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POPULATION STRUCTURE AND ANTIBIOTIC RESISTANCE OF THE GENUS *ENTEROCOCCUS* IN HUMANS, ANIMALS AND THE ENVIRONMENT

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To Henrik, Axel and Klara

ABSTRACT

Enterococci belong to the normal intestinal flora of humans and animals. An increased prevalence of antibiotic resistant enterococci causing nosocomial infections has drawn attention to the epidemiology and emergence of antibiotic resistance in this genus.
In the present thesis we have studied the enterococcal flora in samples from humans, animals, and the environment, in order to be able to follow the movement of bacteria between different ecological niches, as well as to determine the prevalence of antibiotic resistant enterococci in these samples.
In a European study, 17,157 enterococcal isolates from 2,868 samples from humans, animals and the environment in Sweden, Denmark, the United Kingdom, and Spain were studied (study I-III). The diversities of enterococci in environmental samples were generally high. Samples from hospital sewage, urban sewage, and manure contained enterococcal populations that reflected those in faecal samples of hospitalised patients, healthy humans and animals. Thus, such samples could be used as pooled faecal samples and replace cumbersome samplings from many individuals. Vancomycin resistant enterococci (VRE, resistant to 20 mg/L vancomycin), were identified in 8.2% of all samples and most frequently and at similar levels in untreated urban sewage in Sweden, Spain and the UK (in an average of 71% of the samples). In contrast, pig faeces and manure were more often VRE-positive in Spain than in Sweden (30% vs. 1%), most probably reflecting the former use of the vancomycin analogue avoparcin as a feed additive. Most VRE were *E. faecium* carrying *vanA* both among humans and animals. Typing of VRE showed a high degree of polyclonality and no evidence were found for transmission of VRE strains between humans and animals.
The high prevalence of VRE in Swedish sewage samples (19-60% in 118 samples) was unexpected. Typing of 35 isolates revealed a high diversity (D_i 0.97). Four of five VRE from hospital sewage were *E. faecium* with *vanB*, which is the most common type in infections and among hospitalised patients in Sweden. However, the origin of VRE from urban sewage remains unclear. A majority of VRE from urban sewage were *E. faecium* with *vanA* (17 of 29), but a larger proportion than found in the other countries was *E. faecalis* with *vanA* (11of 29). Either these VRE represent a higher carriage rate among healthy individuals in the community than earlier reported or perhaps they harbour in the sewage system.
An ampicillin and ciprofloxacin resistant *E. faecium* (ARE) strain, named FMSE1, was in a previous study found to dominate among faecal ARE isolates from patients in several Swedish hospitals. In study IV, the prevalence of the same PhP-type as the FMSE1 PhP-type, was searched for among typing data from 9676 isolates from Sweden and Denmark. FMSE1 was most common in samples of hospital sewage (50%), surface water (35%), treated sewage (28%), and untreated sewage (17%), but were rare in samples from healthy children (0.8%) and animals (2%). PFGE typing of FMSE1-like isolates from hospital sewage indicated that they were closely related to the nosocomial FMSE1 strain.
According to study I-III the enterococcal flora in sewage and hospital sewage resembled that of the flora in individual faecal samples. This fact led to an idea for a new concept for monitoring antibiotic resistance in the community and in hospitals, based on samplings of sewage water. In study V and VI the feasibility of this concept was evaluated. Up to 24 enterococcal isolates from each sample of hospital sewage (N=9), sewage treatment plants (N=14), and sewage from an anthroposophic village, were screened for resistance, using breakpoint concentrations of antibiotics in microplates. The resistance rates found for ampicillin, ciprofloxacin and erythromycin were markedly higher in hospital sewage (30, 35 and 30%) than in community sewage (4, 6 and 15%l), whereas tetracycline resistance was found at the same level in all sewage types (28%). Differences in resistance rates for enterococci isolated from different types of sewage samples were obvious and easy to monitor using this method.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which in the text will be referred to by their roman numerals:

I. Kühn I, **Iversen A**, Burman LG, Olsson-Liljequist B, Franklin A, Finn M, Aarestrup F, Seyfarth AM, Blanch A, Vilanova X, Taylor H, Caplin J, Moreno M, Dominguez L, Herrero I, Möllby R. Comparison of enterococcal populations in animals, humans, and the environment - A European study. International Journal of Food Microbiology, 2003, 88:133-145.

II. **Iversen A**, Kühn I, Franklin A, Möllby R. High prevalence of vancomycin-resistant enterococci in Swedish sewage. Applied and Environmental Microbiology, 2002, 68(6): 2838-42.

III. Kühn I, **Iversen A**, Finn M, Greko C, Burman LG, Blanch AR, Vilanova X, Manero A, Taylor H, Caplin J, Domínguez L, Inmaculada A. Herrero, Moreno MA, Möllby R. Occurrence and relatedness of vancomycin resistant enterococci in animals, humans and the environment in different European regions. Applied and Environmental Microbiology, 2005, 71(9) 5383-90.

IV. **Iversen A**, Kühn I, Rahman M, Franklin A, Burman LG, Olsson-Liljequist B, Torell E, Möllby R. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium.* Environmental Microbiology, 2004, 6:55-9.

V. **Iversen A** och Kühn I. Screening for antibiotic resistance among environmental bacteria using microplates containing breakpoint concentrations of antibiotics. Manuscript.

VI. **Iversen A**, Guldevall L, Colque-Navarro P, Burman LG, Olsson-Liljeqvist B, Franklin A, Möllby R, Kühn I. Analysis of antibiotic resistant enterococci in sewage, a new approach to monitor antibiotic resistance in the community and in hospitals. Manuscript.

CONTENTS

ABBREVIATIONS

ABSM	Antibiotic breakpoint screening in microplates
ARE	Ampicillin resistant enterococci
C	Cytosine
DD	Disk diffusion
D_i	Diversity index
FMSE1	Arbitrary name of an ampicillin and ciprofloxacin resistant clonal group of *E. faecium* clone
G	Guanine
LAB	Lactic Acid Bacteria
MEA	M Enterococcus agar
MIC	Minimal inhibitory concentration
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PhP-system	Phene Plate system
RHS	Raw hospital sewage
RUS	Raw urban sewage
S_p	Population similarity
STP	Sewage treatment plant
SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring
TUS	Treated urban sewage
UPGMA	Unweighted pair group method using arithmetic averages
VRE	Vancomycin resistant enterococci
VRE20	Enterococci resistant to 20 mg/L vancomycin
VRE8	Enterococci resistant to 8 mg/L vancomycin

INTRODUCTION

THE GENUS *ENTEROCOCCUS*

Characteristics

Enterococci are Gram-positive cocci that occur singly, in pairs, or as short chains (Figure 1). They are facultative anaerobes with an optimum growth temperature of 35˚C and a growth range from 10 to 45˚C. They are all catalase negative, grow in broth containing 6.5% NaCl, and they hydrolyse esculin in the presence of 40% bile salts. Most of them also hydrolyse pyrrolidonyl-β-naphtylamide (PYR) (Facklam et al. 2002). Other characteristics of enterococci that have made them extremely competitive in many areas are their tolerance against disinfectants and heat as well as a promiscuous lifestyle.

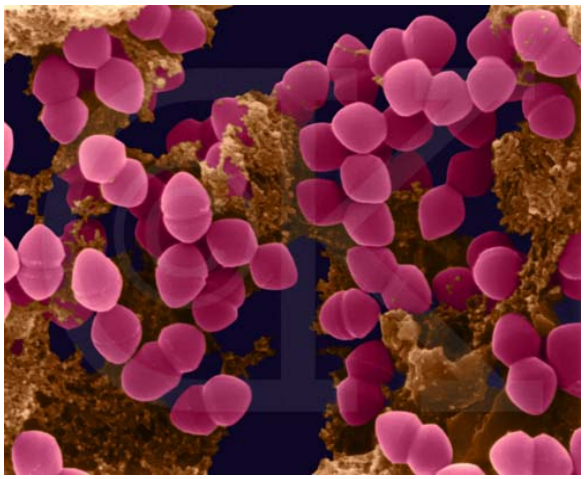


Figure 1. *Enterococcus faecium*. Image copyright Dennis Kunkel Microscopy, Inc.

Taxonomy and history

The genus *Enterococcus*, currently consisting of 34 species (Table 1), belongs to the *Firmicutes* together with other genera of lactic acid bacteria (LAB). All *Firmicutes* are Gram-positive and catalase negative cocci with a low percentage of G + C content (the guanine and cytosine bases are forming the stronger linkage in the DNA molecule). Phylogenetic analysis of 1,400 bases in the 16S rRNA gene separate the genus *Enterococcus* from other LAB belonging to the *Firmicutes* (Klein 2003).

Based on molecular analysis the former faecal streptococci, *Streptococcus faecalis* and *S. faecium*, were separated from the genus *Streptococcus* in 1984 and were included in a new genus, *Enterococcus* (Schleifer et al. 1984). However, already in 1899 a first description of enterococci was published (Thiercelin 1899) and after a few years, in 1903, Thiercelin and Jouhaud gave the name *Enterococcus* to coccoid bacteria from the intestine (Thiercelin et al. 1903). During the following decades the enterococci were referred to as streptococci, but were subdivided into group D streptococci based on serotyping (Lancefield 1933), and into faecal streptococci based on their origin (Sherman 1937).

Table 1. Species included in the genus *Enterococcus* (Euzéby).

Species	Reference	Species	Reference
Enterococcus aquimarinus	(Svec et al. 2005)	*E. hirae*	(Farrow et al. 1985)
		E. italicus	(Fortina et al. 2004)
E. asini	(de Vaux et al. 1998)	*E. malodorous*	(Collins et al. 1984)
E. avium	(Collins et al. 1984)	*E. moraviensis*	(Svec et al. 2001)
E. canintestini	(Naser et al. 2005)	*E. mundtii*	(Collins et al. 1986)
E. canis	(De Graef et al. 2003)	*E. pallens*	(Tyrrell et al. 2002)
E. casseliflavus	(Collins et al. 1984)	*E. phoeniculicola*	(Law-Brown et al. 2003)
E. cecorum	(Williams et al. 1989)	*E. porcinus*	(Teixeira et al. 2001)
E. columbae	(Devriese et al. 1990)	*E. pseudoavium*	(Collins et al. 1989)
E. dispar	(Collins et al. 1991)	*E. raffinosus*	(Collins et al. 1989)
E. durans	(Collins et al. 1984)	*E. ratti*	(Teixeira et al. 2001)
E. faecalis	(Schleifer et al. 1984)	*E. saccharolyticus*	(Rodrigues et al. 1990)
E. faecium	(Schleifer et al. 1984)	*E. saccharominimus*	(Vancanneyt et al. 2004)
E. flavescens	(Pompei et al. 1992)	*E. seriolicida*	(Kusuda et al. 1991)
E. gallinarum	(Collins et al. 1984)	*E. solitarius*	(Collins et al. 1989)
E. gilvus	(Tyrrell et al. 2002)	*E. sulfureus*	(Martinez-Murcia et al. 1991)
E. haemoperoxidus	(Svec et al. 2001)	*E. villorum*	(Vancanneyt et al. 2001)
E. hermanniensis	(Koort et al. 2004)		

Habitat

Enterorococci are commensal organisms for which the natural habitat is the intestinal tract of humans along with other mammals and birds. The most frequently encountered species are *E. faecalis* and *E. faecium*. Enterococci are also common in environments contaminated by human and animal faecal materials, e.g. sewage, recipient water, soil receiving fertilisers of animal origin, as well as in food products derived from animals (Franz et al. 1999). Some species seem to be host-specific as species e.g. *E. columbae* that is specific for pigeons (Devriese et al. 1990) and *E. asini* for donkeys (de Vaux et al. 1998) and other appear to be plant associated e.g. the yellow pigmented *E. casseliflavus*, *E. mundtii*, and *E. sulfureus* (Martinez-Murcia et al. 1991; Ulrich et al. 1998).

Pathogenicity

Enterococci have a limited potential for causing disease as they lack potent toxins and other significant virulence factors. Despite this fact, they can cause bacteraemia, surgical wound infections, urinary tract infections and endocarditis. They are also associated with obligate anaerobes in mixed infections that result in intra-abdominal abscesses. Typically, enterococci cause infections in debilitated and hospitalised patients that often have been treated with broad-spectrum antibiotics. An explanation for their involvement in disease may thus be a combination of "virulence" factors that enhances their ability to colonize, adhere and induce tissue damage (Gilmore *et al.* 2002). Most of these factors have been identified in *E. faecalis* that also is responsible for the majority (90%) of infections caused by enterococci, but due to the higher ability of acquiring antibiotic resistance the proportion of infections caused by *E. faecium* is increasing.

Antibiotic resistance

Antimicrobial therapy for enterococcal infections is complicated. Due to intrinsic low-level of resistance in enterococci to many antibiotics (clindamycin, aminoglycosides and β-lactams) a bactericidal effect cannot be reached at clinically relevant concentrations (Murray 1990). Traditionally, treatment of infections caused by enterococci has consisted of a synergistic combination of an aminoglycoside and a cell wall active antibiotic (e.g. ampicillin and vancomycin). However, emergence of resistance to also these antibiotics has become a problem in many parts of the world. Antibiotic resistance can be obtained either by the acquisition of genes mediating resistance from other organisms or by spontaneous mutations.

β-lactam resistance

β-lactam antibiotics act by inhibiting the cell wall synthesis. Penicillin-binding proteins (PBPs) that are involved in the synthesis and assembly of the peptidoglycan layer in the cell wall are the targets for β-lactam antibiotic (Kak *et al.* 2002). PBPs bind the β-lactam antibiotic the cell wall synthesis is thereby inhibited. Intrinsic resistance towards β-lactam antibiotics in enterococci is due to low affinity of PBPs for the β-lactam agents. This resistance differs between different β-lactams, with penicillins having the most activity against enterococci, carbapenems having slightly less activity, and with the cephalsporins having the least activity. High-level resistance to penicillins is mainly due to either overproduction of a PBP (enterococci have at least five different PBPs) with a natural low affinity for penicillins or to mutations that make the low-affinity PBP even less susceptible to inhibition by penicillins (Fontana et al. 1996).

Aminoglycoside resistance

Aminoglycosides act primarily by interfering with the protein synthesis of bacteria by binding to the 16S rRNA of the 30S ribosomal subunit. The intrinsic low level of resistance found among the enterococci is due to limited drug transport across the cell membrane. High-level aminoglycoside resistance in enterococci involves the acquisition of genes that are encoding aminoglycoside-modifying enzymes, like phosphotranferases, accetyltransferases or nucleotidyltransferases (Chow 2000). The most common gene, *aac(6')-Ie-aph(2")-Ia*, is found in 90% of clinical enterococci with high-level aminoglycoside resistance, and encodes a bifunctional enzyme with both acetylating and phosphorylating activity (Azucena et al. 1997; Chow 2000). This gene, which is located on transposons or plasmids, mediates resistance to a broad range of aminoglycosides and has also been detected in other Gram-positive cocci like *Staphylococcus aureus, S. epidermidis*, and *Streptococcus* spp. (Thomas et al. 1989; Kaufhold et al. 1993; Galimand et al. 1999).

Glycopeptide resistance

The glycopeptide vancomycin is an important antibiotic used in human medicine against multiresistant enterococci and against methicillin resistant *Staphylococcus aureus* (MRSA). Avoparcin is another glycopeptide that has been used extensively as a feed additive given to livestock in Europe.

Glycopeptides are large molecules that inhibit cell wall synthesis through binding to the peptidyl-D-alanyl-D-alanine terminus of the peptidoglycan precursor, and thus

forms a steric hinder that inhibits further cell wall synthesis. Resistance to glycopeptides is mediated by synthesis of modified peptidoglycan precursors to which the glycopeptides cannot bind (Kak *et al.* 2002). Six types of glycopeptide resistances have been described in enterococci that can be distinguished on the basis of sequence of the structural gene for the resistance ligase (*vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG*) (Kak *et al.* 2002). *E. gallinarum, E. flavescens* and *E. casseliflavus* possess *vanC* that confer an intrinsic low-level resistance to vancomycin (MIC 4 - 32 mg/L), but is not transferable (Tannock *et al.* 2002).

The VanA phenotype is characterised by high-level resistance to both vancomycin (MIC 64 - >1,000 mg/L) and teicoplanin (MIC 16 - 512 mg/L). This resistance is mediated by seven genes (*vanR*, *vanS*, *vanH*, *vanA*, *vanX*, *vanY* and *vanZ*), located on the mobile genetic element Tn*1546.* Expression of *vanH*, *vanA* and *vanX* result in a modified peptidoglycan precursor D-alanyl-D-lactate to which the glycopeptides will not bind (Kak *et al.* 2002). Thus, cell wall synthesis is not inhibited by glycopeptides. Tn*1546* is able to direct its own transfer from the chromosome of one strain to another. The VanB resistance phenotype has a varying degree of resistance to vancomycin (MIC 4 - 1,024 mg/L) but not to teicoplanin. The *vanB* gene cluster is also located on a mobile genetic element Tn*1547*(Kak et al. 2002). The transfer of these mobile genetic elements conferring vancomycin resistance between enterococci but also to more potent pathogens such as MRSA has caused much concern.

Other *van*-genes have been described, *vanE* and v*anG* that exhibit low-level resistance to vancomycin in *E. faecalis* and *vanD* located on the chromosome of some *E. faecium* strains that mediates moderate levels of resistance to both vancomycin (MIC 64 - 128 mg/L) and teicoplanin (MIC 4 - 64 mg/L), but these are less frequently found (Kak *et al.* 2002).

Tetracycline resistance

Tetracycline inhibits protein synthesis by interfering with the binding of amionoacyl-tRNA to the ribosome. Tetracycline resistance in enterococci is most commonly encoded by *tet*(M) that usually is carried by Tn*916* or related conjugative transposons that has been found in isolates from both animals and humans (Aarestrup et al. 2000; Haack et al. 2000).

Macrolide resistance

Macrolides is a group of antimicrobials produced by *Streptomyces* spp. Erythromycin and tylosin have been used in treatment of infections caused by Gram-positive cocci in both animals and humans. Tylosin has also together with spiramycin been used as growth promoting agents given to animals. Resistance to macrolides is very common among enterococci isolated from humans and from pigs and is most commonly encoded by the *erm*(B) gene, located on the Tn*917* in humans, but this transposon has also been found in bacteria from other sources (Jensen *et al.* 1999).

Quinolone resistance

Quinolones e.g. ciprofloxacin, inhibit bacteria by interaction with type IV topoisomerases and DNA gyrase that are essential for DNA replication (Shen et al. 1989). Spontaneous mutations in the *parC* gene mediate a conformational change of

the quinolone resistance-determining region that results in an intermediate level of quinolone resistance, whereas an additional mutation in the *gyrA* gene results in high-level resistance (Kanematsu et al. 1998).

MULTIPLE IMPORTANT ROLES OF ENTEROCOCCI

A commensal in the intestine of humans and animals

The microbial ecology of the adult intestine is suggested to be influenced by host characteristics like age, stress, disease, immunity, bile acid and enzymatic secretions and microbial factors, e.g. antagonism and synergism between the colonizers, and environmental factors like diet and intake of antibiotics. The gastrointestinal microflora consists of 400-500 bacterial species. A majority of the bacteria belong to anaerobic genera, such as *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Lactobacillus*, *Clostridium* and anaerobic cocci. Among the facultative anaerobes, the most common bacteria are *Escherichia coli*, *Enterococcus* spp. and *Streptococcus* spp. (Kleessen et al. 2000). The most commonly isolated enterococcal species in adult humans are *E. faecalis*, followed by *E. faecium, E. hirae*, *E. avium* and *E. durans* where these constitute approximately 1% of the intestinal microflora (Sghir et al. 2000). *E. gallinarum* and *E. casseliflavus* seems also to be associated with the intestinal microflora as they have been isolated from human faeces (Edlund et al. 1997). Bacteriocin and superoxide production of enterococci may have an importance in colonization resistance (Franz et al. 2002; Huycke et al. 2002), but due to their relatively low numbers in the intestine their contribution to the microbial stability may be of minor importance. Instead, the focus on enterococci is the role they display acquiring antibiotic resistance and virulence genes in a favourable milieu for genetic exchange between bacteria within the same genus, but also between genera.

The most common species encountered in farm animals are *E. faecium*, *E. faecalis*, *E. hirae* and *E. durans*, although the species distribution may vary between host species and age of the animals (Devriese et al. 1987; Devriese et al. 1994).

Importance in food production

Probiotics are given to improve the microbial balance of the intestine or as treatment of gastroenteritis in humans and animals. The beneficial effect of enterococci is somewhat unclear, but some strains of *E. faecium* and *E. faecalis* are used in probiotic products.

Enterococci are used for fermentation of cheeses and other milk products where they contribute to ripening and development of product flavour (Franz et al. 1999). The most frequently encountered species in such products are *E. faecalis* and *E. faecium*, and more rarely *E. durans*, *E. hirae* and *E. gallinarum* (Suzzi et al. 2000). However, enterococci have also been implicated in food spoilage due to their tolerance against high temperatures (Franz et al. 1999), e.g. strains of *E. faecium* have been able to survive heating to 65˚C for 20 min, 71˚C for 10 min and 80˚C for 3 min (Kearns et al. 1995).

An indicator for faecal contamination

Indicator organisms are used to predict the presence of potential pathogenic organisms. As enterococci constitute a part of the normal intestinal flora of humans

and animals, survive long enough in the environment and are easily isolated, enumerated and identified, they are used as indicators of faecal contamination of recreational waters (WHO 2003).

An indicator for antibiotic resistance

The normal intestinal flora acts as a reservoir of resistant bacterial strains and resistance genes, which may be transmitted further to other pathogenic bacteria. The development of resistance may thus be detected in the intestinal flora before it appears in pathogenic bacteria and in clinical infections. Enterococci are well documented for their ability to adapt to environmental changes. Not the least they are known for their capability to develop or acquire resistance to antibiotics and are therefore suitable to study as indicators of the selective pressure exerted by the use of antibiotics in exposed populations. Surveillance of resistance among indicator bacteria in the normal intestinal flora may thus be of great value to detect trends and to follow effects of interventions. As such indicators, enterococci and also *Escherichia coli* are used in the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) of the intestinal microflora of healthy animals (SVARM 2004).

An emerging pathogen with potential to spread genes

Although enterococci are not regarded as primary pathogens, due to their ability to acquire high-level resistance to antimicrobial agents they have emerged as a nosocomial pathogen worldwide (Linden et al. 1999). In the United States it is estimated that enterococci cause infections in 800,000 cases per year, which cost health care about 500 million dollars annually.

The threat for health care is not only the fact that enterococci themselves are causing infections that are difficult to treat, but they also pose a risk through carriage of resistance genes that might be spread further to more pathogenic bacteria. In year 2002, the serious event that many researchers had predicted a long time ago, finally occurred in the United States. Methicillin resistant *Staphylococcus aureus* (MRSA) that usually are treated with vancomycin had acquired the *vanA* operon and was thus resistant to vancomycin (CDC 2002a; CDC 2002b). The *vanA* operon had probably been transferred from an *Enterococcus* spp.

EMERGENCE OF ANTIBIOTIC RESISTANCE

Antimicrobial compounds, produced by microorganisms, existed in microbial communities long before antibiotics were discovered by man, and are an important tool for controlling the microbial balance in all kinds of microbial communities. Development of resistance to these compounds in previously sensitive bacteria thus gave access to new niches. Since the introduction of antibiotics in humans and animals there has been an accelerated emergence of antibiotic resistance and dissemination of antibiotic resistant strains. Antibiotics are used in humans and animals for treatment of infection, but in animals antibiotics are also used metaphylactic (for treatment of healthy animals belonging to the same flock or pen as animals with clinical signs), prophylactic (treatment of stressed animals to prevent disease) and to promote growth (a continuous inclusion of antibiotics in animal feed to prevent infection and improve growth) (Aarestrup *et al.* 2002).

Usage of antibiotics affects the normal microbial flora of individuals and selects for strains that are resistant. As long as antibiotics are present in the individual the resistant bacteria will have an advantage. Sensitive bacteria may develop resistance by mutations or by acquisition of genes conferring resistance. In addition, several genes mediating resistance are often located on transferable conjugative elements together with other resistance genes and thus, acquisition of these will render the bacteria multiresistant. Multiresistant strains easily become endemic in environments with high usage of antibiotics (e.g. hospitals) and dissemination of these strains is facilitated by high density of people and close contact between patients and staff. As a result, outbreaks of multiresistant strains are common in hospitals. Nosocomial infections caused by resistant bacteria have become more frequent during the past decade and also the mortality in nosocomial infections is increasing and is often associated with drug resistance (Cars 1997; Tokars et al. 1997; Kim et al. 1998; Linden et al. 1999). Strict hygien control is therefore of outermost importance in hospitals.

ANTIMICROBIAL RESISTANCE SURVEILLANCE OF HUMAN ISOLATES

Surveillance of antibiotic resistance in Sweden is performed by annual assembly of data from 30 laboratories covering the whole country (STRAMA 2005). Each laboratory collects susceptibility data from 100 consecutive clinical isolates of *Streptococcus pneumoniae*, *S. pyogenes* and *Haemophilus influenzae* once every year and on occasion resistance data has been collected for other species e.g. *Escherichia coli* and *Enterococcus faecalis/ faecium*. These data are collected in a database (ResNet) and used for monitoring of the resistance frequencies of each county, but also for quality control of the susceptibility testing method.

EARSS (European antimicrobial resistance surveillance system) is a European network of national surveillance systems that collect comparable and validated antimicrobial susceptibility data of e.g. *E. faecalis*, *E. faecium* and *Escherichia coli* that have caused invasive infections (STRAMA 2005). From Sweden 21 laboratories participate in the EARSS network.

EPIDEMIOLOGY OF VANCOMYCIN RESISTANT ENTEROCOCCI

In 1986, the first vancomycin resistant *E. faecium* isolates from patients were reported from France and England (Leclercq *et al.* 1988; Uttley *et al.* 1988). Since then, VRE have emerged worldwide. The epidemiology of vancomycin resistant enterococci is complex and there are some fundamental differences between the VRE epidemiology in parts of Europe, the United States and Sweden (Figure 2).

VRE in Europe

In European countries a high prevalence of VRE, mainly *E. faecium* with the *vanA* genotype, have been reported from farm animals, meat products, farmers, non-hospitalised individuals and from sewage treatment plants (Bates et al. 1993; Klare et al. 1993; Aarestrup et al. 1996; Chadwick et al. 1996; Bates 1997; van den Bogaard et al. 1997). Genetic fingerprinting of European nosocomial VRE isolates has often shown polyclonality, indicating the import of various *vanA* strains from the community (Bonten et al. 1998; Gambarotto et al. 2000). In the Netherlands for

example, 5 to10 % of healthy people were colonized with VRE (Endtz et al. 1997; van den Bogaard et al. 1997), and a study in a cattle rearing region in France conducted in 1997, revealed that 1.8% of the non-hospitalised individuals and 8.6% of the haematology patients carried VRE of the *vanA* type (Gambarotto et al. 2000). Further support for an animal origin of the *vanA* gene clusters in Europe are that those are genotypically indistinguishable in isolates from human and nonhuman sources (Jensen et al. 1998; Stobberingh et al. 1999; Simonsen et al. 2003). The relatively large community reservoir of VRE in Europe has been linked to the use of avoparcin in live stock (Bates et al. 1993; Aarestrup 1995; Klare et al. 1995). Avoparcin is a glycopeptide that is closely related to vancomycin and has been used as a growth promoter in livestock in Europe for many years. In Denmark, for example, about 24,000 kg of avoparcin was used for growth promotion in animals in 1994, while only 24 kg of vancomycin was used to treat humans (Wegener et al. 1998). These facts led to the ban of the use of avoparcin in the European Union in January 1997 (Commission Directive 97/6 EC). Since then a decreased VRE colonization rate have been reported in healthy Europeans (Klare et al. 1999; van den Bogaard et al. 2000), whereas a high prevalence of VRE have been maintained in other populations e.g. broiler flocks in Denmark and Norway (Heuer et al. 2002). The proportion of invasive vancomycin resistant *E. faecium* were below 5% in most countries in Europe in 2003 (EARSS). However, some countries reported outbreaks of invasive VRE in several health care settings and these resulted in higher proportions of VRE e.g. Portugal (50%), followed by Italy (25%) and Greece (23%).

VRE in the US

In contrast, clones of VRE have spread within and between hospitals in the United States (Frieden et al. 1993; Pegues et al. 1997), but VRE of the VanA and VanB types have so far not been reported among humans and animals. In 1997, 23% of the tested enterococci in intensive care units were resistant to vancomycin (Martone 1998). VRE of the *vanA* type have also been isolated from hospital sewage (Harwood et al. 2001). A heavy antibiotic use in hospitals is thus the probable explanation for the concentration of VRE in samples with a connection to hospitals in the United States.

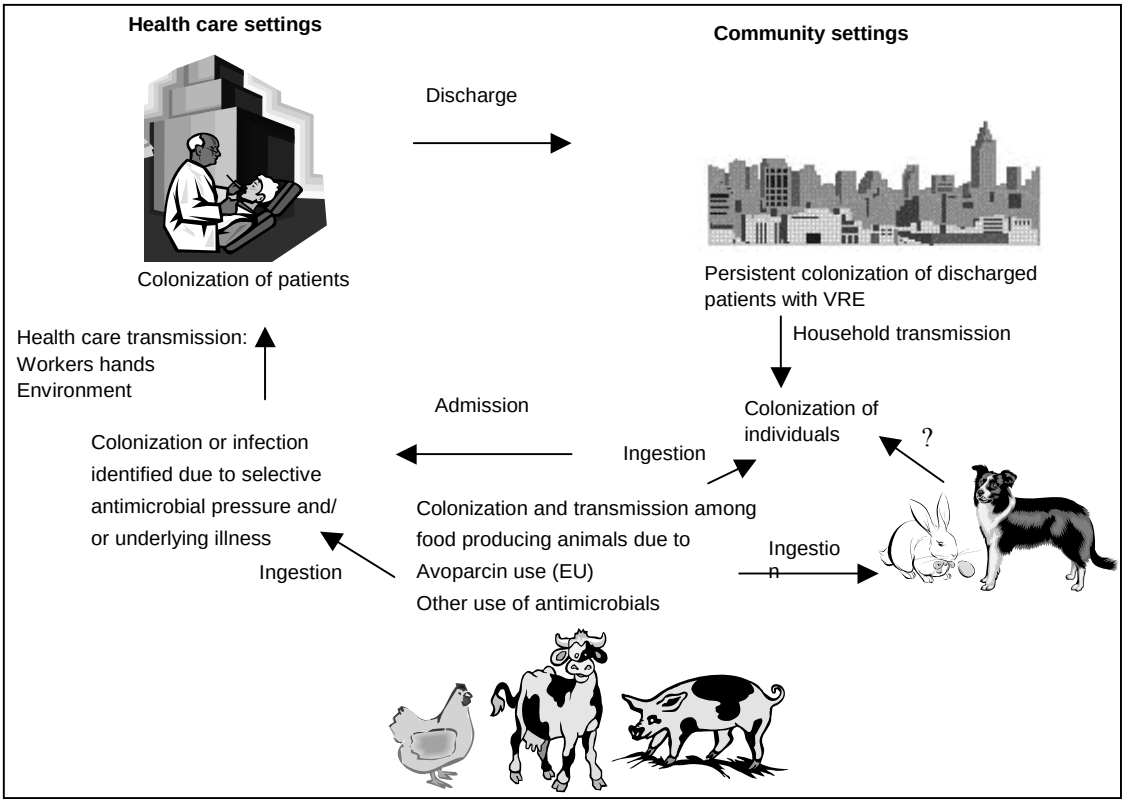


Figure 2. Possible routes for transmission of VRE

VRE in Sweden

Sweden applied a restrictive antibiotic policy in both human and veterinary medicine a long time ago. The usage of antibiotic feed additives was prohibited already in 1986 and vancomycin is only used for treatment of serious human infections caused by multiresistant Gram-positive bacteria, such as methicillin-resistant staphylococci or multiresistant enterococci, and enterocolitis caused by *Clostridium difficile*. VRE was reported for the first time in Sweden in 1995 (Larsson 1995) and after that a few small outbreaks with VRE have been reported (Torell et al. 1997; Torell et al. 1999). An investigation in 1998 revealed a low prevalence of VRE in faeces both from hospitalized patients (9 of 841 patients, all from the same hospital and representing a small outbreak of *E. faecium* of the VanB type) and from one healthy human (1 of 670 individuals carried a VRE of the VanA type and this person had recently returned from Africa) (Torell et al. 1999). Since year 2000 VRE is a notifiable organism and has to be reported to the Swedish Institute for Infectious Disease Control (SIIDC). The number of reported VRE have been around 20 for all years except in year 2003 when 51 VRE were reported. This year the first cases of VRE in bloodstream infections were also reported (STRAMA 2005). A majority of the reported VRE in Sweden has been *E. faecium* carrying the *vanB* gene.

Other resistant enterococci in Sweden

Clonal spread of other antibiotic resistant enterococci has also been documented in Sweden, mostly associated with hospitals. A nationwide study conducted in Sweden on the carriage rates of ampicillin-resistant enterococci (ARE) from patients in 27 hospitals and from outpatients, revealed that 22% of the hospitalised patients and 6% of the outpatients were faecal carriers of ARE (Torell et al. 1999). Typing of the

isolates by the biochemical phenotyping indicated that he majority of ARE in both groups (73% of 180 isolates from hospitalised patients and 54% of 39 isolates from outpatients) belonged to the same type (named FMSE1). In contrast only 1% of 169 sensitive isolates belonged to the same type. This indicated a common clonal origin of the FMSE1 isolates that was verified by PFGE typing on a subset of these isolates (Torell 2003). In another study high-level gentamicin resistant strains of *E. faecalis* in southern Sweden were shown to be clonally related (Hallgren et al. 2003).

PERSISTING ANTIBIOTIC RESISTANCE

Until recently it was generally believed that once the selective pressure of antibiotics disappeared the resistant bacteria would loose their advantage and be outnumbered by the faster multiplying wild-type strains. Recent findings have indicated that some antibiotic resistant bacteria can also persist in the absence of the selective pressure exerted by the antibiotic. One possible reason for this could be ability of these bacteria to adapt through e.g. mutations that enable them to proliferate at similar rates as the corresponding sensitive wild type population. Another mechanism may be coselection with other genes, as has been shown for e.g. vancomycin resistant enterococci among pigs and chicken in Denmark, which carry genes also mediating resistance to macrolides or copper (Aarestrup et al. 2002; Hasman et al. 2002). However, there are still no satisfactory explanations for the recent emergence of a VRE clone in Swedish chicken farms, now prevalent in 18% of the flocks (SVARM 2005). VRE are not continuously present in feed, in flocks of parent birds or in hatcheries. It seems like these VRE have adapted to the environment of chicken farms and proliferate enough to persist also in the absence of any known selective pressure.

AIMS OF THE PRESENT INVESTIGATION

The general aim for paper I-IV was to generate knowledge about enterococcal populations in different parts of the food chain with regard to abundance, species distribution, clonal relations and antibiotic resistance. This was done as a part of a European project in collaboration with Denmark, United Kingdom and Spain. The specific aims for each paper were:

- To study the populations of enterococci in terms of abundance, species distribution, and diversity in different parts of the food chain and in different regions of Europe (Paper I).

- To investigate the relation between VRE isolates found in Swedish sewage and to determine their resistance traits (Paper II).

- To compare the occurrence of VRE in countries where avoparcin have been used until recently, 1997, with Sweden where avoparcin have not been in use since 1986 (Paper III).

- To study the occurrence of a nationwide spread nosocomial strain of ampicillin resistant *E. faecium* among samples collected from animals, healthy humans and the environment in order to find an origin of the clone and/ or to describe a possible transmission route (Paper IV).

The data obtained from studies I, II and IV resulted in an idea about a new concept on how to monitor antibiotic resistance in certain populations, e.g. hospitals or whole communities, by analysis of sewage originating from these populations. The aims for the following paper were:

- To develop and evaluate a rapid screening method in microplates for detection of antibiotic resistance among enterococci in sewage (Paper V).

- To evaluate the new concept for antibiotic resistance surveillance in the society by monitoring antibiotic resistance in enterococci isolated from sewage samples originating from a defined population (Paper VI).

MATERIALS AND METHODS

COLLECTION OF SAMPLES

Human, animal and environmental samples (Paper I, II, III and IV)

All samples included in the European study were collected from Sweden, Denmark Spain and the United Kingdom during the period April 1998 to December 2000. The samples were selected in order to be representative for enterococcal populations in humans, animals and mixed/ environmental origin (Table 2).

Samples of human origin consisted of faecal samples from healthy humans and hospitalized patients, clinical isolates obtained from hospital laboratories, community sewage, and hospital sewage. Samples of animal origin consisted of caecal contents from chicken and faecal material from cattle and pigs that were collected from randomly selected healthy animals at slaughterhouses (Sweden, Denmark, Spain). Other samples of animal origin were pig manure and soil from farmland fertilized with pig manure and these were collected from 12 farms in Sweden and 11 farms in Spain. Samples assumed to be of environmental or other origin were surface water receiving treated sewage, soil and crop from farmland without manure and pig feed. (Paper I for details on how the samples were collected.)

Longitudinal studies in pig farms were conducted in one farm each of Sweden, Spain and the United Kingdom (paper I and III). The aim for this part of the study was to investigate the occurrence and possible transmission of certain clones of enterococci through the food chain. Therefore samples were collected eight times during two years from the same farm. Samples that were collected were pig feed, pig feces, pig manure, liquid manure (Figure 3), soil and crops from farmland fertilized with pig manure as well as soil and crops from farmland where fertilizer of animal origin had not been used.

Table 2. Samples collected for the European study on enterococci in the food chain. All samples and isolates are included in paper I. In paper III VRE that had been stored from these samples were included, except. In paper II all Swedish samples from urban sewage, hospital sewage and surface water were included in the study. In paper IV, PhP-typing data from all Swedish and Danish isolates were included.

	Number of collected samples and in brackets the number of typed enterococci									
Sample origin	**Sweden**		**Denmark**		**Spain**		**UK**		**All**	
Human/environmental										
Urban sewage	67	(1365)			99	(1995)	48	(721)	214	(4081)
Hospital sewage	14	(302)			29	(393)	26	(181)	69	(876)
Humans										
Healthy humans (faecal)	24	(130)					39		63	(130)
Hospitalized patients (faecal)	18	(134)							18	(134)
Clinical isolates	97	(97)			55	(42)	6	(5)	158	(144)
Animals in slaughterhouses										
Broiler chicken (caecal)	150	(941)	137	(836)	100	(98)			387	(1875)
Cattle (faecal)	194	(1004)	134	(850)					328	(1854)
Pig (faecal)	306	(988)	134	(308)	242	(214)			682	(1510)
Animals in farms										
Pig (faecal)	64	(381)			68	(616)	69	(151)	201	(1148)
Pig manure	54	(917)			47	(917)	25	(52)	126	(1886)
Farmland with manure	70	(208)			46	(198)	25	(41)	141	(447
Crop from farmland with manure	4	(12)			13	(84)	14		31	(96)
Farm run-off water							15		15	0
Other farm animals							15	(57)	15	(57)
Sheep milk					65	(65)			65	(65)
Mixed animal/human/other										
Surface water	37	(579)			75	(1281)	37	(225)	149	(2085)
Pig feed	21	(99)			35	(448)	25	(18)	81	(565)
Farmland/crop without manure	24	(34)			66	(170)	35	nd	125	(204)
TOTAL	1144	(7191)	405	(1994)	940	(6521)	379	(1451)	**2868**	**17157**

Figure 3. Views from Funbo where samples for the longitudinal study in Sweden were collected.

Sewage samples (paper V and VI)

Samples were collected ten times during September 2004 to June 2005. Raw urban sewage (N = 10) and treated sewage (N = 6) were obtained from sewage treatment plants (STPs) with permanent equipment, programmed for time or flow proportional sampling during 24 h.

In the anthroposophic village, a sampler was used for time proportional collection of two samples, one from the hospital and one from the community (Figure 4)

Figure 4. Time proportional collection of sewage, from the anthroposophic village Järna, using a sampler

For collection of sewage from hospitals (N = 6) a peristaltic pump with tubings having a diameter of 2.mm was installed in the sewage outlets of wards with high usage of antibiotics (e.g. intensive care units and haematology) or at the main sewage outlet from the hospitals (before the hospital sewage is mixed with sewage from the community on its way to the sewage treatment plants). This pump allowed sampling

over 24 h and the collected sewage was kept cold in a cool box. In some cases, samples of the main outlet from the hospitals could not be collected with this equipment, and had instead to be collected as grab samples, i.e. by lowering a bottle into the sewage (N = 4).

Treatment of samples

All samples were collected with aseptic techniques, kept at 4ºC, and analysed within 24 h. Extraction of faecal material from cotton swabs was done in 2 ml of phosphate buffered saline (PBS). Ten grams of solid samples (pig feed, manure, soil and crop) were mixed with 10 times PBS and stirred in room temperature for 20 minutes. After settling, 20 mL of the liquid was withdrawn and used for cultivation.

ISOLATION OF ENTEROCOCCI (PAPER I-VI)

M *Enterococcus* agar (MEA, Becton Dickinson, Sparks, Md.) was used for the isolation of enterococci. Samples with high concentration of enterococci were subjected to serial dilutions in PBS and 100 µL of suitable dilutions were spread on the agar surface. Samples with lower concentrations of enterococci such as surface water or when we wanted to isolate resistant enterococci also with a lower concentration in the sample, the sample was first filtered through 0.45 µm pore-size membrane filters (Millipore Corporation, Bedford, Mass) and then membrane filters were pre-incubated for 2 h at 37ºC on brain heart infusion agar (BHIA, Becton Dickinson). Membrane filters were then transferred to MEA-plates and incubated at 37ºC for 48 h. MEA is a selective media and the enterococci grow as pink to dark red colonies. For confirmation, enterococci were sub-cultured on bile esculin agar (BEA, Becton Dickinson) at 44º C which resulted in black zones around the esculin hydrolyzing enterococcal colonies and those were also tested for not having catalase activity with 3% H_2O_2. Some uncertain isolates were tested for hydrolysis of L-pyrrolidonyl-β-naphtylamide using a PYR-test.

Antibiotic resistant enterococci were isolated on MEA with added antibiotics. For the European study (Paper II-IV) all samples were also cultured on MEA with vancomycin (8 mg/L, MEA8) and on MEA with erythromycin (8 mg/L). In paper II and III, all isolates growing on MEA8 were also subcultured on MEA with 20 mg vancomycin per liter (MEA20) for confirmation of the VanA and VanB resistance phenotype. In paper VI, MEA with ampicillin (8 mg/L), ciprofloxacin (4 mg/L), gentamicin (64 mg/L), vancomycin (16 mg/L) and erythromycin (4 mg/L) were used for isolation of resistant enterococci.

An enrichment culture consisting of 10 mL of the sample and 10 mL of 2x concentrated Enterococcosel broth (Becton Dickinson) with a final vancomycin concentration of 8 mg/L was used for enrichment of VRE. After incubation of the enrichment culture for 24 h at 37ºC, 10 µL was spread on MEA with vancomycin. Plates were incubated and colonies typical for enterococci were tested as described above for confirmation of genus.

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Broth microdilution (Paper II-IV

A subset of enterococci from the European project (of which some data are included in paper II, III and IV) were susceptibility tested using commercial panels for determination of MICs with the broth microdilution method (VetMic system, National veterinary Institute, Uppsala, Sweden) according to the recommendations of NCCLS (NCCLS 2000). The panels included vancomycin, ampicillin, erythromycin, tetracycline, virginiamycin and avilamycin.

E-test (paper IV)

E-test (AB Biodisk, Solna, Sweden) was used for determination of MICs to vancomycin, teicoplanin, ampicillin, imipenem, ciprofloxacin, netilmicin, clindamycin and erythromycin.

Antibiotic screening in microplates (ABSM, paper V and VI)

An antibiotic screening method in microplates for rapid detection of antibiotic resistant isolates among a "normal" population of bacteria (not selected with antibiotics) was developed and presented in paper V. In this assay, 96 well U-shaped microplates were prepared with antibiotics. The concentration of each antibiotic was selected for detection of only resistant isolates and thus, inhibiting the wild-type population lacking resistance mechanisms. Twenty-four isolates from each sample were inoculated in PBS in a "masterplate". Ten µL of these bacterial suspensions were transferred with a microplate replicator to a dilution plate (Figure 5). Diluted suspensions were further transferred with microplate replicators to a first growth control plate, and then to a set of microplates containing different antibiotics, and finally to a last growth control, all of which contained 100 µL Isosensitest broth (Oxoid, Basingstoke, England). Antibiotics included in the screen were ampicillin (8 mg/L), ciprofloxacin (4 mg/L), gentamicin (64 mg/L), erythromycin (4 mg/L) and tetracycline (4 mg/L). After incubation at 37ºC for 18-20 h, the microplates were scanned in a flatbed scanner and images were stored for interpretation. Isolates showing similar growth rate in the absence and in the presence of antibiotics, were considered resistant, and could be further tested with a conventional method, such as disk diffusion or broth microdilution. The agreement between the ABSM method and disk diffusion in detecting resistant isolates was 99% (Paper V). In one case the ABSM method detected ciprofloxacin resistance that was interpreted as intermediate

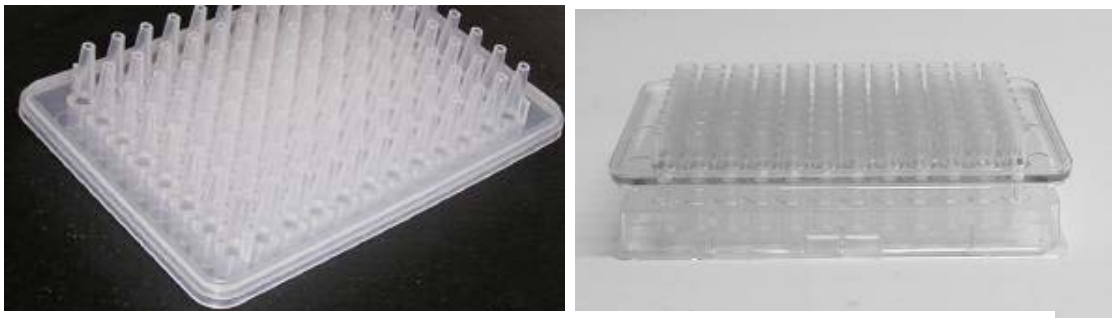


Figure 5. Microplate replicators (Sigma) were used for transfer of 96 bacterial suspensions between microplates, in the antibiotic screening method (ABSM paper V, VI).

Isolate		PhP-data													PhP-	Antibiotic screening				
	BEA	oooo	L-AP	LAC	MEL	MEL	RAF	INO	SOR	MAN	G-L-A	AMY	GLU	Species in PhP	type	amp	van	cip	gen	ery
W4.06-01	pos	99	20	4	21	3	22	7	8	4	4	3	6	E.fcs mean	6	s	s	s	s	s
W4.06-02	pos	99	20	3	4	22	20	23	22	22	22	11	21	E.hirae ATCC49611	3	s	s	s	s	s
W4.06-03	pos	99	19	4	21	3	22	7	3	3	3	3	4	E.fcs mean	6	s	s	s	s	r
W4.06-05	pos	99	21	3	4	24	22	23	22	22	23	8	22	E.hirae ATCC49611	3	s	s	s	s	s
W4.06-06	pos	99	20	4	21	3	22	8	4	3	22	3	5	E.fcs FS18	1	s	s	r	s	s
W4.06-07	pos	99	19	3	4	21	22	22	22	22	23	8	21	E.hirae	3	s	s	s	s	s
W4.06-08	pos	99	20	4	21	3	21	8	4	3	22	3	4	E.fcs FS18	1	s	s	r	s	s
W4.06-09	pos	99	20	3	5	22	23	23	23	23	23	9	22	E.hirae	3	s	s	s	s	s
W4.06-10	pos	99	19	4	21	3	22	7	4	3	4	3	5	E.fcs mean	6	s	s	s	s	r
W4.06-11	pos	99	20	4	3	22	23	23	22	23	23	10	22	E.hirae	3	s	s	s	s	s
W4.06-12	pos	99	3	4	18	5	22	22	4	4	22	9	20	E.avium	Si	s	s	s	s	s
W4.06-13	pos	99	17	10	18	23	23	23	20	7	23	21	23	E.fcm US	Si	r	s	r	s	r
W4.06-14	pos	99	20	3	21	3	22	7	4	4	5	7	10	E.fcs mean	Si	s	s	s	s	s
W4.06-15	pos	99	20	3	4	21	11	23	21	23	22	8	21	E.hirae	7	s	s	s	s	s
W4.06-17	pos	99	20	2	4	23	11	22	22	23	23	9	22	E.hirae	7	s	s	s	s	s
W4.06-18	pos	99	20	4	22	3	22	8	4	3	23	3	4	E.fcs FS18	1	s	s	r	s	s
W4.06-19	pos	99	21	4	22	3	22	8	4	3	23	3	4	E.fcs FS18	1	s	s	r	s	s

Figure 6. Example of data obtained using our approach for analysing enterococci from sewage. PhP-RF data were used for identification of species by comparison to a reference database with known species, as well as for determination of the diversity within each sample and the prevalence of certain resistant types. Resistance data were obtained by antibiotic screening in microplates (ABSM).

according to disk diffusion. To speed up the analysis of environmental isolates (paper VI), bacterial suspension from the ABSM masterplate were also transferred with a multi-channel pipette to the first column in PhP-RF plates for typing (Figure 6).

Disk diffusion method (Paper V and VI)

Resistant isolates that were detected with ABSM were further tested using disk diffusion according to the recommendations of the Swedish reference group for antibiotics (SRGA) and its subcommittee on methodology (SRGA-M) (SRGA). 1-3 colonies were suspended in 5 mL PBS, a cotton swab was soaked with suspension and spread on isosensitest agar (Oxoid). Antibiotic disks (Oxoid) with ampicillin, ciprofloxacin, gentamicin, erythromycin and vancomycin were applied on the agar plate and left for 30 min in room temperature to allow diffusion of the antibiotics into the agar. Plates were examined after 18-20 at 37ºC. Inhibition zones were recorded and interpreted in S I R according to species related zone breakpoints of SRGA. Zone breakpoints for erythromycin, tetracycline and ciprofloxacin were not available at this website, instead the zone diameters were recorded for these antibiotics.

BIOCHEMICAL TYPING WITH THE PHP-SYSTEM (PAPER I-IV, VI)

PhP-RF

The PhenePlate rapid screening system for biochemical typing of enterococci (PhP-RF, PhPlate, Stockholm) (Kühn et al. 1993; Kühn et al. 2003) was used in paper I to IV, and in paper VI. This is a semi-automated typing system based on the measurements of the kinetics of a set of biochemical reactions performed in microplates. In order to obtain a representative collection of isolates from each sample that could yield data suitable for calculations of diversity 24 isolates were picked from each environmental sample (Bianchi et al. 1982) and 8 isolates were picked from each faecal or caecal sample for PhP-typing. Each microplate was inoculated with eight isolates in the first column (one of each row) that were transferred to the other eleven wells containing different chemicals that have been

selected for a high discrimination between enterococci: The PhP-plates were incubated at 37ºC and were read in a microplate reader after 16, 40 and 64 h. After the last reading the mean values of the reactions for each isolate were calculated, resulting in a set of eleven numerical values characterizing each isolate (the biochemical fingerprint).

PhP-FS (Paper II and IV)

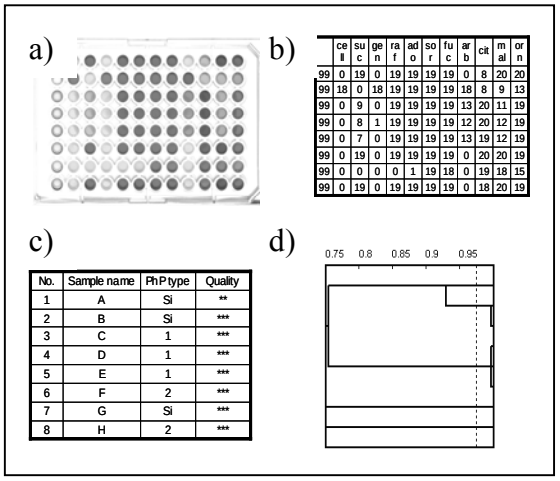
VRE isolates that had a MIC > 16 mg/l of vancomycin were subjected to a more discriminatory biochemical typing of enterococci, using 23 different chemicals (PhP-FS plates) (Kühn et al. 1995).

Species identification

Preliminary species identification was done by comparing the PhP patterns of unknown isolates to the patterns of known strains of various *Enterococcus* species (Manero et al. 1999).

Data analysis

Analysis of the PhP-data, such as calculations of similarities between isolates, diversities within samples and similarities between populations, as well as calculations and construction of dendrograms were done with the PhP software (PhPlate, Figure 7).



	cell	suc	gen	raf	ado	sor	fuc	arb	cit	mal	orn
99	0	19	0	19	19	19	19	0	8	20	20
99	18	0	18	19	19	19	19	18	8	9	13
99	0	9	0	19	19	19	19	13	20	11	19
99	0	8	1	19	19	19	19	12	20	12	19
99	0	7	0	19	19	19	19	13	19	12	19
99	0	19	0	19	19	19	19	0	20	20	19
99	0	0	0	0	1	19	18	0	19	18	15
99	0	19	0	19	19	19	19	0	18	20	19

No.	Sample name	PhP type	Quality
1	A	Si	**
2	B	Si	***
3	C	1	***
4	D	1	***
5	E	1	***
6	F	2	***
7	G	Si	***
8	H	2	***

Figure 7. PhP-typing, from plate image to analysis. a) Reactions of eight isolates in a PhP-plate b) The biochemical fingerprints and c) calculations of the similarity between isolates and d) construction of dendrogram.

Identity level

Data generated by PhP typing were used to calculate pairwise similarities among isolates expressed as correlation coefficient. At least 95% of comparisons between data from replicate assays of the same isolate run at the same occasion yielded a higher correlation coefficient than 0.975, and thus an identity level (ID level) of 0.975 could be defined (intra assay reproducibility) (Kühn et al. 2003). When comparing data obtained from different assays and between different laboratories, an ID level of 0.965 was used (inter assay reproducibility, paper I). One of the purposes using typing of bacteria is to determine whether the occurrence of bacteria is due to clonal spread of one strain or due to release of phenotypically and genotypically diverse bacteria from many sourcesl. Advantages with the PhP-system are the high reproducibility and that large numbers of isolates easily are typed. Thus, differences between types can be regarded as true whereas identity between types may have to be confirmed with a more discriminatory method e.g. pulsed-field gel electrophoresis (PFGE, see below).

Cluster analysis

The unweighted-pair group method using average linkages (UPGMA) was used for cluster analysis of PhP-data and the results were illustrated in dendrograms.

Calculations of diversity and population similarity

The diversity for enterococci at different population levels, e.g. sample, sample type, species type, was calculated as Simpson's diversity index (D_i) (Hunter et al. 1988; Kühn et al. 2003). D_i is a relative measure of the distribution of isolates into different types. It is high, maximum 1.00, if the isolates are evenly distributed into many types and it is low, minimum 0.00, when the bacterial population consists of a few dominating types. In paper VI, calculation of the diversity in each sample was found to be a useful tool in the judgement of the quality of a sample. Sewage samples were regarded as pooled faecal samples of many individuals, and according to studies in paper I, such isolates normally show diversities of 0.96. A low diversity in a sewage sample indicated that it was not representative for the faecal flora of many individuals. Therefore, samples were regarded representative when the diversity exceeded a certain level; for continuously collected samples $D_i > 0.85$ and for grab samples $D_i > 0.7$ were included in the analysis.

The similarity between different populations of enterococci was calculated on PhP-data and was measured as population similarity coefficient S_p (Kühn et al. 1991). The Sp coefficient measures the proportion of isolates that belong to the same type in the two populations that are compared with each other. This type of analysis is convenient when a large set of data has to be analysed (paper I).

Selection of representative isolates

Enterococcal populations normally consist of some common PhP-types containing at least two isolates each and some singletons. One isolate of each PhP type representing at least 10% of the typed isolates, was saved in -70ºC for further studies.

PHP DATA GENERATED IN OTHER STUDIES (PAPER IV)

In a previous nationwide Swedish study on the carriage rates of ampicillin resistant enterococci (Torell et al. 1999; Torell 2003) a certain PhP-type representing a wide-spread clonal group was found to be dominating. For comparisons with data obtained from our studies, the PhP patterns of two isolates representing this clonal group were selected. These patterns were compared to all other patterns generate from the European study (paper I-III) stored in a database. They were also compared to the PhP data from 1232 faecal isolates from 125 healthy children performed in 1999 (Alm et al. 2002).

GENOTYPING WITH PULSED-FIELD GEL ELECTROPHORESIS (PAPER II AND IV)

DNA purification and enzyme digestion for analysis with PFGE on the Swedish VRE (paper II) were performed as described previously (Jensen et al. 1998). Restriction enzyme *Sma*I was used for DNA digestion. Electrophoresis was performed on a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) using 1.4% agarose (Seakem) in 0.5 x Tris-borate-EDTA at 180V. Running conditions consisted of two phases used in sequence. Phase 1 was 2 to 8 s with a run time of 20 h. Phase 2 was 8

to 22 s with a run time of 20 h (Jensen et al. 1998). A slightly different protocol was used for PFGE in paper IV, as described previously (Torell 2003).

Reconstructed PFGE gel images were prepared for paper II, using the Gel compare software (version 4, Applied Maths, Sint-Martens-Latem, Belgium). The DNA banding pattern, in paper IV, was analysed visually and interpreted according to the criteria proposed by Tenover *et al.*, as indistinguishable (no band differences), closely related (1-3 bad differences), possibly related (4-6 band differences) and unrelated (>6 band differences)(Tenover et al. 1995).

DETECTION OF GENES WITH PCR (PAPER II, III)

Confirmation of the species *E. faecium* and *E. faecalis* and determination of high-level vancomycin resistance genotypes was carried out by PCR of the $ddl_{\text{E. faecalis}}$ and $ddl_{\text{E. faecium,}}$ and of *vanA* and *vanB* genes according to the method described by Dutka-Malen (Dutka-Malen et al. 1995).

RESULTS AND DISCUSSION

1. ENTEROCOCCAL POPULATIONS IN VARIOUS SAMPLES FROM THE FOOD CHAIN IN FOUR EUROPEAN COUNTRIES (PAPER I)

A total number of 2,868 samples, of which 522 samples had a presumed human origin, 1,991 samples had a presumed animal origin and 355 samples had a mixed or other origin, were collected from Sweden, Denmark, Spain and the United Kingdom within a European collaboration project, which aimed at generating knowledge on the ecology and epidemiology of enterococci in the food chain (Table 2).

Enterococci are found almost everywhere

Growth of enterococci was detected in 77% of the 2,868 samples. As expected, enterococci were isolated in high numbers from most samples of faeces, caeca, sewage and pig manure. An exception to this was the low detection of enterococci in faecal samples from slaughtered pigs in Sweden (45%) and Denmark (37%). In contrast, enterococci were detected in 86% of the faecal pig samples from one farm in Sweden.

Enterococci were found in most samples that had a presumed mixed human/ animal or other origin, like pig feed (48% to 94%) and surface water (62% to 94%), but in all countries except for pig feed samples in Spain they were recovered in low numbers. Most Spanish crop samples grown on farmland fertilized with pig manure presented growth of enterococci (77%), but they were less common in such samples from UK (21%). As expected, in each of the three countries studied (Sweden, Denmark and Spain) the percentages of samples with enterococci from farmland fertilized with a fertilizer of non-animal origin and crop from such farmland, were lower than the percentages of enterococci-positive samples from farmland and crop fertilized with manure. Furthermore, the positive samples from farmland and crops that had been fertilized with a fertilizer of non-animal origin, presented generally lower counts of enterococci than those fertilized with manure.

Population diversity

PhP-data was generated on 17,157 presumed enterococcal isolates that had been typed with the PhP-RF plates. Diversity indices were calculated both as total D_i, representing the diversity within all isolates of each sample type, and as median D_i, representing the median value of diversity indices among individual samples within each sample type (paper I, table 3).

Although only 18 human faecal samples were analysed, compared with 137 to 306 faecal samples of broiler, cattle or pig, the total D_i for human faecal samples was the highest (D_i 0.96). Among the animals in both Denmark and Sweden the highest total D_i was found in broiler (0.91 and 0.94), followed by cattle (0.73 and 0.79) and pigs (0.90 and 0.83). A plausible explanation to this is that the 18 human individuals carried out more different life styles that affected the enterococcal flora, e.g. food habits and exposure to different enterococci, than the animals were, and this contributed to a higher total D_i for the human faecal samples. Even the similarity between enterococcal populations from the same animal species in Sweden and

Denmark showed high S_p values (0.42 to 0.56), indicating that strains with the same PhP-type were common among animals of the same species in the two countries (Paper I, Figure 4b). Whether this reflects a circulation of strains among animals of the same species or, a common clonal origin of certain strains that once found favourable niches in the intestine of certain animal species, remains unclear. The enterococcal faecal flora of pigs in Spain does however not resemble the ones found in pigs in Sweden (S_p 0.08) and Denmark (S_p 0.15). It thus seems like some strains are specific not only for an animal species, but also for certain regions in Europe.

Clinical isolates from Sweden and Spain showed a lower total diversity (D_i 0.89 and 0.83) than other isolates of human origin, depending on a high prevalence of one PhP-type belonging to *E. faecalis* in both countries (paper I, fig 2). One reason for this could be that this PhP-type represents a clonal group of more virulent *E. faecalis*, something that has been found among clinical isolates of *E. faecium* (Willems et al. 2001). The clinical isolates showed the highest similarity to those from hospital sewage and faecal samples from hospitalised patients (S_p 0.3 to 0.4).

Median diversities were usually lower than the total diversity for each sample type, e.g. for enterococci from Swedish human faecal (median D_i 0.55, total D_i 0.96). The total D_i of human faecal samples was however in agreement with the median and total D_i of urban sewage (both D_i 0.96), each sample here was a pooled sample of many individuals and therefore no differences between the diversity measurements were seen. Also in surface water similar median and total diversities were obtained. This was not surprising since surface water (especially recipients) receives bacterial input from many human and animal sources.

Species distribution

The most common species among the 17,157 isolate were *E. faecium* (33%), followed by *E. faecalis* (29%) and *E. hirae* (24%). Eight percent of the isolates clustered with less common species, mainly other species of the *E. faecium* group (*E. durans* and *E. mundtii*) and the *E. gallinarum* group (*E. gallinarum*, *E. casseliflavus*, and *E. avium*). The remaining six percent did not cluster with any of the strains in the reference database used (Manero et al. 1999).

In Spain and UK, *E. faecium* was found to dominate in all samples with a human origin, such as hospital sewage and urban sewage, and in most samples with a pig origin, such as faeces, manure and, farmland with manure (Table 3). *E. faecium* dominated also in surface water in both countries. In contrast, *E. faecalis* was dominating in all Swedish samples with a human origin, such as clinical isolates, hospitalized patients, hospital sewage, urban sewage and, even in the surface water receiving treated sewage. The species dominating in most samples from pig and cattle

Table 3. Distribution of enterococcal species in samples from the food chain in four European countries Sweden, Denmark, the United Kingdom and Spain. Dominating species in each sample are bold.

		Number of		% of enterococcal species				
Country	Sample type	Samples	Isolates	fs	fm	hir	other	nt
SE	Sewage	66	1365	**40**	26	18	10	6
ES	Sewage	99	1995	35	**39**	18	5	3
UK	Sewage	42	721	31	**51**	6	9	3
SE	Hospital sewage	14	302	**54**	24	8	5	9
ES	Hospital sewage	23	393	21	**49**	23	5	1
UK	Hospital sewage	22	181	30	**46**	2	3	19
SE	Healthy humans	18	130	**39**	15	20	9	17
SE	Hospitalized patients	18	134	**57**	21	13	7	1
SE	Clinical isolates	97	97	**80**	14	0	0	5
ES	Clinical isolates	42	42	**93**	2	0	5	0
SE	Broiler	114	941	31	**35**	23	9	1
DK	Broiler	108	836	**52**	15	23	5	5
ES	Broiler	98	98	**67**	15	15	1	1
SE	Cattle	147	1004	3	22	**48**	20	7
DK	Cattle	117	850	5	23	**53**	16	3
SE	Pigs	138	988	30	14	**44**	10	2
DK	Pigs	50	308	23	29	**39**	6	3
ES	Pigs	214	214	7	**80**	6	1	6
SE	Pig faeces	55	381	29	24	**36**	4	6
ES	Pig faeces	68	616	5	**68**	21	4	1
UK	Pig faeces	64	151	24	25	**36**	7	8
SE	Pig manure	50	917	19	14	**41**	12	14
ES	Pig manure	47	475	**40**	24	26	7	3
UK	Pig manure	25	52	4	**52**	13	31	0
SE	Farmland w. manure	34	208	13	21	**30**	20	15
ES	Farmland w. manure	23	198	13	**35**	21	17	14
UK	Farmland w. manure	5	41	2	**49**	17	22	10
SE	Crop	2	12	0	8	8	0	**83**
ES	Crop	10	84	4	24	35	**36**	2
ES	Sheep milk	65	65	**74**	8	11	3	5
SE	Surface water	35	579	**38**	28	20	9	4
ES	Surface water	67	1281	23	**46**	24	5	2
UK	Surface water	23	225	1	**53**	26	15	6
SE	Farmland/crop w. artificial fertilizer	7	34	3	0	26	15	**56**
ES	Farmland/crop w. artificial fertilizer	28	170	24	**42**	11	21	2
SE	Pig feed	10	99	**52**	16	14	15	3
ES	Pig feed	33	448	15	**54**	10	19	0
UK	Pig feed	17	18	0	**89**	0	0	11

SE = Sweden; ES = Spain; DK = Denmark; UK United Kingdom
fs = *E. faecalis*; fm = *E. faecium*; hir = *E. hirae*; other = other *Enterococcus* spp.; nt = not typeable.

was *E. hirae*, whereas the dominating species in broiler was *E. faecalis* in Denmark and Spain, and *E. faecium* in Sweden.

Also the species distribution for *E. faecalis*, *E. faecium* and *E. hirae* corresponded well between samples from hospitalised patients and hospital sewage, as well as between healthy humans and urban sewage in Sweden (Table 3).

Sewage and manure = Pooled faecal samples

In paper I, samples of manure, urban sewage and hospital sewage, were regarded as pooled faecal samples from their respective corresponding population of pigs, healthy humans and patients and staff in hospitals. Indeed, the analysis of the diversities, population similarities and species distribution of manure and sewage showed that the enterococcal populations in manure mirrored the enterococcal populations found in a set of faecal samples from animals, and sewage samples mirrored the enterococcal populations found in a set of individual faecal samples from and humans. Collecting samples from manure and sewage thus seem to be a convenient way to obtain faecal material from many individuals and this approach has been used in several studies ever since.

2. OCCURRENCE OF VRE IN SAMPLES FROM THE FOOD CHAIN (PAPER II, III)

The glycopeptide antibiotic avoparcin was used as a growth promoting feed additive in Sweden until the early 1980, and was banned in 1986. Avoparcin was used in many other countries in Europe until it was banned in 1997. Sweden was therefore included in a European study as a reference country for its expected low levels of VRE.

A total number of 2,580 samples from the European study were analysed for the presence of VRE8 (enterococci growing on 8 mg/L vancomycin) and VRE20 (enterococci growing on 20 mg/L vancomycin). VRE8 were found in 10.9% of the samples and VRE20 in 8.2% (Paper III, Table 1). Most of these VRE (67%) were detected after enrichment in vancomycin broth.

Sweden - A negative control?

VRE in Swedish sewage (humans)

In spite of this assumption that Sweden would serve as a negative control, VRE were commonly detected in sewage samples from Sweden. In total, 118 samples from sewage treatment plants, hospital sewage and surface water were collected and analysed for the presence of VRE (paper II, III). Even though these sample types may be regarded as environmental they have a major bacterial input from humans. VRE were detected in 21 of 35 (60%) samples from untreated sewage and in 6 of 32 (19%) samples from treated sewage. However, VRE were isolated only from one of the 37 (3%) samples of surface water collected from the recipients for treated sewage water. Fourteen samples of hospital sewage were collected at two major hospitals in Stockholm, but VRE were only detected in samples originating from the larger hospital (1,000 beds), where it was found in the majority of the samples (5 of 7 samples). Interestingly, this hospital also had a presumably higher antibiotic consumption since they purchased ten times more antibiotics than the other hospital.

Further characterization with PCR of the 35 VRE from 33 samples showed that, 30 VRE carried *vanA* (86%) and the remaining five, carried *vanB*. Twenty-four of the VRE were *E. faecium* (69%) and 11 were *E. faecalis* (31%). All five isolates from hospital sewage were *E. faecium* and all but one, carried the *vanB* gene, which corresponds well to the properties of the majority of clinical VRE isolates in Sweden (Torell et al. 1999; Hallgren et al. 2001; STRAMA 2005).

A majority of the VRE isolates (27 of 35) were resistant to at least two of the other tested antibiotics (ampicillin, erythromycin, virginiamycin, avilamycin). Ampicillin resistance was found among a considerable number of *E. faecium* (38%), something that indicates a human source for these isolates. Ampicillin resistance has been commonly found among *E. faecium* isolates from human infections and in hospitalised patients, but less often among healthy humans (6%) (Torell et al. 1999). Further, ampicillin is sparsely used in animal production and thus, ampicillin resistance is rarely found among isolates with an animal origin (< 1%) (Grave et al. 1999; van den Bogaard et al. 2000).

VRE among animals (paper III)

VRE were detected in few samples of slaughtered animals in Sweden, only in 1 of 306 pigs (0.3%) and in 4 of 150 broiler chickens (3%). These VRE were *E. faecium* of the *vanA* type, whereas the one VRE isolated from 1 of 54 manure samples (2%) was an *E. faecalis* of the *vanA* type (PM, Figure 8). Since this manure sample was obtained from a dunghill, one could speculate whether this originates from pig faeces or perhaps from other sources e.g. bird droppings.

Occurrence of VRE in Denmark, Spain and the United Kingdom in comparison to Sweden

VRE in sewage (humans)

VRE20 were also common in raw urban sewage in the UK and Spain (52% and 90% of the samples), and in treated urban sewage (22% and 54%). VRE20 were less common in samples from hospital sewage in the UK and Spain (4% and 17%) than in Sweden, but the large difference between the two Swedish hospitals also indicates individual differences between hospitals that may influence the VRE prevalence. The overall high prevalence of VRE in sewage samples with a human origin in Europe is probably reflecting a human reservoir of VRE in all countries, and it seems as it is maintained also in Sweden despite a restrictive antibiotic policy, and one of the lowest antibiotic consumptions in Europe (Cars et al. 2001; Goossens et al. 2005).

VRE of animal origin

Among the animal samples, VRE20 were most prevalent in samples with a pig origin, especially in Spain and to some extent in Denmark. In Spain, 26% of the faecal samples and 34% of the manure samples collected at pig farms contained VRE20. This should be compared to Sweden where one VRE20 was found in 118 such samples. The faecal pig samples showed a lower prevalence of VRE20 in Denmark (5%) and also in Spain (8%), but still there was a striking difference in comparison to Sweden (0.3%). A low prevalence of VRE20 was found among caecal samples from chicken in Denmark (2%) and Sweden (3%), but VRE were not detected at all in the

100 analysed samples in Spain. In all countries the VRE20 prevalence in cattle was low (0-1%) (Paper III, Table 1). Thirty-nine VRE of animal origin were further characterised, all possessed the *vanA* gene and the majority belonged to *E. faecium* (82%), followed by *E. hirae* (10%) and *E. faecalis* (8%).

VRE in the environment

Interestingly, a small fraction of Spanish soil and crop samples from farmland with artificial fertilizer, pig feed and surface water samples, presented growth of VRE20 (3-12%), as also one of the surface water samples from Sweden did.

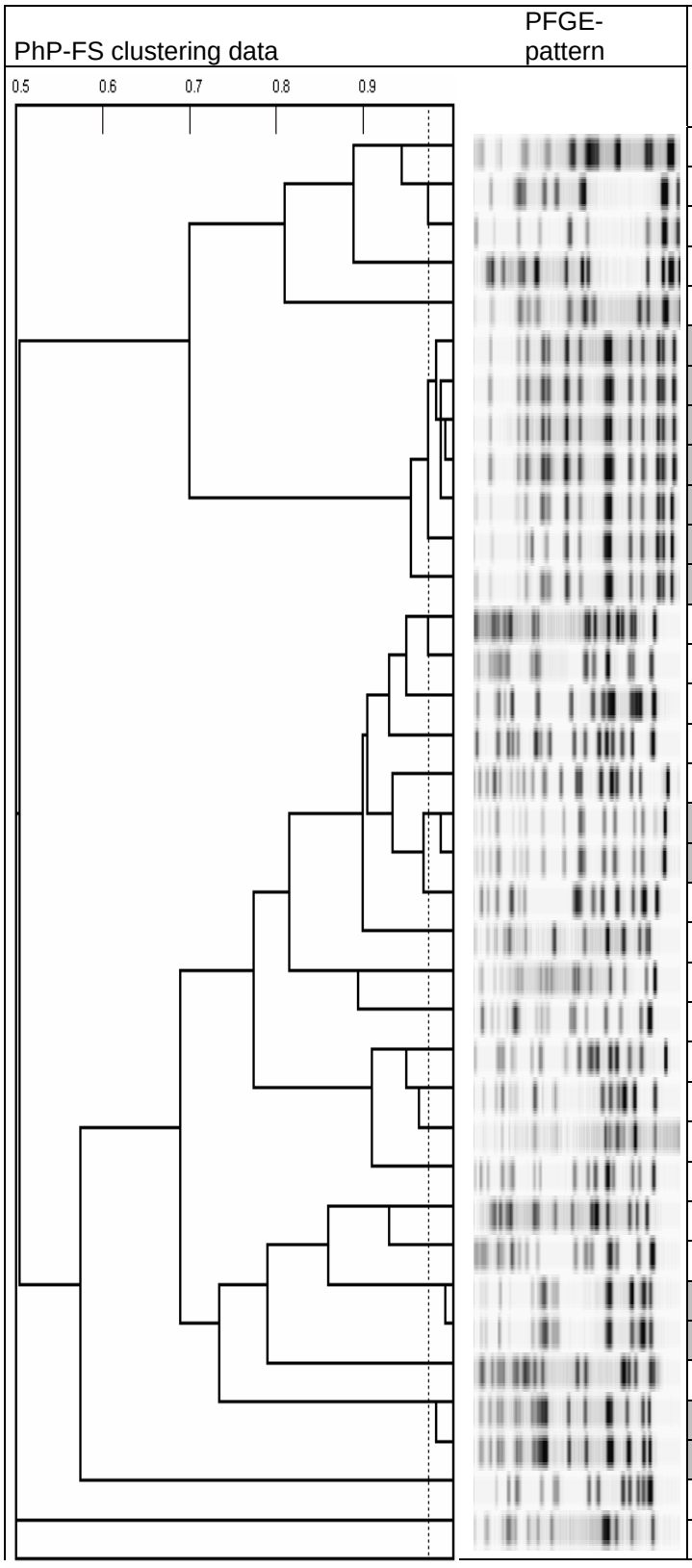
3. POPULATION STRUCTURE OF VANCOMYCIN RESISTANT ENTEROCOCCI

VRE in Sweden

VRE from sewage

Both typing methods, biochemical typing with PhP-FS and genotyping with PFGE, revealed a high diversity (D_i 0.97) among the 35 VRE isolated from hospital sewage, raw sewage, treated sewage and surface water in Sweden, indicating a polyclonal population structure (Figure 8).

One major cluster was found, which comprised of seven *E. faecalis*, all of the *vanA* type that also had similar resistance patterns. These VRE were isolated from raw and treated sewage samples from three different STPs in Stockholm. Four of the VRE originated from the same STP, but the samples were collected on three occasions during one year. In one case, the VRE type was found both in raw and in treated sewage that had been collected on the same occasion. Three other pairs of VRE with identical type, vancomycin resistance type and similar resistance patterns were also found. The first pair of VRE was from hospital sewage, isolated one month apart. The second pair originated from untreated sewage in the same STP, but the isolates were from samples collected with one month apart, and the third pair was from two different plants, collected on the same occasion. These findings of identical strains in the same STP and hospital sewage imply that clonal spread of VRE might have occurred or that some strains are more prone to acquire resistance. Other explanations to these findings could be that strains are persisting in the sewage system or are leaking from a common source, e.g. hospitals. Sewage from the hospitals is mixed with sewage from the community and is led to the STP. Thus, it was not surprising that that seven of the nine VRE that were ampicillin resistant *E. faecium* had an identical or similar PhP-type as FMSE1, a nationwide spread clone of ampicillin resistant *E. faecium* found among hospitalised patients in Sweden (Torell 2003).



Sample type	Isolation site	Isolation date	Species	*vanA/ van B*
PM*		98-05	FS	*vanA*
RS	B	99-06	FS	*vanA*
RS	U	99-12	FS	*vanA*
RS	B	99-06	FS	*vanA*
RS*	N	98-08	FS	*vanA*
RS*	H	98-10	FS	*vanA*
RS	L	99-03	FS	*vanA*
RS	B	99-03	FS	*vanA*
RS	H	99-06	FS	*vanA*
TS*	H	98-10	FS	*vanA*
RS	H	99-10	FS	*vanA*
TS*	B	99-03	FS	*vanA*
RS	L	98-10	FM	*vanA*
HS	A	99-03	FM	*vanA*
RS	H	99-11	FM	*vanA*
HS*	A	99-09	FM	*vanB*
RS	B	00-02	FM	*vanB*
HS	A	98-11	FM	*vanB*
HS	A	98-12	FM	*vanB*
HS	A	99-10	FM	*vanB*
RS	B	98-10	FM	*vanA*
RS	U	99-06	FM	*vanA*
W		99-06	FM	*vanA*
RS	L	99-11	FM	*vanA*
TS	L	98-12	FM	*vanA*
TS*	B	98-13	FM	*vanA*
TS*	B	99-12	FM	*vanA*
RS*	U	98-11	FM	*vanA*
RS	L	98-10	FM	*vanA*
RS	B	99-10	FM	*vanA*
RS	B	99-11	FM	*vanA*
RS	L	99-06	FM	*vanA*
RS	H	98-12	FM	*vanA*
RS	L	98-12	FM	*vanA*
TS	H	99-12	FM	*vanA*
RS	H	98-12	FM	*vanA*

Figure 8. Dendrogram showing UPGMA clustering of PhP-FS data for Swedish VRE isolated from raw sewage (RS), treated sewage (TS), hospital sewage (HS), and surface water (W). The corresponding PFGE patterns of *Sma*I-digested DNA for each isolate, isolation site and date (year-month), species (FS, *E. faecalis*; FM, *E. faecium*) and vancomycin resistance genotype (*vanA* or *vanB*) are also shown. The horizontal axis in the dendrogram shows the similarities between the isolates, and the dotted line indicates the ID-level (0.975). Grey shading indicate identical or highly similar isolates.

VRE from animals

Within the European study (1998-2000), four *E. faecium* carrying the *vanA* gene were isolated from the 150 broiler chickens that were analysed in Sweden. Three of them belonged to the same unique PhP-type (Paper III, Figure 2). Since then, VRE have been isolated from an increasing number of broiler chickens, when using vancomycin enrichment broth, and reached 36% last year (SVARM 2005). All these VRE have been *E. faecium*, all tested carried the *vanA* gene, and the majority were of the same PhP-type, as well as those typed with PFGE differed only by one band. So far, there is no good explanation for this clonal spread of VRE among chicken broilers that is prevalent in 18% of the farms in Sweden. Also other countries in Europe have a high prevalence of VRE among broiler flocks e.g. Denmark (74%) (Heuer *et al.* 2002) and Norway (96% in farms exposed to avoparcin and 64% in farms established after the avoparcin ban)(Sorum *et al.* 2004), but in contrast to Sweden VRE in these countries do not seem to have a common clonal origin.

VRE from infections

The majority of VRE reported to the clinical disease act between years 2000 and 2004 in Sweden were *E. faecium* possessing the *vanB* gene (N = 92). Sixteen *E. faecium* and 5 *E. faecalis* carried the *vanA* gene and one was *E. faecalis* with the *vanB* gene (STRAMA 2005). A subset of 85 *E. faecium* from years 2000 and 2004, were typed using the PhP-FS system and were shown to be highly diverse (D_i 0.97, Figure 9, unpublished data). Most of the VRE belonged to small clusters, usually from the same county. However, one major cluster that comprised 10 isolates was of the same PhP-type as the previously found FMSE1 clone. This could be an indication for acquisition of vancomycin resistance genes among the FMSE1 clone, something that also was found among isolates of the FMSE1 type from hospital sewage (Paper IV). Recently, a genetic lineage of *E. faecium* was found among isolates from five continents (Complex-17) and was characterized by ampicillin resistance, a pathogenicity island, and an association with hospital outbreaks (Willems *et al.* 2005). Further genotyping of the Swedish clinical VRE would thus be required both for confirmation of a clonal relatedness to the FMSE1 strain and to determine whether Complex-17 occurs also in Sweden. None of the clinical VRE isolates were of the same PhP-type as the VRE clone found in chicken in Sweden (Figure 9). This provides a strong evidence for that this clonal group so far has not been involved in any infections in humans.

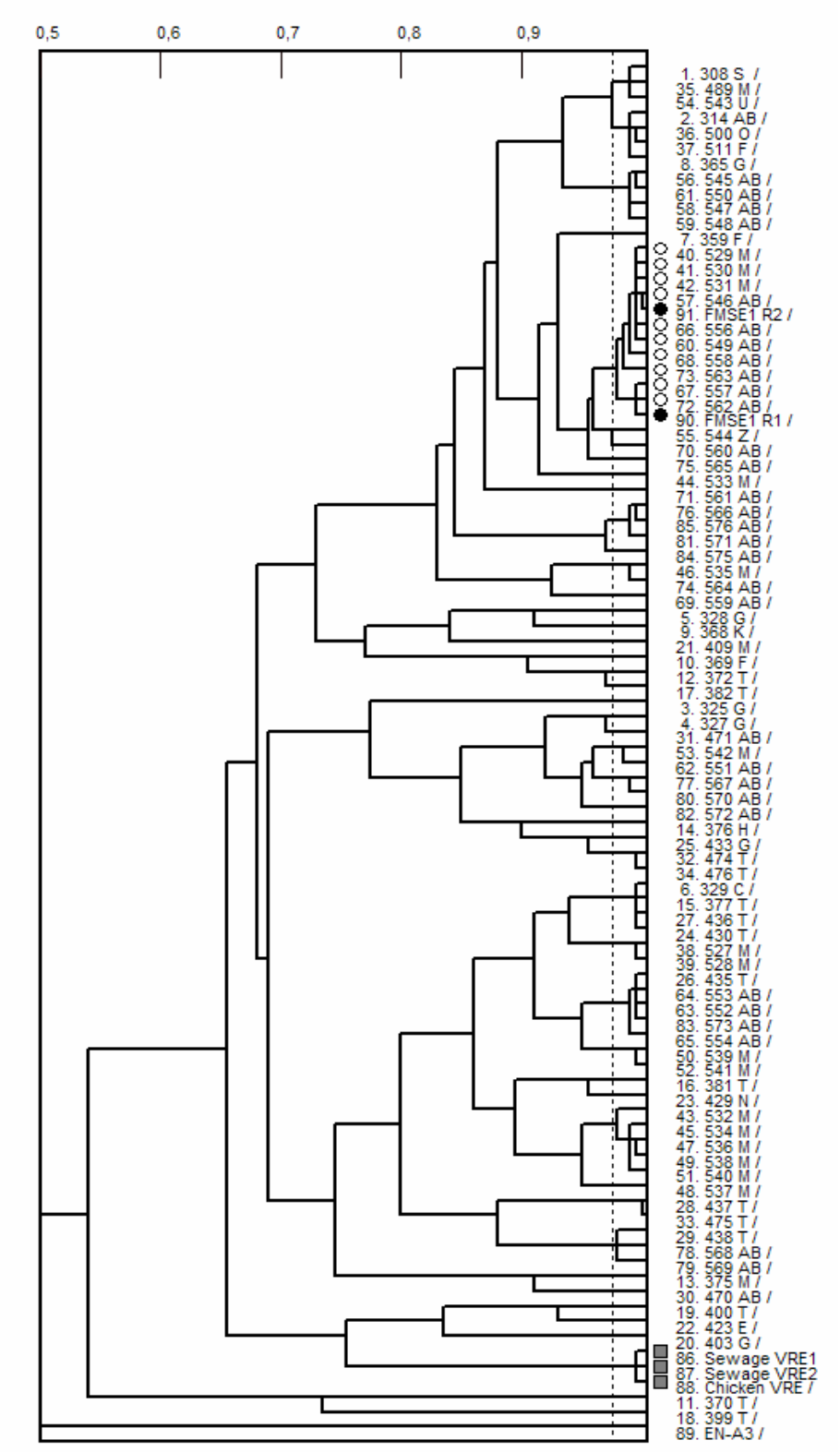


Figure 9. Dendrogram showing UPGMA clustering of PhP-FS data for clinical vancomycin resistant *E. faecium* isolates (n=85) from Sweden from years 2000 to 2004, two representatives of the nationwide spread clone of ampicillin and ciprofloxacin resistant *E. faecium*, FMSE1 (solid circles), one representative of the vancomycin resistant *E. faecium* clone spread among Swedish chicken and two vancomycin resistant *E. faecium* from sewage (grey squares). Open circles indicate clinical VRE of the same PhP-type as the FMSE1 reference strains.

VRE in Europe

All 599 VRE8 isolates were typed using the PhP-RF screening system (paper III). Their diversity measured as Simpson´s diversity index, D_i, was 0.94 and almost as high as that for "normal" unrelated enterococci from the present study (D_i 0.95 - 0.97). Taken together, these data indicated that vancomycin resistance was widespread among enterococci and thus, not confined to certain clones. This supported the hypothesis that vancomycin resistance has emerged more by horizontal spread of the van gene clusters than by transmission of a few clones (Bingen et al. 1991; Jensen et al. 1998).

Characterization of a subset of VRE20 from Europe

A total of 127 isolates confirmed as being enterococci, having vancomycin MICs of ≥32 μg/mL and representing separate PhP-RF types (only one isolate per PhP-RF type and sample was included) were further phenotyped using the more discriminatory PhP-FS system (Paper III, Fig. 1). Of these, 111 belonged to the *E. faecium* group (including *E. hirae*) and 16 were *E. faecalis*. Notably, 12 *E. faecalis* were from Sweden (all but one from urban sewage), whereas the other four were from the UK (3 isolates of human origin) and Spain (1 isolate from broiler) and all of these were *vanA* positive (data not shown). The diversity index (D_i) among these VRE isolates was 0.99 according to PhP-FS, which indicated that most VRE belonged to unique PhP types, further supporting the notion that they were of different clonal lineages. A total of 16 common types, each comprising between 2 and 11 isolates, that could indicate a common origin or a clonal spread of certain VRE strains, were found (paper III, Fig. 1). Most of these types were of solely animal or human origin, but two types included isolates of both animal and human origin (paper III, marked with arrows in Fig. 1).

VRE in humans

Among the human isolates from the European study, eight small clusters of 2-5 identical isolates were identified, and these were usually from the same country (Paper III, Fig. 3). Of the 67 isolates of human origin that were screened for vancomycin resistance genes 21% were *vanB* positive and 79% had *vanA*. All 14 isolates of the *vanB* genotype were from Sweden or the UK, and notably, eight of these were hospital associated (clinical isolates or from hospital sewage, paper III, Fig. 3). Similarly, other workers found the VanB type in clinical VRE isolates (11%) and faecal VRE from hospitalised patients (6%) in Europe (Goossens et al. 2003), whereas it is rare among animal isolates (Del Grosso et al. 2000; Lemcke et al. 2000). Seven FMSE1-like isolates were also found among VRE20 isolates from UK, represented by two clinical isolates, three isolates from hospital sewage and two from urban sewage (Paper III, Fig. 3, shaded), indicating that this clone is international and has acquired *vanB* and *vanA*. When this clone was discovered in Sweden it was consistently vancomycin susceptible (Torell et al. 1999; Torell 2003).

VRE in animals

The PhP-FS data of animal related (N= 36) and human related (N= 72) vancomycin resistant *E. faecium* and *E. hirae* were subjected also to separate cluster analysis (Paper III, Fig. 2-3). The diversity was lower among the animal isolates than among human isolates (D_i = 0.92 versus 0.99). All animal isolates possessed the *vanA* gene

and one major cluster of 11 *E. faecium* was found among the animal isolates, mainly from pigs in Denmark and Spain (Paper III, Fig. 2, shaded).

4. EVIDENCE FOR TRANSMISSION OF STRAINS FROM HUMANS AND ANIMALS TO THE ENVIRONMENT

Humans to environment

In paper IV we present evidence for transmission of the FMSE1-type from the hospital sphere to hospital sewage, and probably further to the sewage treatment plants. FMSE1 was an ampicillin and ciprofloxacin resistant clone of *E. faecium* that was commonly isolated from patients in several hospitals in Sweden (Torell et al. 1999; Torell 2003). This clone was discovered during our work with the EU project and since we had a database comprising PhP-typing data from 9676 enterococci from human, animal and environmental sources in Sweden and Denmark we wanted to investigate if a route for transmission of FMSE1could be identified. Isolates in our database having a PhP-type showing a similarity of ≥ 0.95 to the reference strains of the FMSE1-clone were designated FMSE1-like isolates. FMSE1-like isolates were most commonly identified in samples of hospital sewage (50% of the samples), in which isolates of this type also were present in higher numbers than in other sewage samples. This type was also found in samples of surface water (35%), treated urban sewage (28%) and raw urban sewage (17%, Paper IV, table 1). However, it was found only in few samples of healthy children (0.8%) and in samples of animal origin (2%), but the FMSE1-like isolates found here was not ampicillin resistant. Actually, ampicillin resistance is a property mainly associated with human *E. faecium* from infections (78% (STRAMA 2005)) and hospitalized patients (22%, (Torell et al. 1999) in Sweden, and is rare among enterococci from farm animals (< 1%, (SVARM 2004). The low prevalence of FMSE1-like isolates among healthy children and samples with animal origin is thus a strong evidence of near absence of this clone in those populations, whereas the high prevalence of FMSE1-like isolates in samples from sewage is an indication of rather high prevalence of the nosocomial ARE strain FMSE1.

Only isolates representing dominating phenotypes had been saved from each sample in the European study. Therefore, only eight FMSE1-like isolates from hospital sewage and one from surface water, all of them being ampicillin resistant were thus available for genotyping. PFGE-typing of the isolates resulted in a banding pattern that confirmed FMSE1-like isolates from hospital sewage to be closely related to the FMSE1 references (Paper IV, Figure 1). All of them were multiresistant, showing a similar resistance pattern to that of the FMSE1 reference strains. A different banding pattern was obtained for the isolate from surface water, but could still be regarded as possibly related to the FMSE1 references.

Animals to environment

One of the aims of the European study was to investigate if there is a transmission of enterococcal strains through the food chain. VRE has been suggested to reach humans from animals via meat products (Klare et al. 1995). A related mechanism of spread of VRE could be the use of manure containing VRE as a fertilizer of crops consumed by humans. In our study, VRE were not detected in any sample from soil or crops from farmland with pig manure in Spain even though as much as 34% of pig manure

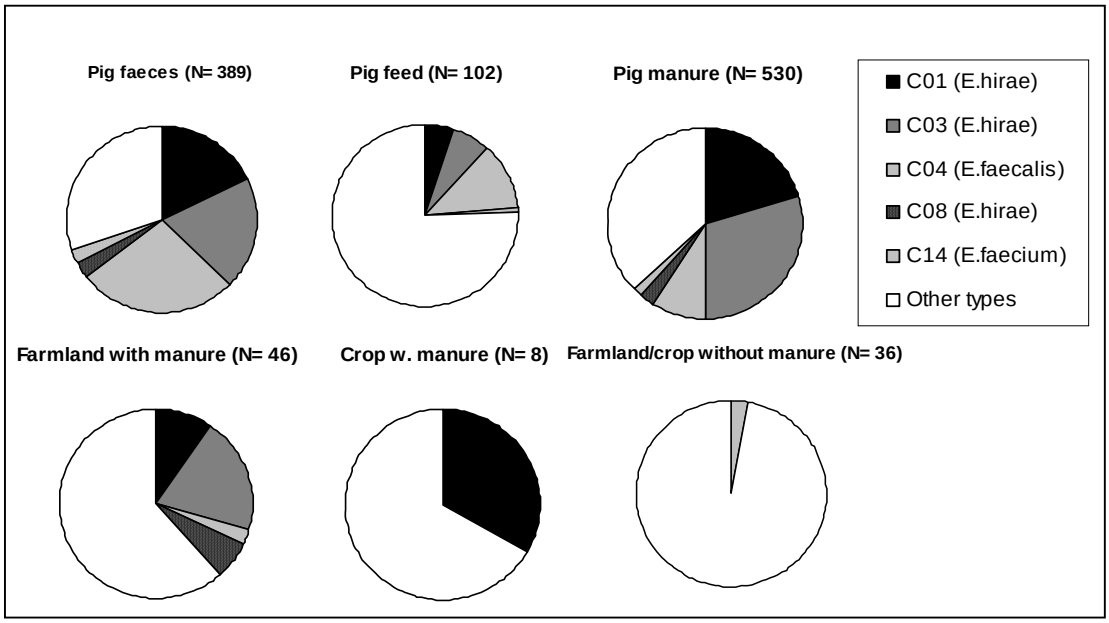


Figure 10. Identical PhP-type in various samples collected during a longitudinal study of one farm in Sweden, Funbo.

samples contained VRE. On the other hand, in the longitudinal study performed at one farm in Sweden (Funbo), we found certain PhP-types in all sample types, from pig feed, via pig faeces and pig manure, to farmland with manure and crop from farmland with manure (Figure 10). These PhP-types could represent strains with a special ability to survive in the agricultural environment. If such strains are antibiotic resistant, there is a possibility to spread antibiotic resistance genes via this route.

In paper I, we found that the species distribution in samples of surface water in UK resembled more the one found in farmland with manure than in urban sewage, as was the case in Sweden and Spain. In fact, it was found that the surface water sampling sites in the UK were the sea adjacent to a sewage treatment plant and the river behind a pig farm. Both the sea and the river received input from rural areas. According to further investigations it seems as a significant bacterial input to the sea could be derived from rural runoff.

Another example of possible transmission from food animals to the environment was found for VRE from chicken. VRE isolates of the same unique PhP-type as found among 18% of the chicken farms in Sweden were detected in a sample of untreated sewage (Figure 9). This at first seemed surprising, since that PhP-type so far only has been found in samples related to chicken. We then discovered that sewage from one of the largest slaughterhouses for chicken was connected to that STP and might well be its source.

Animals to humans

In only two cases, VRE of the same PhP-type were found both in samples from animals and of human origin, but in these cases there were no known epidemiological relationship. This finding is well in line with reports showing that VRE from animals and humans mostly belong to separate genotypes (Willems et al. 2000; Borgen et al. 2002).

5. DISSEMINATION OF ANTIBIOTIC RESISTANT STRAINS AND GENES TO THE ENVIRONMENT

Due to extensive antibiotic usage and that some resistant strains adapt to persist in the absence of a selective drug, a low carriage of antibiotic resistant strains is likely to be found in the normal intestinal flora of humans and animals. The level of this carriage rate in humans is probably influenced also by other factors than antibiotic treatment, such as travels, intake of imported meat products and further transmission in e.g. day-care centers, military camps and households. Taken together these facts indicate that there is a growing reservoir of antibiotic resistant strains and resistance genes in the intestinal flora of humans and animals and that these may finally reach the environment via the e.g. sewage. Drugs excreted and discharged in the sewage system may theoretically have an influence on the resistance development in the bacterial biofilm lining the sewers, or in the liquid phase bacteria. An investigation on this matter reported only sub-inhibitory concentrations of most antibiotics in sewage, except for ciprofloxacin (Johansson *et al.* 2003). Its highest concentration detected in hospital sewage (0.1 mg/L) could be enough to select resistant enterobacteria. Another long-term study from the same hospital found the highest concentrations of quinolones bound to sediments (151 μg ciprofloxacin per g) but without any association with resistance among the studied enterococci or coliforms (Jarnheimer *et al.* 2004).

Our data from paper IV, indicated a higher proportion of the FMSE1 PhP type among isolates from surface water, and treated sewage than in untreated sewage. These data may thus suggest that the FMSE1 type is better fit to survive the bacterial reduction process in treatment plants than other enterococcal strains. These isolates could however not be confirmed neither for resistance to ampicillin and ciprofloxacin nor for their genetic relatedness to FMSE1 since they had not been saved. According to another study it seems though as resistant enterococci are decreasing in numbers or loosing their resistance during the treatment process (Gallert *et al.* 2005).

Soil may become enriched with antibiotic resistant bacteria and resistance genes through the use of fertilizers of animal origin. We found no evidence for VRE in soil that had been fertilized with VRE containing manure. In this study we did not analyse the autochthonous soil bacteria for acquisition of resistance genes. In contrast, VRE were recovered from a few samples of soil that had not been fertilized with manure and also from surface waters (3-12%). These data show that antibiotic resistant bacteria that have emerged due to human activities end up in the environment where their resistance genes may spread further to better adapted, naturally occurring bacteria and thus contribute to an increasing environmental reservoir of resistance genes. Furthermore, glycopeptide producing soil bacteria that have been suggested to be the origin of the *vanA* and *vanB* clusters (Fraimow *et al.* 2005) may possibly have a role in the maintenance of those genes in the environment.

6. PROPOSAL OF A NEW CONCEPT TO MONITOR BACTERIAL ANTIBIOTIC RESISTANCE IN DEFINED HUMAN POPULATIONS (PAPER VI)

Antibiotic resistance is found everywhere, but national and international programs for surveillance of resistance are based on susceptibility data collected from clinical isolates. However, data on the antibiotic resistance situation in the whole community is lacking and also simple tools to perform such a study. Results from other studies included in this thesis led to the development of a new concept where sewage samples are analysed to assess antibiotic resistance rates in bacteria from the corresponding human populations. Important demands that had to be fulfilled were:

1. That the sewage samples (regarded as pooled faecal samples) were representing faecal samples from many individuals in the studied populations. In paper I, we indeed show that the composition of enterococci in sewage water from treatment plants and in hospital sewage mirror the ones found in faecal samples from populations of healthy humans and hospitalized humans, respectively.
2. Usage of a relevant indicator for antibiotic resistance originating from the studied populations that is easy to culture. Enterococci fulfill these demands.
3. A feasible protocol for the analysis. The ABSM method showed a 99% agreement to disk diffusion regarding detection of antibiotic resistance, and thus only resistant enterococci according to the ABSM method needed to be tested using a conventional method like disk diffusion (Paper V). A simultaneous biochemical typing of isolates enabled species identification, calculations of diversities (a tool to evaluate the quality of samples) and also analysis of population structures.

A significant difference was found between enterococcal resistance rates in hospital sewage and community sewage

We analysed the antibiotic resistances of 542 “normal” enterococcal isolates (not selected with antibiotics) from samples of raw urban sewage (RUS, N=10), treated urban sewage (N=4), hospital sewage (N=9) and from two sewage samples from an anthroposophic village, Järna, in order to assess the resistance situation in the human population contributing to the sewage.

As expected, the resistance rates for ampicillin, ciprofloxacin and erythromycin among normal enterococci were significantly higher in RHS (30%, 35% and 30%) than in RUS (4%, 6% and 15%, paper VI, Figure 3). Also multi-resistant enterococci (resistance to more than one antibiotic, except tetracycline) were clearly more prevalent in RHS than in RUS (35% and 7%).

The higher resistance rates in RHS, was to a great extent explained by the high resistance rates to those antibiotics among *E. faecium* (ampicillin (86%), ciprofloxacin (84%) and erythromycin (63%)). In RUS and TUS however, the prevalence of resistance rates to the corresponding antibiotics in *E. faecium* were considerably less common (ampicillin (20%), ciprofloxacin (18%) and erythromycin (26%), paper VI, Figure 4a). In contrast, the most common resistance among *E.*

faecalis was found to be to tetracycline, both in RUS and TUS (44%) and in RHS (29%).

Differences in resistance rates among enterococci from hospital sewage and urban sewage were expected if our approach for monitoring resistance in sewage was to be feasible. In fact, the resistance rates found here among normal *E. faecium* in RHS were in accordance with figures reported by SWEDRES for invasive *E. faecium* isolates in 2004, e.g. for resistance to ampicillin (86% vs. 78%) and aminoglycosides 11% vs. 7%). This was also true for gentamicin resistant *E. faecalis* (6% vs. 15%). It is more difficult to find data on resistance rates among enterococci originating from healthy humans that could be compared to our RUS and TUS data. However, in 1999 Torell *et al* reported that 6% of healthy individuals and 21.5% of hospitalized patients in Sweden carried ampicillin resistant enterococci (Torell et al. 1999), figures which also are comparable to the present ampicillin resistance rates in RUS and TUS (4%) and in RHS (30%).

Similar level of tetracycline resistance in sewage of different origins

Enterococci isolated from the anthroposophic village, Järna, were resistant to tetracycline to about the same extent as enterococci from other sample types (28%), whereas the only other resistance detected among normal isolates there was to erythromycin (1 of 46 isolates, 2 %, paper VI, Figure 3). One could thus speculate if these results reflect a current level of tetracycline resistance among normal enterococcal populations from humans. Similar prevalence of tetracycline resistance have also been reported among enterococci from Swedish broiler chickens (20%) and pigs (30%)(SVARM 2005), as well as among enterococci from European food products (24%)(Huys et al. 2004). Tetracycline is a broad-spectrum antibiotic and in spite of the declining use it is still the second largest group of antibiotics used in out-patient care in Sweden (21%) (STRAMA 2005).

SUMMARY AND GENERAL CONCLUSIONS

The main purpose of this thesis was to generate knowledge about the population structure among enterococcal populations in humans, animals and the environment, with regard to species distribution, diversity, and antibiotic resistance, and to compare thee populations between different countries in Europe (Sweden, Denmark, the United Kingdom and Spain).

As expected, enterococci were isolated in high numbers from most sample types with human or animal origin, such as faecal samples, sewage and manure, but were also detected in lower numbers in samples that were not expected to be contaminated by faecal material, such as pig feed, soil and crop, grown on farmland where no animal fertilizer had been used, as well as in surface water.

A high similarity was found between enterococcal populations originating from infections, hospitalised patients and hospital sewage in Sweden. Likewise, the enterococcal populations found among healthy humans and in urban sewage were found to be similar. Thus, based on these results we concluded that sewage samples, which easily are obtained, could be regarded as pooled faecal samples from the population contributing to the sewage (individuals in a hospital or in the community).

High-level vancomycin resistant enterococci (VRE20) were found in untreated sewage samples with a human origin to the same extent in Sweden (60%) as in the United Kingdom (52%) and in Spain (90%). However, VRE was found in samples associated with pig farms to a larger extent in Spain (26-34%) than in Sweden (0-2%) and faecal samples from pigs in Denmark and Spain also showed a higher prevalence of VRE20 (5 and 8%) than in Sweden (0.3%), probably reflecting a more recent discontinued use of the feed additive avoparcin in these countries. On the other hand, the prevalence of VRE20 in broiler chickens were at the same level in Denmark and Sweden (2-3%), were not detected in samples from Spain. More recent investigations have shown a high prevalence of VRE among broiler flocks in e.g. Sweden and Denmark that seem to persist in the absence of the selective pressure exerted by avoparcin. Typing with PhP-FS revealed a high diversity among the 127 VRE20 isolates from the four European countries. Most VRE belonged to unique PhP-types, which is a strong indication that they represented different clones. Horizontal spread of the *vanA* cluster and to some extent the *vanB* gene cluster seems to be the most likely mechanism for which the emergence of vancomycin resistance in Europe has occurred.

The high prevalence of VRE in Swedish sewage was unexpected in the light of the low prevalence among *Enterococcus* spp. isolated from food animals and humans in hospitals and the community in other studies in Sweden. However, all five VRE strains from hospital sewage were *E. faecium* and all but one that carried *vanA*, were ampicillin resistant and carried the *vanB* gene. The majority of VRE involved in infections in Sweden are also *E. faeciu*m carrying the *vanB* gene and, ampicillin resistance is most strongly associated with *E. faecium* of nosocomial origin. Taken together, these results suggest a human origin from hospital for these VRE. However, the source of the 29 VRE isolated from urban sewage remain unclear. A majority was

E. faecium with *vanA* (17 isolates), followed by eleven *E. faecalis* with *vanA*, and one was *E. faecium* with *vanB*. These VRE could represent a higher prevalence of VRE carried by healthy individuals in the community than earlier reported or there could have been a selection for these strains in the sewage systems.

We presented evidence for transmission of a nosocomial strain of ampicillin and ciprofloxacin resistant *E. faecium,* FMSE1, from the hospital sphere to hospital sewage. The PhP-pattern of this strain was also common among samples from sewage treatment plants but was rarely found in samples with animal origin or among isolates from healthy children. However, we found no evidence for transmission of VRE strains between animals and humans, something that according also to other investigations seem to be rare in the absence of close contact, like the one between animals and their farmers. Further, a low level of VRE in manure seem to represent a negligible risk for further spread to crop and humans, as VRE could not be detected in the corresponding crop.

Based on previous findings, a new approach for monitoring antibiotic resistance by analysis of faecal indicator bacteria in sewage from defined populations could be developed and assessed. The analysis was feasible and rapid and involved isolation of enterococci, screening for resistance in microplates, and biochemical typing using PhP-RF plates. As expected if our approach would be feasible, we found marked differences between urban sewage and hospital sewage that most probably reflected differences in resistance rates in the corresponding human populations. Comparing data of this kind generated over time may thus become a powerful method for surveillance of the emergence, persistence and decline of antibiotic resistance in humans or animals.

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