10 Definition and nature of resistance to avilamycin

The definition of an organism as sensitive or resistant to an antimicrobial is made on clinical grounds and the laboratory determination of resistance is used as a predictor for the probability of successful treatment. Resistance is measured *in vitro* by minimum inhibitory concentration, MIC, which is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation (BSAC, 1991). If the concentration of antimicrobial in the target tissue exceeds the MIC following treatment, then an isolate is categorised as susceptible or sensitive (NCCLS, 1999).

However, this definition is unsuitable for growth-promoting antimicrobials as they are not used to treat infection, they act primarily on commensal organisms and are poorly absorbed from the gastrointestinal tract. Therefore, breakpoints for resistance and sensitivity have been defined by population distributions so that where a bimodal distribution of MICs has been evident, organisms in the upper range have been considered resistant (Aarestrup *et al.*, 1998; Butaye, Devriese and Haesebrouck, 1999b). Breakpoints for enterococci to avilamycin, monensin, salinomycin and flavophospholipol have been described using this method (Aarestrup *et al.*, 1998).

However, an alternative means of defining resistance would be to consider the inhibitory effect of the concentration of growth promoter achieved in the gastrointestinal tract, as this is the target site of these compounds. It has been suggested that inhibitory concentrations should be related to the intestinal concentrations of antimicrobial but these are largely unknown and breakpoints have not been defined in this manner to date (Butaye, Devriese and Haesebrouck, 1999b). There is no evidence to suggest that there has been any reduction in the efficacy of growth promoters due to resistance emerging in the gastrointestinal flora, and

therefore there seems to be little biological significance in adopting this approach to categorise organisms under test.

Resistance to growth-promoting antimicrobials has primarily been considered in enterococci, particularly *E. faecium*, and has been most closely monitored in Denmark and Sweden (Danmap, 1997; Danmap, 1998; Danmap, 1999; Danmap, 2000; SVARM, 2000; Danmap, 2001; SVARM, 2001). Estimates of the prevalence of resistance to avilamycin amongst enterococci from animals have varied from country to country with for example, 12.4 per cent of *E. faecium* isolates from broilers categorised as resistant in a recent Japanese study (Yoshimura *et al.*, 2000) compared to 5 per cent of Danish isolates (Danmap, 2001), whilst all 151 isolates tested in Sweden in 2000 were sensitive (SVARM, 2000). In Denmark in 2001, 175 *E. faecium* isolates from pigs were tested and all were found to be sensitive compared to 2 per cent that were resistant in 1997 (Danmap, 1997; Danmap, 2001) and less than 1 per cent of *E. faecium* isolated from pigs in Sweden in 2000 were resistant compared to 1 per cent in 2001 (SVARM, 2000; SVARM, 2001).

There is some variation in the literature on the breakpoint MIC for avilamycin that defines resistance. Isolates with MICs greater than 12.5μg/ml have been considered resistant (Yoshimura *et al.*, 2000) whereas isolates with MICs greater than 16μg/ml (Danmap, 1997; Danmap, 1998; Danmap, 1999; Danmap, 2000; Danmap, 2001) and isolates with MICs greater than 8μg/ml (SVARM, 2000; SVARM, 2001) have been classified as resistant elsewhere. However, in a recent study (Aarestrup and Jensen, 2000), isolates with genes conferring resistance to avilamycin had MICs of at least 32μg/ml and in most cases 64μg/ml or above. Other authors have classified *E. faecium* strains with MICs of greater than 32μg/ml as resistant (Butaye, Devriese and Haesebrouck, 1999b).

A genetic mechanism of resistance to avilamycin amongst enterococci was first described in Danish *E. faecium* and *E. faecalis* isolates in 2000 (Aarestrup and Jensen, 2000). All the variations observed were within the gene encoding ribosomal protein L16 with the resistant *E. faecalis* isolates all containing the same base pair variation, while the same variation and two additional variations were found in the *E. faecium* isolates. However, recent work has also described mutations in 23S rRNA that confer resistance to avilamycin and evernimicin (Mann *et al.*, 2001) and it seems clear that as for most antimicrobials, multiple mechanisms of resistance may exist.

1.11 The role of antimicrobial growth promoters in transfer of resistance to man

None of the growth promoters licensed for use within the UK in 2003 are related to any concurrently used human therapeutic antimicrobials. However, there is evidence that growth promoters used in the past may have possessed cross-resistance to human medical antimicrobials and thereby contributed to the human antimicrobial resistance problem (Witte, 1997; Bishop, 2001).

Vancomycin is a glycopeptide antimicrobial used primarily in the treatment of resistant Gram-positive infections in man and is structurally related to the growth promoter avoparcin, which was used in food animals in Europe prior to its withdrawal in 1997. Vancomycin-resistant enterococci, first isolated in Europe in 1986, were subsequently reported in the United States in 1987 and have since been documented world-wide where they are considered to be important nosocomial pathogens (Cetinkaya *et al.*, 2000). Five major phenotypes of glycopeptide resistance, designated vanA, vanB, vanC, vanD and vanE, have been described in enterococci (Arthur and Courvalin, 1993; Perichon *et al.*, 1997; Fines *et al.*, 1999) with the vanA phenotype, consisting of high level vancomycin and teicoplanin resistance (Leclercq and

Courvalin, 1997), usually contained in a transposon, *Tn 1546*, as the vanA gene cluster (Wegener *et al.*, 1999).

VanA vancomycin-resistant enterococci have been isolated from food animals and foods in Europe (Bates *et al.*, 1994; Aarestrup, 1995; Bager *et al.*, 1997; Wegener *et al.*, 1997) and there is evidence to suggest that the use of avoparcin selects for vancomycin-resistance (Witte, 1997). This evidence has led to the suggestion that there has been selection for vancomycin resistance in animals through the use of avoparcin and that resistance has been transferred to man via the food chain.

However, the prevalence of vancomycin-resistant enterococci (VRE) in animals has been low in some animal surveys (Ike *et al.*, 1999) and in a study in the Netherlands where the prevalence of VRE amongst farmers, turkeys, slaughterers and suburban residents was compared, only 2-4 per cent of enterococci were resistant in all groups, including farmers who did and did not use avoparcin (Stobberingh *et al.*, 1999). Therefore the relative importance of avoparcin use in animals in terms of the problem of vancomycin resistance in man is hard to interpret. Further conflicting evidence is provided by the fact that the incidence of VRE in American hospitals is greater than that in Europe, despite the fact that avoparcin has never been used as a growth promoter in the USA (van den Bogaard *et al.*, 1997; Wegener *et al.*, 1999).

Resistance to avilamycin has frequently been detected in *Enterococcus faecium* isolated from poultry in Denmark (Aarestrup *et al.*, 1998) and recently a structurally related compound with a similar mechanism of action, evernimicin (Ziracin), has been developed for use in human medicine (Aarestrup and Jensen, 2000; McNicholas *et al.*, 2000). Cross-resistance between avilamycin and evernimicin has been reported in Danish enterococcal isolates (Aarestrup, 1998; Aarestrup and Jensen, 2000) but the

development of evernimicin for clinical use by manufacturers Schering Plough has currently been suspended (Shryock, 2001).

Virginiamycin was widely used as an antimicrobial growth promoter prior to the introduction of legislation banning its use in 1999 and in a Danish study in 1997, 66 per cent of *E. faecium* isolates from broilers were reported to be resistant to virginiamycin (Danmap, 1998). It is a streptogramin antimicrobial that inhibits the 24S ribosomal protein (Witte, 1997) and is structurally related to the compounds quinupristin and dalfopristin, which have been developed for use in human medicine in combination. Quinupristin-dalfopristin (Synercid) is recommended for use against resistant Gram-positive infections including VRE although it is not active against *E. faecalis* (Mulazimoglu *et al.*, 1996; British National Formulary, 2003).

Cross-resistance between virginiamycin and quinupristin-dalfopristin has been demonstrated and several resistance mechanisms described (Welton *et al.*, 1998; Werner *et al.*, 2002) and significantly, high levels of resistance to quinupristin-dalfopristin were described amongst pathogens in Taiwan, including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus*, prior to clinical use (Luh *et al.*, 2000) leading to the suggestion that the use of virginiamycin in animals has selected for resistance to quinupristin-dalfopristin.

1.12 History of use - withdrawal

Currently, the use of antimicrobials as growth promoters in food animals is at the centre of debate, with calls for the practice to be banned (Witte, 1997; Fidler, 1999; Courvalin, 2000) and the main reason for this controversy is the recent worldwide anxiety at the emergence of bacterial antimicrobial resistance as a serious threat to human healthcare (van den Bogaard and Stobberingh, 1999; Aarestrup, 2000a).

The first significant attempt at regulation of antimicrobial feed additives followed the release of the Swann Report in 1969 (Swann, 1969) and this action was prompted by the emergence of evidence suggesting that the use of therapeutic antimicrobials such as tetracycline as growth promoters had led to resistance in bacteria including *E. coli* (Smith, 1975). The purpose of these regulations was to reduce antibiotic resistance in organisms colonising animals and therefore to reduce the risk of antimicrobial resistant organisms being transferred to humans and it was recommended that the use of penicillins, tetracyclines, tylosin and sulphonamides as growth promoters be banned (Gustafson and Bowen, 1997).

A subsequent report in 1986 in Sweden (Stockholm Commission on Antimicrobial Feed Additives, 1997) concluded that the benefits of antimicrobial growth promoters did not outweigh the risks and the use of antimicrobials for growth promotion was banned in Sweden from this time, although monensin and salinomycin were still permitted for use as coccidiostats. The controversy surrounding the issue was heightened in May 1995 when Denmark imposed a unilateral ban on the use of avoparcin in animal feeds, followed by the banning of avoparcin throughout the EU in April 1997 (Wegener *et al.*, 1999). The introduction of this ban was due to concerns that its use in animals was contributing to the problem of vancomycin-resistant enterococci in man (MAFF, 1998; Acar *et al.*, 2000).

In 1998, a voluntary ban on the use of all antimicrobial growth promoters was adopted by the food animal industry in Denmark (Danmap, 1998) and the initial ban on avoparcin was extended in July 1999 when the growth promoters spiramycin, virginiamycin, bacitracin and tylosin were banned throughout the EU (Acar *et al.*, 2000) so that the only antimicrobial compounds currently licensed for use in the EU for growth promotion are avilamycin, flavophospholipol, monensin and salinomycin.

1.13 Antimicrobial use, resistance and bacterial population dynamics

Before examining the effects of such legislative decisions, it is first necessary to consider the effects of antimicrobial use on bacterial populations. The proliferation of antimicrobial-resistant organisms is by some considered to be an example of adaptive evolution (Levin *et al.*, 2000) with gene substitution occurring under the selective pressure of the presence of antimicrobial. In most cases, however, the acquisition of resistance genes in bacteria has an associated fitness cost (Andersson and Levin, 1999) and the magnitude of this fitness cost is considered one of the most important factors governing the frequency of resistance in a bacterial population (Bjorkman *et al.*, 2000). If the fitness cost associated with resistance is high relative to non-resistant organisms, then the surviving non-resistant organisms would be expected to proliferate and become the dominant proportion of the population following withdrawal of the selective pressure.

However, evidence suggests that the effects of antimicrobial therapy on bacterial population dynamics are complex and that fitness costs are not the only factor influencing population dynamics. In the case of some drugs, resistance developed very quickly after their introduction for clinical use (Abraham and Chain, 1940) whereas for other drugs, resistance emerged much more slowly, or not at all, after several decades of use (French and Phillips, 1997). In a recent review (Andersson and Levin, 1999), little evidence was found for the existence of fitness costs associated with resistance. Experimental evidence has also suggested that the fitness costs associated with resistance can be compensated for by additional mutations, e.g., Levin *et al.* showed that although streptomycin resistance in *E. coli* associated with ribosomal mutations (rpsL) had a substantial associated fitness cost, when these strains were serially passaged without the presence of streptomycin, resistance to

streptomycin was maintained and fitness increased by adaptive mutations (Levin *et al.*, 2000). However, it should be noted that these strains were still less fit than wild-type strains.

Evidence from the clinical setting also suggests that although cessation of, or reduction in, antimicrobial use is usually associated with a reduction in antimicrobial resistance, resistant strains may still persist, albeit at a lower level (Nowak, 1994) and a recent survey in London found that 20 per cent of *E. coli* isolates were still streptomycin-resistant despite the fact that streptomycin had not been used against this organism for 25 years (Chiew *et al.*, 1998). However, some hospital programmes aimed at modifying antimicrobial use have been successful in reducing the prevalence of resistant organisms (Monroe and Polk, 2000).

The fact that resistances may be cross-linked and that many resistance elements carry more than one set of resistance genes may also influence the persistence of resistance. For example, plasmids can carry resistance to multiple antibiotics (French and Phillips, 1997) and this has led to the development of multi-drug resistance in pathogens such as *S. enterica* serovar Typhimurium DT 204, DT204c and DT193, which were prevalent in the early 1980s (Threlfall *et al.*, 1978; Wray *et al.*, 1987). Furthermore, following the spread of resistance elements between bacteria, multiple resistance genes have become integrated into the bacterial chromosome in some organisms and this is thought to be the basis of the multiple drug resistance seen in *S. enterica* serovar Typhimurium DT104, for example (Threlfall *et al.*, 1994; Ridley and Threlfall, 1998; Sandvag *et al.*, 1998). This means that the continued use of one antimicrobial can continue to select for resistance to other antimicrobials that have been withdrawn. For example, it has been suggested that glycopeptide resistance

persisted in enterococci following avoparcin withdrawal because tylosin was still in use (Aarestrup *et al.*, 2001).

Therefore, there is evidence to suggest that the fitness costs associated with the maintenance of resistance genes and the co-selection of resistance elements are both factors important in influencing whether antimicrobial resistance will decline following the withdrawal of an antimicrobial. Whilst attempts have been made to predict the effects of treatment regimes on the emergence of resistance in bacterial populations (Lipsitch and Levin, 1997b; Lipsitch and Levin, 1998), the situation in the field is apparently complex. For example, vancomycin-resistant enterococci have been detected in Denmark and Norway several years after the withdrawal of avoparcin (Borgen *et al.*, 2000; Heuer *et al.*, 2002). These findings suggest that even low levels of antibiotic use can lead to the development of resistance that can later persist following withdrawal.

1.14 Effects of the ban on antimicrobial growth promoters

The effects of banning antimicrobial growth promoters on animal health and production, antimicrobial usage and antimicrobial resistance have been monitored in Sweden and Denmark (Danmap, 1998; Danmap, 1999; Danmap, 2000; SVARM, 2000; Danmap, 2001).

In Sweden, there has been a gradual reduction in the total amount of antimicrobials used in farm animals since the ban on growth promoters in 1986 with 17 tonnes of active ingredient sold in 2000 compared to 20.6 tonnes sold in 1996 (SVARM, 2000) whilst in Denmark, there has been an increase in the use of therapeutic antimicrobials between 1996 and 2001 of 17 per cent to 94.2 tonnes active ingredient (Danmap, 2001). It is not possible to relate antimicrobial sales to individual animal species in

either country, but it is felt that these changes in consumption are independent of changes in livestock numbers.

Information from the Danish broiler industry indicates that there have been disease and production problems in the period following the voluntary ban in that country, with mean feed consumption at slaughter increasing from 1.78 to 1.82kg, mean weight at slaughter (42 days) reduced by approximately 30g and an increase in the number of flocks suffering from necrotic enteritis and chronic hepatitis caused by *Clostridium perfringens* (Danmap, 1998). This is in agreement with the findings in Sweden where streptogramins were prescribed as prophylactics for necrotic enteritis in poultry following the growth promoter ban. This practice has now ceased and treatment of poultry in Sweden with therapeutic antimicrobials is reported to be uncommon (SVARM, 2000). However, it should be pointed out that ionophores are used as coccidiostats in most broiler flocks in Sweden (SVARM, 2001) and that these compounds are also active against *Clostridium perfringens*, the causative agent of necrotic enteritis. The amount of ionophoric anticoccidials prescribed for group treatment in Sweden also increased dramatically immediately following the withdrawal of growth promoters and has persisted at approximately 10, 000kg active ingredient per annum since (SVARM, 2001). It is possible that the use of these compounds has masked disease problems that would otherwise have developed following the withdrawal of growth promoters. Information on the prevalence of disease in pigs has not been published but the increase in therapeutic antimicrobials sold in Denmark since the ban suggests that there has been an increase in antimicrobial treatments (Danmap, 2000; Danmap, 2001).

Data on resistance in commensal bacteria in Sweden are not available before 2000 making it difficult to associate trends in resistance with the withdrawal of

antimicrobial growth promoters, but 2 per cent of *E. faecium* isolates tested from pigs were resistant to avilamycin in 2000, indicating that resistance has persisted albeit at a low prevalence (SVARM, 2000). The most recent data available from Denmark indicate that, in general, resistance to antimicrobial growth promoters has reduced in concordance with a decrease in their use (Aarestrup *et al.*, 2001). Resistance to glycopeptides in enterococci isolated from broilers has fallen significantly, from 72.7 per cent before avoparcin was banned in 1995, to 5.8 per cent in 2000 (Bager *et al.*, 1999).

A similar reduction in glycopeptide-resistant isolates from pigs was not seen until use of tylosin as a therapeutic antimicrobial was also decreased. Two reasons have been suggested for this observation. First, that the use of all-in all-out husbandry in broiler farms led to the gradual replacement of vancomycin-resistant enterococci with enterococci sensitive to vancomycin and second, that the continued use of tylosin in pigs co-selected for resistance to vancomycin (Bager *et al.*, 1999). Evidence to support this was initially based on the demonstration of associations between resistance to therapeutic antimicrobials such as tetracycline and penicillin and resistance to vancomycin in enterococci but the association with tylosin resistance in pigs was not statistically significant (Bager *et al.*, 1999). However, it was later shown that genes encoding macrolide and glycopeptide resistance were located close together on the same plasmid in enterococcal isolates from pigs (Aarestrup, 2000b) and the prevalence of vancomycin resistance in enterococci from pigs in Denmark did decline significantly following a reduction in tylosin use in 1998 and 1999 (Aarestrup *et al.*, 2001). The reduction in tylosin use also resulted in a significant reduction in erythromycin resistance in enterococci isolated from pigs (Aarestrup *et al.*, 2001).

Virginiamycin resistance has also decreased in enterococci isolated from broilers since its ban in 1998 but persisted at a prevalence of 33.9 per cent in 2000. Finally, avilamycin resistance in enterococci isolated from broilers peaked in 1996 at 63.6 per cent but has since fallen to 4.8 per cent in 2000, in parallel with a reduction in its use (Aarestrup *et al.*, 2001). These results indicate that the withdrawal of these growth-promoting antimicrobials has generally been followed by a reduction in resistance amongst enterococci, but this has been complicated by the fact that resistances to some antimicrobials are genetically linked and therefore a decline in resistance has not always been immediately apparent.

1.15 Epidemiology of antimicrobial resistance and modelling resistance

In order to improve understanding of resistance in bacterial populations, mathematical models are beginning to be employed to explain differences in patterns of resistance between organisms and also to predict the behaviour of organisms following changes in antimicrobial use (Levin *et al.*, 1999) but these models have primarily been directed towards the treatment of clinical infections in humans and have generally modelled bacterial population dynamics (Lipsitch and Levin, 1997a; Lipsitch and Levin, 1997b; Lipsitch and Levin 1998; Levin et al, 2000; Levin, 2001; Lipsitch *et al.*, 2002). According to the predictions of these models, the most important factors governing the frequency of antimicrobial resistance include the duration of infectiousness of individuals, the incidence of drug treatment, the extent to which treatment reduces transmission, the degree to which resistance reduces the competitiveness of a microorganism in the absence of treatment and the probability that a drug-sensitive infection becomes resistant on treatment (Levin, 2001). These factors have been used to predict the different patterns of resistance development in

genital herpes and influenza A and the population dynamics of bacteria and their response in evolutionary terms to antimicrobial therapy has been modelled quantitatively (Lipsitch and Levin, 1997a; Lipsitch and Levin, 1997b; Levin *et al.*, 1997). The efficacy of different antimicrobial treatment regimes in tuberculosis has also been compared (Lipsitch and Levin, 1998) and the effects of different treatment regimes on resistance considered, with the aim of developing protocols to minimise its development (Bonhoeffer *et al.*, 1997).

However, the situations represented by such models are specific to human medicine and are quite unlike the situation in veterinary medicine where intensive antimicrobial treatment is unusual and concern has not been primarily about successful clinical treatment in animals but about transfer of resistant organisms or resistance determinants to man via the food chain (MAFF, 1998; ACMSF, 1999)

Historically, much of the study of the epidemiology of resistance in animals has been observational, with the presence of genetically similar resistant organisms in animals and man taken as evidence of the spread of resistance (van den Bogaard *et al.*, 1997; Threlfall *et al.*, 1997; Fey *et al.*, 2000) but the magnitude of this effect has been difficult to quantify (Lipsitch *et al.*, 2002) and many sources have suggested that the surveillance of antimicrobial resistance in animals is important and should be improved (Williams and Ryan, 1998; House of Lords Select Committee on Science and Technology, 1998; ACMSF, 1999).

Currently, as far as resistance to growth-promoting antimicrobials is concerned, there are few published data, certainly in the United Kingdom. Surveillance of resistance to growth promoters has, however, been undertaken in Denmark since 1995 as part of a programme to monitor resistance in zoonotic bacteria, non-zoonotic pathogens and indicator (commensal) bacteria in animals, food and humans (Danmap, 1997;

Aarestrup *et al.*, 1998; Danmap, 1998; Danmap, 1999; Danmap, 2000) and several other European countries have recognised the importance of antimicrobial resistance surveillance in animals and are developing their own programmes (Martel *et al.*, 2000; Moreno *et al.*, 2000; Wray and Gnanou, 2000; SVARM, 2001).

However, there are many issues, including variation in laboratory methodologies, discrepancies in categorisation of organisms and lack of demographic data, which have meant that comparison of resistance data and analysis of trends has been difficult (Wray and Gnanou, 2000). The need to quantify resistance in order to estimate prevalence and also to monitor changes over time and in relation to antimicrobial use, has been highlighted (Davison *et al.*, 2000) but a quantitative approach to resistance surveillance has not yet been adopted with current surveillance even in the best programmes based on testing a relatively small number of isolates from a small proportion of the food animal population (Danmap, 2001; SVARM, 2001).

1.16 Conclusions

Antimicrobial resistance is a serious clinical problem in human medicine, which makes the treatment of bacterial disease more difficult, more costly and less likely to be successful and for these reasons, it is necessary that resistance to antimicrobials is minimised in order that as many antimicrobials as possible can be preserved for the treatment of infection. Since the emergence of antimicrobial resistance is usually linked to antimicrobial use, it is important that the necessity of all antimicrobial use is evaluated.

Animal and human bacterial populations are linked by the food chain, so that not only may zoonotic pathogens be transferred, but also commensal organisms or their resistance elements may spread to the human microflora.

Antimicrobial growth promoters have been used in agriculture for many years but a proper assessment of their contribution to antimicrobial resistance is now thought to be appropriate. In order to do this, it is necessary to consider the epidemiology of resistance; its genetic basis; and its biological impact, so that the contribution made to the global problem of antimicrobial resistance by antimicrobial growth promoter use can be properly evaluated. The withdrawal of several antimicrobials from use as growth promoters in Europe has been controversial and such decisions will only be vindicated if they can be shown to have reduced antimicrobial resistance and this, in turn, can only be achieved if resistance can be measured.

The aims of this project were to consider resistance to the growth promoter avilamycin and to assess how resistance could be measured at farm level. Resistance to therapeutic antimicrobials and their relationship to avilamycin use were also to be considered and the effect of avilamycin withdrawal on avilamycin resistance measured. Conventional bacteriological techniques in conjunction with epidemiological modelling and molecular methods were to be used to achieve these aims. The plan of work was based around the following chapter headings:

Sample size requirements for the detection of resistance

Isolation, enumeration and identification of target organisms

Resistance to avilamycin

Resistance to therapeutic antimicrobials

Molecular investigations

Epidemiological models

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Introduction

The materials and methods used can be subdivided into three main subheadings: bacteriological materials and methods, selection of farms and sampling methods and epidemiological modelling methods. Listed below are the equipment, media, reagents and conditions of cultivation used in the isolation, identification and sensitivity testing of the organisms studied as well as an overview of the farms selected for study and the software used to simulate the epidemiological models. A comprehensive list is provided here for completeness but the methods used for specific pieces of work are briefly described in each of the proceeding chapters.

2.2 Bacteriological materials and methods

2.2.1 Equipment used

1. Incubator, (37°C, aerobic), Swallow, Jencons PLS, Forest Row Business Park, Station Rd., Forest Row, East Sussex, RH18 5DW.
2. Incubator, (44°C, aerobic), Mini/75, Genlab Mini-Series Incubator, Jencons PLS, Forest Row Business Park, Station Rd., Forest Row, East Sussex, RH18 5DW.
3. Incubator, (44°C, aerobic), Shake'n'Stack, Hybaid Ltd., Action Court, Ashford Rd., Ashford, Middlesex, TW15 1XB.
4. Incubator, (37°C, microaerobic), MACS-VA500-microaerobic workstation, Don Whitley Scientific Ltd., 14 Otley Rd., Shipley, West Yorkshire, BD17 7SE.

5. Ultra-low temperature freezer, (-86°C), Sanyo Electric Co. Ltd. Refrigeration Products Division, Sakota Oizumi-Machi Ora-Gun, Gunma 370-0596, Japan.

6. Refrigerator (3°C), Indesit, Merloni House, 3 Cowley Business Park, High Street, Cowley, Uxbridge, Middlesex UB8 2AD.

7. Electronic pipettor, Jencons Sealpette, Jencons, Cherrycourt Way, Stanbridge Rd., Leighton Buzzard, Bedfordshire, LU7 4UA.

8. Vortex, Vortex-Genie 2, Jencons, Cherrycourt Way, Stanbridge Rd., Leighton Buzzard, Bedfordshire, LU7 4UA.

9. Water Bath, (50°C), NEI Range Clifton Unstirred Bath, Nickel Electro Ltd., Oldmixon Crescent, Weston-Super-Mare, North Somerset, B824 9BL.

10. Disc dispenser, Oxoid Ltd., Wade Rd., Basingstoke, Hampshire, RG24 OPW.

11. Nephelometer, Biomerieux Ltd., Grafton House, Grafton Way, Basingstoke, Hampshire, RG22 6HY.

12. Multipoint inoculator, A 400 Multipoint Inoculator, Denley Ltd. Billingshurst, Sussex, RH14 9SJ.

13. Balance, Oertling HB63.

14. Safety cabinet, Holliday Fielding Hocking Ltd., Wesley Works, Wesley Place, Leeds, LS9 8HA.

15. Centrifuge, MSE Micro Centaur, model no. MSB010.CX1.5, Sanyo Electric Co. Ltd. Sakota Oizumi-Machi Ora-Gun, Gunma 370-0596, Japan.

16. PCR machine, PCRExpress, Hybaid UK Ltd. Action Court, Ashford Place, Ashford, Middlesex TW15 1XB

17. Sequencer, ABI Prism 3100 Genetic Analyzer, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 U.S.A.

2.2.2 Media and reagents used

The following media and materials were used throughout the study for general cultivation and maintenance. They were prepared according to the manufacturers instructions and used in the form given below.

1. Nutrient agar plates (Oxoid Ltd., Wade Rd., Basingstoke, Hampshire, RG24 OPW, CM0003).
2. 5 per cent horse blood agar.

   20ml defibrinated horse blood (E and O Laboratories Ltd., Burnhouse, Bonnybridge, Stirlingshire, FK4 2HH) was added to 400ml blood agar base No. 2 (Oxoid Ltd., CM0271).
3. 5 per cent sheep blood agar.

   20ml formalised sheep blood, E and O Laboratories Ltd., Burnhouse, Bonnybridge, Stirlingshire FK4 2HH, was added to 400ml blood agar base No. 2 (Oxoid Ltd., CM0271).
4. MacConkey agar plates, Oxoid Ltd., CM0007.
5. Slanetz and Bartley agar plates, Oxoid Ltd., CM0377.
6. Preston campylobacter medium, Oxoid Ltd., CM689, SR0117E.
7. Skirrow campylobacter medium, Oxoid Ltd., CM689, SR0069E.
8. CIN agar plates, Oxoid Ltd., CM0653, SR0109.
9. Mueller Hinton agar pre-poured plates, Oxoid Ltd., P0152A.
10. Mueller Hinton agar with 5% Sheep Blood pre-poured plates, Oxoid Ltd., PB0413A.
11. Phosphate buffered saline.

Dulbecco A tablets (Oxoid Ltd., BR0014a) were dissolved in distilled water according to the manufacturers instructions. The sodium chloride concentration was 0.85 per cent. It was sterilised before use.

12. Physiological saline.

The sodium chloride concentration was 0.85 per cent in deionised water. It was sterilised before use.

13. Glycerol, "AnalaR" about 87 per cent, BDH Ltd., Merck House, Poole, Dorset BH15, 1TD.

14. Tryptone-soya broth, Oxoid Ltd., CM0129.

15. API Identification, Biomerieux Ltd., Grafton House, Grafton Way, Basingstoke, Hampshire, RG22 6HY.

The following API kits were used with associated reagents for the identification of appropriate organisms:

API 20E	Enterobacteriaceae	20100
API Campy	*Campylobacter* species	20800
API 20 Strep	*Streptococcus* and *Enterococcus* species	20600

16. Antimicrobial discs, Oxoid Ltd.

The following antimicrobial discs were used for susceptibility testing throughout the study:

Amoxycillin/clavulanic acid 2:1	30μg	CT0223B
Ampicillin	10μg	CT0003B
Cefuroxime sodium	30μg	CT0127B
Ciprofloxacin	5μg	CT0425B
Erythromycin	15μg	CT0020B
Gentamicin	10μg	CT0024B

Gentamicin	120µg	CT0794B
Linezolid	30µg	CT1650B
Oxacillin	1µg	CT0159B
Penicillin G	10units	CT0043B
Quinupristin/dalfopristin	15µg	CT1644B
Sulphamethoxazole/trimethoprim 19:1	25µg	CT0052B
Teicoplanin	30µg	CT0647B
Tetracycline	30µg	CT0054B
Vancomycin	30µg	CT0058B

2.2.3 Conditions of cultivation

2.2.3.1 Atmospheric conditions

Cultures were incubated aerobically or, where appropriate, in microaerobic conditions.

Aerobic cultures were incubated in the following incubators:

Incubator, (37°C, aerobic), Swallow, Jencons PLS, Forest Row Business Park, Station Rd., Forest Row, East Sussex, RH18 5DW.

Incubator, (44°C, aerobic), Mini/75, Genlab Mini-Series Incubator, Jencons PLS, Forest Row Business Park, Station Rd., Forest Row, East Sussex, RH18 5DW.

Incubator, (44°C, aerobic), Shake'n'Stack, Hybaid Ltd., Action Court, Ashford Rd., Ashford, Middlesex, TW15 1XB.

Microaerobic cultures were incubated in the microaerobic cabinet, Don Whitley Scientific Ltd., 14 Otley Rd., Shipley, West Yorkshire, BD17 7SE.

2.2.3.2 Temperature of incubation

Cultures were incubated at 37°C except Slanetz and Bartley plates that were incubated at 37°C for 2-4 hours and subsequently at 44°C for a further 44-46 hours.

2.2.3.3 Period of incubation

All primary cultures on non-selective media and MacConkey agar plates were incubated for 18-24 hours.

All primary cultures on Slanetz and Bartley agar plates were incubated for 24 hours, examined and then reincubated for a further 18-24 hours.

All primary cultures on campylobacter selective media were incubated for 42-48 hours.

All primary cultures on CIN agar plates were incubated for 18-24 hours.

2.2.4 Identification of bacterial isolates

2.2.4.1 Colonial morphology

The examination of colonies was by the unaided eye and, where necessary, by hand lens. The morphological characteristics of colonies (colour, size, elevation etc.) were noted. Many different colony types were present on initial cultures on non-selective media. On selective media, colonies typical of the organisms of interest were subcultured to purity.

2.2.4.2 Morphology of bacterial cells

This was determined by the microscopic examination of air-dried heat-fixed smears made from cultures and stained by Gram's method.

2.2.4.3 Biochemical tests

Oxidase tests were carried out using sterile swabs dipped in the reagent (1 per cent NNN'N-tetramethyl-P-phenylenediamine dihydrochloride).

Catalase tests were carried out using loopfuls of 24 hour or 48 hour cultures of the organism under test, grown on nutrient agar or the blood medium described above. The organism under test was emulsified with a few drops of 3 per cent hydrogen peroxide (B.D.H. Ltd, Merck House, Poole, Dorset BH15 1TD) on a microscope slide. Suspensions were examined for bubbles of gas immediately and after 5 minutes. Further biochemical tests were carried out using the API identification system (Biomerieux Ltd.).

2.2.5 Examinations for specific bacteria

2.2.5.1 *Enterococcus* species.

Faecal specimens or their dilutions were inoculated on Slanetz and Bartley agar and incubated in aerobic conditions at 37°C for 48 hours in initial investigations. In later investigations, faecal specimens or their dilutions were inoculated on Slanetz and Bartley agar and incubated in aerobic conditions at 37°C for 2-4 hours then at 44°C for a further 44-46 hours. Typical colonies were subcultured on 5 per cent horse blood agar or nutrient agar. Smears were made and stained by Gram's method and catalase tests performed on each isolate. Catalase-negative, Gram-positive cocci were identified to species using the API 20 Strep kit, Biomerieux Ltd.

2.2.5.2 Coliforms

Faecal specimens or their dilutions were inoculated onto MacConkey agar and incubated aerobically for 18-24 hours at 37°C. Typical lactose-fermenting colonies were subcultured and identified biochemically using the API 20E kit, Biomerieux Ltd.

2.2.5.3 *Yersinia* species

Faecal specimens were inoculated onto CIN agar and incubated aerobically for 18-24 hours at 37°C. Faeces were also diluted in phosphate-buffered saline (approximately 5 per cent by volume) and maintained at 3°C in the refrigerator for three weeks, during which time CIN agar was inoculated weekly and incubated as above. Typical red colonies were subcultured and identified biochemically using the API 20E kit, Biomerieux Ltd.

2.2.5.4 *Campylobacter* species.

Skirrow's agar plates and Preston agar plates were inoculated with faeces or faecal dilutions and were incubated at 37°C for 48 hours in microaerobic conditions. Smears of colonies growing on the media were stained by Gram's method to ascertain whether they contained vibrios. All colonies that did contain vibrios were subcultured to purity and incubation in microaerobic and aerobic conditions were used in duplicate for each subculture in order to identify and eliminate organisms other than *Campylobacter* species. The oxidase test was performed on all colonies as described above and further biochemical testing was performed using the API Campy kit, Biomerieux Ltd.

2.2.6 Maintenance of cultures

Each pure culture isolated was maintained by subculture onto blood agar or nutrient agar at appropriate intervals. Pure cultures were then inoculated into vials containing cryopreservative (Microbank, Pro-Lab Diagnostics, 7 Westwood Court Neston, South Wirral, Cheshire CH64 3UJ) and stored at -70°C.

2.3 Selection of farms and sampling

2.3.1 Selection of farms

Four farms were selected for sampling on the basis of their use (or not) of the growth promoter avilamycin. Avilamycin use was recorded and the inclusion of copper and zinc in feeds was also noted (Table 2.1). All four farms were farrow-to-finish units and Farms 1, 2 and 3 were located in west central Scotland whilst Farm 4 was located in north east Scotland. Farms 1 and 2 were commercial units producing pigs for slaughter and there were approximately 200 and 60 breeding sows on these farms, respectively. Farm 3 was a multiplier unit of approximately 200 breeding sows and Farm 4 was a fully integrated breeding and finishing unit of approximately 2000 breeding sows. On Farm 1, avilamycin was included in rations from weaning until the end of the finishing period. On Farm 2, avilamycin was included in feed given to weaners only, salinomycin was included in feed given to growing pigs, and finishing pigs did not receive any growth promoters. On Farm 3, avilamycin was included in feed given to weaners only. Avilamycin was withdrawn from use on Farm 4 two years before the start of the study and none of the pigs received any growth promoters.

In addition, the farmers were asked about their use of therapeutic antimicrobials (Table 2.2). A formal assessment of therapeutic antimicrobial use on these farms was beyond the scope of this work and was not carried out.

2.3.2 Collection and handling of samples

Faecal samples were collected from the floors of pens in sterile plastic universal containers, with freshly voided faeces collected where possible. Slurry samples were also collected into sterile plastic universal containers using sterile plastic pipettes. The samples were placed on ice and transported to the laboratory. The time taken from collection of samples on farm until arriving at the laboratory varied from 1-2 hours for Farms 1-3 and 6-8 hours for Farm 4. For Farms 1-3, samples were kept on ice in the laboratory for approximately 1 hour whilst samples were weighed into 1g aliquots. For Farm 4, samples were kept refrigerated at 3°C overnight before being weighed into 1g aliquots. Where samples were stored before analysis, each 1g aliquot of faeces or 1ml aliquot of slurry was added to 4.5ml sterile glycerol and 4.5ml tryptone-soya broth, mixed and frozen at -70°C.

2.4 Epidemiological modelling methods

All of the models described were written in Microsoft Excel (Microsoft, Redmont, WA 98052-6399, USA) and the stochastic models were simulated using @Risk (Palisade, Newfield, NY), a software package allowing Latin Hypercube simulation.

Table 2.1 Use of avilamycin, salinomycin, zinc and copper as non-nutrient feed additives on the farms selected for testing, mg/kg of feed.

Farm	Compound	Weaners	Growers	Finishers
Farm 1	Avilamycin	40mg/kg	40mg/kg	20mg/kg
	Zinc	2.5g/kg	-	-
	Copper	175mg/kg	165mg/kg	90mg/kg
Farm 2	Avilamycin	40mg/kg	-	-
	Salinomycin	-	50mg/kg	-
	Zinc	2.5g/kg	-	-
	Copper	175mg/kg	165mg/kg	90mg/kg
Farm 3	Avilamycin	40mg/kg	-	-
	Zinc	2.5g/kg	-	-
	Copper	175mg/kg	165mg/kg	90mg/kg
Farm 4	Zinc	2.5g/kg	-	-
	Copper	175mg/kg	165mg/kg	90mg/kg

Table 2.2 Therapeutic antimicrobials used in the previous twelve months on the farms selected for testing.

Farm	Group	Antimicrobial (route of administration)	Reason for use
Farm 1	Piglets	Tetracycline (spray)	Tail-docking
	Piglets	Enrofloxacin (oral doser)	Diarrhoea
	Weaners/Growers	Tetracycline (water)	General debility
	Weaners/Growers	Penicillin (feed)	Meningitis, pleuropneumonia
	Gilts (incoming)	Lincomycin (water)	Arthritis due to *Mycoplasma synoviae*
	Sows	Penicillin/streptomycin (parenteral)	General malaise
Farm 2	Weaners/Growers	Amoxycillin (water)	Meningitis
Farm 3	All groups	Penicillin/streptomycin (parenteral)	General malaise
Farm 4	Piglets	Enrofloxacin (oral doser)	Diarrhoea
	Piglets	Tetracycline (water)	Diarrhoea/Glasser's Disease
	Piglets	Penicillin/streptomycin (parenteral)	Glasser's Disease
	Weaners	Tylosin (feed)	Spirochaetal diarrhoea
	Growers	Tetracycline (water)	Routine, for 1 week

CHAPTER 3

SAMPLE SIZE REQUIREMENTS FOR THE DETECTION OF RESISTANCE

3.1 Introduction

The first stage in designing studies to measure resistance on the farms selected was to consider the necessary sample numbers required. Previously, published data on antimicrobial resistance has been criticised because sample size estimates have not been considered and this has hampered interpretation of the results of surveillance studies (MAFF, 1998; Davison *et al.*, 2000). It has been suggested that rigorous epidemiological studies of resistant bacteria need to be carried out in human and animal populations and that standard statistical formulae should be used to estimate sample sizes for such studies (Davison *et al.*, 2000). Prior determination of the sample size has also been recognised as essential in a well-designed study of any kind (Dawson-Saunders *et al.*, 1994).

In order to decide how many animals or organisms should be sampled, the objectives of the investigation must first be considered. When the aim of a study is to determine the prevalence of a disease or organism, the main factors to be considered from a statistical point of view are the desired precision of the prevalence estimate and the expected frequency of the disease or organism. However, when the aim is to detect the presence or absence of a disease or organism, the prevalence of disease, the desired confidence limits and the population size must be considered (Cannon and Roe, 1982; Thrusfield, 1995).

Prior to conducting the fieldwork involved in the project, sample size estimation was considered and some of the available literature on antimicrobial resistance in pigs was

examined with a view to using prevalence estimates from these data in order to estimate sample sizes for the study.

3.2 Materials and Methods

Two statistical methods of estimating sample size were considered; the sample size needed to estimate the prevalence of resistance (Equation 3.1) and the sample size needed to detect the presence or absence of resistance (Equation 3.2), (Cannon and Roe, 1982; Thrusfield, 1995):

$$n = \frac{1.96^2 P_{\exp}\left(1 - P_{\exp}\right)}{d^2} \qquad \text{Equation 3.1}$$

where n is required sample size, P_{exp} is the expected prevalence, d is the desired absolute precision, simple random sampling is applied, the size of the study population is large in relation to the sample and the confidence level is 95 per cent.

$$n = \left(1 - \left(1 - p_1\right)^{\frac{1}{d}}\right)\left(N - \frac{d}{2}\right) + 1 \qquad \text{Equation 3.2}$$

where n is required sample size, p_1 is the probability of finding at least one case in the sample, d is the number of affected animals in the population and N is the population size.

Surveillance data from Denmark were used as a source of information on the prevalence of resistance amongst enterococci isolated from pigs (Danmap, 1999) and confidence intervals and ideal sample size estimates were calculated using these data.

3.3 Results

First, the sample numbers needed to estimate prevalence of resistance and to detect the presence of resistance were considered and compared for a wide range of expected prevalences (Tables 3.1 and 3.2). Estimates of the prevalence of resistance to various antimicrobials amongst *E. faecium* isolated from pigs (Danmap, 1999) were then used to calculate confidence intervals (Table 3.3).

Table 3.1 Sample numbers required in order to estimate the prevalence of resistance with 95% confidence and 5% absolute precision.

Expected prevalence of resistance	Sample numbers required to estimate prevalence of resistance
1% or 99%	15
2% or 98%	30
3% or 97%	45
5% or 95%	73
10% or 90%	138
20% or 80%	246
30% or 70%	323
40% or 60%	369
50%	384

Table 3.2 Sample numbers required to have a 95% probability of detecting the presence of resistance*.

Population size (bacteria or animals)	Prevalence of resistance, P_l (%)							
	0.1	1	5	10	20	30	40	50
∞	2995	299	59	29	14	9	6	5
5000	2253	290	59	29	14	9	6	5
1000	950	258	57	29	14	9	6	5
100	100	96	45	25	13	8	6	5
50	50	50	35	22	12	8	6	5

*(Thrusfield, 1995).

Table 3.3 Estimated prevalence of resistance amongst *E. faecium* isolated from pigs in Denmark* and associated confidence intervals.

Antimicrobial	Estimated prevalence of resistance	95 per cent confidence interval
Tetracycline	0.53	0.46-0.60
Penicillin	0.39	0.32-0.46
Erythromycin	0.48	0.41-0.55
Streptomycin	0.27	0.21-0.33
Vancomycin	0.06	0.03-0.09
Quinupristin-dalfopristin	0.19	0.14-0.24
Virginiamycin	0.08	0.04-0.12
Avilamycin	0.01	0.00-0.02

* Danmap, 1999, 202 isolates tested.

Sample sizes were then calculated for a total animal population of 2.3 X 10^7 (Danmap, 1999) using the methods described and the data in Table 3.3 (Table 3.4).

Table 3.4 Number of animals to be tested in order to estimate prevalence of resistance with 95% confidence and 5% absolute precision (prevalence estimation) and to have 95% probability of detecting resistance (presence/absence detection). Prevalence data taken from Table 3.3 and total animal population of 2.3 X 10^7*.

Antimicrobial	Sample numbers needed for prevalence estimation	Sample numbers needed for presence/absence detection
Tetracycline	383	5
Penicillin	366	7
Erythromycin	384	6
Streptomycin	303	11
Vancomycin	87	49
Quinupristin-dalfopristin	236	15
Virginiamycin	113	37
Avilamycin	15	299

*Danmap, 1999

3.4 Discussion

The calculation of sample sizes using standard statistical formulae described above has highlighted the important factors to be considered in the design of antimicrobial resistance studies. Prevalence of resistance, population size and the reliability of the conclusions, have all been shown to be important considerations and although these factors are routine issues in the design of animal health surveys, their importance in antimicrobial resistance studies has only recently been suggested (Davison *et al.*,

2000). In a recent paper that aimed at defining the minimum epidemiological requirements for establishing surveillance of antimicrobial resistance in animals throughout Europe, sample size requirements were not even discussed (Caprioli *et al.*, 2000) and the above calculations suggest that this is a glaring omission that will seriously affect the interpretation of surveillance data of any kind and prevent useful comparisons between countries. Unless the population of interest and the confidence level required are defined and the estimated prevalence of resistance is taken into account, then studies will have little statistical power.

In addition, key differences in sample size determination for studies designed to detect the presence or absence of resistant organisms and to determine the prevalence of resistance have also been demonstrated, with the objective of a study having a crucial influence on the sample numbers required. Although the output from most of the surveillance of antimicrobial resistance conducted in animals to date has been prevalence data, legislative changes leading to the withdrawal of certain antimicrobials have resulted in prevalence data being used to monitor the decline in resistance over time and to demonstrate freedom from resistance without a reappraisal of sample size (SVARM, 2000; Danmap, 2001) and given the above findings, this is perhaps inappropriate.

In general, the sample numbers required to detect the presence of resistance were lower than those required to estimate prevalence (Table 3.4). However, this was dependent upon the estimated prevalence of resistance and for avilamycin, the sample numbers required for resistance detection were very high (299) (Table 3.4) because of the very low estimated prevalence of resistance and this should be borne in mind when data from prevalence studies are used to demonstrate the absence of resistance from an animal population. In prevalence studies, sample size estimates were greatest

when prevalence was 50% (Table 3.1), whereas in presence/absence studies, sample size estimates were greatest when prevalence was very close to zero and the estimate of sample size required increased as the study population increased (Table 3.2). However, in small populations, the sample size estimate was a greater proportion of the total population than when the study population was very large (Table 3.2). These features of sampling design have been highlighted before (Cannon and Roe, 1982; Thrusfield, 1995) but have rarely been considered in antimicrobial resistance studies. Notably, for five of the eight antimicrobials listed, sample size estimates for prevalence estimation were greater than the actual number of samples taken in the study referred to (202) (Danmap, 1999) and the confidence intervals calculated for the different antimicrobials tested varied markedly dependent upon the estimated prevalence of resistance. These differences should be taken into account when sample sizes for antimicrobial resistance studies are being calculated and the aims of surveillance should be clearly defined.

In the data used for sample size calculations, the estimated prevalence of resistance varied widely for the different antimicrobials tested. For example, one percent of isolates were resistant to avilamycin compared to fifty-three percent that were resistant to tetracycline (Table 3.3). However, the prevalence of resistance is likely to vary not only for different antimicrobials but also for different organisms and therefore, if a multi-organism or multi-antimicrobial study is to be undertaken, sample numbers should ideally be based on the largest estimate in order to be confident of detecting resistance/estimating prevalence for all organism/antimicrobial combinations. In much of the currently available surveillance data, this has not been considered and confidence intervals surrounding prevalence estimates have not been presented (Danmap, 1997; Danmap, 1998; Danmap, 1999; Danmap, 2000).

The estimates of sample size described apply when simple random sampling is used to select a sample of animals or bacteria from the study population. This means that every animal or bacterium has an equal chance of being sampled. For animals, this usually involves numbering each animal and then using a table of random numbers to select the animals to be sampled. However, for bacteria this would obviously not be possible and it may be that factors influencing the growth of bacteria on isolation media determine which are selected for testing. This could be particularly important in antimicrobial resistance studies as it has been shown that the acquisition and maintenance of resistance genes in a bacterial population can have an associated fitness cost (Andersson and Levin, 1999). If resistant bacteria in a population require a greater concentration of nutrients for growth or grow at a slightly slower rate on culture media than sensitive organisms, then they may be less likely to be selected for testing and although the effect of using visual cues on the selection of animals for testing has been considered, the selection of bacteria for testing could be an equally important source of bias (Singer *et al.*, 2001). The data described from Denmark was obtained from samples taken at abattoirs and therefore does not represent a true random sample of the pig population in Denmark (Danmap, 1999) and if simple random sampling is not applied to the selection of bacteria or animals for sampling, then even larger sample sizes are required (Cannon and Roe, 1982).

In addition, the sample size calculations described do not take account of the variation that can occur between clusters of animals. Clusters of animals could be groups within a farm or different farms, for instance. If cluster sampling is applied then the between-cluster variance must first be calculated (Equation 3.3).

$$V_C = c\left(\frac{K_1 c V}{T^2(c-1)} - \frac{K_2 P(1-P)}{T}\right) \qquad \text{Equation 3.3}$$

where V_c is between cluster variance component, c is the total number of clusters in the sample, T is the total number of animals sampled, K_1 is equal to $(C - c)/C$ where C is the number of clusters in the population, K_2 is equal to $(N - T)/N$ where N is the total number of animals in the population, V is equal to $P^2(\sum n^2) - 2P(\sum nm) + (\sum m^2)$ where P is the estimate of overall prevalence, n is the number of animals sampled in each cluster and m is the number of diseased animals sampled in each cluster (Thrusfield, 1995). The equations then to be used to calculate sample numbers required are dependent on whether cluster sampling is one-stage or two-stage and are given in standard texts (Thrusfield, 1995).

Furthermore, the definition of the population being studied is also important. For example, the distinction between *E. faecium* and other *Enterococcus* spp. may not always be clear on selection of isolates from selective media or on biochemical testing (Devriese *et al.*, 1994) but there may be differences in the prevalence of resistance between different species that affect ideal sample sizes. The definition of the animal population of interest in terms of pen, age-group or farm is also important in estimating sample size requirements as it provides denominator information so that results can be related to the total study population.

The sample size calculations discussed do not take into account the performance of diagnostic tests and although the effect of test performance has been considered for the interpretation of tests performed on a proportion of animals within a herd (Jordan and McEwen, 1998), it has not been considered for the calculation of sample size requirements or for antimicrobial resistance studies. These issues are discussed in Chapter 8.

In summary, sample size estimation is a necessary consideration prior to conducting antimicrobial resistance studies and despite the implications of sample size estimates

on the cost and labour-intensity of such studies, these estimates cannot be ignored. If optimal sample sizes cannot be applied, then an inevitable consequence is that less confidence can be placed in the conclusions of a study, whether it describes the prevalence of resistance or the presence or absence of resistance in a population. However, despite the strength of these conclusions, the labour-intensive nature of the work conducted in the studies discussed in Chapters 4, 5 and 6 and the poor sensitivity of isolation of enterococci did not allow the application of standard sample size estimates to the sampling regimes adopted and the consequences of this are discussed in the relevant chapters.

CHAPTER 4

ISOLATION, ENUMERATION AND IDENTIFICATION OF TARGET ORGANISMS

4.1 Introduction

Having calculated ideal sample size requirements, the next process was to consider the organisms to be studied. Organisms present in the normal faecal flora of pigs were chosen because of their presence in the site of action of avilamycin and their importance in the transfer of resistance to man. Enterococci were considered to be particularly important because of their importance as a reservoir of resistance to growth promoting antimicrobials.

Enterococcus spp. and *Escherichia coli* are commensal organisms of animals and man that are also capable of causing disease and which have been implicated in the transfer of antimicrobial resistance from animals to humans (van den Bogaard and Stobberingh, 2000). They are both present in animal faeces and although the epidemiology of the diseases they cause in humans is quite different, they are both thought to represent reservoirs of antimicrobial resistance and therefore are important indicator organisms in which resistance should be measured (Caprioli *et al.*, 2000; Witte, 2000).

Enterococcus spp. are occasional pathogens of humans implicated particularly in nosocomial infections and which have readily acquired resistance to therapeutic antimicrobials (Cetinkaya *et al.*, 2000) whilst *Escherichia coli* is responsible for a spectrum of disease in animals and man and certain strains are important zoonotic pathogens (Griffin and Tauxe, 1991).

The pathogenic strains of *E. coli* are well described and the isolation, identification and enumeration of *E. coli* is also relatively straightforward (Ogden *et al.*, 2002). The pathogenicity of enterococci and the distinction between disease-causing strains and commensals is not as clear as they are essentially opportunistic pathogens. *E. faecalis* and *E. faecium* are responsible for the majority of cases of disease in man but *E. durans* and *E. hirae* have also been implicated (Knijff *et al.*, 2001). The identification of enterococcal species by biochemical means alone is also acknowledged to be difficult (Singer *et al.*, 1996; Tsakris *et al.*, 1998) and the importance of different enterococcal species in disease is poorly understood.

The aim of this part of the study was to attempt to quantify *E. coli* and *Enterococcus* spp. in pig faeces and to determine the composition of *Enterococcus* species present using simple dilution techniques and commercially available biochemical kits (API, Biomerieux).

4.2 Materials and Methods

4.2.1 Sample collection and handling

Faecal samples were collected from the floors of pens and transported on ice to the laboratory. The time from collection of samples on farm to arrival at the laboratory varied from 1-2 hours for Farms 1, 2 and 3 to approximately 18 hours for Farm 4. In initial investigations, each faecal sample was weighed into 1g aliquots and each 1g aliquot was gently mixed with 4.5ml sterile glycerol and 4.5ml sterile tryptone-soya broth (Oxoid Ltd.) and frozen at -70°C in glass universals. The frozen samples were later thawed and serial tenfold dilutions made in phosphate-buffered saline for the culture and enumeration of enterococci and coliforms. In later investigations, faecal samples were weighed into 1g aliquots and one aliquot from each sample was added

to 9ml phosphate-buffered saline and gently mixed. Serial ten-fold dilutions were made in phosphate buffered saline and plates inoculated directly with fresh faecal dilutions.

4.2.2 Inoculation of media and incubation

Slanetz and Bartley and MacConkey agar plates were inoculated with 20µl of each dilution of faeces from 1×10^{-1} to 1×10^{-6}. Plates were allowed to dry on the bench for no more than fifteen minutes before being inverted and transferred to incubators. MacConkey plates were incubated at 37°C in aerobic conditions for 18-24 hours. In the initial investigations, Slanetz and Bartley plates were incubated at 37°C in aerobic conditions for 48 hours. Following the conclusions from these initial investigations, Slanetz and Bartley plates were incubated at 37°C in aerobic conditions for 2-4 hours before being transferred to another incubator for a further 44-46 hours incubation at 44°C in aerobic conditions.

4.2.3 Enumeration and identification

Counts were made of each morphologically distinct colony-type at the lowest readable dilution on each plate. For isolation on MacConkey agar this included lactose-fermenting colonies of different textures, for example rough or smooth, and for isolation on Slanetz and Bartley agar this included maroon or pink colonies of different sizes. One or more representative colonies of each type were subcultured to purity and identified to species biochemically using the methods described in Chapter 2.

4.3 Results

4.3.1 Availability and nature of sample material

The material available for collection was highly dependent upon the housing systems in which animals were kept. For example, the only animals housed in individual pens on any of the farms visited were lactating sows. Samples collected from these pens were therefore individual animal samples. All other animals, including piglets, were contained in groups within pens. However, the type of flooring present also influenced whether or not the faecal material on the floors of pens was likely to have been voided by individual animals. In pens with slatted floors, which included farrowing pens on Farm 1 and flatdeck accommodation for weaner pigs on Farms 1 and 3, there was very little faecal material present and any faeces that was present was most likely to have been voided by an individual animal, although this could not be confirmed. These samples therefore probably represented individual animals within these pens. Where animals were housed on solid floors with straw bedding, (finishers aged 16-18 weeks on Farm 4), faecal material appeared to have been voided by individual animals as there was no pooling of faeces on pen floors but again, it was not possible to confirm this.

Where animals were housed on solid floors without bedding material, (Farm 1 growers and finishers; Farm 2 finishers; Farm 3 finishers and Farm 4 finishers from approximately 20 weeks of age), faecal material tended to be mixed with urine and was pooled in a corner of the pens. Therefore, samples from these pens were likely to be pooled samples although it was not possible to determine how many animals were represented by a sample.

4.3.2 Influence of incubation temperature on the isolation of enterococci and colonial morphology as an indicator of genus

The first samples to be examined were collected from pens of weaner pigs on Farm 1. In order to maximise the yield of enterococci from the samples and also to culture all species of enterococci that may have been present, Slanetz and Bartley agar plates were inoculated as described above and incubated at 37°C in aerobic conditions for 48 hours. On examination, the majority of the colonies present on the plates were pink although a few maroon colonies were also present. Regardless of colour, colonies were generally less than 0.5mm in diameter. Counts were made of both type of colony (Table 4.1). Where possible, two pink and two maroon colonies were subcultured from each plate and identified as described (Table 4.2). None of the pink colonies were identified as *Enterococcus* spp. whereas the majority of maroon colonies were. The distinction between maroon and pink colonies was not always obvious and colonies classed as maroon/pink from samples one and two were identified as *Enterococcus* spp. However, as maroon colonies had been shown to be more likely to be identified as *Enterococcus spp.*, pink colonies were disregarded in all future examinations.

Table 4.1 Morphology and counts of colonies present on Slanetz and Bartley agar inoculated with the faeces of weaner pigs following incubation at 37°C in aerobic conditions for 48 hours.

Sample	Colony description	Colony count (CFUs/g)
1	Maroon/pink	5.5×10^5
2	Maroon/pink	3×10^7
3	Pink	5.5×10^7
4	Maroon/Pink	3×10^6
5	Pink	7×10^6
6	Maroon	2×10^6
6	Pink	2.25×10^7
7	Maroon	9.5×10^6
7	Pink	1.75×10^7
8	Pink	1×10^7
9	Pink	3.5×10^7
10	Maroon	1×10^7
10	Pink	1.5×10^8

Table 4.2 Identification of colonies cultured from Slanetz and Bartley plates inoculated with weaner faeces and incubated at 37°C in aerobic conditions for 48 hours.

Sample	Colony description	Species identification
1	Maroon/Pink	*Enterococcus faecalis*
1	Maroon/Pink	*E. faecalis*
2	Maroon/Pink	*E. faecalis*
2	Maroon/Pink	*E. faecalis*
3	Pink	*Streptococcus bovis*
3	Pink	*S. bovis*
3	Dark pink	*S. bovis*
3	Dark pink	*S. bovis*
4	Pink	*Aerococcus viridans*
4	Pink	*A. viridans*
4	Maroon	*Enterococcus faecium*
4	Maroon	*E. faecalis/Lactococcus lactis lactis*
5	Pink	*L. lactis lactis*
5	Pink	*Leuconostoc* spp.
6	Maroon	*L. lactis lactis*
7	Maroon	*E. faecium*
7	Maroon	*E. faecium*
7	Pink	*A. viridans*
7	Pink	*A. viridans*
8	Pink	*S. bovis*
10	Maroon	*E. faecium*
10	Maroon	*E. faecium*

The next set of samples to be examined were from piglets and as a poor yield of enterococcal isolates was obtained from weaner faecal samples incubated at 37°C, faeces from these samples were inoculated onto two sets of plates; one set that was incubated at 37°C and one that was incubated at 37°C for 2-4 hours before being incubated at 44°C for a further 44-46 hours.

Following incubation, it was noted that some of the maroon colonies on the plates incubated initially at 37°C and then at 44°C were larger (>0.5mm diameter) (Figure 4.1) than the maroon or pink colonies on the plates incubated at 37°C. Counts of maroon colonies only were performed for the plates incubated at 37°C, and counts of maroon colonies >0.5mm in diameter were performed for the plates incubated at 37°C followed by incubation at 44°C (Table 4.3) (Figure 4.2). The mean and median counts of maroon colonies on plates incubated at 37°C were 5.3 $x10^6$ CFUs/g and 1.8 $x10^5$ CFUs/g, respectively, compared to a mean count of 1.1 $x10^5$ CFUs/g and a median count of 2.8 $x10^4$ CFUs/g of large maroon colonies on plates incubated first at 37°C and then at 44°C.

Figure 4.1 Appearance of large maroon colonies (>5mm diameter) on Slanetz and Bartley agar inoculated with faeces following incubation at 37°C for 2-4 hours followed by incubation at 44°C for a further 44-46 hours.

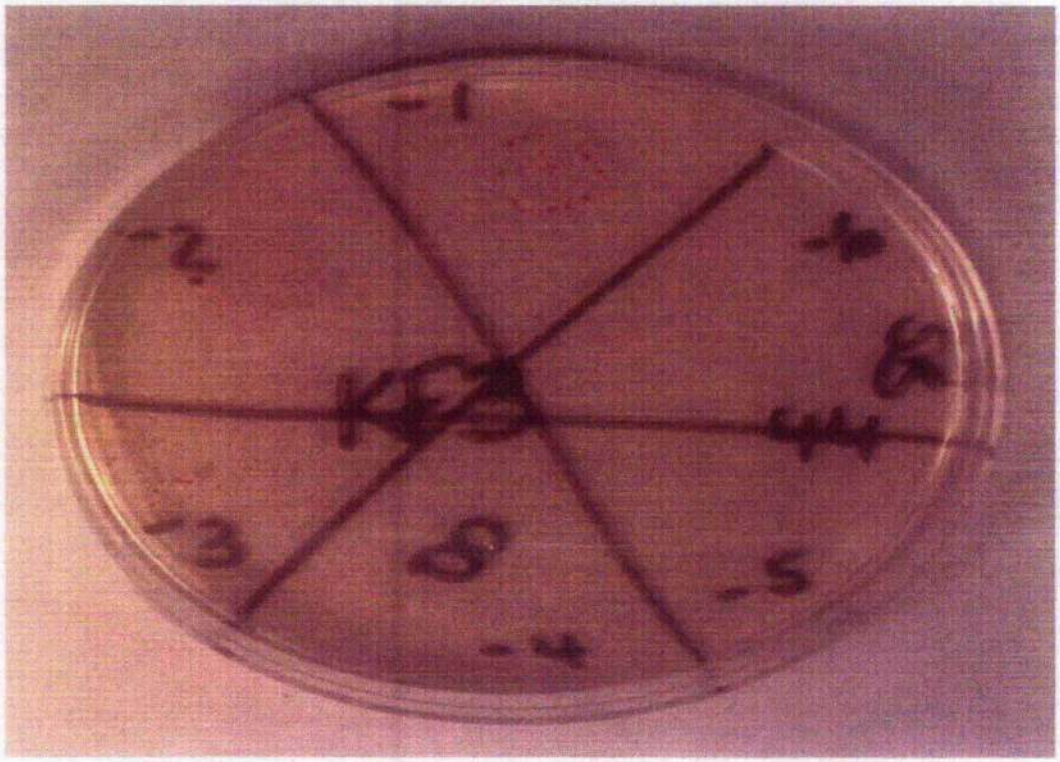


Figure 4.2 Slanetz and Bartley agar plate inoculated with faecal dilutions, after incubation at 37°C for 2-4 hours followed by incubation at 44°C for 44-46 hours, with variation in size of maroon colonies evident.

Table 4.3 Counts of presumptive *Enterococcus spp.* on Slanetz and Bartley plates inoculated with piglet faeces and incubated at 37°C in aerobic conditions for 48 hours and at 37°C for 2-4 hours followed by 44-48 hours at 44°C in aerobic conditions.

Sample	Colony count (maroon) 37°C incubation (CFUs/g)	Colony count (large maroon) 37°C followed by 44°C incubation (CFUs/g)
1	0	1.5×10^3
2	1.6×10^5	1.3×10^5
3	5×10^4	1.5×10^4
4	5×10^7	7×10^5
5	2.5×10^6	0
6	2×10^5	8×10^4
7	2.5×10^5	3×10^4
8	5×10^4	2×10^4
9	2×10^5	1.35×10^5
10	2×10^4	2.5×10^4

Where possible, two to four maroon colonies were subcultured from each plate and the organisms identified to species (Table 4.4). Where a sample is not listed for a particular incubation temperature that is because no gram-positive cocci were isolated. Nine of the ten samples yielded *Enterococcus* spp. when an incubation temperature of 44°C was used and only large maroon colonies were selected compared to only 3 samples from which *Enterococcus* spp. were isolated when an incubation temperature of 37°C was used throughout and only small maroon colonies were available to select for identification. Following these findings, all Slanetz and Bartley plates were incubated at 37°C for 2-4 hours and then at 44°C for 44-46 hours

and only maroon colonies greater than 0.5mm in diameter were counted and subcultured for identification.

Table 4.4 Identification of organisms isolated from piglet faeces collected from Farm 1 using Slanetz and Bartley plates incubated at 37°C and Slanetz and Bartley plates incubated initially at 37°C and then at 44°C.

Sample	Predominant incubation temperature	Identification of isolates obtained
1	44°C	*E. faecium* (3 isolates); *E. durans*
2	37°C	*E. faecium,* (2 isolates)
2	44°C	*E. faecium,* (4 isolates)
3	37°C	*S. bovis,* (2 isolates)
3	44°C	*E. faecium,* (4 isolates)
4	37°C	*E. durans, E. faecium*
4	44°C	*E. faecium,* (4 isolates)
5	37°C	*S. bovis,* (2 isolates)
6	37°C	*E. faecium, E. durans*
6	44°C	*E. faecium,* (4 isolates)
7	44°C	*E. faecium*
8	44°C	*E. faecium*
9	44°C	*E. faecium*
10	44°C	*E. faecalis*

4.3.3 Enumeration of presumptive enterococci

Counts of presumptive *Enterococcus spp.* (maroon colonies of at least 0.5mm diameter) were performed for all the samples collected (Figure 4.3). Samples from sows, piglets, weaners and growers on Farm 1 were frozen at -70°C before use and the other samples were used directly following collection. The counts observed varied widely between different age groups on the same farm and between different farms but this did not appear to be a direct result of sample storage as counts of presumptive enterococci from weaners and growers on Farm 1 were very high. The counts observed in faecal samples from within the same group of animals were also very variable with no presumptive enterococcal colonies detected for some samples and this can be seen from the large standard deviation in counts observed (Figure 4.3).

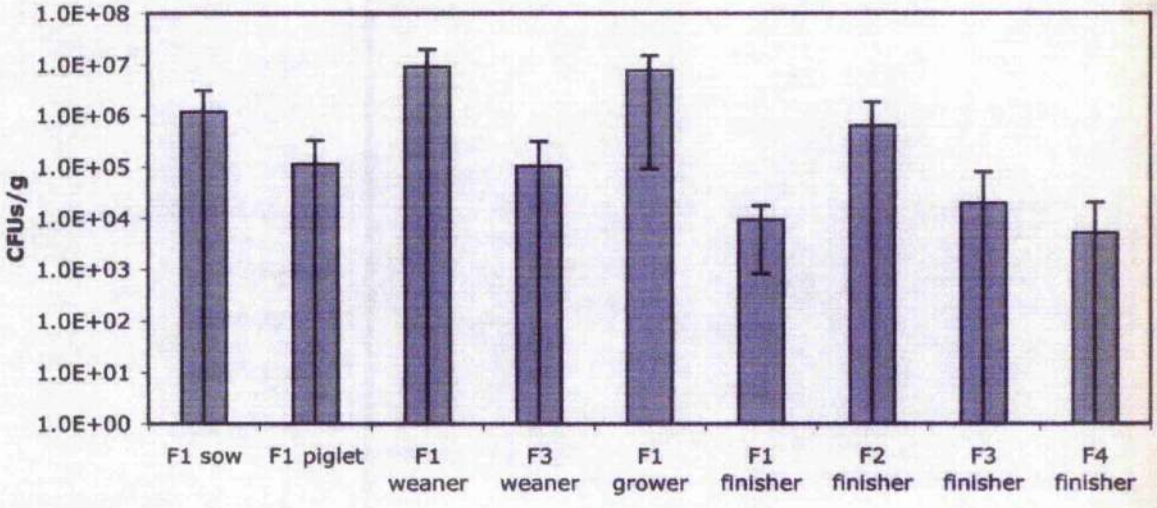


Figure 4.3 Counts of presumptive *Enterococcus spp.* (maroon colonies >0.5mm diameter) in samples collected from Farms 1-4 (F1-F4) with standard deviation shown (CFUs/ml).

4.3.4 Identification of presumptive enterococci

One to four presumptive enterococcal colonies were subcultured from each sample and identified to species (Table 4.5).

Table 4.5 Proportion of faecal samples collected from different groups on Farms 1–4 and cultured on Slanetz and Bartley agar from which *Enterococcus* spp. were isolated and identified.

Farm	Production stage	Proportion of samples from which enterococci were isolated	Enterococcal species isolated (number of isolates)
1	Sow	1 (10 of 10)	*E. faecium* (11); *E. durans* (5); *E. faecalis* (3)
1	Piglet	0.9 (18 of 20)	*E. faecium* (35); *E. faecalis* (5); *E. durans* (2)
1	Weaner	0.5 (5 of 10)	*E. faecium* (6); *E. faecalis* (4)
3	Weaner	0.8 (8 of 10)	*E. durans* (4); *E. gallinarum* (3); *E. faecalis* (1)
1	Grower	0.33 (3 of 9)	*E. faecium* (2); *E. faecalis* (1)
2	Finisher	1 (4 of 4)	*E. faecalis* (6); *E. faecium* (1)
3	Finisher	0.4 (4 of 10)	*E. faecalis* (2); *E. faecium* (1); *E. durans* (1) .
4	Finisher (16-18 weeks)	1 (10 of 10)	*E. faecium* (18); *E. durans* (1)
4	Finisher (20-22 weeks)	0.6 (6 of 10)	*E. faecium* (5); *E. durans* (2)

4.3.5 Enumeration and identification of coliforms

Counts were made of lactose fermenting colonies on MacConkey agar for each sample (Figure 4.4). The counts observed varied between groups but also varied

widely within groups. This can be seen from the large standard deviation in counts observed (Figure 4.4).

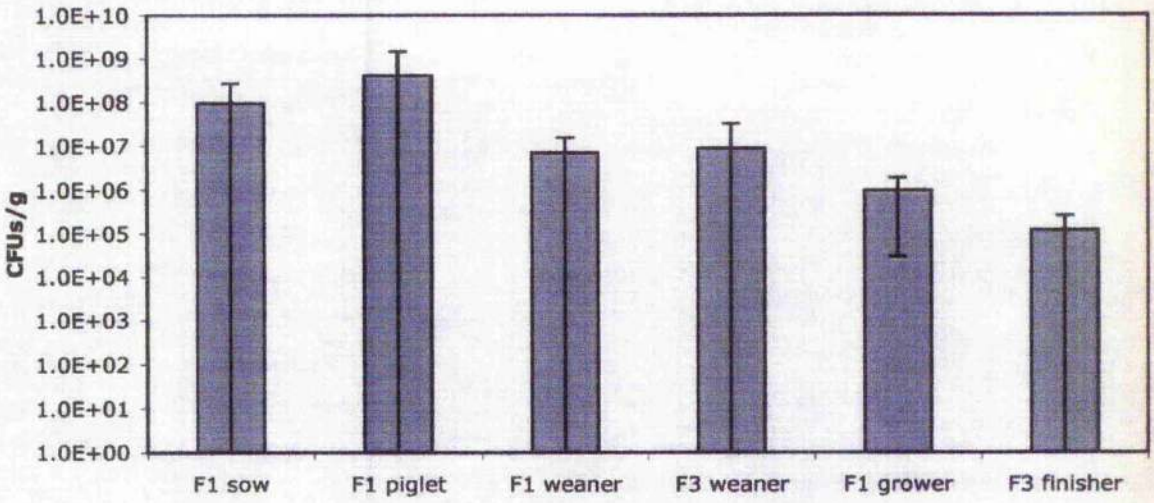


Figure 4.4 Counts of presumptive *E. coli* (lactose-fermenting colonies) in samples collected from Farms 1-3 (F1-F3) with standard deviation shown (CFUs/ml).

One colony of each morphological type (rough, smooth etc.) was then subcultured and identified to species. The vast majority of organisms were identified as *E. coli* but seven isolates from finishers and two from growers on Farm 1 were identified as *E. fergusonnii* (Table 4.6). Other organisms that were occasionally isolated included *Morganella morgani*; *Proteus vulgaris*, *Citrobacter freundii*, *Providencia rettgeri*; *Klebsiella pneumoniae* and *Serratia fonticola*.

Table 4.6 Proportion of faecal samples collected from different groups on Farms 1-3 and cultured on MacConkey agar from which *E. coli* was isolated and identified.

Farm	Production stage	Proportion of samples from which *E. coli* isolated	Other organisms isolated (number of isolates)
1	Sow	1 (10 of 10)	*C. freundii* (1); *P. vulgaris* (2)
1	Piglet	0.85 (17 of 20)	*M. morganii* (1); *P. rettgeri* (1)
1	Weaner	1 (10 of 10)	-
3	Weaner	0.9 (9 of 10)	*S. fonticola* (1); *K. pneumoniae* (1)
1	Grower	1 (10 of 10)	*E. fergusonii* (2)
1	Finisher	1 (10 of 10)	*E. fergusonii* (7)
3	Finisher	1 (10 of 10)	-

4.4 Discussion

One of the aims of these preliminary investigations was to determine the availability of faecal material for sampling on the pig farms under investigation. The findings suggest that this is highly variable and that the inconsistent nature of flooring, bedding and group sizes on pig farms means that the quantification of resistant bacteria in such samples would not be meaningful. Therefore, the results presented are not intended to allow the comparison of numbers or types of organisms present in different groups of animals, but merely to compare the different available sample material in these groups. As a relatively small volume of faeces (10-20ml) was collected and used for analysis, it is possible that a sample could represent only an individual animal, unless thorough mixing of faecal material had occurred within the pens, and if animal-to-animal variation is significant as has been suggested before (Dunlop *et al.*, 1999) this

could be misleading. The frequency of cleaning of pens may also have influenced the age of available faecal material and there was no means of determining this. All of these factors might have influenced the numbers and types of organisms present in faecal samples. Therefore, the only sensible conclusion is that such environmental sampling is suitable only for studies aimed at detecting the presence or absence of resistant bacteria rather than at quantifying them and that individual animal sampling as described elsewhere (Melin *et al.*, 1997; Jensen-Waern *et al.*, 1998) is the only suitable method for the latter. The most straightforward method of ensuring faecal samples are fresh and represent only individual animals is to collect faeces from the rectum but in the UK this procedure can only be performed under Home Office license and therefore this method is unlikely to be suitable for routine monitoring of antimicrobial resistance on farms.

Another aim was to determine the nature of the enterococcal flora and the findings suggest that a variety of enterococcal species are present in pig faeces and that different species predominated on different farms. *E. faecium* has been reported to be the most common enterococcus species present in pig faeces (Devriese *et al.*, 1994) and this was the case in samples from Farms 1 and 4 in this study. However, *E. durans* was the most frequent isolate on Farm 3 and *E. faecalis* was found more commonly in samples from Farm 2. That said, the biochemical methods used have been shown to identify correctly only 77 per cent of enterococcal strains in foods (Devriese *et al.*, 1995) and *E. faecium*, *E. durans* and *E. hirae* are very closely related (Devriese, Pot and Collins, 1993) so it is likely that some strains in this study have been misclassified. The vast majority of coliforms isolated on all farms were *Escherichia coli* and the sensitivity of isolation was much higher for this organism than for enterococci.

These preliminary studies into the isolation, enumeration and identification *Enterococcus spp.* and *E. coli* have highlighted several potential problems when the conventional bacteriological techniques described are used in antimicrobial resistance studies.

First, the use of selective media and colonial morphology as a means of determining species identity had a high specificity for *E. coli* but was of limited use for *Enterococcus spp.* Even when temperature was manipulated to inhibit the growth of other organisms and a strict morphological description of colonies was applied, many of the organisms that were presumed to be enterococci were not. Although the majority of lactose fermenting colonies on MacConkey agar were later confirmed to be *E. coli,* other enterobacterial organisms were also occasionally isolated.

Another problem with this approach was that colonial morphology is very subjective. As described, the distinction between different colours or sizes was not always clear. Therefore, growth on selective media and colony description does not appear to be a reliable method of identifying enterococci. Culture on Slanetz and Bartley agar followed by subculture of up to three colonies on aesculin-azide agar has been used in some prevalence studies to increase the specificity of selection based on colonial morphology (Danmap, 1999; Danmap, 2000; Danmap, 2001; SVARM, 2000; SVARM, 2001) but biochemical identification has still been necessary to confirm that these organisms were enterococci. Enrichment media have also been used prior to culture on Slanetz and Bartley agar to increase the sensitivity of culture (Danmap, 1999; Danmap, 2000; Danmap, 2001) but it would not be possible to enumerate enterococci in faeces using either of these methods. In contrast, faecal counts of *E. coli* have been estimated using selective media and colonial morphology alone (Humphry *et al.*, 2002) and whilst this may be justified by the greater specificity of

the media used, these counts should not be considered to be absolutely specific for this organism.

Second, estimating the numbers of bacteria present in faeces based on colony counts was also difficult. This is of course related to the specificity of the selective media used and counts of presumptive enterococci on Slanetz and Bartley agar were virtually meaningless because of the poor specificity of colonial morphology as an indicator of genus/species. However, for *Enterococcus spp.* in particular there was also significant variation in colony counts between samples from the same groups of animals and there were also occasional samples from most groups of animals from which the colonies selected were not identified as *Enterococcus spp.* or *E. coli.* This variation could represent true pen-to-pen variation, variation in sample age/treatment, variation in performance of culture media or some inherent factor in the bacteria such as tendency to clump. Whatever the reason, these findings cast doubt over the usefulness of colony counting as a means of enumerating enterococci in faecal samples. The reason for attempting morphological identification and enumeration of enterococci was in order that resistant bacteria could be related to the enterococcal population either proportionately or in numbers. These preliminary findings suggest that this is not possible using the methods described.

The identification and quantification of resistant bacteria in animal faeces using spiral plating and colony counting has been described (Dunlop *et al.*, 1998c; Humphry *et al.*, 2002). However, these methods were used to quantify resistant *E. coli* and the problems of colony identification described above would still hinder their usefulness for enterococci. A reliable method of enumerating resistant enterococci in faeces has not yet been described and from the findings of this small study, it seems important

that methods of isolating and identifying enterococci on selective media are improved if this is to be achieved.

CHAPTER 5

RESISTANCE TO AVILAMYCIN

5.1 Introduction

Having determined the limitations of conventional bacteriological techniques for the isolation, identification and quantification of *Enterococcus* spp. and *E. coli* in the faecal samples available, the next aspect to be considered was the measurement of resistance to avilamycin in these bacterial populations. Avilamycin resistance has been reported in *Enterococcus faecium* and *Enterococcus faecalis* isolated from pigs and broilers in Denmark and has been monitored annually in these organisms since 1995 (Danmap, 1997; Aarestrup *et al.*, 1998, Danmap, 1998; Danmap, 1999; Danmap, 2000; Danmap, 2001). No such systematic monitoring is carried out in the UK for resistance to avilamycin or other growth promoter.

As there is no clinical definition of resistance for antimicrobial agents used as growth promoters, breakpoints for resistance to avilamycin based on population distributions have been suggested. A bimodal distribution of avilamycin MICs in *E. faecium* has been described in studies in Denmark and Belgium with the majority of isolates in both studies having minimum inhibitory concentrations (MICs) of 2μg/ml or less whilst a small number of isolates were distinctly less susceptible (one Danish isolate having an MIC of 64μg/ml and two Belgian isolates having MICs of 32μg/ml) (Aarestrup *et al.*, 1998; Butaye, Devriese and Haesebrouck, 1999b). However, in other studies where avilamycin MICs in *E. faecium* from broilers and pigs ranged from 0.12 to greater than 256μg/ml, the strains with high MICs were simply classified as less susceptible rather than resistant because of the lack of breakpoint definitions

for susceptibility and resistance to growth-promoting antimicrobials (Butaye *et al.*, 2001). Susceptibility to avilamycin has also been determined in control strains of enterococci but primarily to assess the effect of different test conditions, (Butaye *et al.*, 1998). The relevance of avilamycin resistance to therapeutic antimicrobial resistance is that it has also been shown that avilamycin-resistant *E. faecium* and *E. faecalis* with MICs of >64μg/ml have reduced susceptibility to evernimicin (SCH 27899), an oligosaccharide antimicrobial that was at one time considered for use in human medicine but there is no evidence that cross-resistance to any currently used therapeutic antimicrobials exists (Aarestrup, 1998; Aarestrup and Jensen, 2000).

Avilamycin resistance in organisms other than enterococci has rarely been considered (Devriese *et al.*, 1993; Aarestrup, 2000a) and longitudinal monitoring of resistance in herds or flocks has not been carried out for avilamycin or other growth promoters. However, changes in the prevalence of avilamycin resistance in *E. faecium* have been monitored at national level in Denmark (Aarestrup *et al.*, 2000). Avilamycin resistance, defined as MIC greater than or equal to 16μg/ml, amongst *E. faecium* isolates tested in Denmark declined from 77.4 per cent in 1996 to 5.0 per cent in 2001in broilers, and from 1.3 per cent in 1996 to 0 per cent in 2001 in pigs, and this decline was associated with the voluntary ban of growth promoter use in 1998 with avilamycin use in farm animals in Denmark declining from 2740kg active compound to 3kg active compound per annum over the same period (Aarestrup *et al.*, 2001).

Resistance to avilamycin in *E. faecium* has also been shown to be associated with antimicrobial use on broiler farms, with farms that used avilamycin having a higher prevalence of resistant isolates (Aarestrup, Bager and Andersen, 2000). However, little is known about the persistence of, or decline in, resistance on individual farms

following the withdrawal of avilamycin either for managemental reasons or for conversion to growth promoter-free production.

Improved surveillance of antimicrobial resistance in farm animals has been called for in the UK (ACMSF, 1999; VPC, 2003) and worldwide (WHO, 2000). Commensal bacteria including enterococci, *E. coli* and *Campylobacter* species have also been identified as important indicator organisms and it has been suggested that the use of antimicrobial growth promoters should be constrained (ACMSF, 1999).

The aims of this part of the study were to determine which organisms in the faecal flora of pigs express resistance to avilamycin; to assess the best method of monitoring resistance in these organisms and, in doing so, to gain information on the epidemiology of resistance to avilamycin within farms and to relate this to patterns of avilamycin use.

5.2 Materials and methods

Two methods of assessing resistance to avilamycin were used in the study.

5.2.1 Breakpoint MICs by agar dilution

First, breakpoint minimum inhibitory concentrations (MICs) were determined for individual organisms isolated in pure culture from faecal samples by the agar dilution/plate MIC method according to NCCLS guidelines (NCCLS, 2000). However, as no such guidelines were available for *Campylobacter spp.*, the method used for these organisms was based on that recommended for *Helicobacter pylori* with a saline suspension equivalent to a 2.0 McFarland standard used without further dilution to inoculate Mueller-Hinton agar plates supplemented with horse blood, 5% v/v (Oxoid Ltd.) and plates read after incubation at 37°C in microaerobic conditions

for 48 hours (NCCLS, 2001). When each batch of isolates was tested, a set of control organisms was included to validate the testing procedure (Table 5.1).

Table 5.1 Sensitivity breakpoints to avilamycin of Gram-positive control strains as determined by agar dilution.

Control organism	Origin	MIC to avilamycin (μg/ml)
E. faecalis 29212	American Type Culture Collection (ATTC)	1
E. faecalis 202	Human hospital strain	1
E. faecalis 203	Human hospital strain	1
E. faecium 206	Human hospital strain	1
S. aureus (MRSA)	Human hospital strain	4
S. aureus 25923	ATTC	4
E. faecium 98-30223-1	Danish broiler strain	>128 *

*MIC on primary isolation as determined by Aarestrup and Jensen, 2000, >64μg/ml.

An additional set of control organisms were used when MICs were being assessed in *Enterobacteriaceae* (Table 5.2) and *Campylobacter* spp. (Table 5.3).

Table 5.2 Sensitivity breakpoints to avilamycin of Gram-negative control strains as determined by agar dilution.

Control organism	Origin	MIC to avilamycin (μg/ml)
E. coli 25922	ATTC	>128
E. coli 35218	ATTC	>128
P. aeruginosa 27853	ATTC	>128
Y. enterocolitica 9610	ATTC	64
S. enteritidis 13076	ATTC	>128

Table 5.3 Sensitivity breakpoints to avilamycin of *Campylobacter* spp. control strains as determined by agar dilution.

Control organism	Origin	MIC to avilamycin (μg/ml)
C. jejuni N82	Human inflammatory diarrhoea	8
C. jejuni O81	Human watery diarrhoea	8
C. jejuni X	Human inflammatory diarrhoea	8
C. jejuni L115	Human inflammatory diarrhoea, causes severe pathology	8
C. jejuni Gl pldA	Human Guillain Barre syndrome isolate	8

Whilst this method provided breakpoint MICs for individual isolates that could then be related to Farm or Group animal populations, it was acknowledged that only a limited number of isolates (usually one to four colonies from any one sample) could be tested in this way due to the labour intensity of isolating organisms in pure culture, identifying them and then testing them. Using this method, it was possible that resistant organisms would be missed and samples or farms misclassified and therefore, a second method of screening samples for avilamycin-resistant isolates was developed.

5.2.2 Avilamycin screening plates

This method was developed and adopted for the organisms of most interest, *i.e.*, *Enterococcus spp.*

5.2.2.1 Preparation of media

Slanetz and Bartley medium was prepared according to the manufacturer's instructions and a measured volume (249ml) poured into each of eight sterile bottles. The bottles were held at 50°C in the waterbath. An additional bottle containing 250ml Slanetz and Bartley agar was also prepared and held at 50°C in the waterbath.

Two hundred and fifty milligrams of avilamycin (potency 1286 units) was weighed and incorporated in 1.953ml acetone to give a solution with a concentration of 128mg/ml. Serial doubling dilutions of this solution were prepared in acetone to give solutions of 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25mg/ml. One millilitre of each solution from 32mg/ml to 0.25mg/ml was added to a different one of the eight bottles of 249ml molten Slanetz and Bartley agar to give bottles with concentrations of 128, 64, 32, 16, 8, 4, 2 and 1μg/ml. Each bottle was mixed thoroughly. Ten to twelve plates were poured from each of the nine bottles. Plates were tested for depth and pH, and sterility and growth controls tested (NCCLS, 2000).

5.2.2.2 Inoculation of media

Faeces were weighed and 1g of each faecal sample or 1ml of each slurry sample suspended in 9ml of phosphate-buffered saline. Serial tenfold dilutions of this suspension were then prepared using phosphate-buffered saline. Twenty microlitres of each dilution (from 1:10 to $1:10^6$) were inoculated onto the surface of each of the series of Slanetz and Bartley plates using a pipette, starting with plain Slanetz and Bartley agar followed by plates containing doubling concentrations of avilamycin from 1μg/ml to 128μg/ml, and finishing with a plate containing plain Slanetz and Bartley agar.

5.2.2.3 Incubation and reading of plates

Plates were left on the bench for not more than fifteen minutes to allow drying, before being inverted, placed in racks and transferred to an aerobic incubator where they were incubated at 37°C for two to four hours. The plates were then transferred to another aerobic incubator where they were incubated at 44°C for a further forty-four to forty-six hours. The plates were then removed from the incubator, growth and sterility controls checked, and counts made of typical large (>0.5mm diameter) maroon enterococcal colonies at each dilution of faeces on each plate. Where possible, for each sample, two typical enterococcal colonies were subcultured from plates of 32μg/ml, 64μg/ml and 128μg/ml. These isolates were subcultured to purity on 7 percent horse blood agar and identified by API as described in Chapter 2.

5.3 Results

5.3.1 Breakpoint MICs, enterococci Farm 1

Enterococci isolated from sows, piglets and weaners on Farm 1, as described in Chapter 4, were tested for susceptibility to avilamycin by the agar dilution method (Tables 5.4-5.7).

Table 5.4 Sensitivity breakpoints to avilamycin of *Enterococcus* spp. isolates from sows on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Sows	1	*E. faecium*	4
Sows	2	*E. faecium*	4
Sows	2	*E. faecium*	4
Sows	3	*E. durans*	4
Sows	4	*E. durans*	4
Sows	4	*E. faecium*	4
Sows	5	*E. durans*	4
Sows	5	*E. faecium*	4
Sows	6	*E. faecium*	4
Sows	6	*E. faecium*	4
Sows	7	*E. faecium*	4
Sows	8	*E. faecium*	4
Sows	9	*E. faecalis*	4
Sows	9	*E. durans*	4
Sows	10	*E. faecalis*	4

Table 5.5 Sensitivity breakpoints to avilamycin of *Enterococcus* spp. isolates from piglets on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Piglets	1	*E. faecium*	64
Piglets	2	*E. faecium*	8
Piglets	3	*E. faecium*	4
Piglets	4	*E. faecium*	4
Piglets	6	*E. faecium*	4
Piglets	7	*E. faecium*	4
Piglets	8	*E. faecium*	4
Piglets	9	*E. faecium*	4
Piglets	10	*E. faecalis*	4
Piglets	11	*E. faecium*	4
Piglets	12	*E. faecium*	4
Piglets	13	*E. faecalis*	4
Piglets	14	*E. faecium*	4
Piglets	15	*E. faecium*	4
Piglets	16	*E. faecium*	>128
Piglets	17	*E. faecalis*	4
Piglets	18	*E. faecium*	4
Piglets	19	*E. faecium*	4
Piglets	20	*E. faecium*	4

Table 5.6 Sensitivity breakpoints to avilamycin of *Enterococcus* spp. isolates from weaners on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Weaners	1	*E. faecalis*	>128
Weaners	2	*E. faecalis*	>128
Weaners	4	*E. faecium*	>128
Weaners	7	*E. faecium*	>128
Weaners	10	*E. faecium*	>128

Table 5.7 Sensitivity breakpoints to avilamycin of organisms isolated on Slanetz and Bartley agar from growers on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Growers	1	*L. lactis*	4
Growers	2	*L. lactis*	8
Growers	3	*L. lactis*	8
Growers	4	*S. salivarius*	32
Growers	5	*L. lactis*	4
Growers	6	*L. lactis*	4
Growers	7	*L. lactis*	2
Growers	8	*L. lactis*	8
Growers	9	*L. lactis*	4
Growers	10	*E. faecium*	>128

All 15 isolates tested from sows were found to be sensitive to avilamycin with MICs of 4μg/ml (Table 5.4). Of the 19 isolates tested from piglet samples, 17 were sensitive

to avilamycin; 16 had an MIC of 4μg/ml and one *E. faecium* isolate had an MIC of 8μg/ml (Table 5.5). Two isolates from piglets were resistant to avilamycin (MICs of 32μg/ml or greater). Both were identified as *E. faecium* and had MICs of 64μg/ml and >128μg/ml.

Three *E. faecium* and two *E. faecalis* isolates from weaners were tested and all were resistant to avilamycin (MIC >128μg/ml) (Table 5.6).

Ten organisms presumed to be enterococci before biochemical testing, that were isolated from grower samples on Slanetz and Bartley plates were also tested but only one of the organisms was identified as belonging to the genus *Enterococcus* (Table 5.7). It was identified as *E. faecium* and was resistant to avilamycin (MIC >128μg/ml). However, eight organisms identified as *Lactococcus lactis lactis* isolated from grower samples on Slanetz and Bartley medium were tested and found to be sensitive (MICs of between 2 and 8μg/ml). The single *Streptococcus salivarius* isolate was resistant (MIC 32μg/ml).

5.3.2 Breakpoint MICs, enterococci Farm 4

In total, 27 isolates of *Enterococcus* spp. from Farm 4 were tested and all were sensitive to avilamycin (MICs of 1μg/ml) (Table 5.8). The majority of these isolates were *E. faecium* but three were identified as *E. durans* and one *E. faecalis* isolate from slurry was also tested and found to be sensitive.

Table 5.8 Sensitivity breakpoints to avilamycin of *Enterococcus* spp. isolates from finishers on Farm 4 as determined by agar dilution.

Group/Origin	Sample number	Species	MIC (μg/ml)
Finishers	1	*E. faecium*	1
Finishers	1	*E. faecium*	1
Finishers	2	*E. faecium*	1
Finishers	2	*E. faecium*	1
Finishers	3	*E. faecium*	1
Finishers	3	*E. faecium*	1
Finishers	4	*E. faecium*	1
Finishers	4	*E. faecium*	1
Finishers	5	*E. faecium*	1
Finishers	5	*E. faecium*	1
Finishers	6	*E. durans*	1
Finishers	6	*E. faecium*	1
Finishers	7	*E. faecium*	1
Finishers	7	*E. faecium*	1
Finishers	8	*E. faecium*	1
Finishers	8	*E. faecium*	1
Finishers	9	*E. faecium*	1
Finishers	10	*E. faecium*	1
Finishers	10	*E. faecium*	1
Finishers	11	*E. faecium*	1
Finishers	14	*E. faecium*	1
Finishers	14	*E. faecium*	1
Finishers	15	*E. faecium*	1
Finishers	17	*E. faecium*	1
Finishers	19	*E. durans*	1
Finishers	20	*E. durans*	1
Slurry	21	*E. faecalis*	1

5.3.3 Breakpoint MICs, coliforms, Farm 1

Several gram-negative control strains including *E. coli*, *Yersinia enterocolitica*, *Salmonella enteritidis* and *Pseudomonas aeruginosa* were tested and they were all resistant to avilamycin, (Table5.2). They had MICs of greater than 128μg/ml except for the *Y. enterocolitica*, which had an MIC of 64μg/ml.

A total of 20 *E. coli* isolates from piglets and 17 *E. coli* and *E. fergusonii* isolates from finishers on Farm 1 were tested and all were found to be resistant to avilamycin (MICs >128μg/ml) (Tables 5.9 and 5.10).

All of the eight *Yersinia* spp. isolates from finishers on Farm 1 were also resistant to avilamycin (Table 5.11). Seven of these isolates had MICs of greater than 128μg/ml and one *Y. pseudotuberculosis* isolate had an MIC of 64μg/ml.

Table 5.9 Sensitivity breakpoints to avilamycin of *Escherichia* spp. isolates from piglets on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Piglets	1	*E. coli*	>128
Piglets	1	*E. coli*	>128
Piglets	2	*E. coli*	>128
Piglets	2	*E. coli*	>128
Piglets	3	*E. coli*	>128
Piglets	3	*E. coli*	>128
Piglets	3	*E. coli*	>128
Piglets	4	*E. coli*	>128
Piglets	5	*E. coli*	>128
Piglets	6	*E. coli*	>128
Piglets	6	*E. coli*	>128
Piglets	7	*E. coli*	>128
Piglets	7	*E. coli*	>128
Piglets	8	*E. coli*	>128
Piglets	8	*E. coli*	>128
Piglets	8	*E. coli*	>128
Piglets	9	*E. coli*	>128
Piglets	9	*E. coli*	>128
Piglets	10	*E. coli*	>128
Piglets	10	*E. coli*	>128

Table 5.10 Sensitivity breakpoints to avilamycin of *Escherichia* spp. isolates from finishers on Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Finishers	1	*E. coli*	>128
Finishers	1	*E. fergusonii*	>128
Finishers	2	*E. coli*	>128
Finishers	2	*E. fergusonii*	>128
Finishers	3	*E. coli*	>128
Finishers	3	*E. fergusonii*	>128
Finishers	4	*E. coli*	>128
Finishers	5	*E. fergusonii*	>128
Finishers	6	*E. coli*	>128
Finishers	7	*E. coli*	>128
Finishers	7	*E. fergusonii*	>128
Finishers	8	*E. coli*	>128
Finishers	8	*E. coli*	>128
Finishers	9	*E. coli*	>128
Finishers	9	*E. fergusonii*	>128
Finishers	10	*E. coli*	>128
Finishers	10	*E. fergusonii*	>128

Table 5.11 Sensitivity breakpoints to avilamycin of *Yersinia* spp. isolates from Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Finishers	2	*Yersinia* spp.	>128
Finishers	3	*Y. enterocolitica*	>128
Finishers	4	*Yersinia* spp.	>128
Finishers	5	*Yersinia* spp.	>128
Finishers	6	*Y. pseudotuberculosis*	64
Finishers	7	*Yersinia* spp.	>128
Finishers	9	*Yersinia* spp.	>128
Finishers	10	*Yersinia* spp.	>128

5.3.4 Breakpoint MICs, *Campylobacter* spp., Farms 1, 3 and 4

First, several control strains of *Campylobacter jejuni* isolated from humans were tested. They were all sensitive to avilamycin with MICs of 8μg/ml (Table 5.3).

Only a small number of *Campylobacter* spp. isolates were available for testing from Farm 1. Of these, the one piglet isolate tested was sensitive to avilamycin (MIC 8μg/ml) and the other six isolates, (one from sows, five from weaners), were resistant, with MICs of 64 to >128μg/ml (Table 5.12).

Table 5.12 Sensitivity breakpoints to avilamycin of *Campylobacter* spp. isolates from Farm 1 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Sows	20	*Campylobacter* spp.	>128
Piglets	16	*Campylobacter* spp.	8
Weaners	2	*C. jejuni jejuni*	>128
Weaners	6	*C. coli*	>128
Weaners	8	*C. coli*	>128
Weaners	9	*C. coli*	>128
Weaners	10	*C. jejuni jejuni*	64

On Farm 3, four *Campylobacter* spp. isolates were tested from piglets, one from weaners on the fourth day of exposure to avilamycin in feed, three from weaners on exposed to avilamycin in feed for two weeks, two from growers from which avilamycin had been withdrawn four weeks previously and one from finishers from which avilamycin had been withdrawn approximately 8 weeks previously. Of these, the piglet isolates were sensitive to avilamycin with MICs of 4 to 8μg/ml and all other isolates were resistant with MICs of >128μg/ml (Table 5.13).

On Farm 4, two *Campylobacter* spp. isolates were tested from each of two samples from finishers. The two isolates from the first sample were resistant to avilamycin, with MICs of 64μg/ml and the two isolates from the second sample were sensitive with MICs of 8μg/ml (Table 5.14).

Table 5.13 Sensitivity breakpoints to avilamycin of *Campylobacter* spp. isolates from Farm 3 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Piglets	B1	*C. coli*	8
Piglets	B2	*C. coli*	8
Piglets	B5	*C. coli*	8
Piglets	B6	*C. coli*	4
Weaners, 4 days on Maxus	A1	*C. coli*	>128
Weaners, 2 weeks on Maxus	C6	*C. coli*	>128
Weaners, 2 weeks on Maxus	C7	*C. coli*	>128
Weaners, 2 weeks on Maxus	C8	*C. coli*	>128
Growers, off Maxus 4 weeks	D3	*C. coli*	>128
Growers, off Maxus 4 weeks	D6	*C. coli*	>128
Finishers	E5	*C. coli*	>128

Table 5.14 Sensitivity breakpoints to avilamycin of *Campylobacter* spp. isolates from Farm 4 as determined by agar dilution.

Group	Sample number	Species	MIC (μg/ml)
Finishers	14	*C. coli*	64
Finishers	14	*Campylobacter* spp.	64
Finishers	20	*C. coli*	8
Finishers	20	*C. coli*	8

5.3.5 Avilamycin screening plates, Farm 1

From Farm 1, nine faecal samples collected from grower pens and six faecal samples collected from finisher pens were screened for avilamycin-resistant enterococci. Of the nine grower samples, eight were classified as resistant based on examination of the screening plates (typical maroon enterococcal colonies present on plates with avilamycin concentrations of 32μg/ml or above), and counts of typical enterococcal colonies were recorded at each concentration of avilamycin (Figure 5.1). All six of the finisher samples were classified as resistant using the same criteria (Figure 5.2).

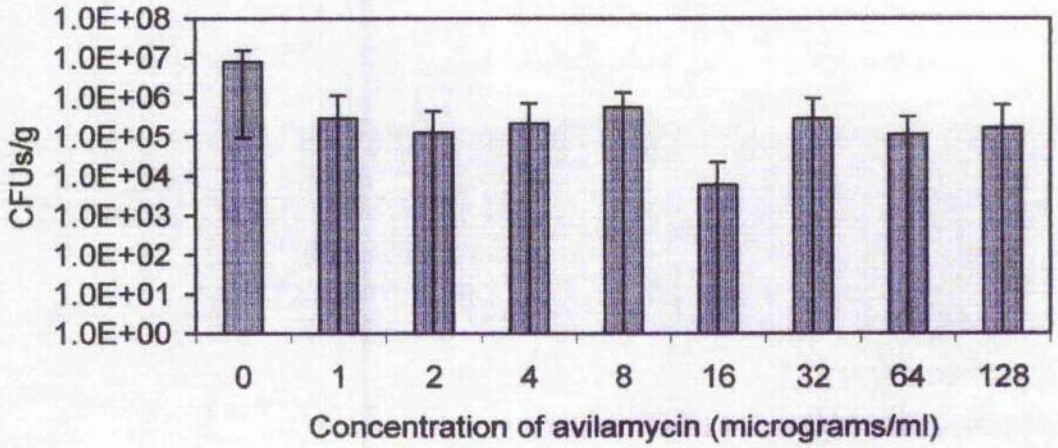


Figure 5.1 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar containing doubling concentrations of avilamycin inoculated with faeces from growers on Farm 1.

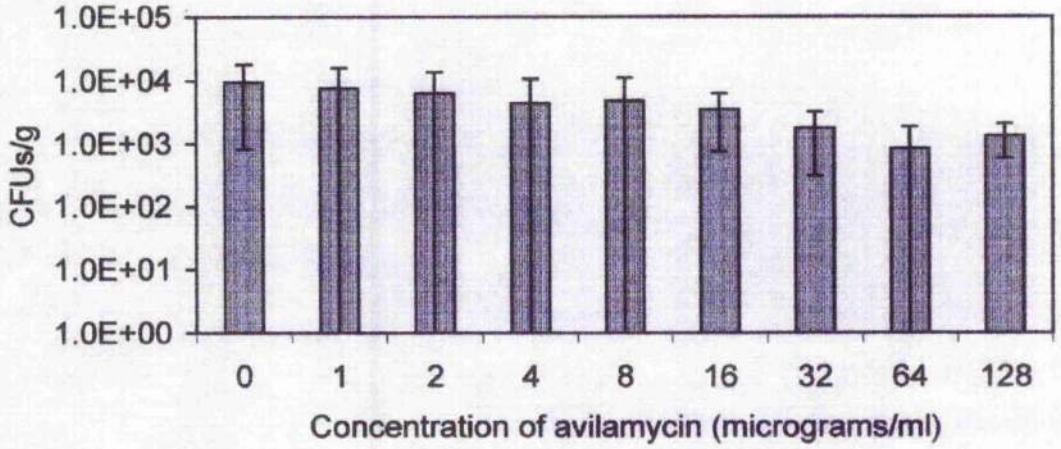


Figure 5.2 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar inoculated with faeces from finishers on Farm 1.

However, following subculture of resistant colonies, (where possible, two colonies from plates with concentration 32, 64 and 128μg/ml were subcultured for each grower sample and two colonies from plates with concentration 128μg/ml for each finisher sample), only seven of the grower samples and five of the finisher samples yielded isolates identified as enterococci (Table 5.15). Where there is no isolate identified for a sample or isolation plate, it is either because the organisms cultured from that plate and sample were not Gram-positive cocci and were therefore not identified to species, or, they failed to grow on subculture. Of the 24 avilamycin-resistant enterococcal isolates cultured from Farm 1, 15 were identified as *E. durans*, six as *E. faecium* and three as *E. faecalis*.

Table 5.15 Species identification of presumptive resistant enterococci isolated from avilamycin screening plates inoculated with faeces from Farm 1.

Group	Sample	Avilamycin concentration of isolation plate (μg/ml)	Species identification
Grower	3	32	*E. faecium*
Grower	4	64	*Leuconostoc* spp.
Grower	5	32	*E. faecalis*
Grower	6	32	*E. durans*
Grower	6	32	*E. durans*
Grower	6	64	*E. faecium*
Grower	7	128	*E. durans*
Grower	8	64	*E. durans*
Grower	9	32	*E. durans*
Grower	9	32	*E. durans*
Grower	9	64	*E. faecium*
Grower	9	128	*E. durans*
Grower	10	32	*E. faecalis*
Grower	10	32	*L. lactis*
Grower	10	64	*E. faecium*
Grower	10	64	*E.durans*
Grower	10	128	*E. faecalis*
Finisher	1	128	*E. durans*
Finisher	1	128	*E. durans*
Finisher	2	128	*E. durans*
Finisher	3	128	*E. durans*
Finisher	3	128	*E. durans*
Finisher	5	128	*E. faecium*
Finisher	5	128	*E. faecium*
Finisher	6	128	*E. durans*
Finisher	6	128	*E. durans*

5.3.6 Avilamycin screening plates, Farm 2

Four pooled faecal samples from finisher pens and two whole-farm slurry samples from Farm 2 were screened for avilamycin-resistant enterococci. Both slurry samples were classified as resistant based on the examination of screening plates, but no typical enterococcal colonies were present on finisher sample plates containing 4μg/ml of avilamycin or above (Figure 5.3).

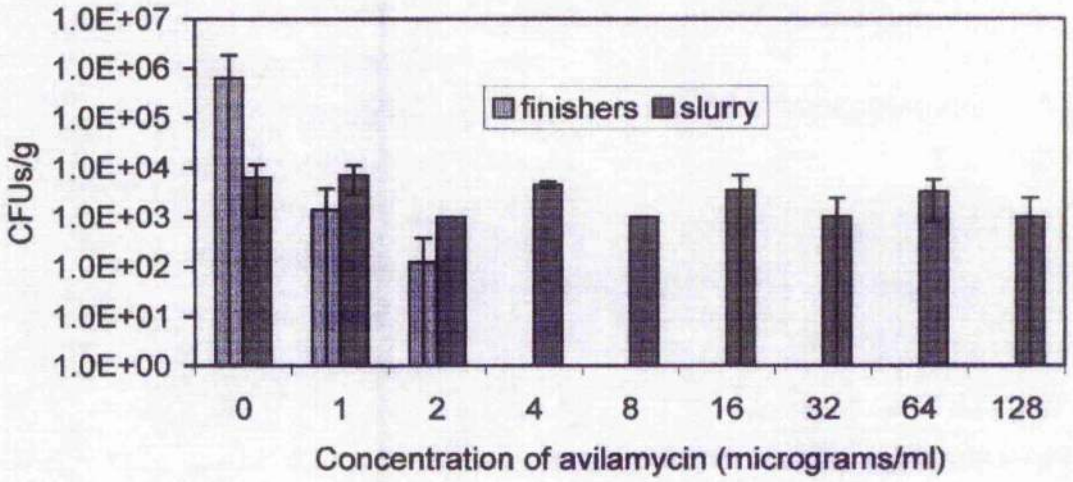


Figure 5.3 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar containing doubling concentrations of avilamycin inoculated with faeces from finishers and slurry from Farm 2.

Following subculture of resistant colonies, *E. faecium* was isolated from the first slurry sample from plates containing 32, 64 and 128μg/ml, and *E. faecium* was also isolated from the second slurry sample from a plate containing 64μg/ml.

5.3.7 Avilamycin screening plates, Farm 3

Ten faecal samples from weaner pens on Farm 3 were screened for avilamycin-resistant enterococci. Five of the ten samples were classified as resistant (presumptive enterococcal colonies on plates containing 32μg/ml of avilamycin or above) based on the examination of screening plates and counts were recorded as before (Figure 5.4).

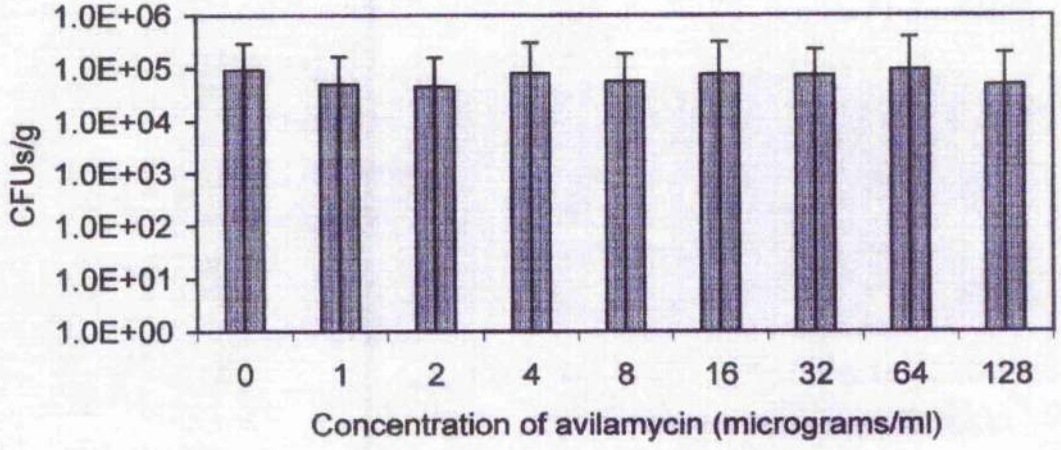


Figure 5.4 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar containing doubling concentrations of avilamycin inoculated with faeces from weaners on Farm 3.

Enterococci were cultured from all five of these resistant samples (Table 5.16). Of the eleven isolates, ten were identified as *E. durans* and one as *E. faecalis*.

Table 5.16 Species identification of presumptive resistant enterococci isolated from avilamycin screening plates, Farm 3.

Group	Sample	Avilamycin concentration of isolation plate (μg/ml)	Species identification
Weaners	2	32	*E. durans*
Weaners	2	64	*E. faecalis*
Weaners	2	128	*E. durans*
Weaners	3	32	*E. durans*
Weaners	5	32	*E. durans*
Weaners	5	64	*E. durans*
Weaners	5	128	*E. durans*
Weaners	7	32	*E. durans*
Weaners	7	64	*E. durans*
Weaners	7	128	*E. durans*
Weaners	8	32	*E. durans*
Finishers	2	64	*E. faecalis/L. lactis*
Finishers	2	128	*E. faecalis/L. lactis*
Finishers	3	32	*L. lactis*
Finishers	3	64	*E. faecalis/L. lactis*
Finishers	4	128	*E. faecalis/L. lactis*
Finishers	6	128	*E. durans*

Ten faecal samples from finisher pens on Farm 3 were also screened for avilamycin-resistant enterococci. Four samples were classified as resistant based on examination of screening plates, and counts recorded (Figure 5.5).

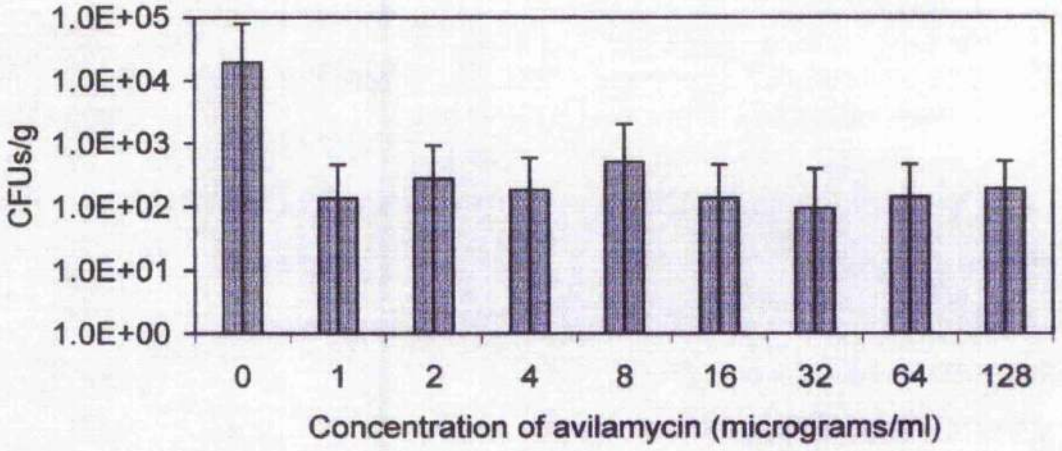


Figure 5.5 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar containing doubling concentrations of avilamycin inoculated with faeces from finishers on Farm 3.

However, when resistant colonies were subcultured, only one isolate was identified clearly by biochemical testing as belonging to the genus *Enterococcus* (Table 5.16). This organism was isolated from a plate containing 128µg/ml of avilamycin inoculated with faeces from sample number six and was identified as *E. durans*. The organisms successfully cultured from the other three samples were poorly discriminated by biochemical testing and were most likely to be *E. faecalis* or *L. lactis* isolates. Repeated biochemical testing of these isolates produced the same equivocal results.

5.3.8 Avilamycin screening plates Farm 4

Twenty faecal samples from finisher pigs and one whole-farm slurry sample from Farm 4 were screened for avilamycin-resistant enterococci. Ten of the faecal samples were collected from animals of 16 to 18 weeks of age bedded on straw and these

samples were most likely to be individual animal samples, although this could not be confirmed. The other ten faecal samples were pooled faecal samples collected from the floor of concrete pens; five were collected from pens containing animals of twenty weeks of age and five were collected from pens containing animals of twenty-two weeks of age.

Four faecal samples were classified as resistant based on the examination of screening plates and all four were samples from the sixteen to eighteen week old animals but there were no typical enterococcal colonies on plates of 16μg/ml or above inoculated with the other faecal samples or the slurry sample. Counts of typical enterococcal colonies were recorded (Figure 5.6).

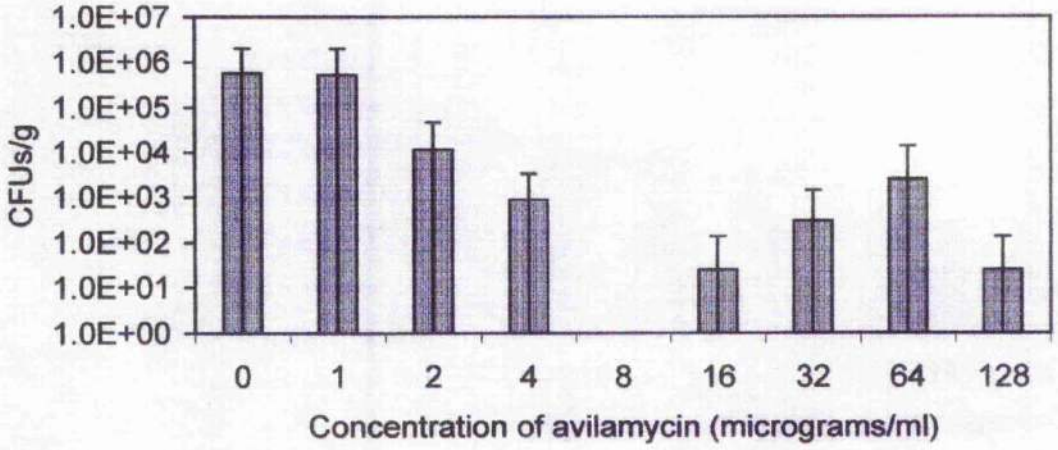


Figure 5.6 Mean counts (CFUs/g) and standard deviation of typical enterococcal colonies on Slanetz and Bartley agar containing doubling concentrations of avilamycin inoculated with faeces from finishers on Farm 4.

However, only two of the four samples from Farm 4 classified as resistant yielded enterococci when resistant colonies were subcultured and identified (Table 5.17). *E. faecium* was isolated from two samples from plates containing 32μg/ml avilamycin and also from one of these samples from a plate containing 128μg/ml of avilamycin.

Table 5.17 Species identification of presumptive resistant enterococci isolated from avilamycin screening plates inoculated with faeces from finishers on Farm 3.

Group	Sample	Avilamycin concentration of isolation plate (μg/ml)	Species identification
Finishers, 16-18wks	1	32	*E. faecium*
Finishers, 16-18wks	1	128	*E. faecium*
Finishers, 16-18wks	8	32	*E. faecium*

5.4 Discussion

From the results obtained, it is clear that whilst the majority of enterococci in pig faeces are sensitive to avilamycin, there are some strains with reduced susceptibility and this is in agreement with the findings of previous authors, (Aarestrup *et al.*, 2000; Aarestrup and Jensen, 2000; Mann *et al.*, 2001). However, there did not always appear to be a clear bimodal distribution in MICs as described in Danish and Belgian studies where individual isolates rather than the faecal population of enterococci were tested (Aarestrup *et al.*, 1998; Butaye, Devriese and Haesebrouck, 1999b). Therefore, it is difficult to say whether isolates with intermediate MICs, say 8 or 16μg/ml, should be considered to be resistant or not, although for the purposes of this study only

organisms with MICs of 32μg/ml or above were considered to be resistant based on the findings of previous authors (Aarestrup and Jensen, 2000).

On Farms 1 and 3 counts of presumptive enterococci from weaners, growers and finishers were relatively constant throughout the range of avilamycin concentrations tested (Figures 5.1, 5.2, 5.4 and 5.5) whilst the distribution of counts from finishers on Farm 4 (Figure 5.6) did appear to be bimodal with the highest counts of presumptive enterococci observed on Slanetz and Bartley agar without avilamycin and a second peak in presumptive enterococci at around 64μg/ml. On Farm 2, counts of presumptive enterococci were greatest on Slanetz and Bartley agar without avilamycin and decreased to zero by 4μg/ml (Figure 5.3) suggesting that the population was uniformally sensitive. These findings are interesting and may suggest that the distinction between sensitive and resistant populations is unclear during avilamycin use and in the period immediately following withdrawal. However, as discussed below, the poor sensitivity of Slanetz and Bartley agar and the wide variation in counts observed within groups means that these findings should be interpreted with caution, e.g., the enterococcal population on Farm 2 may not have been truly unimodal as resistant enterococci may have been missed due to the small number of samples tested.

This study has also shown that reduced susceptibility to avilamycin is present not just in *Enterococcus faecium* and *Enterococcus faecalis*, but also in *Enterococcus durans*. Indeed, when avilamycin screening plates were used, a large proportion of the resistant isolates were identified as *E. durans*. This finding is significant as *E. durans* has been associated with disease in animals (Cardona *et al.*, 1993; Cheon and Chae, 1996) and is an occasional cause of bacteraemia in humans (Watanakunakorn and Patel, 1993) and also as surveillance for antimicrobial resistance in enterococci has to

date focussed exclusively on *E. faecalis* and *E. faecium* (Danmap, 1998; Danmap, 1999; Danmap, 2000; Danmap, 2001).

The findings also suggest that resistance to avilamycin can be acquired by enterococci, either by vertical or horizontal transmission, since the enterococcal control strains isolated from humans were sensitive to avilamycin, whereas resistant strains were isolated from all of the pig farms in the study and all of these farms had at some point in the past used avilamycin for growth promotion. These findings are similar to those of authors who have described sensitive control strains and resistant isolates of *E. faecium* and *E. faecalis* from pigs and poultry (Aarestrup and Jensen, 2000).

However, all of the *Escherichia* spp. and *Yersinia* spp. isolates tested, including control strains isolated from humans, displayed high MICs to avilamycin. This strongly suggests that resistance is inherent and is not influenced by avilamycin use and this is to be expected since avilamycin is reported to have a relatively narrow spectrum of activity, being active mainly against Gram-positive organisms (Aarestrup, 2000a). However, an *E. coli* strain susceptible to the related antimicrobial, evernimicin has been described, (McNicholas *et al.*, 2000).

A range of sensitivity to avilamycin was found amongst *Campylobacter* spp. isolates. Control strains of *C. jejuni* isolated from humans were sensitive whereas many isolates from test samples were resistant. This suggests that avilamycin resistance may also be acquired by *Campylobacter* spp. and although there is no published information in the literature on avilamycin resistance in *Campylobacter spp.*, the findings of a small study using an oral model of colonisation in chickens also suggested that resistance in *C. jejuni* can be induced by exposure to avilamycin (Stapleton *et al.*, 2002).

The pattern of resistant isolates on Farms 1 and 3 also suggests that resistance to avilamycin is acquired by *Campylobacter* spp. following the use of avilamycin in feed. All of the isolates from weaners, growers and finishers on Farms 1 and 3 were resistant to avilamycin whereas piglet isolates were sensitive. The isolation of an avilamycin-resistant *C. coli* from animals that received rations medicated with avilamycin for four days suggests that resistant organisms are quickly acquired or if they are present at a low prevalence initially, quickly proliferate to an easily detectable prevalence. However, the results suggest that avilamycin resistance may not be so quickly lost, as both an isolate from sows on Farm 1 and an isolate from finishers on Farm 4 were also resistant. The sows on Farm 1 were not receiving any avilamycin at the time of sampling but may have done previously, and none of the animals on Farm 4 had ever received avilamycin but it had been used routinely on that farm up until two years previously. This suggests that avilamycin-resistance in *Campylobacter* spp. has either persisted on the farm or been re-introduced following withdrawal. However, the small numbers of isolates tested means that these findings should be interpreted cautiously.

Several studies have described a higher prevalence of resistance to other antimicrobials including erythromycin and ciprofloxacin in isolates of *C. coli* from pigs and meat than in *C. jejuni* isolates (van Looveren *et al.*, 2001; Ge *et al.*, 2003; Pezzotti *et al.*, 2003) and as the majority of the isolates tested in this study were *C. coli* it is possible that there is also a higher prevalence of resistance to avilamycin in this species. However, the numbers of *Campylobacter* spp. isolates tested were very small and without further testing it is not possible to confirm the suggestions made about the epidemiology of avilamycin resistance in *Campylobacter spp.*. Nonetheless,

the results suggest that further study of avilamycin resistance in *Campylobacter* spp. is warranted.

The determination of individual isolate MICs combined with the use of avilamycin screening plates were useful in describing avilamycin-resistance in *Enterococcus* spp. on the farms tested. Based on the testing of enterococci isolated from non-selective medium, the presence of avilamycin resistance was confirmed on Farm 1 in piglets (two of 19 isolates resistant), weaners (all 5 isolates resistant) and growers (1 isolate resistant). Using the same method, 27 isolates tested from Farm 4 were all sensitive to avilamycin, suggesting that resistance had disappeared following the withdrawal of avilamycin two years previously and this is in common with the findings in Denmark where the same methodology has been used to describe the decline in avilamycin resistance following its withdrawal on a national basis (Aarestrup *et al.*, 2001).

When avilamycin screening plates were used, avilamycin resistance was confirmed in grower and finisher samples from Farm 1. However, using the same samples from Farm 4 from which the sensitive enterococcal isolates were cultured, 4 of the 20 samples appeared to contain avilamycin-resistant enterococci. The presence of avilamycin-resistant enterococci was confirmed on culture from 2 of these 4 samples. This suggests that avilamycin-resistant enterococci have persisted or been reintroduced on Farm 4 following the withdrawal of avilamycin. Avilamycin-resistant *E. faecium* have been isolated from avilamycin-free broiler farms before but it was not known whether or when avilamycin had been used previously on these farms (Aarestrup, Bager and Andersen, 2000).

However, avilamycin-resistance was not detected even when 27 isolates from Farm 4 were tested by plate MIC. This suggests that the prevalence of avilamycin resistance amongst enterococci is low on Farm 4 and that sample numbers of this order are

insufficient to detect any resistant bacteria. This, in turn, suggests that using individual isolate MICs to determine whether or not resistance is present in a group of animals or a bacterial population is poorly sensitive and this finding is significant given that this is the method currently most commonly used for monitoring antimicrobial resistance in both pathogens and commensal organisms (SVARM, 2000; Danmap, 2001; Humphry *et al.*, 2002)

On Farm 2, the four faecal samples tested did not appear to contain avilamycin-resistant enterococci and yet resistance was confirmed in two slurry samples. This suggests that either resistance is present on Farm 2 but is lost following withdrawal of avilamycin at the end of the weaner stage, or, resistant organisms were not detected because of the low prevalence of resistance and small number of samples tested.

The screening plates also indicated that resistance was present on Farm 3 in both weaner and finisher samples, suggesting that resistance has persisted in animals on Farm 3 following the withdrawal of avilamycin at the end of the weaning stage. However, only one resistant isolate from finisher samples was confirmed as belonging to the genus *Enterococcus*. The decline of resistance to avilamycin following its withdrawal has not been studied before except at national level where results suggest that the prevalence of resistance has reduced following withdrawal (Aarestrup *et al.*, 2000; Aarestrup, Bager and Andersen, 2000). However, Aarestrup *et al.*'s findings are based on the examination of a relatively small number of isolates (one per animal sampled) and a small proportion of the animal population and as the findings above suggest, this could be misleading.

Throughout the study when the screening plate method was used, regardless of the origin of the sample, some samples were classified as resistant but the presence of resistant enterococci could not be confirmed. This was because some of the organisms

presumed to be resistant enterococci on the screening plates did not belong to the genus *Enterococcus*. This suggests that because of the subjectivity of identifying enterococcal colonies on Slanetz and Bartley agar, the screening plate method of detecting resistance is of high sensitivity but poor specificity. This should be borne in mind when interpreting the results of screening plates, particularly if the presence of resistant enterococci is not confirmed by biochemical or molecular testing. The use of Slanetz and Bartley medium for the isolation of enterococci from food and water is described elsewhere and has been found to be a sensitive method which compares favourably with other media for enterococci (Butaye, Devriese and Haesebrouck, 1999a and 1999b). However, it has also been acknowledged that further testing is required to confirm the identity of isolates (Niemi and Ahtiainen, 1995) and this is supported by the findings in this study. These findings also demonstrate the importance of accurate speciation of resistant enterococci in antimicrobial resistance studies.

Another potential problem of using this method is the possibility that by inoculating sample material onto successive concentrations of antimicrobial, resistance could somehow have been induced. However, as culture on plates containing antimicrobial was not repeated and as care was taken not to allow pipette tips to come into contact with the plate surface, this seems unlikely to have been significant.

Finally, although counts of organisms were made at each dilution, the variability of these counts, as discussed in Chapter 4, and the poor specificity of Slanetz and Bartley agar, reduced their usefulness as a measure of either the numbers of resistant enterococci present, or, of the proportion of enterococci expressing resistance. For example, in many cases typical enterococcal colonies were absent at low concentrations of avilamycin (as shown by a standard deviation greater or equal to the

mean count of presumptive enterococci) and yet present at higher concentrations inoculated with the same sample, suggesting that there is variation in the performance of the isolation technique used or that enterococci were not uniformly distributed in the sample material following thorough mixing. This made the proportional relationship between avilamycin-resistant enterococci and the enterococcal population as a whole difficult to assess and therefore the method was viewed as a means of determining whether or not resistant enterococci were present rather than as a quantitative method. Nonetheless, the use of avilamycin screening plates has been shown to be a useful method of determining the presence or absence of avilamycin-resistant enterococci in faecal and slurry samples with a higher sensitivity than determining MICs for a small number of individual enterococcal isolates. Whilst carrying out MIC tests on individual isolates may be the only way of quantifying the extent to which an individual organism is resistant, this method has a low sensitivity when used on a limited number of individual isolates from a sample and the findings suggest that this is of particular importance when the prevalence of resistance is low. This should be borne in mind when resistance in commensal organisms is being monitored because the commensal bacterial population may be more heterogenous than a rapidly expanding clonal population of pathogenic organisms and the prevalence of resistant organisms may therefore be low (Humphry *et al.*, 2002). The poor sensitivity of this method also has implications for the longitudinal monitoring of resistance following the withdrawal of a growth promoter, as it seems likely that low numbers of resistant organisms would be missed.

CHAPTER 6

RESISTANCE TO THERAPEUTIC ANTIMICROBIALS

6.1 Introduction

Another aspect of resistance monitoring in relation to antimicrobial growth promoters is consideration of the relationship between their use and resistance to therapeutic antimicrobials. Since the late 1960s, there has been concern surrounding the use of antimicrobials as growth promoters because of the possibility that their use may contribute to resistance to antimicrobials used for therapy in animals and humans (Swann, 1969). For example, evidence suggesting that the use of the growth promoter avoparcin contributed to the pool of vancomycin-resistant enterococci in humans (Stobberingh *et al.*, 1999; Bonten *et al.*, 2001) lead to the banning of avoparcin in the EU in April 1997 and it has since been shown that the prevalence of glycopeptide resistance in *E. faecium* isolated from broilers in Denmark has declined (Bager *et al.*, 1999).

A relationship between growth promoter use and resistance to a related therapeutic antimicrobial has also been suggested for avilamycin and evernimicin (Aarestrup, 1998; Aarestrup and Jensen, 2000), tylosin and erythromycin (Aarestrup and Cartensen, 1998) and virginiamycin and quinupristin-dalfopristin (Welton *et al.*, 1998). Of these, avilamycin is the only growth promoter still licensed for use within the EU. Cross-resistance to evernimicin, an antimicrobial considered for clinical use against Gram-positive infections in humans, has been demonstrated (Aarestrup and Jensen, 2000) but the development of evernimicin for clinical use has been suspended for other reasons.

It is also possible for growth promoter use to co-select for resistance to unrelated antimicrobials: It has been suggested that the prevalence of glycopeptide resistance amongst *E. faecium* in pigs in Denmark did not decline significantly until tylosin use decreased because genes encoding resistance to glycopeptides and macrolides were located close together on the same plasmid (Aarestrup, Bager and Andersen, 2000; Aarestrup *et al.*, 2001). This suggests that the influence of growth promoter use on resistance to therapeutic antimicrobials is not restricted to structurally related compounds and whilst cross resistance between avilamycin and evernimicin has been demonstrated, the effect of avilamycin use on resistance to other therapeutic antimicrobials has not been considered.

The aim of this part of the study was to carry out farm studies on antimicrobial resistance in commensal organisms, primarily *E. coli* and enterococci but also *Yersinia* spp., and to see if the use of avilamycin on farms had any influence on patterns of resistance to therapeutic antimicrobials.

6.2 Materials and Methods

6.2.1 Selection of Bacterial Isolates for Testing

The organisms selected for susceptibility testing were isolated in pure culture as described in Chapter 2 and identified to species using the API system (Biomerieux). Attempts were made to isolate enterococci from every sample taken from each of the four study farms and to isolate *Escherichia* spp. from samples on Farms 1, 2 and 3 but these were not always successful due to the poor sensitivity and specificity of the isolation techniques used as discussed in Chapter 5.

6.2.2 Susceptibility Testing Methodology

The method used for determining susceptibility to therapeutic antimicrobials was disc diffusion and the testing was carried out following NCCLS guidelines (NCCLS, 1999), using discs containing known amounts of antimicrobial (Oxoid Ltd., Basingstoke). Zones of inhibition were measured and recorded in millimetres and this information used to classify organisms as sensitive, resistant or intermediately resistant (NCCLS, 1999; NCCLS, 2000a). Control organisms, (*S. aureus* ATTC 25923, *E. coli* ATTC 25922, *E. coli* ATTC 35218 and *P. aeruginosa* ATTC 27853) were tested daily for 30 days initially and then weekly to validate the testing procedure. Where there was any doubt about the integrity of the testing procedure or the interpretation of results, the isolate was re-tested. Similarly, where individual colonies were present within a zone of inhibition, these were subcultured and re-tested.

Results were described in terms of prevalence of resistance amongst isolates and prevalence of resistance amongst samples. In some cases, more than one isolate of the same species was cultured from a sample and conversely, in some cases, culture did not yield the organism of interest.

Although zone size measurements were interpreted as indicating sensitive, intermediately resistant or resistant, for the purpose of analysis, all intermediately resistant and resistant isolates were grouped together as resistant.

6.3 Results

6.3.1 Enterococci, Farm1

Enterococcal isolates were grouped according to their origin on the farm. The number of organisms tested for susceptibility to each antimicrobial is shown in Table 6.1.

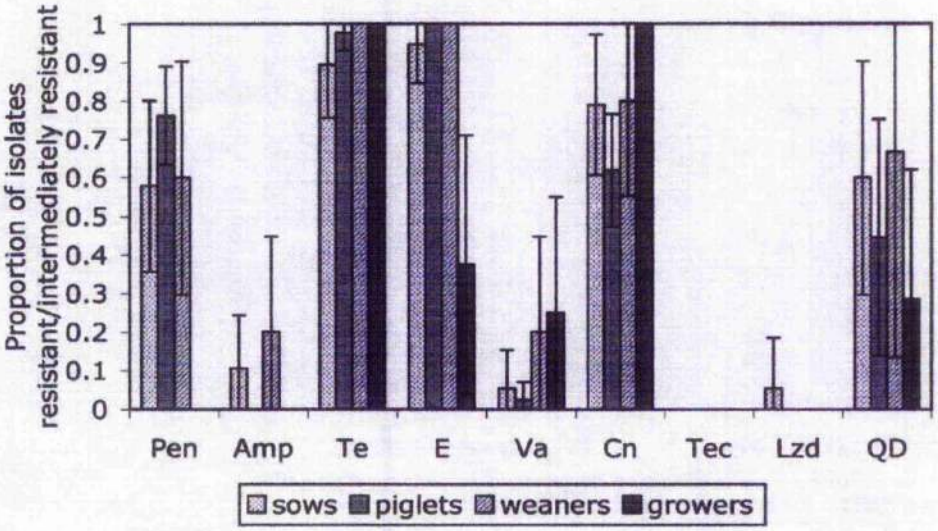
Only a selection of isolates were tested for resistance to teicoplanin, linezolid and quinupristin/dalfopristin.

Table 6.1 Number of samples taken and enterococcal isolates tested from different production stages, Farm 1.

Production stage	Sows	Piglets	Weaners	Growers
Number of samples taken	10	20	10	10
Proportion of samples from which enterococci were isolated	1	0.9	0.5	0.8
Number of isolates tested for susceptibility to Tec, Lzd and QD	11	10	4	8
Number of isolates tested for susceptibility to all other antimicrobials	19	42	10	8

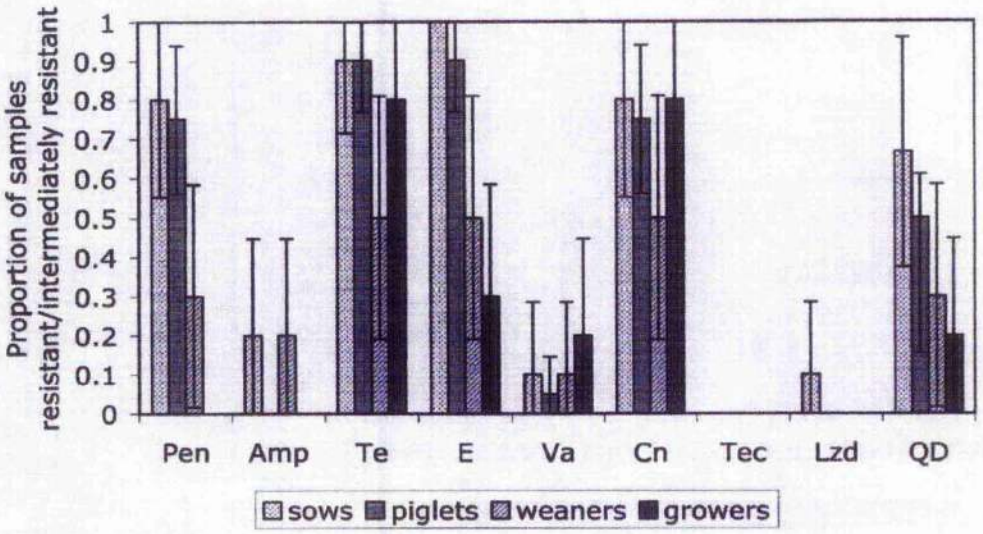
KEY: Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Resistance was considered within several different population levels: The number of resistant/intermediately resistant enterococcal isolates as a proportion of the total number of enterococcal isolates (Figure 6.1); the number of samples from which resistant/intermediately resistant enterococci were isolated as a proportion of the total number of samples (Figure 6.2); and the number samples from which resistant/intermediately resistant enterococci were isolated as a proportion of the number of samples from which enterococci were isolated (Figure 6.3).



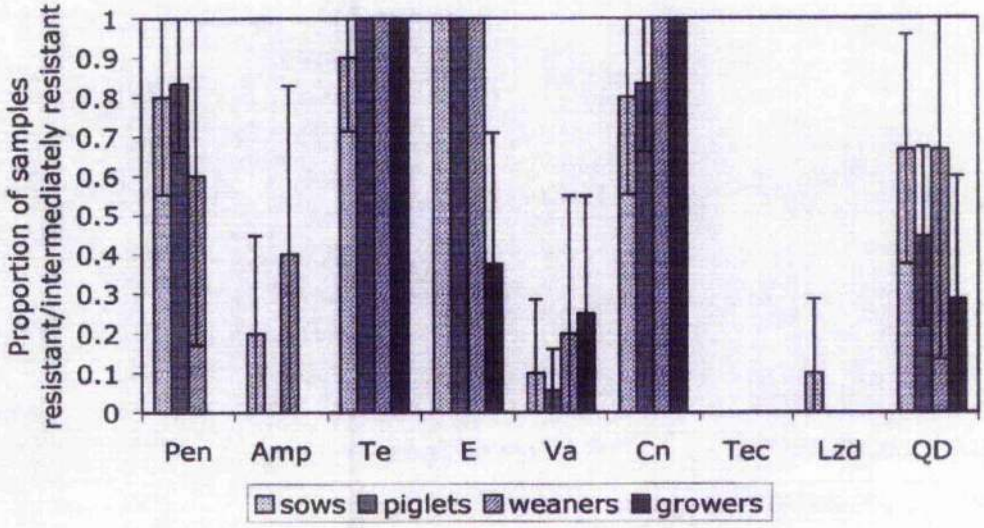
KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.1 Bar chart of prevalence of resistance/intermediate resistance amongst enterococcal isolates from Farm 1. 95% confidence intervals are shown.



KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.2 Bar chart of the proportion of total samples from which resistant/intermediately resistant enterococci were isolated from Farm 1. 95% confidence intervals are shown.



KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.3 Bar chart of the proportion of samples from which enterococci were isolated from Farm 1 from which at least one isolate tested resistant/intermediately resistant. 95% confidence intervals are shown.

As some samples failed to yield any enterococcal isolates, the prevalence of resistance amongst the total sample population (Figure 6.2) was generally lower than the prevalence of resistance amongst samples that yielded enterococci (Figure 6.3) or the prevalence of resistance amongst enterococcal isolates (Figure 6.1).

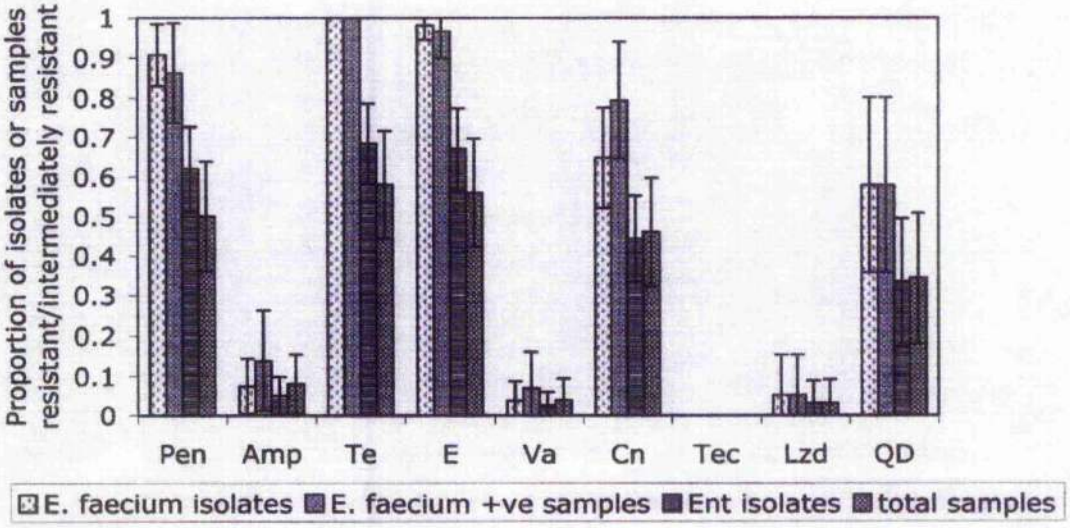
In general, high prevalences of resistance to penicillin, tetracycline, erythromycin and quinupristin-dalfopristin were observed throughout the farm, although no penicillin-resistant isolates were cultured from the samples taken from growers. Confidence intervals around the estimates of quinupristin-dalfopristin resistance are particularly large because *E. faecalis* is intrinsically resistant to this compound and all such isolates were excluded.

A high proportion of isolates were resistant to gentamicin. However, these isolates were subsequently tested for high-level gentamicin resistance and found to be sensitive, indicating that only low-level gentamicin resistance was prevalent.

Six isolates (four *E. faecalis* and two *E. faecium*) were intermediately-resistant to vancomycin but none of the isolates were classified as resistant by the test. Similarly, although intermediate-resistance to quinupristin-dalfopristin was common (sixteen isolates), a smaller number of isolates, (nine), were classified as resistant by the test.

None of the isolates from Farm 1 were resistant to teicoplanin and all the organisms isolated from the growers were also sensitive to penicillin and ampicillin.

Results were also expressed in relation to *E. faecium* for the whole farm (Figure 6.4).



KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.4 Bar chart of the prevalence of resistance/intermediate resistance amongst *E. faecium* isolates and *Enterococcus* spp. isolates, and number of samples from which at least one *E. faecium* isolate tested resistant/intermediately resistant as a proportion of *E. faecium*-positive samples and total samples for Farm 1.

For all of the antimicrobials tested, the prevalence of resistance amongst *E. faecium* was higher than amongst the total number of isolates of all enterococcal species. Resistance was not expressed in relation to other individual enterococcal species due to the small number of isolates.

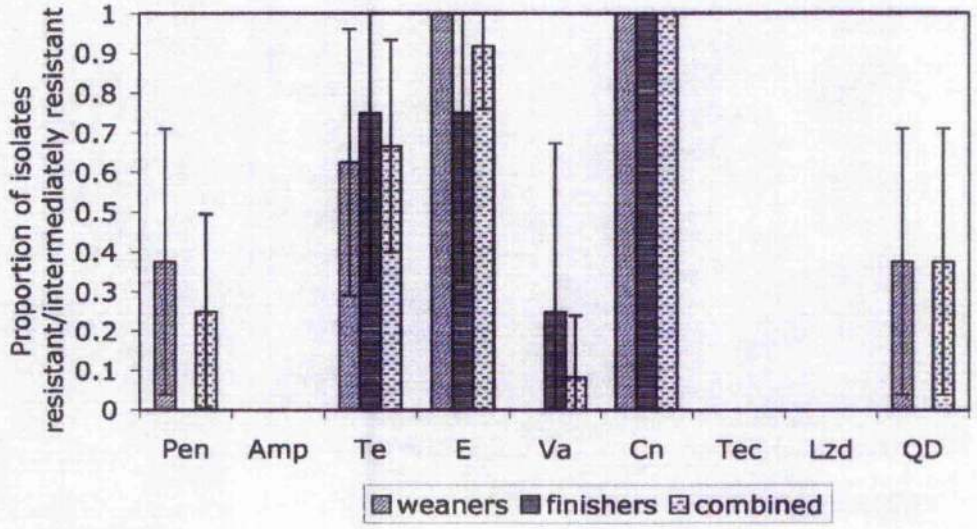
6.3.2 Enterococci, Farm 2

Twelve enterococcal isolates were cultured from two faecal samples from finisher pens and one whole-farm slurry sample. Seven *E. faecalis* and one *E. faecium* were isolated from the faecal samples and two *E. faecium*, one *E. faecalis* and one *E. durans* were isolated from the slurry. All twelve isolates were sensitive to penicillin, ampicillin and vancomycin, and the four isolates tested were susceptible to teicoplanin and linezolid. All twelve isolates exhibited low-level gentamicin resistance, four were resistant to erythromycin and the only isolate sensitive to tetracycline was an *E. durans* isolated from slurry.

6.3.3 Enterococci Farm 3

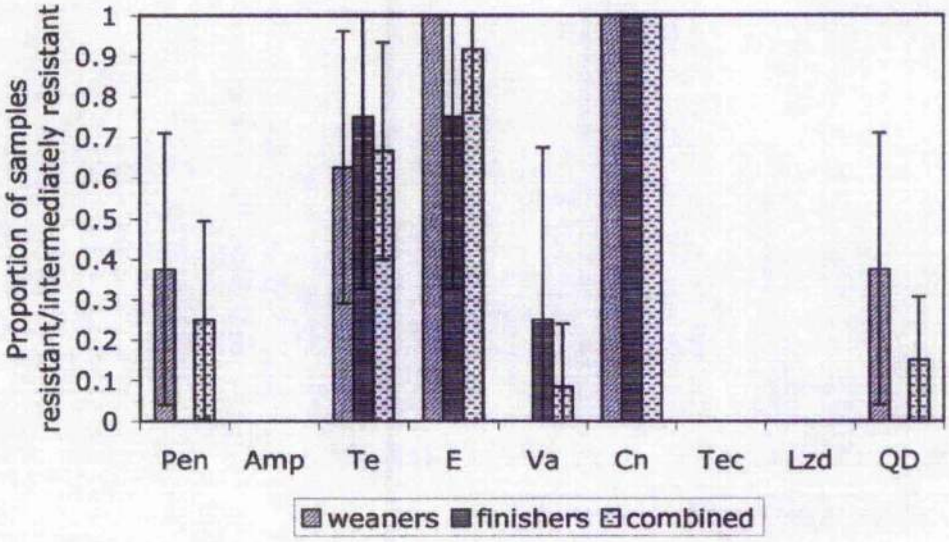
Enterococci were cultured from eight of ten samples taken from weaner pens, of which four isolates were identified as *E. durans*, three as *E. gallinarum* and one as *E. faecalis*. Of the ten samples taken from finisher pens, only four yielded enterococci, of which two were identified as *E. faecalis*, one as *E. durans* and one as *E. faecium*. Due to the small number of isolates, the prevalence of resistance is described for the total isolates and samples from both groups as well as on a group basis, (Figures 6.5 and 6.6). All of the enterococcal isolates from Farm 3 were sensitive to ampicillin, teicoplanin and linezolid and the four isolates from the finisher samples were also

sensitive to penicillin. An *E. faecalis* isolated from the finisher samples was classified as intermediately resistant to vancomycin and three *E. durans* isolates from weaner samples were intermediately resistant to quinupristin-dalfopristin. There was a high prevalence of resistance to tetracycline, erythromycin and low-level gentamicin amongst isolates and samples from Farm 3.



KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.5 Bar chart of the prevalence of resistance/intermediate resistance amongst enterococcal isolates from Farm 3. 95% confidence intervals are shown.



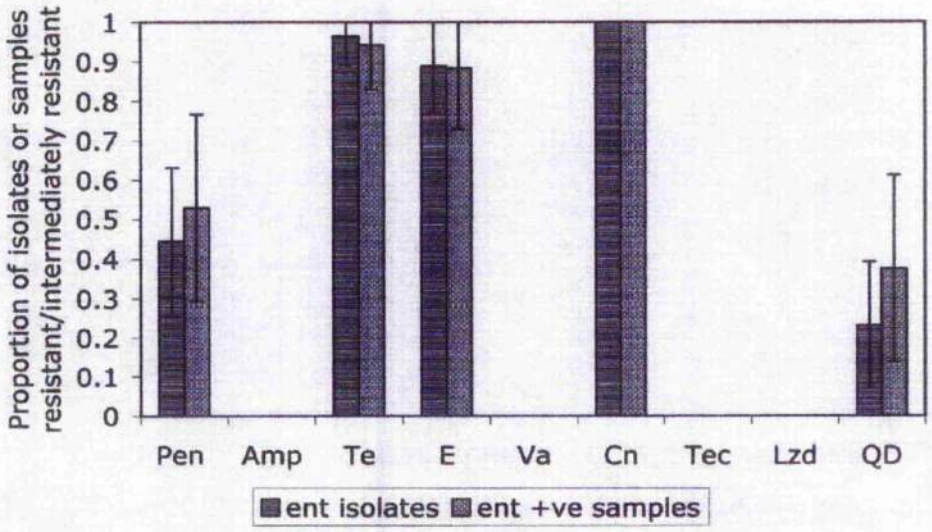
KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.6 Bar chart of the proportion of samples from which enterococci were isolated from Farm 3 from which at least one isolate tested resistant/intermediately resistant. 95% confidence intervals are shown.

6.3.4 Enterococci, Farm 4

Ten *E. faecium* were isolated from five samples taken from pens containing gilts aged 16-18wks of age and nine *E. faecium* and one *E. durans* were isolated from five samples taken from pens of castrates aged 16-18 weeks. All of the samples yielded at least one enterococcal isolate. Of ten faecal samples taken from pens containing a mixture of gilts and castrates aged 20-22weeks of age, enterococci were cultured from only six. Five *E. faecium* isolates and two *E. durans* isolates were cultured in total from these two groups and an *E. faecalis* was isolated from a whole-farm slurry sample. Due to the low microbiological sensitivity of isolation in the samples taken from the pens of older animals and the proximity of all four groups in terms of

production stage, all isolates and samples from Farm 4 were considered together (Figure 6.7).



KEY: Pen = penicillin; Amp = ampicillin; Te = tetracycline; E = erythromycin; Va = vancomycin; Cn = gentamicin; Tec = teicoplanin; Lzd = linezolid; QD = quinupristin-dalfopristin.

Figure 6.7 Bar chart of the prevalence of resistance/intermediate resistance amongst enterococcal isolates and of the proportion of samples from which enterococci were isolated from which at least one isolate tested resistant/intermediately for Farm 4. 95% confidence intervals are shown.

Of the twenty-seven isolates tested from Farm 4, all were sensitive to ampicillin, vancomycin, teicoplanin and linezolid and all were resistant to low-level gentamicin. There was a high prevalence of resistance to tetracycline and twenty-four isolates were resistant or intermediately resistant to erythromycin. Twelve isolates were resistant to penicillin and 53 percent of samples from which enterococci were isolated yielded at least one resistant isolate. Two isolates were resistant to quinupristin-dalfopristin and four were intermediately resistant.

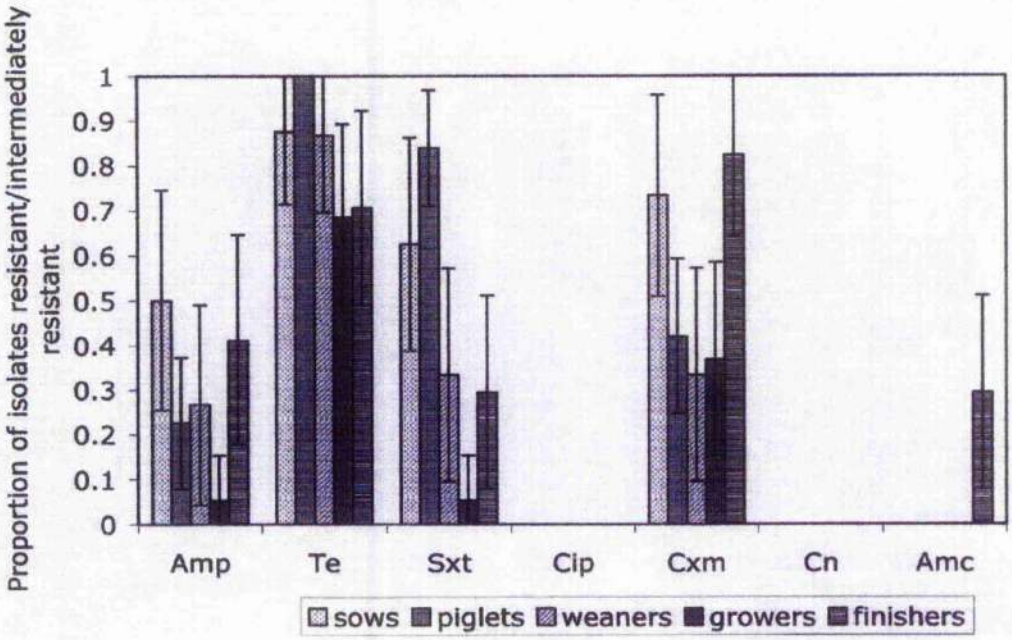
6.3.5 Coliforms Farm 1

The sensitivity of isolation of *Escherichia spp.* was much higher than for *Enterococcus* spp. with the vast majority of samples yielding at least one isolate (Table 6.2).

Table 6.2 Number of samples taken and *Escherichia* spp. isolates tested from Farm 1.

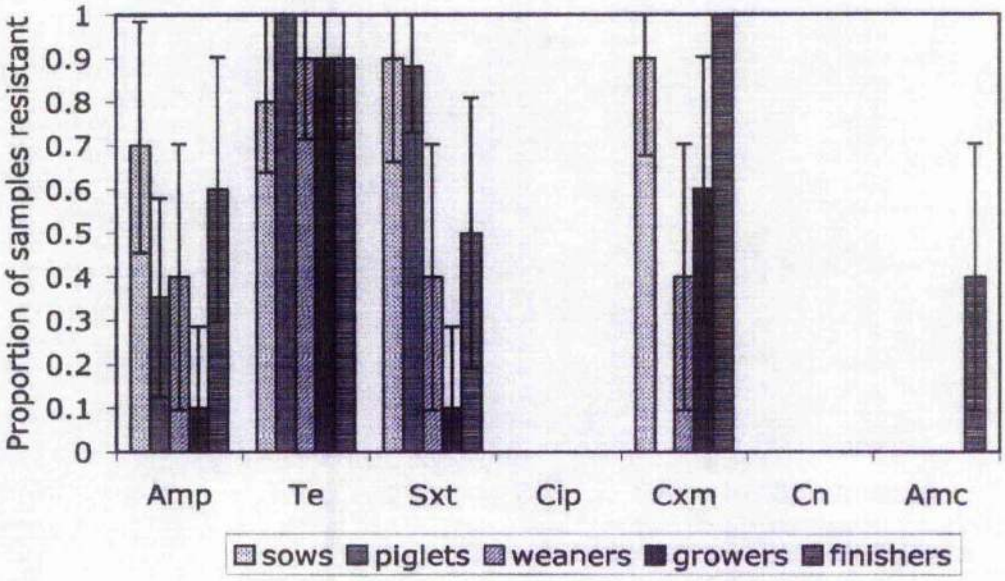
Production stage	Sows	Piglets	Weaners	Growers	Finishers
Number of samples taken	10	20	10	10	10
Proportion of samples from which *Escherichia* spp. was isolated	1	0.9	1	1	1
Number of *Escherischia* spp. isolates tested	16	33	15	19	17

Results are described in terms of the number of resistant/intermediately resistant *Escherichia* spp. isolates as a proportion of the total number of *Escherichia* spp. isolates (Figure 6.8) and the number samples from which resistant/intermediately resistant *Escherichia* spp. were isolated as a proportion of the number of samples from which *Escherichia* spp. were isolated (Figure 6.9).



KEY: Amp = ampicillin; Te = tetracycline; Sxt = trimethoprim-sulphamethoxazole; Cip = ciprofloxacin; Cxm = cefuroxime; Cn = gentamicin; Amc = amoxicillin-clavulanic acid.

Figure 6.8 Bar chart of the prevalence of resistance amongst *Escherichia* spp. isolates from Farm 1. 95% confidence intervals are shown.



KEY: Amp = ampicillin; Te = tetracycline; Sxt = trimethoprim-sulphamethoxazole; Cip = ciprofloxacin; Cxm = cefuroxime; Cn = gentamicin; Amc = amoxicillin-clavulanic acid.

Figure 6.9 Bar chart of the proportion of samples from which *Escherichia spp.* were isolated from Farm 1 from which at least one isolate tested resistant. 95% confidence intervals are shown.

In general, there was a high prevalence of resistance to tetracycline, trimethoprim-sulphamethoxazole and cefuroxime amongst *Escherichia* spp. isolates from Farm 1 and the prevalence of resistant isolates from grower samples was lower than most other groups for most antimicrobials. In particular, there was a higher prevalence of resistance to tetracycline and trimethoprim-sulphamethoxazole amongst isolates from piglets and a higher prevalence of resistance to cefuroxime amongst isolates from finishers, than amongst isolates from other production stages. All the organisms tested were sensitive to gentamicin and ciprofloxacin and only five isolates from finishers were resistant to amoxicillin-clavulanic acid.

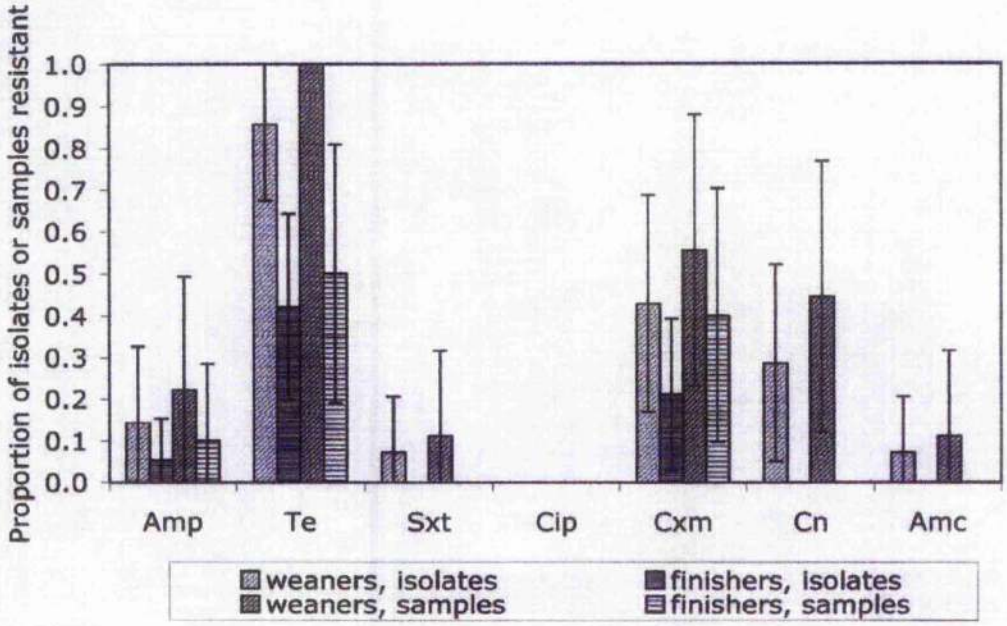
When considered at the sample level (Figure 6.9) the prevalence of resistance to the antimicrobials tested was generally higher than when considered at isolate level (Figure 6.8). For example, 50 per cent of *Escherichia* spp. isolates from sow samples were resistant to ampicillin but at least one resistant organism was isolated from 70 per cent of samples.

6.3.6 Coliforms Farm 2

Twelve *E. coli* were isolated from four finisher samples and two whole-farm slurry samples on Farm 2 and all were sensitive to ampicillin, ciprofloxacin, gentamicin and amoxicillin-clavulanic acid. Eight of the twelve isolates were resistant to tetracycline, two isolates from two finisher samples were resistant to cefuroxime and one isolate from slurry was resistant to trimethoprim-sulphamethoxazole.

6.3.7 Coliforms Farm 3

Nineteen *E. coli* isolated from ten samples from finisher pens and fourteen *E. coli* isolated from ten samples from weaner pens were tested (Figure 6.10).



KEY: Amp = ampicillin; Te = tetracycline; Sxt = trimethoprim-sulphamethoxazole; Cip = ciprofloxacin; Cxm = cefuroxime; Cn = gentamicin; Amc = amoxicillin-clavulanic acid.

Figure 6.10 Bar chart of the prevalence of resistance amongst *Escherichia* spp. isolates and the proportion of samples from which *Escherichia spp.* were isolated from which at least one isolate tested resistant for Farm 3. 95% confidence intervals are shown.

In general, the prevalence of resistance was higher amongst isolates and samples from weaners than from finishers, and resistance to trimethoprim-sulphamethoxazole, gentamicin and amoxicillin-clavulanic acid was only found in weaner samples. There was a very high prevalence of resistance to tetracycline amongst weaner isolates with twelve out of fourteen isolates resistant and at least one resistant organism isolated from every sample. All the isolates tested from Farm 3 were sensitive to ciprofloxacin.

6.3.8 Yersinia spp.

Eight isolates of *Yersinia* spp. from finisher samples on Farm 1 were tested for susceptibility to the same panel of antimicrobials as *Escherichia* spp. isolates and three of these isolates (two *Y. enterocolitica* and one *Yersinia* spp.) were resistant to ampicillin but all eight isolates were sensitive to all other antimicrobials tested. Three of four *Yersinia* spp. isolates from finisher and slurry samples from Farm 2 were resistant to ampicillin and all four isolates were sensitive to the other antimicrobials tested. One *Yersinia enterocolitica* was isolated from a finisher sample from Farm 3 and it was resistant to ampicillin but sensitive to all other antimicrobials tested.

6.3.9 Comparisons Between Groups and Farms

As described above, different prevalences of resistance were observed on testing samples from different farms and different groups within farms. However, the sample and isolate numbers were such that confident comparison between such groups could not be made using descriptive statistics alone.

As the number of isolates of each enterococcal species were small and prevalence of isolation relatively poor, it was considered that the best comparison to use between groups was the proportion of enterococcus-positive samples or *Escherichia* spp.-positive samples from which at least one resistant *Enterococcus* spp. or *Escherichia spp.* was isolated and these data were used for further analysis. The prevalence of resistance amongst *Enterococcus* spp. and *Escherichia* spp. isolates was also compared. Chi-squared analysis was used to compare different groups on Farm 1 and Fisher's exact test was used to compare the same groups on different farms, with a p-value of less than 0.05 considered significant.

Amongst enterococci (isolates and samples) from Farm 1, there was a significant difference ($p<0.05$) between groups in prevalence of resistance to penicillin (100 per cent of grower isolates sensitive), ampicillin (100 per cent of grower and piglet isolates sensitive) and erythromycin (only 37.5 per cent of grower isolates and samples resistant). Amongst *Escherichia* spp. isolates and samples from Farm 1, there was a significant difference ($p<0.05$) in the prevalence of resistance to trimethoprim-sulphamethoxazole (83.3 per cent of piglet isolates and samples resistant and 90 per cent of sow samples resistant), cefuroxime (100 per cent of finisher and 90 per cent of sow samples resistant) and amoxicillin-clavulanic acid (29.4 per cent of isolates and 40 per cent of samples from finishers resistant).

When different farms were compared, the prevalence of resistance on Farm 1 was significantly higher for some, but not all, antimicrobials. The prevalence of resistance amongst enterococci to tetracycline was significantly higher in samples from weaners and growers on Farm 1 (100 per cent of samples resistant) than in samples from weaners (62.5 per cent of samples resistant) and finishers (75 percent of samples resistant) on Farm 3 ($p<0.05$). However, the prevalence of resistance to penicillin (100 per cent of isolates and samples sensitive) and erythromycin (37.5 per cent of isolates and samples resistant) from Farm 1 growers was significantly lower than in samples (52.9 per cent resistant to penicillin and 88.2 per cent resistant to erythromycin) and isolates (44.4 per cent resistant to penicillin and 88.9 per cent resistant to eryhtromycin) from Farm 4 finishers ($p<0.05$).

Amongst *Escherichia* spp. there was a significantly higher prevalence of resistance to several antimicrobials in samples and isolates from Farm 1 than from other farms. The prevalence of resistance to ampicillin amongst *Escherichia spp.* was significantly higher in Farm 1 weaners (26.7 per cent of isolates and 40 per cent of samples

resistant) and finishers (41.2 per cent of isolates and 60 per cent of samples) than in weaners (14.3 per cent of isolates and 22.2 per cent of samples resistant) and finishers (5.3 per cent of isolates and 10 per cent of samples resistant) from Farm 3 ($p<0.05$). The prevalence of resistance to ampicillin in finishers on Farm 2 (100 per cent of isolates and samples sensitive) was also significantly lower than in finishers on Farm 1(41.2 per cent of isolates and 60 per cent of samples) ($p<0.05$). On Farm 1, the proportion of isolates and samples resistant to trimethoprim-sulphamethoxazole (33.3 per cent of weaner isolates, 40 per cent of weaner samples, 29.4 per cent of finisher isolates and 50 per cent of finisher samples resistant) and tetracycline (86.7 per cent of weaner isolates, 90 per cent of weaner samples, 70.6 per cent of finisher isolates and 90 per cent of finisher samples resistant) was also significantly higher than in isolates and samples from the same groups on Farm 3 (7 per cent of weaner isolates and 11.1 per cent of weaner samples resistant, 100 per cent of finisher isolates and samples sensitive) ($p<0.05$). The prevalence of resistance to cefuroxime was significantly higher in Farm 1 finishers (82.3 per cent of isolates and 100 per cent of samples resistant) than in finishers from Farm 2 (16.7 per cent of isolates and 33 per cent of samples resistant) and 3 (21 per cent of isolates and 40 per cent of samples resistant) ($p<0.05$). However, the prevalence of resistance to gentamicin was significantly higher in weaners (28.6 per cent of isolates and 44.4 per cent of samples resistant) and finishers (100 per cent of isolates and samples sensitive) from Farm 3 when these were considered together than in samples from the same groups on Farm 1 (100 per cent of isolates and samples sensitive) ($p<0.05$).

6.4 Discussion

The reason for carrying out studies on resistance to therapeutic antimicrobials on the four selected farms was first to estimate the prevalence of resistance on each farm and secondly to identify any differences between farms or groups on farms that might be explained by or related to avilamycin use.

The prevalences of resistance amongst enterococci to therapeutic antimicrobials observed are similar to those described by previous authors, with a high proportion of isolates resistant to erythromycin and tetracycline (Aarestrup *et al.*, 2002). Similarly, several authors have described low prevalences of 7 per cent or less of resistance to vancomycin amongst enterococci isolated from pigs (Bager *et al.*, 1997; Danmap, 2000; Herrero *et al.*, 2000) and this is in agreement with the findings of this study. However, much higher prevalence levels of vancomycin resistance of up to 17 per cent have been described amongst *E. faecium* in Denmark (Aarestrup *et al.*, 2002) and it is recognised that enrichment is a much more sensitive method of detecting vancomycin-resistant enterococci than the method used in this study (Butaye, Devriese and Haesebrouck, 1999a). The prevalences of resistance to most of the antimicrobials tested are higher than those described in Swedish enterococcal isolates and it has already been suggested that the low proportion of resistant bacteria isolated from farm animals in Sweden is a consequence of the low antimicrobial use in that country (SVARM, 2000; Aarestrup *et al.*, 2002).

It should also be borne in mind that isolates classified as intermediately resistant were considered resistant for the purposes of this study. The rationale behind this approach was that in a clinical setting, these antimicrobials would not be considered effective for treatment. This approach may have lead to higher estimates of prevalence than those described elsewhere if different methodologies or criteria were used. However,

even with more accurate methods of sensitivity testing such as breakpoint MIC determination, some isolates would still have been classified as intermediately resistant.

The prevalences of resistance observed amongst the *Escherichia spp.* isolates were similar to those described elsewhere in commensal *E. coli* isolates, with a high proportion of isolates resistant to trimethoprim-sulphamethoxazole, tetracycline and ampicillin (Das, 1984; Dunlop *et al.*, 1998a). Although lower prevalences of resistance have been described in Denmark (Danmap, 1999; Danmap, 2000) the isolates tested were taken from abattoirs and therefore the animals sampled were unlikely to have been treated recently with therapeutic antimicrobials.

The prevalence of resistance on the four study farms has been estimated, but the large confidence intervals surrounding all of the estimates means that there is uncertainty about the estimated prevalences, making comparisons between groups and farms difficult, and this has highlighted one of the greatest problems in carrying out farm studies on antimicrobial resistance: attaining sufficient sample numbers to achieve accuracy and statistical power. One of the factors that contributed to the relatively small numbers of isolates tested in these studies was the fact that enterococci were not cultured from every sample (Table 6.1) and this is in common with the findings of other authors who have described prevalences of *E. faecium* in pig faecal samples ranging from as low as 9 per cent (Devriese *et al.*, 1994) to 29 per cent (Danmap, 2000). However, even for *Escherichia* spp. where the prevalence of isolation was much higher (Table 6.1) sample numbers were still generally insufficient to allow comparisons between groups or farms to be made with confidence using descriptive statistics alone.

The surveillance of antimicrobial resistance in pigs and other farm animals has largely been directed towards animals of slaughter age because of their proximity to the food chain (Danmap, 1998; Danmap, 1999; Dunlop *et al.*, 1999; Danmap, 2000; SVARM, 2000; Danmap, 2001) but one of the objectives of this study was to compare the prevalence of resistance in different age-groups of pigs. When the prevalence of resistance amongst groups on Farm 1 was compared using Chi-squared analysis with a p-value of less than 0.05 considered significant, the results suggested that resistance to penicillin and erythromycin was less common in grower pigs than in piglets and that ampicillin and erythromycin resistance were both more common in sows than in grower pigs on this farm. The results for *Escherichia spp.* isolates suggest that trimethoprim-sulphamethoxazole resistance was more prevalent in sows and piglets but that the prevalence of resistance to cefuroxime and amoxicillin-clavulanic acid was higher in finishers than in other groups on Farm 1. Although the surveillance of slaughter-age animals is a priority for public health this information suggests that there are significant differences between resistance patterns in different age groups of animals within farms and this is in agreement with the findings of similar studies in cattle that suggested the prevalence of resistance was significantly higher in young calves (Gunn, 2000).

When the prevalence of resistance on different farms was compared using Fisher's exact test with a p-value of less than 0.05 considered significant, several significant differences between farms were identified. However, there was no clear relationship between avilamycin use on farms and the proportion of resistant isolates observed, with the prevalence of resistance higher on Farm 1 to certain antimicrobials e.g. trimethoprim-sulphamethoxazole and tetracycline but lower for others, e.g., penicillin and erythromycin. One possible explanation for the higher prevalence of resistance to

tetracycline on Farm1 is the fact that this antimicrobial has been used on the farm for the medication of hospitalized pigs, but as penicillin has been used on all the farms, the reason for the higher prevalence of resistance on Farm 4 is not known. Significant differences in the prevalence of antimicrobial resistance according to antimicrobial usage between farms have been described for vancomycin-resistant enterococci (Aarestrup, 1995; Bager *et al.*, 1997) and differences between the prevalence of vancomycin-resistant enterococci have been described in countries with different antibiotic policies. Antimicrobial usage in pigs has also been associated with resistance in *E. coli* (Dunlop *et al.*, 1998b) but the findings of this small study suggest that the development and persistence of resistance to antimicrobials on farms is complex and multifactorial and therefore it is difficult to draw firm conclusions from such farm studies because of the many potential variations in therapeutic antimicrobial use and management practices that may affect antimicrobial resistance. However, based on the results for both enterococci and *Escherichia spp.*, there seems little evidence to suggest that exposure to avilamycin has led to resistance to any of the other antimicrobials tested as, in general, the prevalence of resistance in animals exposed to avilamycin for the longest time (growers and finishers) was lower than that observed in the younger animals or sows (Figures 6.3, 6.6, 6.9 and 6.10). The only exception to this was resistance to amoxicillin-clavulanic acid, which was present only in isolates from finishers on Farm 1 and as there is no record of use of this antimicrobial on the farm, there is no obvious explanation for this observation. Cefuroxime resistance was also prevalent in finishers on Farm 1 but a high proportion of isolates from sows were resistant as well and, again, there is no record of use of any cephalosporin on the farm. The only difference in resistance patterns that can be explained by therapeutic antimicrobial use is the higher prevalence of resistance to

tetracycline on Farm 1 where this antimicrobial had been used to medicate hospitalized pigs.

It is possible that resistance to some of the antimicrobials tested may be linked as has been described before for other antimicrobials (Aarestrup, Bager and Andersen, 2000; Aarestrup *et al.*, 2001) and therefore resistance to tetracycline may be driving resistance to other antimicrobials, e.g., trimethoprim-sulphamethoxazole on this farm, or vice versa. It is also notable that despite the use of enrofloxacin to treat diarrhoea in piglets on Farm 1, no ciprofloxacin-resistant organisms were isolated, indicating that the relationship between antimicrobial use and resistance is not straightforward.

From an epidemiological point of view, the studies have highlighted some of the issues to be addressed when considering testing regimes for determining the prevalence of antimicrobial resistance and also when relating results to underlying sample and animal populations. One of the features of the antimicrobial resistance patterns described is the heterogeneity of the underlying bacterial population, which is indicated by the higher prevalence of resistant samples than resistant isolates and this has been suggested before in commensal bacterial populations but is not normally taken into account when isolates are selected for testing (Humphry *et al.*, 2002). Although on most occasions the sensitivity pattern of isolates from an individual sample were identical, on some occasions different sensitivity patterns were observed and this occurred even where organisms were identified as belonging to the same species and where samples were taken from an individual animal. This suggests that multiple isolates of the same species should be tested from an individual sample, rather than classifying a sample or as sensitive or resistant based on the classification of an individual isolate.

This raises the question of how best to express resistance in relation to the underlying bacterial, sample and animal populations. In the studies described, multiple enterococcal isolates were cultured and tested where possible but there were samples from which enterococci were not isolated and this meant that some samples were over-represented when results were expressed in terms of prevalence of resistant isolates and this is significant because this is the usual method by which the results of surveillance data are expressed (SVARM, 2000; Danmap, 2001). Conversely, expressing results in terms of the number of samples from which resistant enterococci were isolated as a proportion of the total number of samples underestimated the prevalence of resistance in the enterococcal population because prevalence of isolation was poor. For these reasons, results were also expressed in terms of the number of samples from which resistant/intermediately resistant enterococci were isolated as a proportion of the total number of samples from which enterococci were isolated. However, although this approach went some way to addressing the low prevalence of isolation, it did not incorporate the heterogeneity in the underlying bacterial population and by describing resistance on the farm by sample alone, information about the underlying bacterial population was lost.

Furthermore, the results suggest that resistance should ideally be related to a particular bacterial species rather than a genus and that accurate speciation is important because species-specific differences in resistance exist. For example, *E. faecalis* is intrinsically resistant to quinupristin-dalfopristin whereas *E. faecium* is not (Singh *et al.*, 2002) and although insufficient numbers of each enterococcus species were isolated to evaluate interspecies differences with statistical power, it would appear that on Farm 1, *E. faecium* isolates were more likely to be resistant to most antimicrobials tested than *E. durans* isolates, for instance. This means that unless

organisms are speciated accurately then comparisons are inaccurate, but the different composition of enterococcus species isolated from each farm in these studies made species-specific comparisons between farms difficult. However, it should be borne in mind that the significant differences in resistance patterns highlighted incorporated all enterococcal species isolated and therefore may be a reflection of the enterococcal flora present on a farm rather than a true comparison of the prevalence of resistance and this may also be the case where isolates are not carefully speciated.

In summary, the farm studies conducted have highlighted some of the problems of quantifying antimicrobial resistance in commensal organisms, of relating the data generated to underlying bacterial and animal populations and of making meaningful comparisons between farms and groups in field studies. Finally, although a small number of significant differences in resistance patterns were noted between farms and groups, there was no evidence to suggest that these differences were related to the use of avilamycin and any such relationship was also difficult to assess because of the many other factors that may have influenced the resistance patterns observed, including the complex relationship between therapeutic antimicrobial use and resistance and the large confidence intervals surrounding prevalence estimates.

CHAPTER 7

MOLECULAR INVESTIGATIONS

7.1 Introduction

It was apparent from the studies discussed in Chapters 4, 5 and 6 that the application of conventional bacteriological methods to the measurement of resistance to both avilamycin and therapeutic antimicrobials had limitations. More sensitive and specific techniques would be required to quantify resistance and particularly to monitor changes over time. One approach that was considered was the detection of genotypic resistance instead of phenotypic resistance and in order to develop this approach, molecular biological techniques including polymerase chain reaction (PCR) and DNA sequencing were used to determine the genetic basis of avilamycin resistance in enterococci isolated from Farm 1 as described in Chapter 5.

Following the first description of the polymerase chain reaction (PCR) in 1987 (Mullis and Faloona, 1987) it has been widely applied in microbiological research. The technique involves temperature cycling to cause the repeated dissociation and annealing of specific oligonucleotide primers to a DNA template. A thermostable DNA polymerase elongates the primers using deoxyribonucleoside triphosphates (dNTPs) thus amplifying the target sequence. The specific nature of the primers means that only the target sequence increases exponentially and by choosing primers that will anneal to unique regions, PCR can be used to detect the presence of DNA from bacteria or viruses.

In clinical laboratories, PCR has been used both to detect and identify microorganisms as well as to detect the presence of antimicrobial resistance (Fluit *et*

al., 2001). For example, several PCRs have been developed in the last decade for the detection of methicillin resistant *Staphylococcus aureus* (MRSA) (Archer and Pennell, 1990; Ligozzi *et al.*, 1991; Murakami *et al.*, 1991; Brakstad *et al.*, 1993 and Towner *et al.*, 1998). These techniques have been shown to be both rapid and sensitive and the mecA PCR is now considered the gold standard technique for the detection of this organism (Kampf *et al.*, 1997). Similarly, both single and multiplex PCRs have been developed for the detection of glycopeptide resistance in enterococci where detection of the van gene cluster has been useful in the surveillance of vancomycin resistance (Dutka-Malen *et al.*, 1995; Miele *et al.*, 1995; Sahm *et al.*, 1997 and Reed *et al.*, 1999).

DNA sequencing is also being more frequently used in diagnostic laboratories (Fluit *et al.*, 2001). The most commonly used method is the chain termination method first described in 1977 (Sanger *et al.*, 1977). In this method, DNA is sequenced by the synthesis of DNA fragments using DNA polymerase and dNTPs as described above for PCR. However, in each reaction one of the dNTPs is designed to halt elongation of the newly synthesised DNA. Each reaction thus produces chains of various lengths terminating at one of the dNTPs. When fluorescence detection is used, each of the 4 dNTPs is primed with a tag that fluoresces at a different wavelength. The DNA sequence can thus be determined by fluorescence measurements at the four different wavelengths.

Some of the genes responsible for antimicrobial resistance in clinically important organisms have been sequenced. For example, at least 24 different tetracycline resistance (Tet) determinants have been described (Levy *et al.*, 1988; Roberts, 1996 and Taylor and Chau, 1996), and the sequence of several different beta-lactamase

genes is now known (Bush *et al.*, 1995). New resistance determinants are being identified continually in a variety of organisms.

The growth-promoting antimicrobial avilamycin inhibits protein synthesis by binding to bacterial ribosomes (Wolf, 1973). A related compound, evernimicin (SCH27899), has been considered for use in human medicine (Nakashio *et al.*, 1995; Urban *et al.*, 1996; Marshall *et al.*, 1999), and it has been shown that the binding sites of avilamycin and evernimicin overlap. Resistance to evernimicin and avilamycin has also been shown to be co-transferable in *E. faecium* in vitro (Aarestrup and McNicholas, 2002). Following the description of mutations in ribosomal protein L16 conferring resistance to evernimicin in *Streptococcus pneumoniae* (Adrian *et al.*, 2000), similar mutations conferring high-level avilamycin and low-level evernimicin resistance were described in enterococci isolated from pigs and broilers in Denmark (Aarestrup and Jensen, 2000).

The aim of this part of the study was to amplify and sequence the L16 gene in avilamycin-resistant enterococcal isolates obtained from the farm studies (Chapter 5) and to assess whether molecular methods could be used in the detection of avilamycin-resistant enterococci.

7.2 Materials and methods

7.2.1 Selection of bacterial strains

Five bacterial isolates identified to species by biochemical testing and exhibiting phenotypic resistance to avilamycin were selected from a bank of strains isolated from Farm 1. They were isolated on Slanetz and Bartley plates containing 64 or 128μg/ml of avilamycin as described in Chapter 5. They were identified to species using commercial biochemical kits (API, Biomerieux) and subsequently by tRNA intergenic

spacer PCR. Susceptibility to avilamycin was determined by culturing on Mueller-Hinton agar plates containing twofold serial dilutions of avilamycin at dilutions ranging from 1 to 128μg/ml, according to NCCLS guidelines, (NCCLS, 2000b). These isolates were chosen to include different enterococcal species and were derived from different age groups on the farm. They were recovered from storage on Microbank beads at -70°C and grown overnight on nutrient agar before use. The strains selected are shown in Table 7.1.

Table 7.1 Identification, origin and susceptibility to avilamycin of enterococcal isolates selected for molecular analysis.

Origin	API identification	Identification by tRNA intergenic spacer PCR*	Avilamycin MIC (μg/ml)
Piglets, Farm 1	*E. gallinarum*	*E. faecium*	>128
Finishers, Farm 1	*E. faecium*	*E. faecium*	>128
Piglets, Farm 1	*E. durans*	*E. hirae*	>128
Weaners, Farm 1	*E. faecium*	*E. faecium*	128
Weaners, Farm 1	*E. faecalis*	*E. faecalis*	>128

*Molecular identification of strains carried out by An Martel, DVM, Laboratory of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium.

7.2.2 Primers

The primers used were identical to those described by Aarestrup and Jensen (2000) and were purchased from MWG Biotech (UK) Ltd., Mill Court, Featherstone Road, Wolverton Mill, South Milton Keynes MK12 5RD. These primers were used to amplify a 414 base pair sequence of the L16 gene.

Primer 1: 5'-AAA CGT GTA AAA CAC CGT CG-3'

Primer 2: 5'-CAT TCG ATT CAC CAC CCA TT-3'

The primers were diluted in sterile distilled water to a concentration of 100-pmol/µl before use.

7.2.3 Preparation of template DNA

Several colonies from overnight growth of a pure culture were suspended in 100µl of sterile distilled water and boiled for five minutes. The suspension was then centrifuged at 15000g for two minutes. The supernatant was pipetted off and 1µl was added to 45µl of Reddymix™ Reaction Buffer (10X) (750mM Tris-HCl, 200mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween®20 and 15mM $MgCl_2$, red dye and precipitant, supplied by Abgene, Blenheim Road, Epsom, Surrey KT19 9AP) and 2µl of a one in ten dilution of each primer. The resultant suspension was mixed by pulsing in the micro-centrifuge.

7.2.4 Polymerase chain reaction (PCR)

DNA amplification was performed in a PCRExpress thermal cycler, (Hybaid UK Ltd., Action Court, Ashford Place, Ashford, Middlesex TW15 1XB). Initial denaturation was carried out by incubation at 95°C for 2 minutes followed by 35 cycles of amplification. Each cycle consisted of 30 seconds at 95°C to denature the DNA, 30 seconds at 55°C to anneal the primers to the template and 30 seconds at 72°C for primer extension. After the last cycle, a further incubation for 15 minutes at 72°C was performed to allow extension of any partially completed product.

7.2.5 Analysis and Purification of PCR products

On completion of the amplification, 5µl of each reaction mixture was electrophoresed through a 1 per cent agarose gel and the size of DNA fragments estimated by comparison with DNA markers of known length (1KB and ϕX DNA ladders, Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley, UK). The DNA was visualised by staining with ethidium bromide and exposure to ultraviolet light (312nm). The DNA fragments were excised from the agarose gel using a scalpel blade, transferred to a microcentrifuge tube and weighed. Amplified products were recovered from the gel fragments using the QIAquick Gel Extraction Kit (Qiagen Ltd. (UK), Boundary Court, Gatwick Road, Crawley, West Sussex RH10 9AX).

7.2.6 Preparation of single stranded DNA for sequencing

Sequencing reaction mixtures were prepared using 8µl of purified PCR product, 3.2µl of a 1pmol/µl dilution of primer, 4µl of ABI Prism® BigDye™ Terminator Ready Reaction Mix (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404 U.S.A), 2µl of 5X buffer and 2.8µl of sterile distilled water. The control reaction mixture was prepared using 1µl of DNA from the pGEM®-13Zf(+) Vector (Promega UK Ltd., Delta House, Chilworth Science Park, Southampton SO16 7NS), 4µl of 0.8pmol/µl primer, 4µl of Big Dye Terminator Ready Reaction Mix, 2µl of 5X buffer and 9µl of MQ water. Twenty five cycles of amplification were performed using a PCRExpress thermal cycler (Hybaid) with an initial temperature of 96°C for 30 seconds to denature the DNA, an annealing temperature of 55°C for 30 seconds and an extension temperature of 60°C for 4 minutes. The amplified DNA fragments were

purified using the Spin Performa DTR Gel Filtration System (Edge BioSystems, 19208 Orbit Drive, Gaithersburg, MD 20879-4149, U.S.A).

7.2.7 DNA Sequencing

The purified DNA was freeze dried (Edwards Pirani 501 Freeze Drier, Edwards High Vacuum International Ltd., Manor Royal, Crawley, West Sussex, RH10 2LW) and then resuspended in 25µl of deionised Hi-Di™formamide (Applied Biosystems) before being transferred to a 96 well plate. The sequencing reactions were carried out in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

7.3 Results

7.3.1 Amplification of target DNA by polymerase chain reaction (PCR)

Amplified products were generated from all 5 isolates and corresponded to the target sequence of the L16 gene (Figure 7.1).

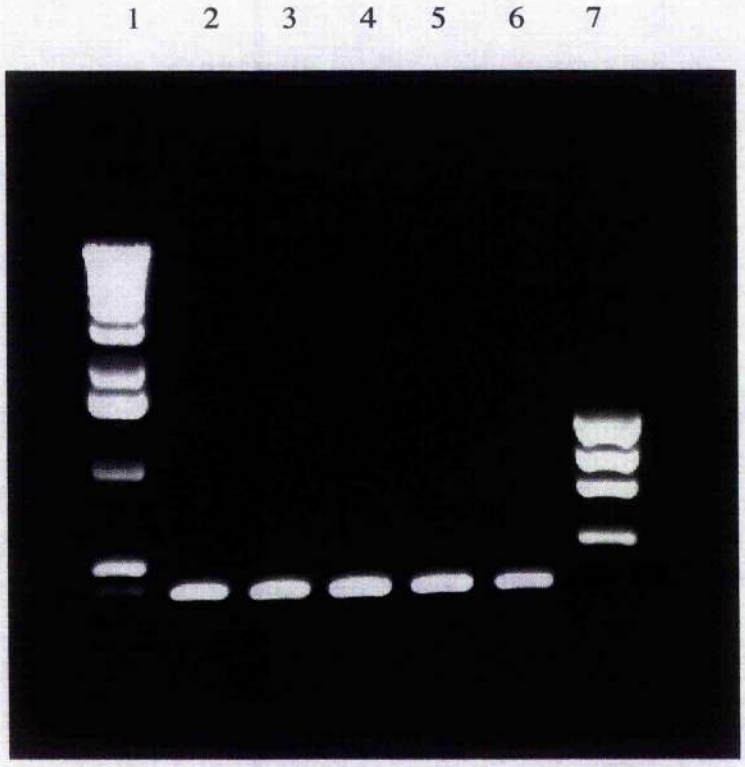


Figure 7.1 PCR products after electrophoresis through a 1 per cent agarose gel. The gel was stained with ethidium bromide and photographed when transilluminated with UV light (312nm). Lanes 1- 7: 1KB ladder, Piglet *E. faecium*, Weaner *E. faecium*, Finisher *E. faecium*, Weaner *E. faecalis*, Piglet *E. hirae* and ϕX ladder, respectively.

7.3.2 DNA sequencing

The PCR products were each sequenced using forward and reverse primers identical to those used in the initial PCR. Sequences were analysed and compared with the published L16 sequence of the human derived reference strain *E. faecium* CCUG (Culture Collection of the University of Goteborg, Sweden) using the Vector NTI software program (InforMax Inc., The Magdalen Centre, Robert Robinson Avenue, The Oxford Science Park, Oxford OX4 4GA) (Figures 7.2 and 7.3). In all 10 cases, sequences of between 350 and 400 base pairs were produced with overall good correlation of sequence data between primers and a high degree of homology with the reference strain.

13 **AAACGTGTAA AACACCGTCG** TGAATTCCGC GGAAAAATGC

GCGGGGAAGC TAAAGGCGGA AAAGAAGTAG CATTCGGTGA

ATACGGTTTG CAAGCTGTTG

113 ATTCACATTG GATCACAAAC CGCCAAATCG AAGCTGCTCG

TA**TC**GCAATG ACT**CGT**TACA TGAAACGTGG TGGGAAAGTA

TGGATTAAAA TTTTCCCTCA

213 CAAATCTTAT ACTGCCAAAG CAATTGGGGT ACGTATGGGT

TCTGGTAAAG GGGCACCTGA AGGATGGGTT GCACCAGTAA

313 GAAATCGCAG GCGTTCCTGA AGAAGTAGCT CGTGAAGCGT

TACGTCTAGC TTCTCACAAA TTACCAATGA AAACTAAGAT

CGTAAAACGT GAGGAA**ATGG**

413 **GTGGTGAATC GAAT**

Figure 7.2 L16 sequence of *E.faecium* CCUG542. Position of primers indicated (bold and underlined). Position of amino acids 52 and 56 indicated in bold.

E. faecium CGUG542
142
GAAGCTGCTCGT**ATC**GCAATGACT**CGT**TACATGAAACGTGGTGGGAAAG

Weaner *E. faecium* Forward Primer
112
GAAGCTGCTCGT**ATC**GCAATGACT**CAT**TACATGAAACGTGGTGGGAAAG

Weaner *E. faecium* Reverse Primer
130
GAAGCTGCTCGT**ATC**GCAATGACT**CAT**TACATGAAACGTGGTGGGAAAG

Finisher *E. faecium* Forward Primer
116
GAAGCTGCCCGT**ATN**GCAATGACT**CGT**TATATGAAACGTGGCGGGAAAG

Finisher *E. faecium* Reverse Primer
130
GAAGCTGCTCGT**ACC**GCAATGACT**CGT**TACATGAAACGTGGTGGGAAAG

Weaner *E. faecalis* Forward Primer
125
GAAGCAGCCCGT**ATT**GCAATGACT**CAT**TACATGAAACGTGGCGGGAAAG

Weaner *E. faecalis* Reverse Primer
131
GAAGCAGCCCGTATTGCAATGACT**CAT**TACATGAAACGTGGCGGGAAAG

Piglet *E. faecium* Forward Primer
110
GAAGCTGCCCGTATTGCAATGACT**CGT**TATATGAAACGTGGCGGGAAAG

Piglet *E. faecium* Reverse Primer
132
GAAGCTGCCCGTATTGCAATGACT**CGT**TATATGAAACGTGGCGGGAAAG

Piglet *E. hirae* Forward Primer
110
GAAGCTGCTCGT**ATC**GCAATGACT**CGT**TACATGAAACGTGGTGGGAAAG

Piglet *E. hirae* Reverse Primer
131
GAAGCTGCTCGT**ATC**GCAATGACT**CGT**TACATGAAACGTGGTGGGAAAG

Figure 7.3 Alignment of L16 sequences of test isolates and reference strain *E. faecium* CCUG542, with position of amino acids 52 and 56 indicated in bold type. Base pair number is indicated on the left hand side. Nucleotide substitutions leading to amino acid substitutions are underlined.

7.4 Discussion

The sequence data described in this chapter shows that considerable variation exists between the isolates tested in codons 52 and 56 of the L16 gene (Figure 7.3). The effect of these variations on amino acid sequence is shown in Table 7.2.

Table 7.2 Amino acids corresponding to nucleotide sequences of control strain *E. faecium* CCUG542 and test isolates.

Isolate	Amino acid 52	Amino acid 56
E. faecium CCUG542	Isoleucine	Arginine
E. faecium (weaner)	Isoleucine	Histidine
E. faecium (finisher)	Threonine /Isoleucine	Arginine
E. faecalis (weaner)	Isoleucine	Histidine
E. faecium (piglet)	Isoleucine	Arginine
E. hirae (piglet)	Isoleucine	Arginine

The weaner *E. faecium* isolate had a nucleotide substitution at codon 56, (CGT – CAT), which would have the effect of substituting arginine with histidine at this residue. The weaner *E. faecalis* isolate had the same nucleotide substitution at amino acid 56, (CGT, Arg – CAT, His). The weaner *E. faecalis* isolate and the piglet *E. faecium* isolate exhibited a different sequence from the other isolates at amino acid 52, (ATC – ATT). However, this substitution would not have any effect on the corresponding amino acid. The finisher *E. faecium* isolate may also have possessed a substitution at amino acid 52, (ATC – ACC), which would result in the substitution of isoleucine with threonine. However, the nucleotides present at the second residue of

this codon were different in the forward and reverse primer sequences and it was unclear from the sequence data which nucleotide was present in the third residue of the forward primer sequence. Finally, the sequence obtained from the piglet *E. hirae* isolate appeared to be identical to codons 52 and 56 of the reference strain.

Therefore, examination of the effect of the nucleotide substitutions observed on the corresponding amino acid sequences revealed that only 3 of the 5 avilamycin-resistant isolates, (weaner *E. faecium*, finisher *E. faecium* and weaner *E. faecalis*), had different amino acids from the sensitive reference strain at these residues (Table 7.2).

Furthermore, the sequence data obtained was used to predict the amino acid sequence over a larger portion of the L16 protein for each of the isolates. As the L16 sequence is known for several reference organisms (Adrian *et al.*, 2000), the amino acid sequences could be compared (Table 7.3). Even over this larger region of the L16 protein, only 3 of the five avilamycin-resistant isolates, (weaner *E. faecium*, finisher *E. faecium* and weaner *E. faecalis*) exhibited differences in amino-acid sequence from the evernimicin-sensitive reference strains, suggesting that the molecular basis for avilamycin resistance is not in this region of L16 for the piglet *E. hirae* and *E. faecium* isolates.

Table 7.3 Comparison of amino acid sequence of the L16 protein from residues 43 to 60 for test isolates and reference strains, (*S. pneumoniae*, *E. faecalis*, *S. aureus* and *E. coli*). Amino acid residues differing from the evernimicin-sensitive *S. pneumoniae* and *E. faecalis* are highlighted in bold type and underlined.

Organism	Amino Acid Sequence
S. pneumoniae	43 TNRQIE AARIAM TRYMKR 60
E. faecalis	43 TNRQIE AARIAM TRYMKR 60
S. aureus	43 T**S**RQIE **S**ARIAM TRYMKR 60
E. coli	43 T**A**RQIE AAR**R**AM TR**AV**KR 60
Piglet *E. hirae*, Piglet *E. faecium*,	43 TNRQIE AARIAM TRYMKR 60
Weaner *E. faecium*, Weaner *E. faecalis*	43 TNRQIE AARIAM T**H**YMKR 60
Finisher *E. faecium*	43 TNRQIE AAR**T**AM TRYMKR 60

Key: T=threonine; N=asparagine; R=arginine; Q=glutamine; I=isoleucine; E=glutamic acid; A=alanine; M=methionine; Y=tyrosine; K=lysine; S=serine; V=valine; H=histidine.

In summary, the sequence data obtained indicates that phenotypic avilamycin-resistance cannot be explained by a single mutation in the sequence of the L16 gene or even by different substitutions at the same locus in this gene. Even when three isolates of the same bacterial species (*E. faecium*) isolated from the same animal species and from the same farm were tested, three different nucleotide substitutions were observed. Moreover, two of the 5 resistant isolates including one *E. faecium*, exhibited sequences identical to the sensitive reference strain and would be indistinguishable from sensitive isolates if molecular detection was aimed at these two residues of L16 (52 and 56). The assertion that different molecular determinants of avilamycin resistance exist has since been confirmed by recent work describing mutations in helices 89 and 91 of rRNA in *Halobacterium halobium* that confer

resistance to evernimicin. These regions lie close to the L16 region of 23S rRNA and the mutations described have also been shown to give cross-resistance to avilamycin (Kofoed and Vester, 2002). In addition, a gene encoding a methyltransferase (*emtA*) that confers high-level evernimicin resistance by methylation of 23S rRNA and that is plasmid-borne has been cloned from an avilamycin-resistant *E. faecium* strain isolated from a broiler in Denmark (Mann *et al.*, 2001). However, the prevalence of high-level evernimicin resistance mediated by the *emtA* gene in human and animal isolates in Denmark is thought to be low (Aarestrup and McNicholas, 2002).

The findings of the small study described in combination with the recent descriptions of avilamycin resistance mechanisms in enterococci suggest that the detection of avilamycin resistance in enterococci by PCR would be difficult. As several different mutations are responsible for avilamycin resistance, a single PCR would have a poor sensitivity as some resistant organisms would be misséd. The development of a multiplex PCR to detect resistance would depend on all the molecular determinants of avilamycin resistance in enterococci being fully described and this has not yet been accomplished.

However, the substitutions identified at residues 52 and 56 in the *E. faecium* and *E. faecalis* isolates are identical to those described in Danish pig and broiler isolates (Aarestrup and Jensen, 2000) and this suggests that these may be the most prevalent genetic determinants of avilamycin resistance in *E. faecium* and *E. faecalis* in pigs.

The study has also highlighted the problems of detecting subtle differences at the molecular level, such as the substitution of one nucleotide, in antimicrobial resistance studies. When the finisher *E. faecium* isolate was sequenced, even using both forward and reverse primers, it was not possible to say with certainty that a substitution was present at residue 52 without further sequencing analysis taking place. If sample size

calculations suggested that a large number of isolates needed to be tested and a large proportion of tests then had to be repeated, this approach to resistance detection would become quite labour intensive.

The conversion of nucleotide sequences to the corresponding amino acid sequence also illustrates some of the problems of relying on molecular differences for resistance detection. First, nucleotide substitution does not necessarily lead to amino acid substitution. For example, the weaner *E. faecalis* and piglet *E. faecium* isolates tested above have a different sequence from the other organisms tested but the same amino acid at residue 52. Second, even when amino acid substitution does take place, the effect on antimicrobial sensitivity may be dependent on which amino acid is inserted. For instance, three different amino acid substitutions at residue 52 in *S. pneumoniae* and their effect on the MIC of evernimicin have been described (Adrian *et al.*, 2000) and whilst substitution of isoleucine with serine or asparagine caused an increase in MIC from 0.03 to 1.5μg/ml, clones with arginine at this residue were sensitive to evernimicin and the replacement of isoleucine with threonine caused a much smaller increase in MIC to 0.38μg/ml. The substitution with threonine is identical to the finisher *E. faecium* isolate described above and that described in avilamycin-resistant *E. faecium* isolates from Danish broilers (Aarestrup and Jensen, 2000). These findings suggest that the phenotypic determination of resistance is still important and that genotypic determinants should be related to this.

It is also important to consider the likely functional effects of mutations on antimicrobial action. Avilamycin inhibits protein synthesis by preventing the attachment of tRNA to the ribosome (Wolf, 1973), and has a binding site close to L16 (Kofoed and Vester, 2002). Therefore, substitutions in the sequence of L16 are likely to be responsible for resistance by disrupting avilamycin binding.

It can be seen from Table 7.3, that *E. coli*, which is naturally resistant to avilamycin, differs from naturally sensitive organisms such as *E. faecalis* at several residues even in this short sequence of amino acids in L16. Any or none of these differences could be responsible for resistance to avilamycin. In addition, the amino acid sequence of *S. aureus* differs at several residues from that of *E. faecalis* and yet is also sensitive to avilamycin and evernimicin. This suggests that molecular analysis of resistant organisms must be coupled with phenotypic determination of resistance and that molecular determinants should be related to functional effects.

However, despite the problems of using molecular analysis as a tool for detecting resistance, examining the genetic composition of resistant organisms can be useful in epidemiology. By describing the genetic basis for resistance, organisms that were phenotypically indistinguishable have been shown to have different genetic mechanisms of resistance and it therefore seems unlikely that resistance to avilamycin in these organisms has arisen by transfer between different enterococcal species. The genetic basis for avilamycin resistance in the piglet *E.faecium* and *E. hirae* isolates remains unknown. However, the absence of a common genetic mechanism conferring resistance to avilamycin in the enterococci analysedemphasises the importance of speciating enterococcal isolates accurately and, as demonstrated by the discrepancies between biochemical and molecular identification of these isolates, this is not always straightforward.

In summary, the mechanisms of resistance to avilamycin and their molecular basis appear to be relatively complex, with both intra and inter-species differences. Although sequencing of resistant isolates has provided additional information on the mode of action of avilamycin and mechanisms of resistance to it, it would not at

present be possible to use this information to screen enterococcal isolates for avilamycin resistance by molecular means alone.

CHAPTER 8

EPIDEMIOLOGICAL MODELS

8.1 Introduction

Having applied standard statistical techniques to determine the sample numbers required to detect resistance (Chapter 3), it was apparent from the findings of the farm studies discussed in Chapters 4, 5 and 6 that the sensitivity and specificity of some of the conventional bacteriological techniques routinely used in antimicrobial resistance studies were poor and that even the genotypic detection of resistance as discussed in Chapter 7 would have a low sensitivity. It was also clear that resistance could be estimated within several different populations including farm, production group, animal and bacterial species. Epidemiological modelling was an alternative approach considered to determine sample sizes whilst taking into consideration the different population levels and incorporating some of the features of the tests applied.

Modelling is the representation of physical processes, designed to increase appreciation and understanding (Thrusfield, 1995) and models can range from simple pictorial representations of a process to complex mathematical algorithms that require computers to be implemented. Epidemiology is the study of disease in populations and thus the epidemiological models developed relate resistance to the underlying animal and bacterial populations.

In veterinary medicine, the use of modelling has largely been directed towards infectious disease and several different types of mathematical model have been applied to disease transmission in different animal species including differential equation models (Cherry *et al.*, 1998), matrix models (Lesnoff *et al.*, 2000), network

models and simulation models (Horst *et al.*, 1999; Cohen *et al.*, 2000; de la Rua-Domenech *et al.*, 1999; Perez *et al.*, 2002). These models have been used to explore the dynamics of disease transmission in endemic and epidemic situations and also to predict the effects of different intervention strategies such as vaccination (Vonk Noordegraaf *et al.*, 2000; Mangen *et al.*, 2001). An epidemiological model is defined as a mathematical model, which may be a computer simulation model, of a disease for the purpose of studying the behaviour of the disease in an animal population under variable conditions of climate, density of population, mix of population and so on, (Blood and Studdert, 1996). However, recently, simulation models have also been applied to veterinary public health and this approach has been a useful adjunct to risk assessment in quantifying the microbial hazards associated with meat production (Cassin *et al.*, 1998; Jordan *et al.*, 1999; Hartnett *et al.*, 2001).

In simulation modelling, scenarios as similar as possible to reality are reproduced by selecting inputs, either deterministically or stochastically, and calculating the model output. This process can be repeated a large number of times using iterations with different input values in order to assess changes in model output. The benefits of developing simulation models are twofold in that not only is it possible to imitate reality and thereby predict outcomes in statistical terms, but all the factors important in determining the output of the model are highlighted as the model is developed. This improves understanding of the disease process or production process being modelled.

The importance of surveillance of antimicrobial resistance in farm animals is well documented (Williams and Ryan, 1998; House of Lords Select Committee on Science and Technology, 1998; ACMSF, 1999) but it is acknowledged that the understanding of the development, persistence and dynamics of antimicrobial resistance in bacterial and animal populations needs to be improved (Lipsitch and Levin, 1997a; Lipsitch *et*

al., 2002; Humphry *et al.*, 2002). The deficiencies in currently available data have also been described and the need for a population based approach to the design of antimicrobial resistance studies highlighted (Davison *et al.*, 2000). However, there is little published information in the scientific literature on the application of epidemiological models to the design of sampling regimes.

The consideration of test performance in infectious disease models has also been limited. The importance of test sensitivity and specificity when the health status of a herd is determined by tests applied to individual animals has been highlighted (Martin *et al.*, 1992) and herd level sensitivity and specificity have been investigated using a modelling approach (Jordan and McEwen, 1998). However, this approach has not been applied to antimicrobial resistance.

Modelling of antimicrobial resistance has been limited to the population dynamics of resistant bacteria and the effect of different antimicrobial treatment regimes on the development and spread of resistant organisms, (Bonhoeffer *et al.*, 1997; Lipsitch and Levin, 1997b; Levin *et al.*, 1997; Lipsitch and Levin, 1998; Levin, 2001). Whilst the same principles used in infectious disease modelling apply, the modelling of antimicrobial resistance requires the consideration of an additional population level, the bacterial population. Transferable resistance can be considered to act like an infectious agent within bacterial populations, which, may, in turn act as infectious agents within animal populations, or may be part of the normal flora.

This chapter describes the development of epidemiological models that consider the underlying animal and bacterial populations and the performance of diagnostic tests in the detection of antimicrobial resistance. It is envisaged that such an approach could be used to help structure and interpret future surveillance programs for antimicrobial resistance in farm animals.

8.1.2 Models Developed

Two types of model were considered. In probability models, a single point estimate is used for each input variable. Various possible scenarios can then be explored by combining different estimates for each variable and considering their effect on the model outcome. Commonly, minimum, maximum and expected values are estimated for each variable in order to model best case, worst case and most likely scenarios. However, the drawbacks of adopting this probabilistic approach include the fact that all possible values for each variable are not represented and the likelihood of each input variable taking an estimated value is not taken into account. In addition, in order to represent some of the various possible scenarios that may arise, large numbers of combinations of inputs become necessary and this becomes quite cumbersome to compute.

In stochastic models, random variation in each input variable is taken into account, such that the model outcome occurs with an associated probability distribution. In order to achieve this, each input variable consists of a probability distribution rather than a point estimate. One method of modelling stochastic processes is to use simulation (Vose, 2000). This technique involves sampling from each probability distribution within the model to produce many scenarios or iterations. In Monte Carlo sampling, the distribution of each input parameter is sampled at random without taking into account previous samples, whilst in Latin Hypercube sampling each distribution is split into sections of equal probability and the same number of samples is taken from each section. Latin Hypercube sampling ensures that all portions of the distribution are represented with the appropriate probability and was therefore the

method used in the stochastic simulation models presented here. Such sampling is also computationally more efficient.

8.2 Probability Models

8.2.1 Individual Animal Probability Model

8.2.1.1 Model Description

The first step in developing the model was to consider the situation for an individual animal. In its simplest form, the probability of an individual animal being detected as harbouring or excreting resistant bacteria depends upon the prevalence of resistant bacteria within the animal and the number of those bacteria that are tested for resistance. The prevalence of resistant bacteria in the animal can also be considered as the proportion of the bacterial population that is resistant. The probability of the animal being detected as harbouring or excreting resistant bacteria is effectively the probability that at least one bacterium isolated from that animal tests positive for resistance. Assuming that the test for resistance is accurate, in other words that it correctly identifies resistant bacteria every time and does not misclassify a sensitive bacterium as resistant, then the probability of detection can be represented by Equation 8.1.

$$P = 1 - (1 - p)^b$$ **Equation 8.1**

where P is the probability of detecting at least one resistant bacterium in an individual animal, p is the proportion of bacteria in the animal that are resistant and b is the number of bacteria tested.

The principle behind this model is that the probability of an event occurring must lie between 0 and 1, where a probability of 1 means it will certainly occur and a probability of 0 means it will certainly not occur. Furthermore, where two events are mutually exclusive, in this case a bacterium testing resistant or not, then the probability of either is equal to 1 minus the probability of the other. Therefore, the probability of at least one bacterium testing resistant is equal to one minus the probability that all of the bacteria tested are not resistant. For each bacterium, the probability of not being resistant is equal to one minus the probability of being resistant and when a number of bacteria are tested, this is the same as one minus the proportion of bacteria that are resistant.

8.2.1.2 Analysis of the Model

The simplest application of this model is to estimate values for the input parameters and consider how these would affect the model output. Where one bacterium from the animal is tested for resistance, the probability of detecting resistance is equal to the proportion of resistant bacteria. As the number of bacteria tested is increased, the probability of at least one testing resistant increases (Figure 8.1).

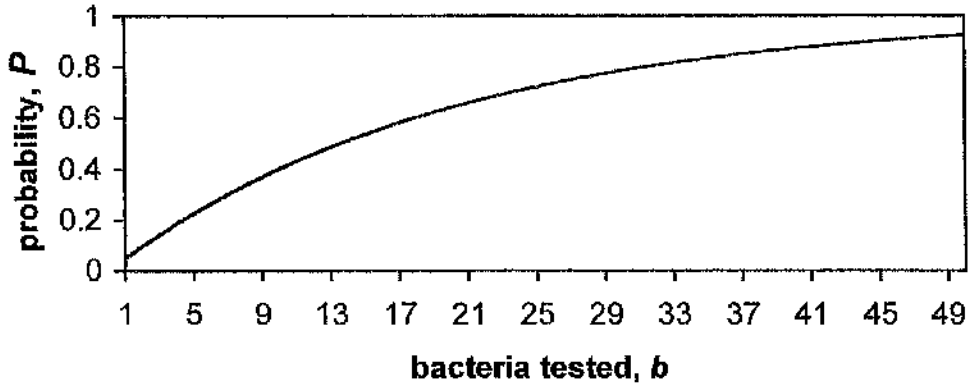


Figure 8.1 Plot showing the relationship between probability of detection, *P*, and number of bacteria tested, *b*, in the individual animal probability model when prevalence, *p*, is 0.05.

When the proportion of resistant bacteria is 0.05, 45 or more bacteria must be tested to attain a probability of detection of 0.9. When the proportion of resistant bacteria is reduced to 0.01 (Figure 8.2), testing as many as 50 bacteria only gives a probability of detection of less than 0.4.

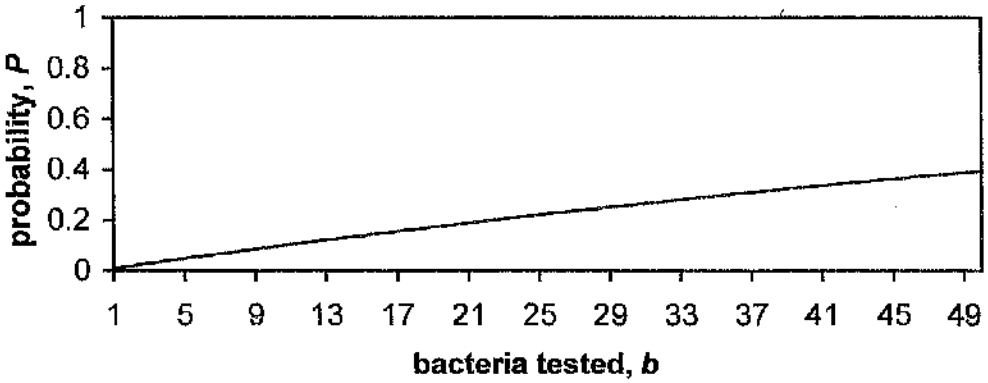


Figure 8.2 Plot showing the relationship between probability of detection, *P*, and number of bacteria tested, *b*, in the individual animal probability model when prevalence, *p*, is 0.01.

In contrast, testing 22 bacteria gives a probability of detection of 0.9 when the proportion of resistant bacteria is increased to 0.1, but 29 must be tested to increase the probability to 0.95 (Figure 8.3).

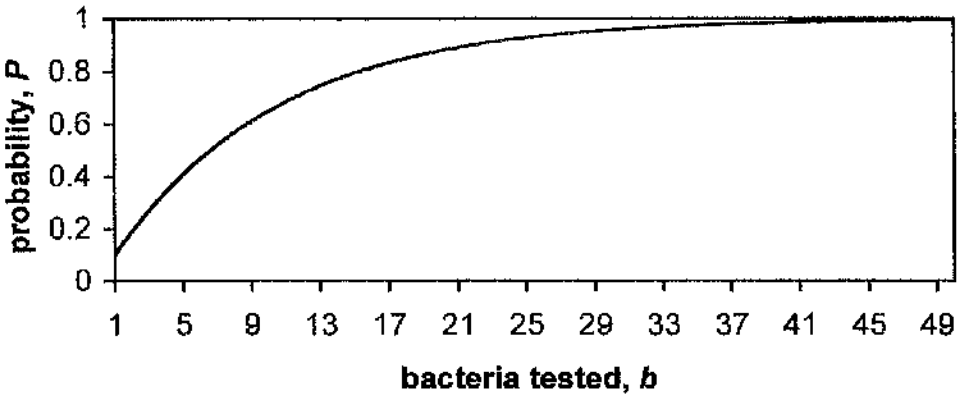


Figure 8.3 Plot showing the relationship between probability of detection, *P*, and number of bacteria tested, *b*, in the individual animal probability model when prevalence, *p*, is 0.1.

Thus, it can be seen from the application of this individual animal probability model that in order to be confident of detecting resistance, high numbers of bacteria should be tested, particularly when the proportion of resistant bacteria is low.

8.2.2 Farm and Region Probability Models

Second, the detection of resistance in a group of animals was considered. The group of animals could represent a pen or batch of animals within a farm or an entire herd or flock.

8.2.2.1 Model Description

The individual animal probability model in Equation 8.1 was expanded to model the situation in a group of animals. The probability of detecting resistance in a group is modelled by Equation 8.2.

$$P = 1 - \left[(1 - p_1)^{b_1} x \ldots\ldots x (1 - p_n)^{b_n} \right] \qquad \textbf{Equation 8.2}$$

where P is the probability of detecting at least one resistant bacterium in a group of animals, p is the prevalence of resistant bacteria, b is the number of bacteria tested from each animal and n is the number of animals tested.

In this instance, the probability of detecting resistance in the group is equal to one minus the probability that for each animal sampled, all the bacteria tested are not resistant and p and b may be different for individual animals. The group model was further developed to model the situation in a region (Equation 8.3).

$$P = 1 - (1 - p)^{bnf} \qquad \textbf{Equation 8.3}$$

where P is the probability of detecting resistance in a region, p is the prevalence of resistant bacteria and assumed constant for each animal and farm, b is the number of bacteria tested from each animal, n is the number of animals tested from each farm and f is the number of farms tested.

Note that in this model it is assumed that there is no animal to animal variation in the prevalence of resistant bacteria.

8.2.2.2 Model Analysis

Again, the effect of alterations in the prevalence of resistance and in the numbers of bacteria tested per animal on the probability of detecting resistance can be assessed. When the aim was to detect resistance at the farm level with the number of bacteria tested per animal set at 1, the probability of detection could be improved by increasing the number of animals tested (Figure 8.4), whilst at region level, with the number of bacteria tested per animal set at 1 and the number of animals tested per farm set at 2, the probability of detection could also be improved by increasing the number of farms tested (Figure 8.5). However, when the prevalence of resistance was low, e.g., 0.05, even when up to 7 animals (Figure 8.4) or 8 farms (Figure 8.5) were tested, the probability of detection was still poor, at 0.30 and 0.56 respectively.

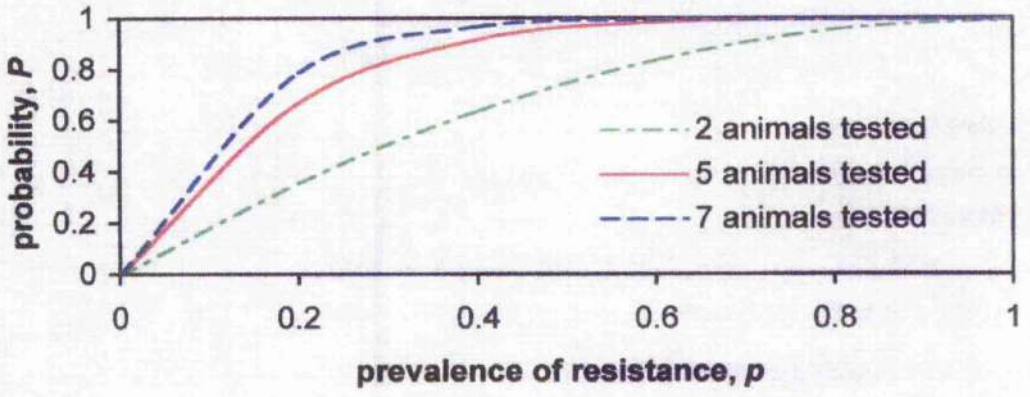


Figure 8.4 Probability of detecting resistance on a farm with one bacterium tested per animal.

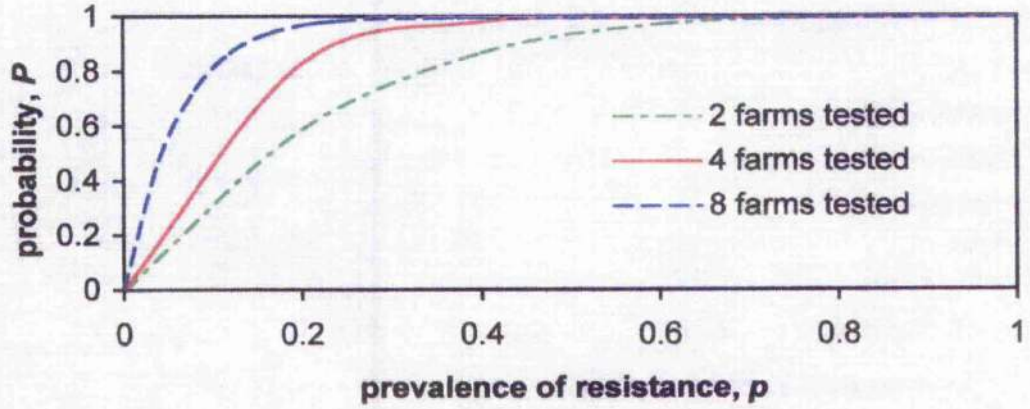


Figure 8.5 Probability of detecting resistance in a region with one bacterium tested per animal and two animals tested per farm.

8.3 Stochastic Models

8.3.1 Group Model with Variation in Prevalence

Stochastic models allow for the incorporation of random variation to input variables. Therefore, any of the probability models described above can be converted to stochastic models by introducing variation. For example, using the group probability model (Equation 8.2) the effect of using three different sampling regimes on the probability of detecting resistance (sampling 2 animals, 5 animals or 7 animals) was considered. At each point, the probability of detecting resistance was calculated for a given prevalence of resistance using each sampling regime. However, in this approach the assumption was made that the prevalence of resistant bacteria was the same for every animal in the group.

In reality, it is likely that there will be random variation in the prevalence of resistant bacteria between animals so that if the mean prevalence in the group is 0.01, some animals will have a higher proportion of resistant bacteria and some will have a lower proportion. This variation can be modelled by substituting the point estimates of prevalence with a probability distribution. For example, if one considers the mean prevalence in the group of animals to be 0.01 and to be Normally distributed, the model output becomes as shown (Figure 8.6). As the output generated is also a probability distribution, probability intervals can be placed around it. The spread of the output is dependent on the variance used for the Normal distribution. In the models that follow, when the Normal distribution is used with a mean proportion of resistant bacteria p, the binomial variance $p(1-p)$ has been assumed.

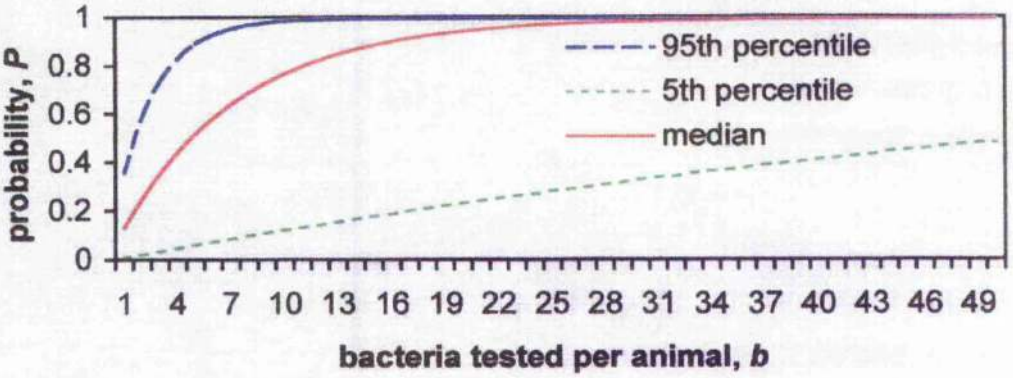


Figure 8.6 Probability of detecting resistance on a farm with two animals tested and prevalence Normally distributed with a mean of 0.01.

The model suggests that if 10 bacteria are tested from each of two animals from a group where the prevalence is Normally distributed with mean 0.01, on average the probability of detection will be 0.66. However, as the model output was generated by simulation, on 5 percent of occasions the same sampling regime gave a probability of detection of 0.98 or above and on another 5 percent of occasions the probability of detection was 0.11 or less. Thus, incorporating variability in one of the inputs has enabled the modelling of the magnitude and probability of the variation that might be seen in the model output and in this case there is considerable variability in the probability of detection.

8.3.2 Incorporating Test Performance

Having explored various sampling regimes for different population levels: animal, farm and region, and also having examined how variation in the prevalence of resistance might affect the probability of detection, the next phase in developing the model was to consider what other sources of variation contributed to the model

output. In all the models discussed thus far, the test for resistance was assumed to be perfect. In reality, it is likely that whatever test is used will not be perfect and will detect resistant bacteria with an associated sensitivity and specificity. Therefore, the model was developed further to incorporate test performance.

Two approaches were considered.

8.3.2.1 Algebraic Solution

Taking test performance into account, the probability of at least one bacterium testing resistant is equal to 1 minus the probability that all the bacteria tested test negative for resistance. The probability of a bacterium testing negative for resistance is equal to the probability that it is truly not resistant (1-p) and is correctly identified as such by the test (the test specificity Sp), or, it is truly resistant (p) and is incorrectly identified as not resistant (1- Se where Se is the sensitivity), (Equation 8.4).

$$P = 1 - \left[(1-p)Sp + p(1-Se)\right]^N \qquad \textbf{Equation 8.4}$$

where P is the probability of detecting resistance in a group of animals, p is the prevalence of resistance, Sp is the test specificity, Se is the test sensitivity and N is the number of bacteria tested.

8.3.2.2 Modelling Approach

An alternative approach considered was to use stochastic simulation to model the number of truly resistant bacteria or true positives, and truly sensitive bacteria or true negatives, in a bacterial population and to use this as a basis for estimating the

probability of detection. In this instance, the probability that at least one bacterium will test positive for resistance is equal to one minus the probability that all the truly sensitive bacteria test negative for resistance and all the truly resistant bacteria also test negative for resistance (Equation 8.5).

$$P = 1 - Sp^{y}\left(1 - Se\right)^{x} \qquad \textbf{Equation 8.5}$$

where P is the probability of detecting resistance in a group of animals, Sp is the test specificity, Se is the test sensitivity, x is the number of truly positive bacteria tested and y is the number of truly negative bacteria tested.

The number of truly negative bacteria and truly positive bacteria tested could then be modelled by sampling from a Binomial distribution where p is the probability of a bacterium being truly resistant and b is the number of bacteria tested. The number of resistant and sensitive bacteria simulated, x and y, could then used as model inputs (Equation 8.5). However, by considering the factors influencing the numbers of resistant and sensitive bacteria sampled, prevalence of resistance and number of bacteria tested, it was possible to adjust the model so that stochastic simulation was not required in order to estimate the number of truly positive and truly negative bacteria in a sample and it was felt that this was a more accurate approach (Equation 8.6).

$$P = 1 - Sp^{N(1-p)}(1 - Se)^{Np}$$ **Equation 8.6**

where P is the probability of detecting resistance in a group of animals, p is the prevalence of resistance, Sp is the test specificity, Se is the test sensitivity and N is the number of bacteria tested.

When the exact model (Equation 8.4) and approximate model (Equation 8.6) were compared, given the same inputs for prevalence, bacteria tested, test sensitivity and test specificity, slightly different outputs were generated, as expected (Figure 8.7).

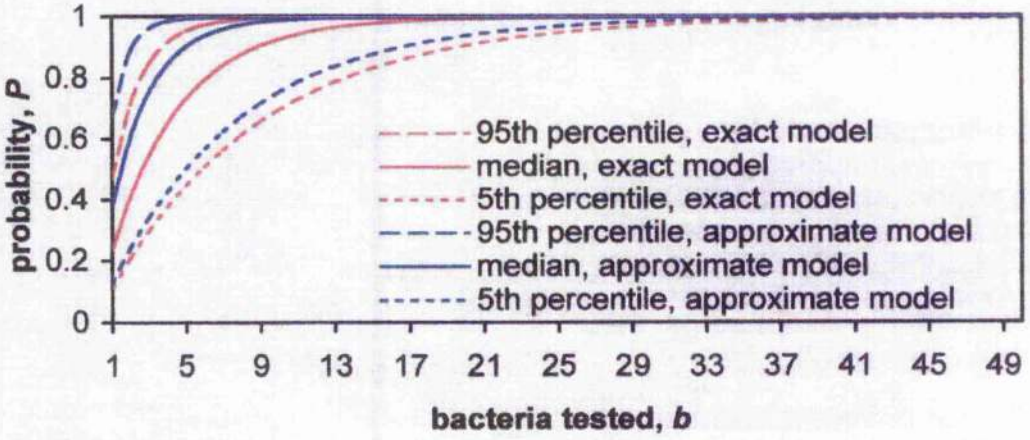


Figure 8.7 Probability of detecting resistance in a group of animals when 1 animal is tested, prevalence of resistance is Normally distributed with mean 0.05, sensitivity is 0.9 and specificity is 0.9.

This can be explained by the fact that the algebraic model is more accurate as it is not dependent upon the numbers of truly positive and truly negative bacteria in a sample and the approximate model is simply an average model.

It was decided that, as the precise model was more accurate, this model should be the one used as a basis for further development and as the model output is the probability

of at least one bacterium testing resistant it will be referred to as the test positive-model.

8.3.3 Variation in prevalence, sensitivity and specificity

Having developed a basic model that incorporated test performance, it was possible to manipulate the model inputs and analyse the effect on model output. Just as prevalence of resistant bacteria is likely to vary from animal to animal, there is also likely to be variation in the estimated test sensitivity and specificity. This variation includes uncertainty as well as variability. Variability refers to the random variation in test performance each time the test is used and may be influenced by the type of test used - several different methods of testing bacteria for resistance are available (Greenwood, 2000), and also by variation in test conditions. In disc diffusion testing for instance, this might include variation in inoculum density and volume, depth of agar, incubation temperature and time, and operator variation. Uncertainty refers to the level of ignorance about test performance. In the case of antimicrobial resistance testing, there is very little quantitative data available on test performance or variability. For this reason, variability and uncertainty were considered together in the models that follow and the probability distributions chosen modelled the total uncertainty surrounding parameter estimates.

The incorporation of variability in prevalence made it possible to simulate what might happen when animals from a population such as a pen or farm, are sampled as opposed to an individual animal. When a probability distribution was used as the input prevalence in the test positive-model the probability of detection modelled was the probability of detecting resistance in a group of animals when a number of bacteria from one animal were tested, or, a number of bacteria from a thoroughly

mixed sample from all the animals in the group were tested. An example of a mixed group sample could be slurry. One of the components of variation in the model output is therefore the variation that exists in the underlying animal population.

By introducing variation in test performance parameters as well as variation in prevalence, the magnitude of variation in model output was also increased (Figure 8.8).

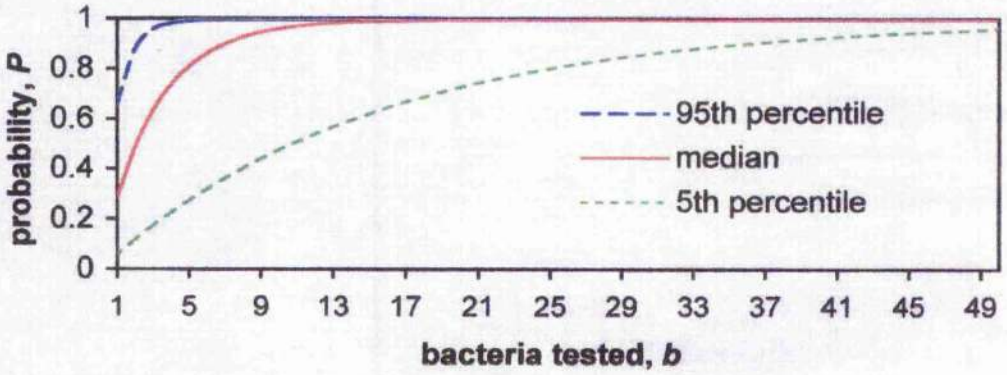


Figure 8.8 Probability of detecting resistance when prevalence is Normally distributed with mean 0.01, sensitivity is Normally distributed with mean 0.9, Binomial variances are assumed for the Normal distribution and specificity is Normally distributed with mean 0.9.

For example, the model suggests that if 5 bacteria were tested, the 90 percent probability interval for detecting resistance would be large, lying between approximately 0.279 and 0.996. Therefore, if the uncertainty surrounding test parameters could be reduced, the variability in model output could also be reduced.

8.3.4 Estimating number of bacteria to be tested

All the models discussed have been manipulated to estimate the probability of detecting resistance given a certain set of conditions such as prevalence, bacteria

tested, animals tested and test performance etc. However, a more useful manipulation of the model is to consider the design of a sampling regime to detect resistance with a given probability. In order to do this, the test positive-model (Equation 8.4) was rearranged, (Equation 8.7).

$$N = \frac{\log(1-P)}{\log\left[(1-p)Sp + p(1-Se)\right]}$$ **Equation 8.7**

This model was then used to estimate the number of bacteria that must be tested to attain a 95% probability of detecting resistance, for three different prevalence levels with a Normal distribution (mean 0.001, 0.01 and 0.05) and three different levels of test sensitivity and test specificity (Normal distribution with mean 0.4, 0.7 and 0.9), (Table 8.1). The Normal distribution was chosen to model prevalence of resistance because it was thought to be a good representation of a commensal bacterial population in the absence of selection pressure from antimicrobial use, with very few animals having a high proportion of resistant bacteria and most having a low proportion of resistant bacteria close to the mean value (Danmap, 2001; Humphry *et al.*, 2002). As before, the variance used was associated with the binomial distribution i.e. if mean is p, variance is $p(1-p)$. The Normal distribution was chosen to model test sensitivity and specificity to account for random variation in test performance and the median number of bacteria to be tested was considered the most appropriate measure because the distribution of sample size from the simulation was skew.

Table 8.1 To nearest whole number, median (5^{th} percentile, 95^{th} percentile) number of bacteria that must be tested in order to attain a probability of detection of at least 95% for various prevalences, test sensitivities and test specificities modelled using Normal distributions with binomial variance.

		Test Specificity, *Sp*		
Prevalence, *p*	Test Sensitivity, *Se*	0.4	0.7	0.9
0.001	0.4	4 (1, 31)	6 (1, 48)	10 (3, 86)
0.01	0.4	4 (1, 25)	6 (2, 37)	10 (3, 53)
0.05	0.4	4 (1, 21)	5 (2, 29)	9 (3, 41)
0.001	0.7	4 (1, 30)	6 (1, 46)	10 (3, 79)
0.01	0.7	4 (1, 25)	5 (1, 34)	10 (3, 53)
0.05	0.7	4 (1, 17)	5 (2, 24)	8 (3, 40)
0.001	0.9	4 (1, 29)	6 (1, 44)	10 (3, 72)
0.01	0.9	4 (1, 21)	5 (1, 29)	9 (3, 42)
0.05	0.9	3 (1, 16)	5 (1, 20)	7 (3, 33)

However, in order to represent a commensal population following the withdrawal of an antimicrobial from use, a Gamma distribution was used to account for small numbers of animals with a higher proportion of resistant bacteria that may persist following withdrawal. The analysis was repeated using two prevalence levels with a Gamma distribution (mean 0.05, mode 0.001 and mean 0.05, mode 0.01) (Table 8.2).

Table 8.2 To nearest whole number, median (5th percentile, 95th percentile) number of bacteria that must be tested in order to attain a probability of detection of at least 95% for various prevalences, test sensitivities and test specificities with prevalence modelled by Gamma distributions and test sensitivity and specificity modelled by Normal distributions with binomial variance.

		Test Specificity, *Sp*		
Prevalence, *p*: mode, mean	Test Sensitivity, *Se*	0.4	0.7	0.9
0.001, 0.05	0.4	4 (1, 30)	6 (2, 46)	10 (3, 82)
0.010, 0.05	0.4	4 (1, 29)	6 (1, 42)	10 (3, 67)
0.001, 0.05	0.7	4 (1, 27)	5 (1, 43)	10 (3, 66)
0.010, 0.05	0.7	4 (1, 26)	6 (1, 42)	10 (3, 66)
0.001, 0.05	0.9	4 (1, 26)	5 (1, 36)	9 (3, 56)
0.010, 0.05	0.9	4 (1, 23)	5 (1, 39)	9 (3, 63)

This manipulation of the model highlighted a number of points. First, the number of bacteria to be tested was greatest when specificity was high, sensitivity was low and prevalence was low. Increasing mean prevalence from 0.001 to 0.05 had only a limited effect on the median number of bacteria it was necessary to test. For example, reducing the number to be tested from 6 to 5 when sensitivity was 0.9 and specificity was 0.7 (Table 8.1). However, increasing prevalence did significantly reduce the number it was necessary to test to have a probability of detection on 95% of occasions - from 44 to 20 using the same test parameters.

Similarly, increasing test sensitivity had very little effect on the median number of bacteria to be tested but had a greater effect on the 95th percentile. For example, increasing sensitivity from 0.4 to 0.9 reduced the bacteria to be tested from 53 to 42 when specificity was 0.9 and mean prevalence was 0.01, (Table 8.1).

However, the model suggests that test specificity is very important in determining the number of bacteria it is necessary to test. When specificity was 0.9 and mean prevalence was 0.001, 86 bacteria had to be tested to be confident of detecting resistance as compared to 31 bacteria when specificity was 0.4 (Table 8.1).

Finally, the estimates for the number of bacteria that must be tested (median, 5th percentile and 95th percentile) were very similar in Table 8.1 and Table 8.2, suggesting that the choice of underlying distribution for prevalence (Normal or Gamma) is less important than the mode prevalence itself.

8.3.5 Decay of Resistance

In the models described thus far, a static estimate of mean prevalence, with and without an underlying distribution, has been used as an input. This has enabled the consideration of what sampling regimes might be appropriate at a particular point in time. However, unless antimicrobial use in a population of animals is constant, it is likely that the proportion of resistant bacteria will change with time, either reducing or increasing depending on factors influencing bacterial growth and fitness. A particular scenario in which the prevalence of resistance might change is following the withdrawal of an antimicrobial that has previously been in constant use such as an antimicrobial growth promoter.

Two types of change in the prevalence of resistance were considered – linear decay (Figure 8.9) and log linear decay (Figure 8.10).

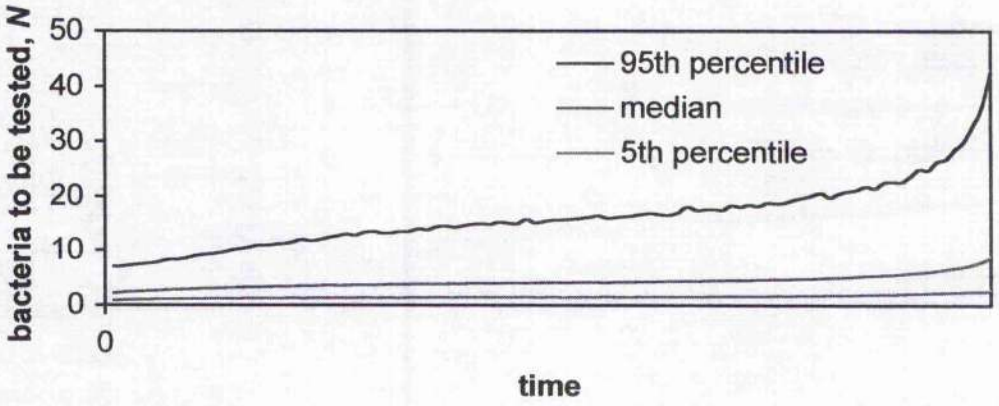


Figure 8.9 Number of bacteria that must be tested to attain 95% probability of detection when prevalence is Normally distributed and decays linearly from a mean of 1; sensitivity is Normally distributed with a mean of 0.9 and specificity is Normally distributed with a mean of 0.9.

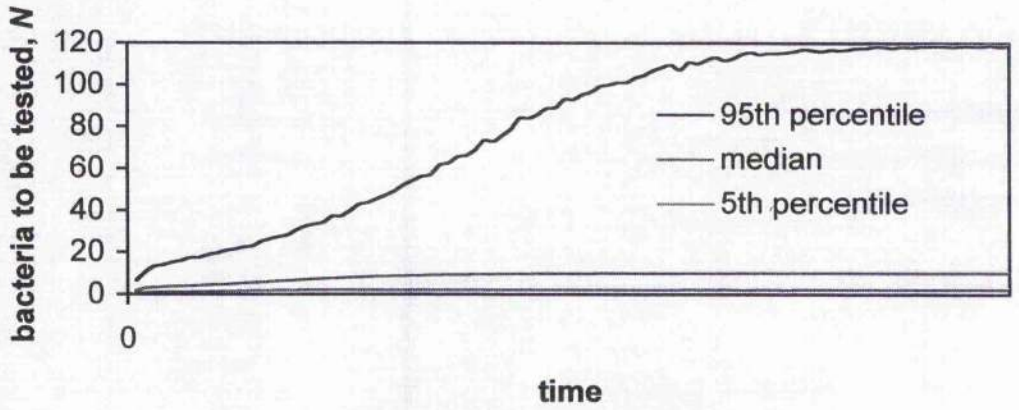


Figure 8.10 Number of bacteria that must be tested to attain 95% probability of detection when prevalence is Normally distributed and decays log linearly from a mean of 1; sensitivity is Normally distributed with a mean of 0.9 and specificity is Normally distributed with a mean of 0.9.

Regardless of how mean prevalence decays, more bacteria need to be tested as mean prevalence decreases and for the same mean prevalence, the same number of bacteria

must be tested in either case. Prevalence reaches a minimum of only 0.01 in Figure 8.9 compared to 1.721×10^{-8} in Figure 8.10 and therefore the number of bacteria that must be tested is much higher at this point in Figure 8.10.

As prevalence declines, the variability in probability of detection also increases. Therefore, the number of bacteria it is necessary to test to be confident of detecting resistance increases not only because prevalence is lower but also because uncertainty about model output is higher. When mean prevalence was 0.99, the 90 percent probability interval of detection lay between 1 and 7 bacteria whereas when mean prevalence was only 0.03 it lay between 3 and 33 bacteria.

Just as in the models considering a static prevalence, test performance is very important in determining how many bacteria must be tested. When specificity was poor, the number of bacteria to be tested actually decreased initially as prevalence decreased before rising again (Figure 8.11).

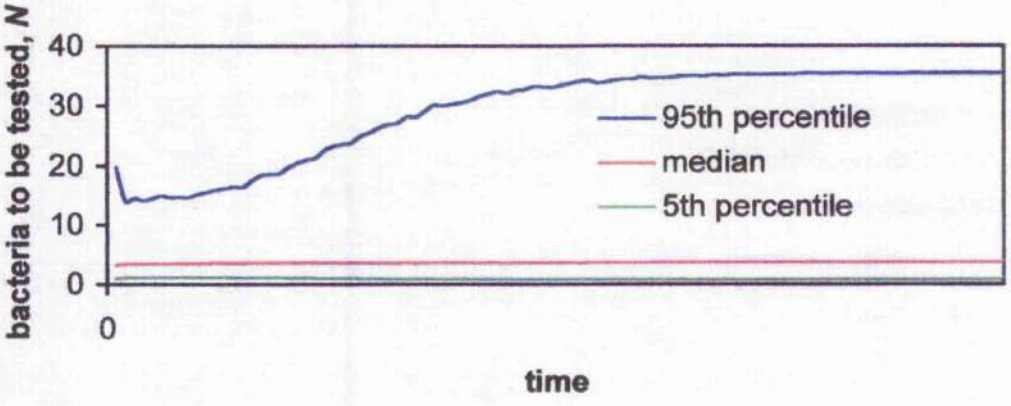


Figure 8.11 Number of bacteria that must be tested to attain 95% probability of detection when prevalence is Normally distributed and decays log linearly from a mean of 1; sensitivity is Normally distributed with a mean of 0.7 and specificity is Normally distributed with a mean of 0.4.

At high prevalence levels, very few truly negative bacteria are present and the detection of resistance depends largely on test sensitivity whereas as prevalence decreases, some truly negative bacteria are misclassified as resistant, reducing the number of bacteria that need to be tested for at least one to test resistant. This effect was less apparent when test sensitivity was increased to 0.9 and test specificity was increased to 0.7 (Figure 8.12). However, it was still important at very high prevalence levels, where, for example 9 bacteria must be tested to be confident of detecting resistance when mean prevalence was 0.99 compared to 7 when mean prevalence was 0.83.

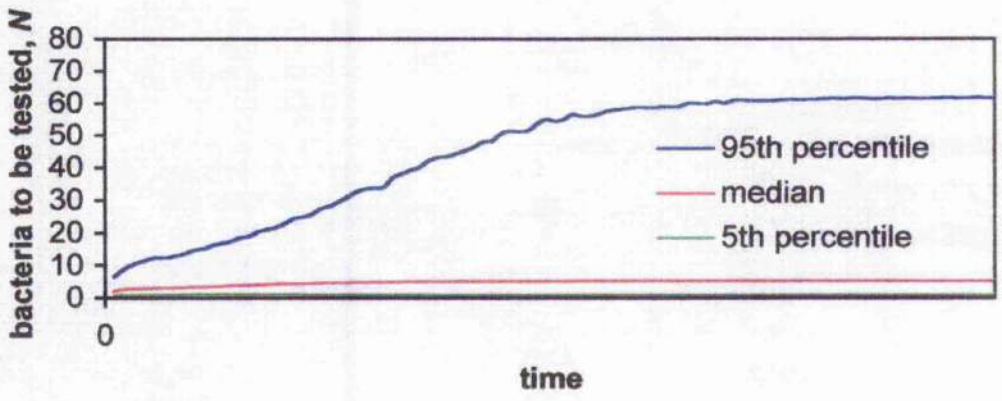


Figure 8.12 Number of bacteria that must be tested to attain 95% probability of detection when prevalence is Normally distributed and decays log linearly from a mean of 1; sensitivity is Normally distributed with a mean of 0.9 and specificity is Normally distributed with a mean of 0.7.

Higher test specificity generally increases the number of bacteria it is necessary to test. For example, when test specificity was 0.7 and sensitivity was 0.9 (Figure 8.12), 51 bacteria must be tested when the mean prevalence was 0.01. When test specificity and sensitivity were both 0.9 (Figure 8.10) 91 bacteria must be tested. However, in the former case, the requirement is reduced simply because of falsely positive test results.

As in the previous models, increasing test sensitivity also reduced the number of bacteria it was necessary to test but when the criterion for detection was that at least one bacterium tests resistant, this was less important than test specificity.

8.3.6 Detecting True Positives

When the definition of detecting resistance is that at least one bacterium tests resistant and the test specificity is not perfect, it is inevitable that resistance will, on occasion, be wrongly "detected" as described in the above models. This is how antimicrobial resistance data are currently interpreted. However, when imperfect tests are used to detect resistance, predictive values of the test should be considered. In the context of antimicrobial resistance, the positive predictive value of a test is the proportion of bacteria that test resistant that are truly resistant. This depends on test sensitivity, test specificity and prevalence and is represented by Equation 8.8 (Thrusfield, 1995).

$$PPV = \frac{pSe}{pSe + (1-p)(1-Sp)} \qquad \textbf{Equation 8.8}$$

If the test specificity is low, sensitivity is low, or prevalence is low, then the positive predictive value of the test will be poor.

However, if the concern is the probability of detecting truly resistant bacteria only, test specificity does not matter. For an individual bacterium, the probability of it being resistant and being detected as resistant is dependent upon test sensitivity and prevalence, (Equation 8.9).

$$P = pSe$$ **Equation 8.9**

where P is the probability of a bacterium being resistant and testing resistant.

For a group of bacteria, the probability of at least one bacterium being resistant and being detected is equal to one minus the probability that they are all sensitive or not detected, (Equation 8.8).

$$P = 1 - \left(1 - pSe\right)^{n}$$ **Equation 8.8**

where P is the probability that at least one bacterium is resistant and tests resistant and n is the number of bacteria tested.

The model of the probability that at least one bacterium is resistant and is detected was compared with the test positive model of the probability that at least one bacterium tests resistant (Figure 8.13). When the model output was the detection of at least one true positive rather than at least one test positive, unsurprisingly, the probability of detection was poorer for the same number of bacteria tested, sensitivity and prevalence. Using the test positive model, on average testing 9 bacteria gave a probability of detection of 95% but on average approximately 60 bacteria had to be tested to achieve a probability of detection of 95% when the true positive model was used (Figure 8.13). In order to achieve a similar probability of detection on 95% of occasions (5^{th} percentile), using the test positive model testing 71 bacteria gave a probability of detection of 95.0% whereas using the true positive model, even testing 100 bacteria only gave a probability of detection of 34.3%. Therefore, the models developed suggest that if the detection of resistance depends upon the detection of at least one true positive rather than at least one test positive, the numbers of bacteria tested must be increased dramatically. In consideration of decay, the same principles

hold good for the true positive model as did for the test positive model, i.e., time merely reflects reduced prevalence and the arguments regarding sampling must be built around a consideration of acceptable prevalence.

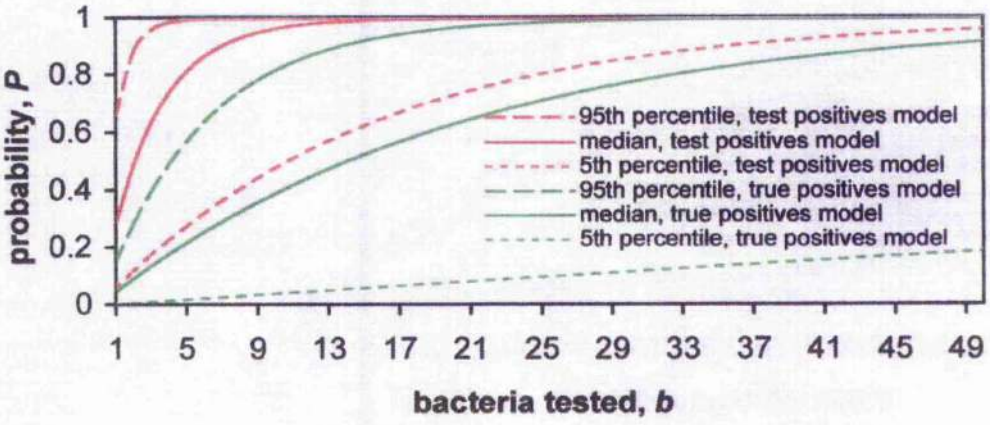


Figure 8.13 Probability of detecting resistance based on the test positives model and the true positives model when prevalence of resistance is Normally distributed with a mean of 0.01, test sensitivity is Normally distributed with a mean of 0.9 and for the test positives model, test specificity is Normally distributed with a mean of 0.9.

8.4 Discussion

The models described have shown how the application of mathematical and statistical techniques such as simulation modelling to the problem of antimicrobial resistance is essential if test systems are to be properly implemented.

First, it demonstrates the factors that should influence the design and interpretation of antimicrobial resistance studies. The prevalence of resistant bacteria and the numbers of bacteria and animals tested have been shown to be important considerations. Moreover, the importance of considering the different population levels, such as

bacterium, animal, farm or region, and of defining the output of antimicrobial resistance studies in relation to them, have been demonstrated and this is in agreement with the suggestions of other authors (Davison *et al.*, 2000; Humphry *et al.*, 2002).

The performance of diagnostic tests used in antimicrobial resistance studies has also been shown to be a significant consideration. The sensitivity and specificity of these tests have been shown to have a strong influence on the number of bacteria it is necessary to test and the probability of detection and although test performance in survey design has been considered before, this has mainly been to determine the number of animals to be tested to be confident that a herd is free from disease (Cannon, 2001) and has largely been ignored in the design of antimicrobial resistance studies in animals. The problems of standardisation of susceptibility testing methods have been acknowledged and it has been suggested that even if this were achieved, "standard" does not mean "correct" (Greenwood, 2000). However, many diagnostic tests used in veterinary and human medicine are not perfect but their interpretation is aided by knowledge of their sensitivity and specificity and the absence of a perfect test for resistance should not preclude attempts to define test performance, as this information is essential if accurate conclusions are to be drawn from surveillance studies.

Second, the models have highlighted how variability in these contributing factors has a significant effect on resistance detection. Animal to animal variation and the uncertainty and variability surrounding estimates of test performance must be considered. Calculating sample numbers based on an "average" situation could lead to a significant under-estimate of the numbers required and allow the findings to be misinterpreted. The variability in proportion of resistant bacteria between sub-populations of animals and between individual animals within these populations has

rarely been considered but is thought to be significant (Dunlop *et al.*, 1999) and given the findings of the models described, such variation would significantly influence the estimated prevalence of resistance and should influence the design of sampling regimes. The degree of heterogeneity in commensal bacterial populations with respect to resistance has not often been quantified but recent data suggest it is significant in *E. coli* (Dunlop *et al.*, 1998c and 1999; Humphry *et al.*, 2002) and the current practice of selecting small numbers of colonies from a bacterial population of millions is wholly inadequate.

Furthermore, variation in the prevalence of resistance also occurs with time and the dynamics of bacterial populations are complex (Lipsitch and Levin, 1997a). The effects of both linear and log-linear decay in resistance were considered but the rate of decay was not defined because this would vary for different antimicrobials and different organisms and would be influenced by many factors as discussed in Chapter 1. Applying the models to the detection of resistance when prevalence is declining has indicated that the longitudinal monitoring of resistance in populations requires careful consideration of sample size in relation to prevalence. Although statistical methods such as time series analysis have been used to monitor changes in the prevalence of resistance over time in hospitals (Lopez-Lozano *et al.*, 2000), this has been based on clinical isolates alone, and has not taken into account the bacterial population as a whole. The findings also suggest that resistant bacteria that persist at low levels in a population may be extremely difficult to detect even if large numbers of bacteria are tested. This means that studies attempting to quantify the decline in resistance following the withdrawal of an antimicrobial from use should be interpreted with caution and findings related to the number of bacteria tested. Failure to detect resistant

bacteria may be just that, a failure of detection rather than a true reflection of the resistance status of the population.

The comparison of models that defined the detection of resistance as the detection of at least one test positive with models that defined the detection of resistance as the detection of at least one true positive, highlighted important differences. The test positive model reflects current practice in the interpretation of antimicrobial resistance data (BSAC, 1991; NCCLS, 1999; Bager, 2000; Martel *et al.*, 2000; Wray and Gnanou, 2000). However, the true positive model suggests a more appropriate way to interpret these data given that the tests used are imperfect. Just as resistant bacteria may be missed due to insufficient sample sizes and poor test sensitivity, susceptible bacteria may be misclassified as resistant and therefore it is unreasonable to accept that the testing of one bacterium from an animal can confirm the presence of resistance. This is likely to be particularly important when commensal bacteria are being considered, as the bacterial population does not necessarily expand clonally as happens when a pathogenic organism infects an animal and therefore the variation in the underlying bacterial population is likely to be greater (Craven and Barnum, 1971; Linton *et al.*, 1978; Langlois *et al.*, 1983). The true positive model also suggests that ideal sample sizes for antimicrobial resistance studies should be further increased to account for the occurrence of bacteria that falsely test positive.

Although the models described have been shown to be useful in highlighting current deficiencies in the design and interpretation of antimicrobial resistance studies, epidemiological modelling techniques are of limited use when considered alone. Whilst these techniques have been used as described to explore theoretical scenarios, the usefulness of the models developed would be increased by the generation of accurate, quantifiable data from laboratory testing and this is a recognised deficiency

of current antimicrobial resistance surveillance (Wray and Gnanou, 2000). Model inputs could be based on these data and sample size estimates calculated with greater accuracy. Currently, the lack of consensus on definitions of resistance, the poor knowledge of the performance of diagnostic tests and the failure to relate organisms tested with underlying bacterial and animal populations are limiting the utility of field data for this purpose.

Nonetheless, the models developed are significant because they clearly demonstrate the epidemiological factors to be considered when designing sampling regimes for antimicrobial resistance studies, including the consideration of different population strata and the performance of diagnostic tests. In addition, they indicate the magnitude of testing required in order to ensure that such studies withstand statistical scrutiny. Although the technology and resources to carry out the level of testing suggested may not be available, ideal sample sizes should not be ignored.

Some sensible conclusions from the models developed that should be considered in studies to detect antimicrobial resistance are as follows: 1. the clear definition of the aims of a study at the outset, including the bacterial species and antimicrobial of interest, as this influences many inputs from the prevalence of resistance to the most suitable susceptibility testing method; 2. knowledge of the performance of the tests used for resistance are also essential to the design of an appropriate sampling regime, with the best test available being used as poor tests make the interpretation of test results very difficult as well as increasing sample numbers; 3. ideally, the degree of variation in the underlying bacterial and animal populations of interest should also be taken into account and the difference between pathogenic and commensal bacteria in this respect demonstrates the importance of this; 4. the acceptable level of detection is also critical to the sample numbers required and a sensible aim would perhaps be to

detect resistance at the 5 percent prevalence level; 5. where the aim is to detect smaller numbers of resistant bacteria, the sample numbers required and therefore financial resources necessary increase dramatically and this must be accepted as the over-interpretation of data based on insufficient sample numbers is misleading; 6. finally sample numbers should be calculated using standard statistical methods once these factors have been taken into account.

Testing on the scale suggested is perhaps unlikely to be implemented widely due to the nature of current laboratory practices and the financial implications of testing large numbers of bacteria and animals. Although some consideration has been given to assessing resistance in bacterial populations using novel methods such as spiral plating and hydrophobic grids (Dunlop *et al.*, 1998c; Humphry *et al.*, 2002) laboratory techniques must be advanced in order to reduce labour-intensity as well as to improve accuracy. However, despite such practical problems, the application of epidemiological models and statistical theory must be considered when designing rational surveillance programs for antimicrobial resistance in animal populations.

CHAPTER 9

GENERAL DISCUSSION

9.1 General Discussion

The aim at the outset of this work was to consider resistance to the growth promoting antimicrobial avilamycin, including the measurement of resistance in the field. To some extent these aims have been achieved but, more importantly, in attempting to meet these objectives several methodologies have been explored and many important questions relevant to antimicrobial resistance studies of any kind have been highlighted.

First, the application of standard statistical formulae to sample size calculations for resistance studies suggested the scale of sampling required if the measurement of resistance is to be addressed seriously. Although the application of standard statistical formulae has been suggested before (Davison *et al.*, 2000) the consequences for resistance surveillance have not been explored. This requires the clear definition of the aims of surveillance at the outset including the animal population, the bacterial species and the antimicrobial of interest, the achievement of simple random sampling or where this is not achievable the necessary adjustments to sample calculations, and the consideration of test performance. Test performance has been considered with regard to herd disease status (Jordan and McEwen, 1998) but has received only very limited consideration in antimicrobial resistance studies (NCCLS, 1999). This not only has significant cost and labour implications for future surveillance but also suggests the inadequacy of much of the surveillance data on which conclusions on antimicrobial resistance transfer via the food chain and legislative decisions have been

based (Fidler, 1999; Acar *et al.*, 2000). In a small study with limited resources in terms of labour one could not hope to achieve the sample numbers suggested and therefore the sample design became a compromise between what was ideal and what was practical but there is no question that the strength of the conclusions were weakened as a result. The practical problems of adhering to sampling designs were also highlighted with the variability and inconsistent availability of faecal material on farms a major barrier to achieving suggested sample numbers as well as to achieving simple random sampling. The findings suggest that the best that can be hoped for from on-farm environmental sampling is detection of the presence of antimicrobial resistance rather than quantification in any way.

Second, several important conclusions from the initial studies related specifically to *Enterococcus spp.* and these are significant because this genus has been the focus of much of the surveillance of resistance to antimicrobial growth promoters in food animals (Wegener *et al.*, 1999; SVARM, 2000; Danmap, 2001). The use of Slanetz and Bartley medium was shown to be effective in the isolation of enterococci with sensitivity of isolation at least as high as that reported by other authors (Devriese *et al.*, 1994; Danmap, 2001). However, colonial morphology was shown to be a poor indicator of species and to be insufficient in itself to confirm genus and this seriously affected the use of conventional colony-counting techniques for enumerating bacteria. Counts were shown to be unreliable not only because of the poor specificity of the medium but also because of the subjectivity of assessing the visual appearance of colonies These factors made the quantification of resistant enterococci, either actual or proportionate, very difficult and although these methods have been applied to assessing faecal contamination of water supplies (Pagel and Hardy, 1980) this suggests that they are inadequate for accurately quantifying resistant bacteria.

Improved methods of enumerating bacteria for the purpose of antimicrobial resistance studies have been suggested (Dunlop *et al.*, 1998c; Humphry *et al.*, 2002) but these have focussed on *E. coli* and the limitations of the isolation media available for enterococci may limit their adaptation to this genus.

A variety of enterococcal species including *E. faecium*, *E. faecalis* and *E. durans* was isolated with different species apparently predominating on different farms. Variation in the enterococcal flora of pigs has been suggested before (Devriese *et al.*, 1994) but the majority of surveillance of antimicrobial resistance has been based on *E. faecium* and *E. faecalis* (SVARM, 2000; Danmap, 2001). The confirmation of avilamycin resistance in species other than *E. faecium* and *E. faecalis* and in particular in *E. durans* was also significant because it again emphasises the importance of accurately speciating enterococci but also raises the question of the relevance of these species to public health. It would seem logical that species other than *E. faecium* and *E. faecalis* are capable of being a reservoir of resistance even if they are less common pathogens (Gilad *et al.*, 1998; Devriese *et al.*, 2002) and this also suggests a possible deficiency in the current approach to resistance surveillance. Although insufficient enterococci were isolated to carry out a detailed comparison of resistance patterns amongst individual species, the findings suggest that resistance to therapeutic antimicrobials is perhaps more prevalent in *E. faecium* than in other enterococcal species and therefore that resistance data should not be compared unless isolates have been carefully speciated.

The majority of enterococci isolated were sensitive to avilamycin by conventional classification (MIC ≤ 16μg/ml) (Danmap, 2001), but the fact that no clear bimodal distribution of MICs was observed highlighted the problem of choosing a breakpoint MIC for resistance to growth-promoters. This method has been described as the only

suitable means of determining breakpoints to growth promoting antimicrobials but it is accepted that the distinction between resistant and sensitive populations may not always be clear (Butaye *et al.*, 2003) and this is an issue that must be clarified if resistance is to be measured in bacterial populations. It might be agreed that the breakpoint chosen should depend on the aims of the study, with a low breakpoint of say 8μg/ml used when the aim is to detect all resistant organisms. However, these data cannot then necessarily be used to quantify the proportion of resistant bacteria in a sample.

The conventional technique of MIC determination in individual isolates on which most surveillance data is based was shown to be wholly inadequate to detect resistance when the prevalence of resistance was low. This suggests that this method is unsuitable for the longitudinal monitoring of resistance as low numbers of resistant organisms would be missed. However, these are exactly the sort of data that have been used to examine the effects of the removal of antimicrobial growth promoters from use on bacterial resistance and to justify their withdrawal (Boerlin *et al.*, 2001; Aarestrup *et al.*, 2001).

Although no resistant isolates were detected using conventional MIC testing of individual isolates, avilamycin resistance was detected on a farm that had not used avilamycin for at least two years when screening plates were used, suggesting that resistance had either persisted for this time or been reintroduced and was present at a low prevalence. A similar method has recently been applied to the detection of vancomycin-resistant enterococci in broiler flocks and has also suggested that there has been no significant decline in vancomycin resistance in the five years following the ban on this growth promoter (Heuer *et al.*, 2002). These findings not only suggest that resistance can persist in commensal bacterial populations but also emphasise the

importance of using a sensitive method of detection if such resistant isolates are not to be missed.

The use of screening plates containing avilamycin was useful in confirming the presence of resistant organisms when the prevalence of resistance was very low but unfortunately the poor specificity of the media made this a highly sensitive but poorly specific test that could not be relied on without confirmation of the identity of the presumptive enterococcal isolates. A similar method has recently been used to assess resistance in faecal *E. coli* populations in conjunction with molecular methods to detect resistance genes, with the authors also suggesting that this approach offers advantages over conventional individual isolate MICs in assessing resistance in commensal bacteria (Blake *et al.*, 2003). However, despite the use of automated counters and the availability of isolation media highly specific to *E. coli* meaning that further confirmation of species identity was not performed, the labour intensity of performing such detailed analyses meant that faeces from only three animals could be examined fully or where animal numbers were increased to twenty, molecular analysis could be performed on only a small number of bacterial colonies. Therefore, whilst this method offers a means of improving bacterial sample numbers, it is not suitable for application to realistic animal sample numbers due to the labour involved in preparing media and in particular in culturing many colonies to purity and identifying them to species.

The small-scale studies on resistance to therapeutic antimicrobials (Chapter 6) highlighted the problem of attaining sufficient sample numbers to achieve statistical power. In these studies, standard statistical techniques were strictly applied to the results and very few firm conclusions could be drawn. It has been suggested elsewhere that legislative decisions regarding antimicrobial growth promoters have

been taken based on small data sets (Acar *et al.*, 2000). There was no evidence to suggest that avilamycin use was associated with resistance to therapeutic antimicrobials but the most important finding was probably the difficulty in proving clear association because of the many factors possibly influencing antimicrobial resistance. Again, it has been suggested elsewhere that these other factors have not always been taken into account before conclusions have been drawn from antimicrobial resistance surveillance data (Phillips, 1997). These findings highlight some of the limitations of interpreting field data, particularly when only small sample numbers are available or when resources are limited, and also when many uncontrolled factors may be influencing resistance.

In addition to the problems of confidently estimating the prevalence of resistance, an equally significant problem was how best to express the findings. Although it has been suggested that resistance should be related to underlying bacterial and animal populations (Davison *et al.*, 2000), the most appropriate way to do this has not been described. Should resistance be related to isolates or samples, or, in the case of commensal organisms that are not consistently isolated, to samples from which the species of interest was isolated? Whilst this may seem pedantic, the findings suggest that the prevalence of resistance suggested were quite different and could easily influence interpretation of resistance data. Perhaps the most appropriate measure is dependent on the aim of the study and the relevance of the organism. For instance, for enterococci the main significance of resistance is the potential for transfer to humans via faecal contamination of meat and therefore a faecal sample from which no resistant enterococci were isolated could be considered a significant negative finding. However, when dealing with commensal organisms it is assumed that they are

omnipresent in all animals and therefore a failure to isolate resistant enterococci may be due to poor isolation technique rather than the true absence of these organisms.

Another important finding of the farm studies on both enterococci and *Escherichia spp.* was the heterogeneity in the bacterial population with regard to resistance. This is a feature that has clearly been ignored by conventional microbiological methods designed to measure resistance in a clonally expanding pathogen (Hedges *et al.*, 1977; Humphry *et al.*, 2002; Blake *et al.*, 2003). The measurement of resistance in commensal organisms has been suggested for some time but only recently has the importance of measuring resistance in the bacterial population rather than in individual isolates been suggested (Blake *et al.*, 2003) and although the problem of how best to achieve this has not yet been resolved it should not be ignored. The recent application of mathematical modelling techniques to the problem of antibiotic resistance in commensal organisms in animals and man has suggested that very small increases in the prevalence of resistant bacteria in animals could have a significant and irreversible impact on the spread of resistant bacteria to humans and this has again highlighted the inadequacies of current surveillance practices suggesting that they would fail to detect such a change (Smith *et al.*, 2002).

Having attempted to quantify resistance using conventional microbiological techniques, the possibility of measuring resistance to avilamycin in enterococci by molecular techniques was explored following the detection of substitutions in ribosomal protein L16 conferring resistance to avilamycin by other authors (Aarestrup and Jensen, 2000). However, the findings suggest that resistance to avilamycin in commensal enterococci is very complex with more than one genetic mechanism responsible and this has since been confirmed by the description of other resistance mechanisms (Mann *et al.*, 2001). With the small time and labour available, it was not

possible to consider the genetic mechanism of resistance in a large population of isolates but the heterogeneity observed in the small number of isolates tested was perhaps surprising, if not daunting and effectively precluded the possibility of screening isolates or samples for resistance by PCR. Classically, resistance has been viewed as an all or nothing event with isolates classified simply as resistant or sensitive (NCCLS, 1999; BSAC, 2003) but a practical consequence of the multiple genetic mechanisms of resistance in many organisms is the increasing complexity of probes for the genetic detection of resistance (Phillips, 1997). Although such techniques have offered important advances in our understanding of resistance and its detection and offer advantages in specificity over conventional microbiological techniques, phenotypic detection of resistance remains more sensitive and is therefore currently more suitable for the surveillance of avilamycin resistance in enterococci.

Finally, taking all the factors discussed into account, epidemiological modelling techniques were applied to the design of sampling regimes and this again highlighted the importance of applying standard statistical theory to antimicrobial resistance studies if quantification is to be achieved. The definition of the population of interest – region, farm, animal, bacterial species was shown to be critical as was consideration of prevalence and test performance. Exploration of these modelling techniques highlighted several gaps in our knowledge of antimicrobial resistance that must be addressed – the proportion of commensal bacteria expressing resistance, the variability in bacterial and animal populations and the performance of diagnostic tests. Some of these aspects have been raised before (Davison *et al.*, 2000; Dunlop *et al.*, 1999) but have not been thoroughly explored. The use of epidemiological modelling again highlighted the inadequacy of current antimicrobial resistance surveillance, with the testing of a few colonies from a commensal bacterial population of millions with

the aim of monitoring the prevalence of resistance over time shown to be futile and the ignorance of test specificity and sensitivity when surveillance data is interpreted potentially misleading.

In summary, the measurement of antimicrobial resistance in bacterial and animal populations has been shown to be extremely complex with many factors including the calculation of suitable sample sizes, the definition of bacterial and animal populations and the consideration of diagnostic tests all critical to improving the quantification of resistance. Avilamycin resistance has been shown to be present in the enterococcal flora of pigs in the UK and to be detectable on pig farms where it has been withdrawn from use. The relevance of this finding to public health is uncertain but it is clear that conventional surveillance is of very limited use in monitoring resistance in commensal bacterial populations of animals.

9.2 Future Work

In order to improve understanding of the relevance of resistance in enterococci in farm animals to public health, there are several areas of work considered in this thesis that need to be expanded upon.

First, the quantification of resistant enterococci has not been fully achieved and there are several reasons for this. It would be useful to improve the specificity of the media for the isolation of enterococci perhaps by utilising some of their biochemical properties such as the hydrolysis of aesculin in conjunction with a chromogenic medium such as Slanetz and Bartley and then to develop improved counting techniques. These could perhaps be based on the work of other authors on *E. coli* (Dunlop *et al.*, 1998c; Humphry *et al.*, 2002) and involve hydrophobic grids or spiral plating technology.

Molecular technology also offers possibilities for the quantification of bacteria with techniques such as real-time PCR described (Ibekwe and Grieve, 2003). If molecular methods for the speciation of enterococci (Dutka-Malen *et al.*, 1995; Baele *et al.*, 2000) could be combined with the molecular detection of resistance genes then this would offer exciting opportunities to improve the quantification of resistance in commensal enterococci. However, it would first be necessary to further characterise the genetic basis for avilamycin resistance in enterococci, perhaps paying particular attention to inter-species differences. This would involve screening a large number of isolates of each species, including *E. durans*, *E. hirae* and other less commonly considered enterococci for the presence of the resistance genes currently described (Aarestrup and Jensen, 2000; Kofoed and Vester, 2002) as well as considering other as yet undescribed genetic mechanisms of resistance.

A useful extension of the epidemiological modelling work would be to develop a quantitative risk assessment of the probability of antimicrobial growth-promoter use in farm animals leading to increased morbidity and mortality in humans due to infectious disease. However, it would perhaps be more useful to consider an antimicrobial closely related to a human therapeutic antimicrobial, such as avoparcin, in this risk analysis as the relevance of avilamycin resistance to human health remains uncertain.

All of the future work suggested would require considerable financial resources as well as labour. Whilst it would help to address some of the questions raised in this thesis, it seems unlikely that such resources will be available as there is little evidence to suggest that avilamycin use is a significant threat to public health. Finally, following recommendations made in a recent report by the World Health Organisation (WHO, 2003), an EU regulation enforcing the phasing out of the four remaining

antimicrobial growth promoters, avilamycin, monensin, salinomycin and flavophospholipol, was adopted in July 2003 (EC, 2003). This legislation was agreed as part of the EU strategy to combat the threat to human, animal and plant health posed by antimicrobial resistance and will come into force later this year although the time-scale for withdrawal has not been firmly agreed. This legislation will perhaps reduce the need for surveillance of resistance to avilamycin in the longer term, but some of the work described could be applied to monitoring the effects of avilamycin withdrawal, and many of the findings are relevant to antimicrobial resistance surveillance of any kind in animal populations.

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