Vancomycin Resistant Enterococci in Swedish Broilers

Emergence, Epidemiology and Elimination

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Vancomycin Resistant Enterococci in Swedish Broilers. Emergence, Epidemiology and Elimination.

Abstract

Vancomycin resistance enterococci (VRE) are an important cause of nosocomial infections. Presence of VRE in farm animals constitute a reservoir of resistance that can spread to humans via the food-chain and is due to extensive use of the growth promoter avoparcin conferring cross-resistance to vancomycin.

Since 2000, the occurrence of VRE among Swedish broilers has increased in the absence of any obvious selective pressure as avoparcin has not been used since 1984. Also, the increased occurrence seemed to be due to spread of one clone of VRE.

The work of this thesis confirms that the increased occurrence of VRE is caused by the spread of one predominant clone. Both the predominant and the minority clones are *E. faecium* with plasmid mediated *vanA* gene.

The *vanA* gene is transferrable from the predominant and several of the minority clones. Thus, the predominance of one clone is not due to that the resistance is untransferable from the clones. It also confirms that VRE among Swedish broilers have a potential for zoonotic spread of the *vanA* gene. Plasmid addiction systems are most likely not involved in the retention of the *vanA* gene as there is a near absence of such systems among VRE from Swedish broilers. Decreased susceptibility to the ionophore narasin can be co-transferred with the *vanA* gene from the predominant and some of the minority clones. Thereby the use of narasin for coccidial prophylaxis could contribute to retention of the *vanA* gene. The traits are probably located close to each other so when retaining the decreased susceptibility to narasin, the enterococci also retain the *vanA* gene.

Broilers arriving to the farms are colonized with VRE persisting in the broiler houses. Differences in occurrence of VRE among farms indicate that the occurrence of VRE can be reduced. Furthermore, a reduction in the contamination of the broiler houses can be achieved by disinfection with a method combining steam and formaldehyde. Subsequently, this could reduce the occurrence of VRE in Swedish broiler production on the whole.

Further research should focus on the role of narasin in the VRE epidemiology as well as exploring the possibility to eliminate VRE from Swedish broiler production.

Keywords: VRE, epidemiology, broiler, vancomycin, *vanA*, *Enterococcus faecium*, disinfection, gene transfer, narasin, plasmid addiction system

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Dedication

Till hen – antagligen inte sista gången du ändrar på mina planer.

Evolution of bacteria towards antibiotic resistance is unavoidable as it represents a particular aspect of the general evolution of bacteria. Thus, at the very best, the only hope we can have in the field of resistance is to delay dissemination of resistant bacteria or resistance genes.

Patrice Courvalin

I drink champagne when I'm happy and when I'm sad. Sometimes I drink it when I'm alone. When I have company I consider it obligatory. I trifle with it if I'm not hungry and drink it when I am. Otherwise I never touch it - unless I'm thirsty.

Madame Lilly Bollinger

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nilsson, O., Greko, C., Top, J., Franklin, A., Bengtsson, B. (2009). Spread without known selective pressure of a vancomycin-resistant clone of *Enterococcus faecium* among broilers. *Journal of Antimicrobial Chemotherapy* 63(5), 868-872.

II Nilsson, O., Greko, C., Bengtsson, B., Englund, S. Genetic diversity among VRE isolates from Swedish broilers with the coincidental finding of transferrable decreased susceptibility to narasin. (manuscript).

III Nilsson, O., Greko, C., Bengtsson, B. (2009). Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production. *Acta Veterinaria Scandinavica* 51, 49.

IV Nilsson, O., Greko, C., Vågsholm, I., Bengtsson, B. Survival of vancomycin resistant *Enterococcus faecium* under laboratory and field conditions – a pilot study to investigate if eradication from broiler farms is possible? (manuscript).

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Abbreviations

ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
MLVA	multiple locus variable number tandem repeat analysis
MRSA	meticillin resistant *Staphylococcus aureus*
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
RAPD-PCR	random amplified polymorphic DNA-PCR
SPMA	Swedish Poultry Meat Association
ST	sequence type (for MLST)
SVARM	Swedish Veterinary Antimicrobial Resistance Monitoring programme
VRE	vancomycin resistant enterococci

1 Introduction

Epidemiological knowledge is an important factor in reducing any infectious disease. For a zoonotic agent the need for knowledge is not limited to its spread from animals to humans. Instead, all aspects of the diseases persistence and spread among animals are at least as important.

1.1 Foodborne zoonoses

The World Health Organisation defineds a zoonosis as "any disease or infection that is naturally transmissible from vertebrate animals to humans and vice-versa" (WHO, 2011). The transmission from animals to humans can be either direct or indirect (Figure 1). One route for indirect transfer is contaminated animal or vegetable food products (Schjorring & Krogfelt, 2011; Giraffa, 2002). The scenario regarding spread of zoonotic agents via the food chain, i.e. foodborne zoonoses, is contamination of human food products by organisms from animals which are pathogenic to man. Meat products could for example be contaminated by faecal material at the slaughterhouses whereas vegetables may be contaminated in the field by manure or sewage water used for fertilization and irrigation.

Normally, it is the agent *per se* that is zoonotic (e.g. Norovirus, *Salmonella* and *Campylobacter* spp.). However, regarding antimicrobial resistant bacteria it is not only pathogens that could have a zoonotic potential, but also the genes encoding antimicrobial resistance in commensals such as *Escherichia coli* and *Enterococcus* spp. which may transfer to more pathogenic organisms (Schjorring & Krogfelt, 2011; EFSA, 2008b). Here, the scenario is that once in the human intestine, the bacteria might colonize and persist or their presence may only be transient (Trobos *et al.*, 2009; Berchieri, 1999). Even if the animal derived bacteria colonize the human intestine only for a short time, this can be sufficient for resistance genes to be transferred to other

strains better adapted to colonize humans (Lester *et al.*, 2006). These human adapted strains can then persist for long periods and also spread to other people. The zoonotic potential of certain antimicrobial resistance genes means any resistant bacteria present in farm animals may be considered a reservoir for resistance that can spread affecting both veterinary and human medicine (Witte, 2000).

This zoonotic potential is demonstrated by the emergence of resistance to streptothricin in East Germany (Witte, 2000; Tschape, 1994; Hummel *et al.*, 1986). In 1983, when a streptothricin antimicrobial (nourseothricin) was introduced for animal growth promotion in East Germany no resistance to that antimicrobial was seen in *Enterobacteriaceae* from humans or animals. After a couple of years, resistance to streptothricin mediated by a transposon located gene coding for streptothricin acetyltransferase was found in *E. coli* from pigs. In the subsequent years, resistance spread further and was found not only in *E. coli* from pig farmers but also from healthy people in the community as well as in clinical isolates of *E. coli*. Finally, resistance to streptothricin spread to other bacteria and was found in clinical isolates of *Salmonella* and *Shigella* spp.

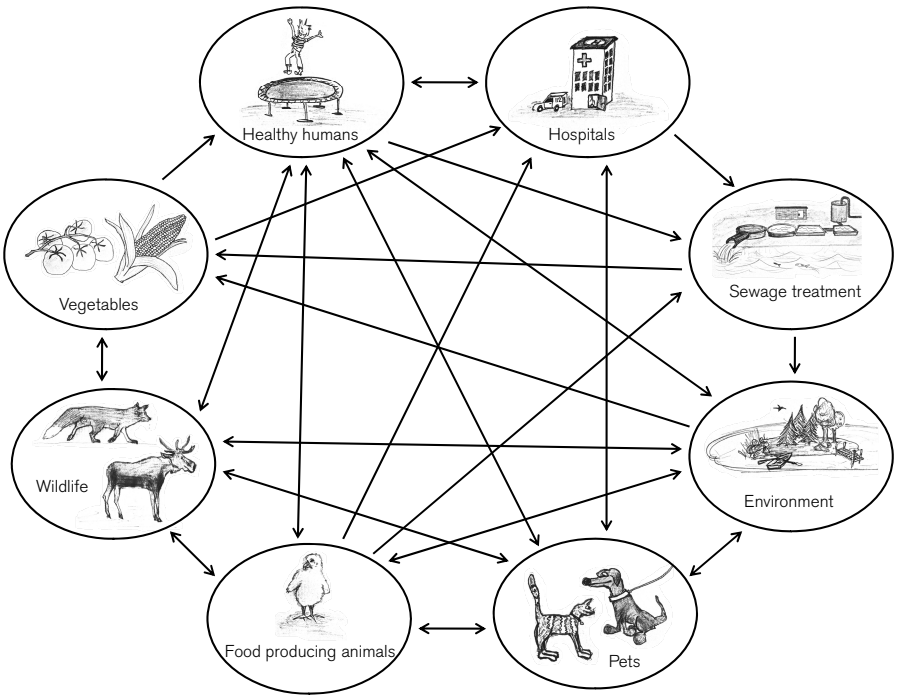

Figure 1. Various routs by which zoonotic bacteria can spread between animals and humans. The same routes apply also for resistance genes. Illustrations by K. Dahl.

1.2 Swedish broiler production

Broiler production is often illustrated as a pyramid (Figure 2) with the elite animals and pedigrees at the top and the broilers in the bottom. The production is highly organized and the breeding is controlled by a few large international companies. The Swedish Poultry Meat Association (SPMA) organises companies within Sweden involved in all parts of the production chain (i.e. feed manufacturers, producers of hatching eggs, hatcheries, broiler producers and slaughterhouses). The association has around 145 broiler producers as members. Members of SPMA account for 98-99% of the domestic production of about 75 million broilers each year, which is about 65-70% of the broiler meat consumed annually in Sweden.

The Swedish broiler production is dependent on import of day-old grandparent birds for breeding parent birds, and later broilers. Currently this import is managed by only two companies, Aviagen SweChick AB and Blenta AB importing the hybrids Ross from Scotland and Cobb from England respectively. In total, there are two hatcheries for parent birds and three hatcheries for broilers in Sweden. The broilers and the by-products (males from the female line and females from the male line) from the parent level are delivered as day-old chickens to the broiler producers. After the grow-out period, the broilers are sent to slaughterhouses which are also associated to the companies delivering the day-old chickens to the broiler producers.

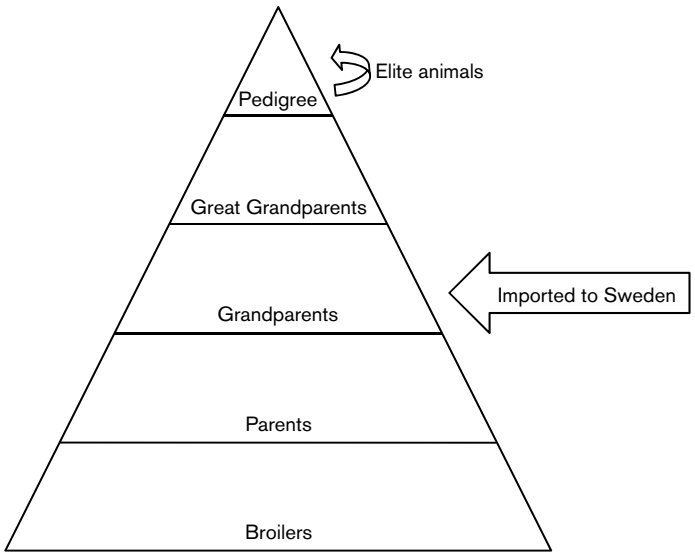

Figure 2. "The broiler pyramid" illustrating the hierarchical structure of commercial poultry production.

A standard Swedish broiler farm consists of one or several houses, each consisting of one to four compartments with a separate service room (Figure 3). Before entering the service room, personnel should change coverall and footwear and wash their hands. At the average farm, around 20 000 chickens are raised within each compartment and the broilers are sent for slaughter when they are about 35 days old. Litter material used is sawdust, wood shaving or short cut straw. During the growing period the broilers have *ad lib* access to water and feed. Normally the feed consists of pelleted cereals but most producers also feed their broilers with whole wheat in amounts increasing by age. When the broilers have been sent for slaughter, the houses are thoroughly cleaned and disinfected. Then follows an empty period of about one week when there are no broilers at the farm before the next batch of day-old chickens arrives. This production system is normally referred to as an all-in all-out procedure or batch production.

All members of SPMA are also, apart from practicing all-in all-out procedure, obliged to be enrolled in various health programmes such as the *Salmonella* control programme and the *Campylobacter* programme.

Figure 3. A compartment at a standard Swedish broiler farm. Photo by Eva Berntsson.

1.3 Vancomycin resistant enterococci

The term vancomycin resistant enterococci (VRE) includes many forms of both bacteria and resistances. This is best elucidated by further exploring the three words in the term.

1.3.1 Vancomycin

Vancomycin is a glycopeptide antimicrobial produced by the soil bacteria *Streptomyces orientalis* (McCormick *et al.*, 1955). It was developed and introduced in the 1950s (Reynolds, 1989). Another glycopeptide authorised for use in humans in Sweden is teicoplanin (Läkemedelsverket, 2011).

Glycopeptides interfere with the cell wall production resulting in a destabilized cell wall and lysis of the bacteria (Reynolds, 1989). When the bacterial cell wall is synthesized, polysaccharide-pentapeptide complexes are linked together via a transpeptidation reaction in which the end amino acid of the pentapeptide is removed (Reynolds, 1989). Glycopeptides interfere with this process by binding tightly to the D-Alanyl-D-Alanin (D-Ala-D-Ala) end of the pentapeptide and hiding it from the transpeptidase that is to catalyse the cross-linking in the peptidoglycan synthesis (Reynolds, 1989).

Vancomycin is active against most Gram positive bacteria whereas the majority of Gram negatives are resistant (Walsh, 2003; French, 1998). It is considered a drug of "last resort" and has been classified as critically important for human medicine for treatment of patients with severe infections with multi-drug resistant *Enterococcus* spp. and meticillin resistant *Staphylococcus aureus* (MRSA) as the main indications (WHO, 2009). Vancomycin is also used for intestinal infections, especially pseudomembranous colitis caused by *Clostridium difficile* where the poor absorption of vancomycin when administered orally is advantageous (Kirst *et al.*, 1998).

1.3.2 Enterococci

Enterococci are intestinal bacteria colonizing humans and other mammals as well as birds, reptiles and insects (Martin & Mundt, 1972; Mundt, 1963; Sherman, 1937). They are Gram positive, facultative anaerobes, catalase negative and non sporeforming cocci occurring either as single bacteria, in pairs or in short chains (Fisher & Phillips, 2009; Schleifer & Kilpper-Bälz, 1984). They can sustain various adverse conditions and can survive for several months in the environment (Kramer *et al.*, 2006; Barnes, 1959).

Until 1984 enterococci were considered a part of the genus *Streptococcus*, even though they were first described and tentatively named enterococci (*entérocoque*) in 1899 (Schleifer & Kilpper-Bälz, 1984; Thiercelin, 1899).

Today, 40 different species of enterococci have been described (Euzeby, 2011). The species most frequent in the intestines of humans are *Enterococcus faecalis,* and to a lesser extent *E. faecium* whereas the most common species in various farm animals are *E. faecium* together with *E. cecorum*, *E. faecalis*, and to some extent *E. hirae* (Klein, 2003; French, 1998; Devriese *et al.*, 1991).

Even though the first description in 1899 referred to enterococci as potential pathogens they were for a long time regarded as harmless intestinal bacteria without clinical importance (Fisher & Phillips, 2009; Thiercelin, 1899). Nowadays however, enterococci are recognised as important opportunistic pathogens, especially causing nosocomial infections such as urinary tract infections, wound infections and endocarditis (Fisher & Phillips, 2009). The clinically most important species in human medicine are *E. faecalis* and *E. faecium* (Werner *et al.*, 2008). Of these, *E. faecalis* is the most pathogenic species but *E. faecium* is of increasing importance as it is generally more frequently resistant to antimicrobials (French, 1998).

1.3.3 Enterococcal resistance to vancomycin

Until today, nine different variants of vancomycin resistance in enterococci have been described (vanA, B, C, D, E, G, L, M and N; Table 1) (Hegstad *et al.*, 2010; Lebreton *et al.*, 2010; Xu *et al.*, 2010; Boyd *et al.*, 2008; Courvalin, 2006). Among those, the three most common variants are the vanA, B and C types with *E. faecium* carrying the *vanA* genotype as the most common combination (Fisher & Phillips, 2009; Werner *et al.*, 2008). An additional variant (vanF) has also been described but thus far only in *Paenibacillus popilliae* (Patel *et al.*, 2000). Since the vanF variant has a high similarity in amino acid sequences to the vanA variant, *P. popilliae* has been suggested as a possible origin for vancomycin resistance in enterococci (Patel *et al.*, 2000). Other plausible sources are various glycopeptide producing organisms, even if genetic differences make an older common source more likely (Patel, 2003).

Common to all variants of vancomycin resistance in enterococci is the ability to cause a change in the structure of the pentapeptide incorporated in the three dimensional web of peptidoglycans composing the bacterial cell wall: from the original D-Ala-D-Ala to either D-Ala-D-Lactate (D-Ala-D-Lac) or D-Ala-D-Serine (D-Ala-D-Ser) (Courvalin, 2006). This shift results in a reduced affinity for vancomycin by 1000 and 7 times respectively (Fisher & Phillips, 2009).

Table 1. *Characteristics of different types of vancomycin resistance described among* Enterococcus *spp.*

	Sort	Modified target	Range of MIC (mg/L)		Expression	Location	Transferable
			vancomycin	teicoplanin			
vanA	Acquired	D-Ala-D-Lac	64-1000	16-512	Inducible	Chromosome or plasmid	Yes
vanB	Acquired	D-Ala-D-Lac	4-1000	0.5-1	Inducible	Chromosome or plasmid	Yes
vanC	Intrinsic	D-Ala-D-Ser	2-32	0.5-1	Constitutive or inducible	Chromosome	No
vanD	Acquired	D-Ala-D-Lac	64-128	4-64	Constitutive or inducible	Chromosome	No
vanE	Acquired	D-Ala-D-Ser	(6) 8-32	0.5	Inducible	Chromosome	No
vanG	Acquired	D-Ala-D-Ser	16	0.5	Inducible	Chromosome	Yes
vanL	Acquired	D-Ala-D-Ser	8	<8	Inducible	Chromosome	No
vanM	Unknown	D-Ala-D-Lac	>128	64->256	Inducible	Unknown	Yes
vanN	Acquired	D-Ala-D-Ser	8 (12)	2	Constitutive	Plasmid	Yes

MIC = minimum inhibitory concentration (mg/L).

D-Ala-D-Lac = D-Alanyl-D-Lactate, D-Ala-D-Ser = D-Alanyl-D-Serine

Adapted from CLSI, 2010; Hegstad et al., 2010; Lebreton et al., 2010; Xu et al., 2010; Boyd et al., 2008 and Courvalin, 2006.

In all different variants of vancomycin resistance are several genes involved in the alteration of the cell wall structure which results in the resistance. The number and organisation of these genes are somewhat similar among the different variants. For the vanA variant, the genes are organized as in Figure 4. *VanS* is a sensor gene which in the presence of a glycopeptide phosphorylate, and thus activate the regulator gene *vanR* (Courvalin, 2006). After activation of the gene complex, *VanH* mediates production of lactate from pyruvate which *vanA* uses to synthesize the alternative D-Ala-D-Lac end of the pentapetide (French, 1998). It is essential for resistance that production of the normal D-Ala-D-Ala end of the pentapetide does not continue. This is resolved by the *vanX* and *vanY* genes where *vanX* hydrolyzes and thereby interrupts the production of the pentapeptides, and *vanY* cleaves the pentapeptides that might still be produced (French, 1998; Arthur *et al.*, 1996). In the absence of a glycopeptide, *vanS* initiates dephosphorylation of *vanR* resulting in deactivation of the gene (Courvalin, 2006).The function of the *vanZ* gene is not understood (Courvalin, 2006).

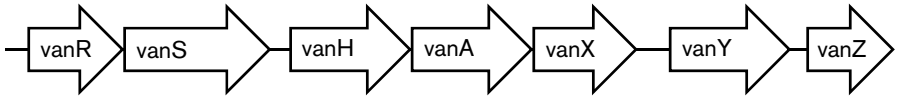

Figure 4. Organization of the genes involved in the vanA variant of vancomycin resistance in enterococci. Adapted from Courvalin, 2006.

1.4 Clinical impact of VRE

The first cases of infection with vancomycin resistant entrerococci were seen in 1986 (Leclercq *et al.*, 1988; Uttley *et al.*, 1988). Since then, VRE have spread around the world and are now one of the most important causes of nosocomial infections, even if asymptomatic intestinal colonization is much more common than morbidity (Patel, 2003). When infections with VRE do occur, the consequences are often worse compared to infections with vancomycin susceptible enterococci. Associations have been seen between infections with VRE and increases in therapy failure, length of hospital stay as well as mortality (DiazGranados *et al.*, 2005; Patel, 2003). However, studies where no significant difference is seen also exist (Garbutt *et al.*, 2000).

1.5 VRE in farm animals

Feeding animals low doses of antimicrobials may in certain conditions increase their productivity, for example by improving feed conversion and decreasing morbidity and mortality caused by clinical and subclinical infections (Butaye *et al.*, 2003). The growth promoting effect of antimicrobials was first discovered when broiler chickens were fed the fermentation leftovers after production of antimicrobials (Stokstad & Jukes, 1950; Stokstad *et al.*, 1949). Notable is that all use of growth promoters in Sweden was forbidden in 1986 by the Feedingstuffs Act (SFS 1985:295).

The glycopeptide avoparcin was first introduced for growth promotion in 1975 (Hammerum *et al.*, 2010). At that time it was used extensively in most parts of Europe and the rest of the world with the notable exception of Canada and USA where avoparcin never has been approved for animals (McDonald *et al.*, 1997). Avoparcin was mainly used for broilers and pigs but to some extent also for turkeys, veal calves and other animals (Hammerum *et al.*, 2010; Wegener, 1998; Bates, 1997). The extent of avoparcin use is demonstrated by data from Denmark where 24 kilograms of vancomycin was used in human medicine in 1994, and in the same year more than 24 000 kilograms of avoparcin was used for growth promotion (Wegener, 1998). A similar example is Australia, where from 1992 to 1996 less than 600 kilograms of vancomycin but over 62 000 kilograms of avoparcin was imported (Witte, 1998).

As avoparcin confers cross-resistance to vancomycin the (mis)use of avoparcin selected for VRE (Bager *et al.*, 1997). Hence, VRE, i.e. *E. faecium* carrying the *vanA* genotype was common in the intestinal flora of farm animals in Europe during the 1990's (Aarestrup, 1995; Klare *et al.*, 1995b). By contrast, since avoparcin has never been approved in Canada and USA, VRE had until 2008 never been isolated from farm animals in USA (Donabedian *et al.*, 2010).

When the connection between avoparcin and VRE in farm animals was confirmed, the use of avoparcin was discontinued as a precautionary measure to avoid further spread of VRE to the community and into hospital settings (Anonymous, 1997b). Apart from Sweden, the use of avoparcin in Europe ceased first in Denmark, Finland and Norway (Aarestrup *et al.*, 2000). Later it ceased in Germany and finally in the whole of the European Union as a consequence of the Commission Directive 97/6/EC (Anonymous, 1997b). Avoparcin has later been banned or phased out also in other parts of the world (Hammerum *et al.*, 2010; Lauderdale *et al.*, 2007; Yoshimura *et al.*, 1998). Once the use of avoparcin had been discontinued,

the prevalence of VRE in farm animals rapidly declined (Aarestrup *et al.*, 2001; van den Bogaard *et al.*, 2000; Klare *et al.*, 1999).

The decreased occurrence of VRE in animals after the use of avoparcin was discontinued reinforces the theory that if the selective pressure is removed, the antimicrobial resistance will disappear (Andersson & Hughes, 2010; Andersson & Levin, 1999). However, when using selective media (i.e. media with vancomycin) VRE could and can still be readily detected (Ghidan *et al.*, 2008; Garcia-Migura *et al.*, 2007; Lim *et al.*, 2006; Sorum *et al.*, 2006; Novais *et al.*, 2005; Heuer *et al.*, 2002a; Borgen *et al.*, 2000a). Furthermore, a recent study modelling persistence of VRE indicates that it will be present among farm animals for a long period of time which is also in agreement with today's view on the timeframe of reversal of antimicrobial resistance (Johnsen *et al.*, 2011; Andersson & Hughes, 2010).

Different theories about why VRE persist among farm animals have been presented. In Denmark, the use of the macrolide tylosin in pigs was suggested to co-select for vancomycin resistance among enterococci since the genes encoding the two resistances were located on the same plasmid (Aarestrup, 2000). A similar but weaker correlation with co-selection by copper resistance has also been suggested (Hasman & Aarestrup, 2005). Another explanation that has been suggested is that plasmid addiction systems located on the same plasmid as the *vanA* gene would force the bacteria to retain the resistance (Johnsen *et al.*, 2005).

1.6 Spread of vancomycin resistance

Spread of vancomycin resistance can be due to dissemination of resistant bacteria and for some of the variants also to horizontal transfer of the resistance genes (Bonten *et al.*, 2001). Both ways are possible for the vanA variant and is among other things of importance for the zoonotic potential.

Similar strains of VRE have been isolated from pigs and humans (Freitas *et al.*, 2011). Furthermore, when the use of avoparcin was discontinued in Europe, not only did the occurrence of VRE among farm animals decrease but there was also a subsequent decrease in the occurrence of VRE in food of animal origin and in the prevalence of human colonization with VRE (Klare *et al.*, 1999; Pantosti *et al.*, 1999).

Transfer of the *vanA* gene is associated with mediation by a transposon, a mobile genetic element that can be incorporated either in the bacterial chromosome or on plasmids (Courvalin, 2006; Tamarin, 2004). More specifically the genes are located in and transferred by the transposon Tn*1546* or closely related genetic elements (Courvalin, 2006). Also, the

possibility for *in vivo* transfer of vancomycin resistance from VRE of animal origin to enterococci of human origin in the intestines of humans has been described (Lester *et al.*, 2006). Furthermore, *in vivo* transfer of the vanA gene to hospital adapted enterococci has also been demonstrated (Lester & Hammerum, 2010).

1.7 The Swedish situation, human side

Cases of vancomycin resistant *E. faecalis* and *E. faecium* within human medicine in Sweden became notifiable according to the Swedish Communicable Diseases Act in 2000. From then and until 2006, the number of persons colonized or infected varied from 18 to 47 per year, and over that period a total of 185 cases were reported (Smittskyddsinstitutet, 2011). In the autumn of 2007 the number of people colonized or infected with VRE increased considerably. The outbreak involved several counties over various parts of Sweden and from 1th August 2007 to the end of 2009 a total of 986 domestically acquired cases were reported (SWEDRES, 2010).

In most parts of the world, VRE of the vanA variant are the most prevalent among human cases (Werner *et al.*, 2008). In contrast, the majority of the Swedish cases (87%) were *E. faecium* with the vanB variant of vancomycin resistance (SWEDRES, 2010). Moreover, most of these isolates belonged to one clone as indicated by identical or closely related profiles when analysed with pulsed-field gel electrophoresis (Soderblom *et al.*, 2010; SWEDRES, 2010). Notably, Australia also has the majority of their cases among humans of the vanB type (Johnson *et al.*, 2010; Bell *et al.*, 1998). The reasons for the dominance of the vanB type resistance in Sweden and Australia are unknown (Soderblom *et al.*, 2010; SWEDRES, 2010; Bell *et al.*, 1998). Notably, in 2010 the number of cases with *vanB* decreased considerably whereas the number of cases with *vanA* was stable (SWEDRES, 2011).

There are also indications that VRE disseminate from humans and into the environment. VRE of both vanA and vanB type have been detected in various samples of sewage (Sahlstrom *et al.*, 2009; Iversen *et al.*, 2002). The distribution between *E. faecium* with the *vanA* or the *vanB* gene differed between the two studies. The source of the *E. faecium* with the *vanB* gene is most likely humans. The source of *E. faecium* with the *vanA* gene is more uncertain even though human cases of vanA type VRE had already been reported in Sweden (Torell *et al.*, 1997).

1.8 The Swedish situation, farm animal side

In Sweden, avoparcin was only used for a short period of time from the end of the 1970's until 1984 and the yearly usage by the end of that period was between 7 000 and 9 000 kilograms (Wierup *et al.*, 1987). Furthermore, all use of growth promoters in Sweden was forbidden in 1986 by the Feedingstuffs Act (SFS 1985:295).

In accordance with the low selective pressure for vancomycin resistance by avoparcin use that enterococci in Swedish farm animals had been exposed to, VRE were not isolated in samples from Swedish broilers or pigs in the middle of the 1990's (Quednau *et al.*, 1998; Anonymous, 1997a). However, in a study conducted 1998 to 2000 the first VRE from Swedish farm animals were isolated (Kuhn *et al.*, 2005). In that survey, VRE were isolated from 4 out of 150 faecal samples from broilers and 1 out of 306 faecal samples from pigs.

In the following years, the occurrence of VRE increased and in samples analysed within the Swedish Veterinary Antimicrobial Resistance Monitoring programme (SVARM), the proportion of broilers colonized with VRE increased from less than 1% in 2000 to over 40% in 2005. In addition, all of the isolates were *E. faecium* with high level resistance to vancomycin and the vast majority did also display similar resistance patterns. This situation called for further investigations and a number of pilot studies were initiated. These studies investigated for example the epidemiology of the observed increase including possible ways of introduction, the persistence of the VRE at farm level including possible ways of reducing the occurrence, as well as prevalence of VRE both at farm level and on broiler meat in retail stores (Jansson *et al.*, 2009a; Jansson *et al.*, 2009b; Franklin *et al.*, 2005 and unpublished results).

Until present in Sweden, apart from the single isolate from a pig mentioned above, VRE have only been isolated from broilers. Furthermore, the proportion of VRE among randomly selected enterococci from Swedish broilers is very low with only 4 VRE found among 1666 investigated enterococci out of which 1040 are *E. faecium* (SVARM, 2011).

2 Aims of the thesis

The motivation for this project was a desire to eliminate, or at least reduce the occurrence of VRE in Swedish broiler production. In order to succeed with this, knowledge about the epidemiology is vital.

The more specific aims of the project were:

- To study the increase in the proportion of Swedish broilers colonized with VRE, with the hypothesis that it was caused by the spread of one clone of VRE.
- To study the diversity among VRE from Swedish broilers, with the hypothesis that similarities exist among the clones.
- To study if vancomycin resistance in VRE from Swedish broilers is transferrable, with the hypothesis that it is transferrable.
- To study why vancomycin resistance is retained by enterococci in Swedish broilers, with the hypothesis that the resistance is linked to traits ensuring stable inheritance.
- To study environmental contamination with VRE on farms during grow-out, with the hypothesis that the contamination decreases but is not eliminated when broiler houses are cleaned and disinfected between batches.
- To study possibilities for VRE control in Swedish broilers, with the hypothesis that elimination from the broiler houses is possible if sufficient exposure of the VRE to the disinfectant is achieved.

3 Materials and Methods

3.1 Sampling procedures

3.1.1 Caecal samples

In Paper I, III and IV of this thesis, caecas from broilers were used to investigate VRE colonization. The caecas sampled in Paper III and IV were obtained through the Swedish *Campylobacter* programme, whereas the caecas sampled in Paper I initially were obtained directly from the slaughterhouses and from 2005 and onwards through the *Campylobacter* programme. Only one slaughter group from each broiler house was included, i.e. if all broilers within a compartment were not slaughtered on the same day only samples from the first group sent for slaughter were included. In addition, only one caecum per slaughter group was analysed in Paper I whereas all ten caecas available from each slaughter group were analysed in Paper III and IV.

The Swedish *Campylobacter* programme is organized by the SPMA and covers the majority of broilers raised in Sweden. Within the programme, caecas from ten birds in each slaughter group are collected by personnel at the slaughterhouses. The caecas are then analysed for the presence of campylobacter at the National Veterinary Institute. The rationale for using caecas from the *Campylobacter* programme is that it is a convenient way to get samples that are also representative of the Swedish broiler production.

3.1.2 Environmental samples

The environment within the broiler houses were sampled with two different commercial kits; "Sterisocks humid" and "Sterile cloth" (SodiBox). These products were originally developed for the French Agency for Food, Environmental and Occupational Health and Safety to sample animal farms and food industries for salmonellae and other bacteria. Socks were used to

sample the floor of the compartments and cloths were used to sample air inlets, air outlets, feed lines and water lines.

In Paper III, environmental samples were taken from all the sites mentioned above. In Paper IV, samples were taken only from air outlets, feed lines and water lines.

3.2 Culturing methods

3.2.1 Selective media

All caecal and environmental samples analysed were cultured on selective media, i.e. Slanetz–Bartley agar supplemented with vancomycin, and incubated at 37°C for 48 hours. Slanetz–Bartley is a medium developed to favour enterococci (Slanetz & Bartley, 1957). Depending on the concentration of vancomycin used in the media the inhibition of other bacteria than VRE is more or less stringent. But the selection of different variants of VRE is also affected since they exhibit diverse levels of minimum inhibitory concentration (MIC) of vancomycin (see Table 1, page 15).

In the SVARM programme (hence also in Paper I) the concentration in the Slanetz–Bartley agar was 8 mg/L from 2000 to 2002. From 2004 and onwards in the SVARM program (and Paper I) as well as in Paper III and IV, the concentration of vancomycin in the agar was increased to 16 mg/L.

That a vancomycin concentration of 16 mg/L was used probably inhibited some variants of VRE, for example enterococci with intrinsic vanC type resistance. A major concern would be a possible elimination of some VRE of the vanB variant, as they can have MIC of vancomycin as low as 4 mg/L. However, over 88% of the VRE with vanB variant in Swedish healthcare during 2009 had MIC of vancomycin of at least 16 mg/L[1]. Thus, it is unlikely that VRE of the vanB type was consistently missed.

3.2.2 Prepreparation of samples

Depending on the sample and study, the handling of the samples before culture on Slanetz–Bartley agar has varied.

For caecal samples, the first step has always (i.e. in Paper I, III and IV) been to suspend approximately 0.5 grams of faecal material in 4.5 mL of saline. For the samples from 2000 to 2002 included in Paper I, the suspension was enriched before cultured on selective media. Thus, the faecal suspension was mixed with Enterococcosel broth containing vancomycin (8

[1] Barbro Liljequist, SMI, personal communication.

mg/L final concentration) and incubated at 37°C for 24 hours. The samples in Paper I collected after 2002, as well as the samples in Paper III and IV were however not enriched but cultured directly on selective media.

Environmental samples, i.e. socks and cloths, in both Paper III and IV were treated essentially the same way. Broth was added to the samples before they were placed in a stomacher and treated for one minute. Then, the wash fluid was (i) cultured directly on selective media and (ii) enriched before cultured on selective media. In Paper III, the broth used was Enterococcosel whereas in Paper IV buffered peptone water was used. The reason for not using Enterococcosel in Paper IV was that a more gentle treatment of the samples was desired as the samples were taken after disinfection and were anticipated to contain damaged but still viable bacteria.

3.3 Species identification

Potential VRE on selective media in Paper I, III and IV were first subcultured on horse blood agar and then on Bile-Esculine agar. If morphological appearance also on these media indicated that the isolates were enterococci, all (Paper I) or a subset (Paper III and IV) were identified to species level by fermentation of various sugars, e.g. arabinose, manitol, ribose, sacarose and sorbitol (Devriese *et al.*, 1993). The species identification of selected isolates were confirmed using PCR (Dutka-Malen *et al.*, 1995). For quality control, either of the reference strains *E. faecalis* ATCC 29212 or 33186 was used.

3.4 Antimicrobial susceptibility testing

All antimicrobial susceptibility testing (Paper I-IV) was performed by determining of MIC with a microdilution method using cation-adjusted Mueller–Hinton broth according to the standards of the CLSI (2008). Tests were performed in VetMIC™ E-cocci panels produced by the National Veterinary Institute and the same reference strains as for species identification were used for quality control.

Obtained MIC values were interpreted according to epidemiological cut-off values issued by EUCAST (2011).

3.5 Molecular typing methods

3.5.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to investigate the presence of various genes in three of the papers included in this thesis.

In Paper I and II, PCR was used for confirmation of the vanA resistance genotype in the VRE (Dutka-Malen *et al.*, 1995). In addition, the Tn*1546* transposon in which the genes responsible for the vancomycin resistance are located was typed using ten different PCRs amplifying the whole transposon (Woodford *et al.*, 1998). Lastly, PCR was also used in Paper II to look for two different plasmid addiction systems and six enterococcal plasmid replicon types (Jensen *et al.*, 2010; Rosvoll *et al.*, 2010; Sletvold *et al.*, 2008).

3.5.2 Multilocus sequence typing

Multilocus sequence typing (MLST) is a typing scheme used in Paper I-III to investigate the genetic relationship among VRE isolates.

In MLST, a number of housekeeping genes (seven for *E. faecium*) are amplified with PCR, sequenced and then compared to previously known sequences of the genes (Homan *et al.*, 2002). Based on the nucleotide sequences, each gene is assigned an allele number and the generated seven digit code is interpreted to a sequence type (ST).

In Paper I and II, MLST was used since it is a typing method especially useful when strains isolated over long time periods are investigated (Homan *et al.*, 2002; Enright & Spratt, 1999). Furthermore, the possibility for comparison of the variants of VRE identified among Swedish broilers to isolates of enterococci from other parts of the world also contributed to the choice of MLST as typing method in those two studies.

However, in Paper III the aim for the molecular typing was to confirm that it was indeed the clone dominating among Swedish broilers that was persisting. This could have been done with several other methods (e.g. RAPD-PCR, PFGE, MLVA). But then the variation among isolates of the dominating clone for the alternative method would first need to be established. Hence, MLST was chosen in order to avoid that extra work.

3.5.3 Pulsed-field Gel Electrophoresis

In Paper II, mating experiments was performed to assess the transferability of vancomycin resistance. The isolates obtained could either be tranconjugants, i.e. originating from the recipient strain that had received the *vanA* gene, or mutants, i.e. originating from the donor strains that had become resistant to rifampicin and fusidic acid. To confirm that they did indeed originate from

the recipient strain a typing method with high discriminatory ability was desirable. Therefore pulsed-field gel electrophoresis (PFGE) with *Sma*I as restriction enzyme was chosen.

When performing PFGE, bacteria are incorporated in agarose gel plugs and lysed before the DNA is digested with restriction enzymes cleaving at specific, and preferably rare, nucleotide sequences. The resulting DNA fragments are then separated in a gel by applying an electrical current switching between two different directions with an angel of, for example, 120°. Finally, the bands are visualised by staining and the band patterns are analysed (van Belkum *et al.*, 2007; Sambrook & Russell, 2001).

For epidemiological typing of VRE, PFGE has been considered as the "gold standard" (Homan *et al.*, 2002). However, in some situations it can be too discriminatory and isolates that are actually the same strain can differ from each other with several bands (Morrison *et al.*, 1999).

In Paper II, a variation of PFGE designed to detect and determine the size of plasmids was used. Instead of digesting the DNA with a restriction enzyme it was treated with S1 nuclease. The S1 nuclease converts the circular or supercoiled three dimensional structures of plasmids into full length linear molecules (Barton *et al.*, 1995). When separated on a gel, instead of three possible bands one plasmid then results in only one band.

3.6 Filter mating

To assess the possibility that vancomycin resistance will spread from VRE in Swedish broilers to other enterococci a number of isolates were subjected to mating experiment as a part of Paper II.

In mating experiments the potential donor strain and the recipient are mixed and cultured together before screening for transconjugants, i.e. recipient strains that have become resistant to the antimicrobial of interest. Mating experiments can be performed either as filter or broth mating. For filter mating, broth cultures of the donor and recipient strains are mixed, added to a filter and cultured over night on blood agar. Then, the bacteria are suspended and cultured on media selecting for transconjugants. The difference in broth mating compared to filter mating is that the strains are mixed and kept in broth when incubated over night (Toomey *et al.*, 2009)

In Paper II, filter mating was done and performed as described by Hammerum et al. (1998) and with *E. faecium* 64/3 as recipient. Since *E. faecium* 64/3 is resistant to rifampicin and fusidic acid and the resistance of interest in this case was vancomycin resistance, the agar plates used for screening for transconjugants contained all these three antimicrobials.

3.7 Fitness studies

In Paper IV, the ability of three VRE from Swedish broilers to survive and/or grow in various physical conditions was investigated. Assessed parameters were growth at different pH and temperatures, survival at different temperatures, tolerance to high temperatures and susceptibility to disinfectants.

To investigate growth at different pH and temperatures, bacteria were inoculated in Mueller-Hinton broth (normal or with adjusted pH) and incubated (at test temperature or 37°C) before growth was determined as visible turbidity.

To investigate survival at different temperatures, bacteria were pipetted into empty Petri dishes and allowed to dry. After various times of storage at 6, 21.5 or 31°C, the number of surviving bacteria was determined by dissolving them in saline, pouring on agar and incubating over night.

To investigate tolerance to high temperatures, bacteria were inoculated in pre-heated Mueller-Hinton broth and incubated for four hours at test temperature, cooled and incubated over night before growth was determined as visible turbidity.

To investigate the susceptibility to disinfectants, a quantitative suspension test was used for the liquid agents (Reybrouck, 2004). Whereas for the gaseous agent, bacteria were pipetted into empty Petri dishes and allowed to dry and exposed to the agent before the number of surviving bacteria was compared to the number in unexposed dishes.

3.8 Field disinfection experiment

In Paper IV, a disinfection method that combines steam and formaldehyde was evaluated. Both the effect on occurrence of VRE in the broiler houses and on colonization of the birds sent for slaughter was investigated.

The method was originally developed to sanitize layer hen farms from salmonellae but its efficacy towards enterococci had been shown under both laboratory and field conditions (Gradel *et al.*, 2004a; Gradel *et al.*, 2004b).

3.9 Statistical analysis

Statistical analyses were used in Paper I and III to investigate significant changes and associations. Since the data in both Paper I and III are mainly descriptive, only basic analyses, i.e. Pearson's χ^2 test, were performed. All statistical work was done using Stata software (release 10, Stata, College Station, TX, USA).

4 Results and Discussion

The extensive use of avoparcin for growth promotion in farm animals can be seen as a full scale experiment of survival of the fit. Furthermore, the long term impact of the selection for VRE among farm animals is illustrated by the predicted persistence for at least forty years in Danish broiler production after the removal of the selective pressure exerted by avoparcin (Johnsen *et al.*, 2011).

4.1 Emergence of VRE

In Sweden an increased occurrence of VRE among broilers was observed between 2000 and 2005 within the SVARM programme (Figure 5). All isolates are *E. faecium* with high level vancomycin resistance and all isolates where the resistance genotype has been investigated carry the *vanA* gene.

The increase in VRE occurrence in Sweden is peculiar in that it appeared in the absence of any obvious selective pressure. The use of avoparcin in Sweden was discontinued about 15 years earlier (Wierup *et al.*, 1987). Moreover, all use of antimicrobial growth promoters to farm animals was forbidden in 1986 (SFS 1985:295). Today antimicrobials are seldomly used for treatment of bacterial diseases but ionophores, mainly narasin, are regularly used for coccidial prophylaxis.

Apart from the increased occurrence in the apparent absence of selective pressure, also other findings indicated that the situation was peculiar. The majority of the isolates have identical resistance patterns with low level resistance to erythromycin and decreased susceptibility to narasin in addition to vancomycin resistance. This indicated that isolates were clonally related, which was further strengthened by investigations regarding phenotypical characteristics (Franklin *et al.*, 2005).

In order to confirm the clonality, VRE isolates were typed with molecular methods (i.e. MLST) in Paper I. The typing confirmed that the increased occurrence was caused by the spread of one clone of *E. faecium*. The clone was of the *vanA* genotype, belonged to ST310 and had the prototype Tn*1546* transposon of *E. faecium* BM4147. Such a predominance of one clone is in contrast with the situation in most other countries, where several different clones of VRE are isolated (Lim *et al.*, 2006; Garcia-Migura *et al.*, 2005; Novais *et al.*, 2005; Borgen *et al.*, 2002; Heuer *et al.*, 2002b).

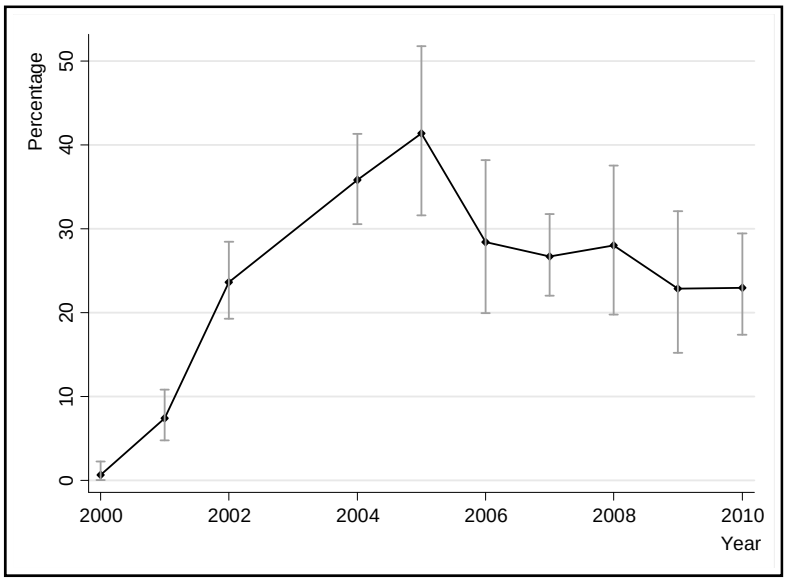

Figure 5. Proportion of Swedish broilers colonized with VRE from 2000 to 2010. Whiskers indicate 95% confidence intervals. Data from the SVARM programme.

The presence of VRE isolates with other resistance patterns among Swedish broilers was taken as an indication that these isolates belong to other clones. This was confirmed in Paper II where molecular typing revealed several different sequence types (STs). Moreover, a further analysed subset of isolates also differed in their *Sma*I PFGE digestion patterns. Compared to other countries, the situation in Sweden is special in that one clone is predominant and the others are in clear minority.

Additional molecular characterization of the predominant and the minority clones was done in Paper II. These investigations showed that in all of the clones the *vanA* gene was plasmid mediated, and all but one had the prototype variant of the Tn*1546* transposon. Both the plasmid localization of the *vanA* gene and the fact that the prototype variant of the

Tn*1546* transposon was the most common are in agreement with previous knowledge about VRE of the vanA type (Biavasco *et al.*, 2007; Courvalin, 2006; Woodford *et al.*, 1998).

The number of plasmids detected in the different clones varied from one to four. Furthermore, the only plasmid replicon type detected among the clones was pRE25, although in five of the eleven clones none of the investigated plasmid replicon types was detected. In addition, under the investigated conditions vancomycin resistance was transferrable from six of the eleven clones. However, there does not seem to be any correlation between the varying traits, e.g. presence of pRE25 is not correlated to transfer of vancomycin resistance.

All together, the molecular characterization of the different clones does not explain why one clone of VRE is predominant in Swedish broiler production.

4.2 Spread of VRE

The rapid increase in VRE occurrence from 2000 to 2005 and the predominance of one clone indicate that VRE did not emerge among Swedish broilers during the years when avoparcin was used. If that had been the case, one clone would probably not be predominant. Also, VRE would probably have been isolated in studies performed in the middle of the 1990's (Quednau *et al.*, 1998; Anonymous, 1997a). Instead, an introduction of VRE to Swedish broilers after the use of avoparcin had been discontinued is a more plausible scenario. This probably happened by the end of the 1990's since the first isolate was found in 1998 (Kuhn *et al.*, 2005).

Regardless of whether VRE was introduced into, or less likely arose within the Swedish broiler production, once there, VRE spread to more and more farms. In 2002–2003, 18% of the farms were positive for VRE in faecal droppings collected about two weeks prior to slaughter (unpublished results). When a similar study was done in 2011, 27% of the farms were positive (unpublished results). Notably, the occurrence of VRE appears to have peaked in 2005. Since then, the proportion of broilers colonized with VRE has decreased and varied between 20 and 30% (Figure 5) (SVARM, 2011).

There are several ways by which both an introduction of VRE into the Swedish broiler production and the subsequent spread to more and more farms could have occurred. One source could be poultry feed or feedstuff where the presence of VRE has been described (Martins da Costa *et al.*,

2007). Contrary, VRE have never been isolated from broiler feed in Sweden (Nilsson *et al.*, 2008 and unpublished results).

Another way for introduction and spread of VRE could be via import of grandparent birds and a subsequent vertical spread within the broiler pyramid (see Figure 2, page 11). Spread with day old chickens have been described for various resistant bacteria, e.g. fluoroquinolone resistant *Campylobacter coli* and *E. coli* resistant to ampicillin and nalidixic acid (Bortolaia *et al.*, 2010; Idris *et al.*, 2006). Also indications of the presence of VRE in day old chickens have been reported (Garcia-Migura *et al.*, 2007).

On the other hand, VRE was not isolated at Norwegian hatcheries when sampled four years after the use of avoparcin was discontinued in Norway (Borgen *et al.*, 2000b). Furthermore, VRE have never been isolated at hatcheries in Sweden (Nilsson *et al.*, 2008 and unpublished results). Notable though is that VRE of the same type as the clone dominating among broilers in Sweden was isolated from broilers in Norway in 1999 (Rosvoll *et al.*, 2010). This could indicate that there has been a vertical spread within the broiler pyramid since there has been trade with birds between the countries, mainly export of parent birds from Sweden to Norway.

In summary, the studies performed in Sweden show that there is no continuous introduction of VRE, neither by feed nor by day old chickens into Swedish broiler production. Nevertheless, these routes could still have been the original source(s) of introduction.

Another possible vehicle for the spread between farms is trucks used for collecting and loading birds for slaughter. These trucks are affiliated with the respective slaughterhouses and are used at all farms associated to the respective slaughterhouses. In a small study, VRE was found in five out of six samples taken from two different trucks as they were ready to leave the slaughterhouses to collect birds at production sites (Jansson *et al.*, 2009b). These findings indicate that also other equipment used when collecting birds for slaughter could be contaminated with, and introduce VRE into a broiler house, as described for other bacteria (Slader *et al.*, 2002; Mead *et al.*, 1994).

Still, once the birds have been slaughtered, the compartments are cleaned and disinfected and an introduction of VRE with these trucks and related equipment should therefore theoretically be eliminated directly. However, if an all-in all-out procedure is not strictly practiced there is a potential for proliferation of VRE in the remaining birds before the compartments are emptied and can be cleaned and disinfected. Previously, so called thinning, where a proportion of the broilers within a compartment are slaughtered about one week before the remaining birds, was practiced in Swedish

broiler production. Thinning was however associated with increased likelihood for isolating campylobacter from the broiler flocks (Hansson *et al.*, 2004) and it is therefore no longer practiced. But birds in one compartment are still, due to slaughterhouse capacity, sometimes slaughtered on two consecutive days, or exceptionally with a week-end in between. Also such "split slaughter" could potentially lead to proliferation of VRE, although to a lesser extent than if thinning is practiced.

4.3 Persistence of VRE

Persistence of VRE in the broiler houses between batches was seen in Paper III and IV, as well as in a study related to this thesis (Jansson *et al.*, 2009b). That VRE persists in the broiler houses is an important aspect of the epidemiology which has also been described in previous studies (Garcia-Migura *et al.*, 2007; Heuer *et al.*, 2002b; Borgen *et al.*, 2000b). In addition, in one of the farms sampled in Paper III, VRE could be isolated from all environmental samples from cleaned and disinfected compartments. This shows how widespread VRE can be within the broiler houses.

Differences among farms with regard to the degree of environmental VRE contamination were shown in Paper III. These differences were also reflected in the proportion of birds colonized with VRE at slaughter. Variations in contamination and colonization could be due to differences in management routines among the farms but this was not investigated in Paper III. A study looking at management routines at Swedish broiler farms with or without VRE was done previously but no consistent differences were identified (Jansson *et al.*, 2009b). Nevertheless, the differences among farms demonstrated in Paper III indicate the possibility to influence the degree of environmental VRE contamination. If the factor(s) causing these differences were identified, it would be possible to reduce the occurrence of VRE within the broiler houses. This would then lead to a decrease in the proportion of broilers colonized with VRE.

Another aspect of persistence of VRE is the fact that the enterococci retain the vancomycin resistance. Elsewhere, linkage to plasmid addiction systems as well as co-selection by the use of other antimicrobials has been suggested as reasons for the persistence of the *vanA* gene among enterococci (Johnsen *et al.*, 2005; Aarestrup, 2000).

In VRE isolated from broilers in Norway, the *vanA* gene has been shown to be located on the same plasmid as the ω-ε-ζ plasmid addiction system (Johnsen *et al.*, 2005). In a VRE of animal origin from Italy was both

the ω-ε-ζ and the axe-txe systems detected (Rosvoll *et al.*, 2010). The presence of these plasmid addiction systems among the different clones of VRE in Swedish broiler production was investigated in Paper II.

The ω-ε-ζ system was detected only in one of the minority clones whereas the axe-txe system was not detected in any of the clones. This indicates that plasmid addiction systems do not play as an important role in the persistence of VRE in Swedish broiler production as the ω-ε-ζ system has been suggested to do in Norwegian broiler production (Johnsen *et al.*, 2005). This was also indicated by the fact that in the clone were the ω-ε-ζ system was present it did not seem to be closely linked to the *vanA* gene, as the genes were not co-transferred in the conjugation assay in Paper II.

A coincidental finding of the conjugation assay in Paper II was that in four of the six clones with transferrable vancomycin resistance, decreased susceptibility to narasin was co-transferred. The ionophore narasin is widely used narasin for coccidial prophylaxis and decreased susceptibility is common among both vancomycin susceptible *E. faecium* as well as VRE from Swedish broilers. Therefore co-selection of VRE by the use of narasin is unlikely. Neither the mechanism of decreased susceptibility to narasin, nor its connection to the vancomycin resistance has been investigated but the genes involved could for example be located close to each other. Thereby the use of narasin could actually play a role in the persistence of the *vanA* gene among enterococci in Swedish broilers.

4.4 Potential for zoonotic spread

One negative implication of the presence of VRE in farm animals is the potential for zoonotic spread via the food chain. VRE contamination of animal derived food products reaching consumers was shown at the time when avoparcin was still used (van den Braak *et al.*, 1998; Klare *et al.*, 1995a). In Sweden, VRE was isolated from 36% of imported and 14% of domestically produced chicken fillets sampled in a pilot study preceding this thesis (unpublished results).

The implication of VRE present on broiler meat depends partly on whether the VRE has transferrable vancomycin resistance or not. A broiler adapted enterococcus will only transiently stay in humans and it is also less likely than hospital adapted enterococci to cause disease (Sorensen *et al.*, 2001; Willems *et al.*, 2000; Berchieri, 1999). Nevertheless, it has been demonstrated that the *vanA* gene can be transferred from VRE of animal origin to human enterococci in the intestines of humans (Lester *et al.*, 2006).

To investigate if VRE from Swedish broilers can transfer their vancomycin resistance, a conjugation assay with representatives of both the predominant and the minority clones of VRE was performed in Paper II. Under the investigated conditions, the *vanA* gene was transferrable from both the dominating and five of the ten minority clones. The *vanA* gene is commonly transferrable (French, 1998), thus it was expected that the vancomycin resistance would be transferrable from all the investigated clones. It is possible that the vancomycin resistance is transferrable also from the remaining clones if the experimental conditions, e.g. temperature and recipient, are altered.

Nevertheless, the majority of VRE from Swedish broilers belongs to one predominant clone and the ability of that clone to transfer vancomycin resistance is proven (Paper I and II). But when assessing the potential for zoonotic spread of vancomycin resistance from Swedish broilers, it is also important to remember that VRE only constitutes a minute proportion of all enterococci in the intestine of a broiler. This is demonstrated by the fact that only 4 VRE have been found among 1666 randomly selected enterococci from broilers (out of which 1040 are *E. faecium*) investigated within the SVARM programme since 2000 (SVARM, 2011).

In accordance, the presence of VRE among Swedish broilers does not seem to have affected the situation in Swedish healthcare where there is a predominance of VRE isolates with the *vanB* gene (SWEDRES, 2010). Nonetheless, the presence of VRE in Swedish broiler production constitutes a reservoir of vanomycin resistance that is unwanted and if possible should be eliminated.

4.5 Ways to reduce the occurrence of VRE

The results of Paper II confirm that VRE among Swedish broiler have the potential for zoonotic spread of vancomycin resistance genes. Furthermore, the absence of a continuous introduction of VRE to Swedish broiler production together with the results of Paper III indicates that the occurrence of VRE could be reduced. Possibly, VRE might even be eliminated from Swedish broiler production – an approach that has probably never been attempted elsewhere before.

Currently, birds are colonized by VRE persisting in the broiler houses. This leads to proliferation of VRE and an increase in the contamination, apparently to such an extent that the cleaning and disinfection does not manage to eliminate the contamination. Contrary, a reduced contamination of the broiler houses would lead to fewer colonized birds and subsequently

to a reduction in the contamination of the broiler houses. The intervention could be at two points: hindering the birds from becoming colonized with VRE and reducing the number of VRE that can potentially colonize the birds.

Competitive exclusion with probiotics has been used to prevent intestinal colonization of various bacteria in both human and veterinary medicine. Promising results have been reported regarding reduction of VRE colonization of broilers (Sakai *et al.*, 2006) but studies regarding the effect of probiotics on intestinal VRE colonization in humans have been discouraging (de Regt *et al.*, 2010; Vidal *et al.*, 2010). Furthermore, futile experiments on use of probiotics to protect broilers from being colonized with VRE have been done in work related to this thesis (Jansson *et al.*, 2009a). Due to these discouraging findings it was considered unlikely that occurrence of VRE among Swedish broilers could be reduced by the means of competitive exclusion.

A reduction in the number of VRE that can colonize the broilers is obviously achieved by the normal cleaning and disinfection routines practiced between batches, as they for example include removing all the litter from the broiler houses. However, the findings in Paper III and IV show that VRE persist in the houses. There can be various reasons why the VRE persist. One explanation could be that VRE among Swedish broilers, and especially the predominant clone, have a reduced susceptibility to the commonly used disinfectants. Decreased susceptibility to disinfectants is known to occur among various bacteria and has also been described in association with antimicrobial resistance (Cerf *et al.*, 2010; SCENHIR, 2009; EFSA, 2008a).

In Paper IV, the ability of three VRE isolates from Swedish broilers to sustain various physical conditions including exposure to commonly used disinfectants was investigated. The disinfectants were found to be fully effective at recommended concentrations of the products. This, together with the fact that no differences in the stated management routines could be detected between VRE positive or negative farms (Jansson *et al.*, 2009b), implies that the problem lies within difficulties to fulfill the disinfection protocols. The explanation could be problems to (i) adequately clean the broiler houses before disinfection and/or (ii) apply the disinfectant into all equipment and at all locations in the houses. Thus, a disinfection protocol that circumvents these problems is needed.

In Paper IV, a pilot study was performed to evaluate the possibility to eliminate VRE with a disinfection method that combines steam and

formaldehyde. The method was originally developed to sanitize layer hen farms from salmonellae (Gradel *et al.*, 2004a).

Although the disinfection experiment was only performed at two farms, the results show that the tested method was more efficient in reducing the environmental VRE contamination than the normal routines on these farms. The predicted reduction in colonization of the birds was however not conclusive. Nevertheless, the results indicate a possibility to reduce the occurrence of VRE in Swedish broiler production.

5 Conclusions

This work demonstrates that the occurrence of VRE in Swedish broiler production can probably be reduced. The project has also generated deeper understanding about the epidemiology of VRE among Swedish broilers.

The specific conclusions of the project are:

- The increase in proportion of broilers colonized with VRE from 2000 to 2005 was caused by the spread of one clone which still persists and is predominant among VRE from Swedish broilers.
- Several clones of VRE have been isolated from Swedish broilers but one clone is predominant.
- All the clones are *E. faecium* with plasmid mediated *vanA* gene.
- The *vanA* gene is transferrable from the predominant as well as from several of the minority clones. This shows that VRE among Swedish broilers have a potential for zoonotic spread of the resistance genes.
- There is a near absence of the investigated plasmid addiction systems, indicating that such systems are not responsible for retaining the *vanA* gene among enterococci in Swedish broiler production.
- Decreased susceptibility to narasin can be co-transferred with the *vanA* gene. This indicates that the traits are linked and that the use of narasin might contribute to the persistence of vancomycin resistance among enterococci in Swedish broiler production.
- Broilers are colonized with VRE persisting in the broiler houses between batches of birds. However, variations among farms in degree of contamination of houses as well as in proportion of colonized birds indicate that the occurrence of VRE can be reduced.
- The VRE contamination of the broiler houses can be reduced by disinfection with a method combining steam and formaldehyde. This method could be useful to eliminate VRE persisting at farms.

6 Perspectives for the future

The persistence, spread and increased occurrence of one clone of VRE, apparently without selective pressure within the Swedish broiler production shows how complex and unpredictable the epidemiology of antimicrobial resistance can be. It also shows that no matter when and where antimicrobials are used without caution, it can have implications on resistance both geographically and temporally remote.

Even though VRE with transferrable vancomycin resistance are present in Swedish broiler production, the situation in Swedish healthcare does not seem to have been affected. Nevertheless, VRE among Swedish broilers constitute a reservoir of resistance genes that, if possible should be eliminated.

Elimination of VRE from the Swedish broiler production could possibly be achieved by reducing persistence in the broiler houses with adequate cleaning and disinfection. Ways to investigate this could include large scale disinfection studies where all of the houses at farms are disinfected.

Also the role of narasin in the persistence of the vancomycin resistance in enterococci in Swedish broilers needs to be further studied. Possibly, the use of narasin acts as a sort of plamid addiction system and ensures stable inheritance of the *vanA* gene. Objectives for the future would include clarifying the genetic background and mechanism of the decreased susceptibility to narasin as well as its linkage to the *vanA* gene.

7 Populärvetenskaplig sammanfattning

7.1 Introduktion

Vankomycinresistenta enterokocker (VRE) är ett samlingsord för många olika kombinationer av enterokocker och gener. Den vanligaste varianten är *Enterococcus faecium* med *vanA* som den gen som kodar för resistens mot vankomycin. Men bland människor i Sverige är *E. faecium* med *vanB* vanligast. VRE är en viktig orsak till sjukhusassocierade infektioner men att bara bära bakterierna i tarmen är mycket vanligare än att bli sjuk och få kliniska symptom.

VRE finns också hos livsmedelsproducerande djur. Det beror på att antibiotikumet avoparcin i många Europeiska länder använts i stor omfattning för att öka djurens tillväxt. Både avoparcin och vankomycin är antibiotika i gruppen glykopeptider. Likheten mellan molekylerna ger korsresistens, det vill säga resistens mot den ena substansen innebär resistens också mot den andra.

När det blev känt att VRE var vanligt hos livsmedelsproducerande djur i Europa förbjöds avoparcin. Detta skedde först i Danmark, Norge och Finland, sedan i Tyskland och slutligen, 1997, i hela EU efter ett beslut i EU kommissionen (Kommissionsdirektiv 97/6/EC).

Förbudet i Europa mot att använda avoparcin berodde på en oro för att VRE skulle sprida sig från djur till människor via livsmedel (Figur 1, sidan 10). De gener som ger vankomycinresistens kan nämligen föras över från enterokocker hos djur till enterokocker anpassade till människor och göra att människor under längre tid bär på VRE. Om antalet friska människor som bär på VRE ökar skulle även antalet infektioner med VRE i sjukvården kunna öka. Sedan avoparcin förbjöds har förekomsten av VRE bland livsmedelsproducerande djur minskat.

I Sverige användes avoparcin endast från mitten av 1970-talet till 1984. Dessutom är det sedan 1986 förbjudet att över huvud taget använda antibiotika för att öka djurs tillväxt. Därmed har enterokocker hos livsmedelsproducerande djur i Sverige inte på samma sätt som i övriga Europa haft någon fördel av att vara vankomycinresistenta. I mitten på 1990-talet, när förekomsten av VRE hos livsmedelsproducerande djur i Europa var som högst, hittades inte heller VRE i Sverige.

Först 1998 isolerades VRE från livsmedelsproducerande djur i Sverige. Efter det upptäcktes i resistensövervakningsprogrammet SVARM (Svensk Veterinär Antibiotika Resistens Monitorering) en tydlig ökning i andelen slaktkycklingar med VRE i tarmen (Figur 5, sidan 30). Ökningen var oväntad eftersom det inte fanns något som uppenbart gynnade VRE framför enterokocker känsliga för vankomycin. Dessutom verkade det som att ökningen berodde på spridning av en enda klon av VRE eftersom nästan alla isolat var känsliga för, respektive resistenta mot samma antibiotika.

Trots att andelen slaktkycklingar som bär på VRE ökat är det bara en liten del av alla enterokocker i tarmen hos en slaktkyckling som är VRE. Sedan 2000 har bara 4 VRE hittats bland 1666 slumpmässigt utvalda enterokocker från slaktkyckling som undersökts i SVARM. Ökningen av VRE upptäcktes bara för att prover odlades så att enbart VRE kunde växa.

7.2 Sammanfattning av avhandlingsarbetet

Målet för avhandlingsarbetet var att undersöka varför förekomsten av VRE i svensk slaktkycklingproduktion ökade i början av 2000-talet samt om förekomsten kan minskas.

Arbetet har visat att ökningen av VRE beror på att en klon av *E. faecium* av typen ST310 har spridit sig i svensk slaktkycklingproduktion (Artikel 1 och 2). Även andra kloner finns i svensk slaktkycklingproduktion men alla är *E. faecium* med *vanA* som den gen som ger vankomycinresistens (Artikel 1 och 2). I alla kloner sitter genen på en plasmid, det vill säga en cirkelformad, ofta rörlig, bit arvsmassa (Artikel 2).

Både från den dominerande och från flera av de övriga klonerna kunde generna som är inblandade i vankomycinresistensen föras över till andra enterokocker (Artikel 2). Därmed är inte oförmåga att föra över generna förklaringen till varför en klon dominerar bland VRE hos svensk slaktkyckling. Men det betyder att VRE bland svensk slaktkyckling är en reservoar för resistensgener som kan sprida sig till enterokocker anpassade till människor.

Trots att avoparcin sedan länge inte används finns genen för vankomycinresistens kvar bland enterokocker hos slaktkycklingar, både i Sverige och i övriga Europa. En förklaring skulle kunna vara att generna är kopplade till ”plasmid addiction system”. Sådana system är en kombination av ett toxin och ett antitoxin där toxinet bryts ner långsammare än antitoxinet. Om bakterien som har systemet gör sig av med det kommer toxinet fortfarande att finnas kvar när antitoxinet försvunnit. Bakterien kommer då att dödas av toxinet den själv har bildat. Detta gör att bakterien måste behålla den plasmid som systemet sitter på och därmed även andra gener på samma plasmid. Men sådana system verkar inte finnas i någon större grad bland VRE i svensk slaktkycklingproduktion (Artikel 2).

Ionoforer, som narasin, används i nästan all slaktkycklingproduktion för att förebygga tarminfektioner med koccidier. Minskad känslighet för narasin kan föras över till andra enterokocker tillsammans med resistens mot vankomycin (Artikel 2). Att de förs över samtidigt beror troligen på att de kodande generna sitter nära varandra i enterokockernas arvsmassa. Därmed skulle alltså användningen av narasin inte bara göra så att den minskade känsligheten för narasin utan även resistensen mot vankomycin blir kvar i enterokockerna.

Att VRE kan finnas kvar i slaktkycklinghusen mellan uppfödningsomgångar trots rengöring och desinficering är känt sedan tidigare och visades även i avhandlingsarbetet (Artikel 3 och 4). Mängden VRE i husen ökar under en omgång slaktkycklingar för att sedan minska när husen rengörs och desinficeras efter att kycklingarna slaktats (Artikel 3).

Mängden VRE som finns kvar i slaktkycklinghusen skiljer sig mellan olika gårdar och det avspeglas i andelen kycklingar med VRE (Artikel 3). Att det finns skillnaderna mellan olika gårdar tyder på att mängden VRE på gårdarna kan påverkas och att förekomsten i svensk slaktkycklingproduktion skulle kunna minskas med rätt åtgärder.

Att VRE finns kvar efter rengöring och desinficering beror inte på att de är okänsliga för de desinficeringsämnen som oftast används inom svensk slaktkycklingproduktion (Artikel 4). Anledningen är istället troligtvis svårigheter i att lyckas med rengöringen och desinficeringen. Problemet kan till exempel vara att lyckas avlägsna all smuts från sprickor och utrustning samt att få tillräcklig mycket desinficeringsämnena under tillräckligt lång tid till alla delar av husen.

För att om möjligt lösa problemet med rengöring och desinficering av husen testades i ett fältförsök en desinficeringsmetod som kombinerar hög temperatur från vattenånga med formalin (Artikel 4). Den testade metoden

minskade mängden VRE i slaktkycklinghusen mer än de metoder som normalt användes på gårdarna. Detta betyder att det borde gå att minska förekomsten av VRE i svensk slaktkycklingproduktion i stort och att det kanske till och med går att helt få bort dem.

7.3 Konklusion och betydelse för framtiden

Avhandlingsarbetet har ökat kunskapen om varför VRE finns i svensk slaktkycklingproduktion och vad det eventuellt har för betydelse för människors hälsa. Dessutom har arbetet gett uppslag till hur förekomsten av VRE kan minskas.

Att förekomsten av VRE har ökat beror på att en klon spridit sig i svensk slaktkycklingproduktion. Det finns även andra kloner men de utgör bara en liten andel av alla VRE. Både den klon som dominerar och de övriga är *E. faecium* med *vanA* som den gen som ger vankomycinresistens. I alla klonerna sitter *vanA* genen på en plasmid.

Både från den dominerande klonen och från flera av de övriga kan genen för vankomycinresistensen föras över till andra enterokocker. Detta betyder att VRE i svensk slaktkycklingproduktion är en reservoar för resistensgener som kan spridas och påverka människors hälsa.

Att genen för vankomycinresistensen behålls av enterokocker i svensk slaktkycklingproduktion beror inte på att resistensen är kopplad till ”plasmid addiction systems”. Däremot verkar resistensen vara nära kopplad till minskad känslighet för ionoforen narasin vilken används för att förebygga tarminfektioner med koccidier. Därmed skulle alltså användningen av narasin inte bara göra så att den minskade känsligheten för narasin utan även resistensen mot vankomycin blir kvar i enterokockerna.

De slaktkycklingar som kommer till gårdarna koloniseras troligen av VRE som finns kvar i slaktkycklinghusen sedan föregående uppfödningsomgång. Att det finns skillnader mellan gårdar i mängd VRE i husen tyder på att förekomsten kan minskas och detta antagande förstärks av resultaten från undersökningen med en ny desinficeringsmetod.

Framtida forskning bör undersöka vilken roll användningen av narasin har för att enterokocker i svensk slaktkycklingproduktion behåller de gener som kodar för vankomycinresistens. Viktigt är också att ytterligare undersöka möjligheten att helt få bort VRE från slaktkycklinggårdar.

References

Aarestrup, F.M. (1995). Occurrence of glycopeptide resistance among Enterococcus faecium isolates from conventional and ecological poultry farms. *Microb Drug Resist* 1(3), 255-7.

Aarestrup, F.M. (2000). Characterization of glycopeptide-resistant enterococcus faecium (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. *J Clin Microbiol* 38(7), 2774-7.

Aarestrup, F.M., Kruse, H., Tast, E., Hammerum, A.M. & Jensen, L.B. (2000). Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among Enterococcus faecium from broilers and pigs in Denmark, Finland, and Norway. *Microb Drug Resist* 6(1), 63-70.

Aarestrup, F.M., Seyfarth, A.M., Emborg, H.D., Pedersen, K., Hendriksen, R.S. & Bager, F. (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 45(7), 2054-9.

Andersson, D.I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature reviews. Microbiology* 8(4), 260-71.

Andersson, D.I. & Levin, B.R. (1999). The biological cost of antibiotic resistance. *Curr Opin Microbiol* 2(5), 489-93.

Anonymous (1997a). *Antimicrobial Feed Additives; SOU 1997:132, Annex D*. Stockholm, Sweden: Fritzes Bookshop. ISBN 91-38-20707-9.

Anonymous (1997b). Commission directive 97/6/EC. *Official journal of the European Communities* L35 5.2.97, 11-13.

Arthur, M., Reynolds, P.E., Depardieu, F., Evers, S., Dutka-Malen, S., Quintiliani, R., Jr. & Courvalin, P. (1996). Mechanisms of glycopeptide resistance in enterococci. *J Infect* 32(1), 11-6.

Bager, F., Madsen, M., Christensen, J. & Aarestrup, F.M. (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant Enterococcus faecium on Danish poultry and pig farms. *Prev Vet Med* 31(1-2), 95-112.

Barnes, E.M. (1959). Differential and selective media for the faecal streptococci. *J Sci Food Agric* 10(12), 656-662.

Barton, B.M., Harding, G.P. & Zuccarelli, A.J. (1995). A general method for detecting and sizing large plasmids. *Anal Biochem* 226(2), 235-40.

Bates, J. (1997). Epidemiology of vancomycin-resistant enterococci in the community and the relevance of farm animals to human infection. *J Hosp Infect* 37(2), 89-101.

Bell, J.M., Paton, J.C. & Turnidge, J. (1998). Emergence of vancomycin-resistant enterococci in Australia: phenotypic and genotypic characteristics of isolates. *J Clin Microbiol* 36(8), 2187-90.

Berchieri, A. (1999). Intestinal colonization of a human subject by vancomycin-resistant Enterococcus faecium. *Clin Microbiol Infect* 5(2), 97-100.

Biavasco, F., Foglia, G., Paoletti, C., Zandri, G., Magi, G., Guaglianone, E., Sundsfjord, A., Pruzzo, C., Donelli, G. & Facinelli, B. (2007). VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* 73(10), 3307-19.

Bonten, M.J., Willems, R. & Weinstein, R.A. (2001). Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis* 1(5), 314-25.

Borgen, K., Simonsen, G.S., Sundsfjord, A., Wasteson, Y., Olsvik, O. & Kruse, H. (2000a). Continuing high prevalence of VanA-type vancomycin-resistant enterococci on Norwegian poultry farms three years after avoparcin was banned. *J Appl Microbiol* 89(3), 478-85.

Borgen, K., Sorum, M., Kruse, H. & Wasteson, Y. (2000b). Persistence of vancomycin-resistant enterococci (VRE) on Norwegian broiler farms. *FEMS Microbiol Lett* 191(2), 255-8.

Borgen, K., Wasteson, Y., Kruse, H. & Willems, R.J. (2002). Vancomycin-resistant Enterococcus faecium (VREF) from Norwegian poultry cluster with VREF from poultry from the United Kingdom and The Netherlands in an amplified fragment length polymorphism genogroup. *Appl Environ Microbiol* 68(6), 3133-7.

Bortolaia, V., Bisgaard, M. & Bojesen, A.M. (2010). Distribution and possible transmission of ampicillin- and nalidixic acid-resistant Escherichia coli within the broiler industry. *Vet Microbiol* 142(3-4), 379-86.

Boyd, D.A., Willey, B.M., Fawcett, D., Gillani, N. & Mulvey, M.R. (2008). Molecular characterization of Enterococcus faecalis N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, vanL. *Antimicrob Agents Chemother* 52(7), 2667-72.

Butaye, P., Devriese, L.A. & Haesebrouck, F. (2003). Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clin Microbiol Rev* 16(2), 175-88.

Cerf, O., Carpentier, B. & Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int J Food Microbiol* 136(3), 247-54.

CLSI (2008). *Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria Isoalted from Animals; Approved Standard - Third Edition*. Wayne, PA: Clinical and Laboratory Standards Institute. CLSI document M31-A3.

CLSI (2010). *Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement*. Wayne, PA: Clinical and Laboratory Standards Institute. CLSI document M100-S20.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* 42 Suppl 1, S25-34.

de Regt, M.J., Willems, R.J., Hene, R.J., Siersema, P.D., Verhaar, H.J., Hopmans, T.E. & Bonten, M.J. (2010). Effects of probiotics on acquisition and spread of multiresistant enterococci. *Antimicrob Agents Chemother* 54(7), 2801-5.

Devriese, L.A., Hommez, J., Wijfels, R. & Haesebrouck, F. (1991). Composition of the enterococcal and streptococcal intestinal flora of poultry. *J Appl Bacteriol* 71(1), 46-50.

Devriese, L.A., Pot, B. & Collins, M.D. (1993). Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* 75(5), 399-408.

DiazGranados, C.A., Zimmer, S.M., Klein, M. & Jernigan, J.A. (2005). Comparison of mortality associated with vancomycin-resistant and vancomycin-susceptible enterococcal bloodstream infections: a meta-analysis. *Clin Infect Dis* 41(3), 327-33.

Donabedian, S.M., Perri, M.B., Abdujamilova, N., Gordoncillo, M.J., Naqvi, A., Reyes, K.C., Zervos, M.J. & Bartlett, P. (2010). Characterization of vancomycin-resistant Enterococcus faecium isolated from swine in three Michigan counties. *J Clin Microbiol* 48(11), 4156-60.

Dutka-Malen, S., Evers, S. & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 33(1), 24-7.

EFSA (2008a). Scientific opinion of the Panel on Biological Hazards on a request from DG SANCO on the assessment of the possible effect of the four antimicrobial treatment substances on the emergence of antimicrobial resistance. *EFSA Journal* 659, 1-26.

EFSA (2008b). Scientific Opinion of the Panel on Biological Hazards on a request from the European Food Safety Authority on foodborne antimicrobial resistance as a biological hazard. *The EFSA Journal* (765), 1-87.

Enright, M.C. & Spratt, B.G. (1999). Multilocus sequence typing. *Trends Microbiol* 7(12), 482-7.

EUCAST *EUCAST, Clinical Breakpoints*. [online] Available from: http://www.srga.org/eucastwt/MICTAB/index.html. [Accessed 2011-02-21].

Euzeby, J.P. *List of Prokaryotic names with Standing in Nomenclature - Genus Enterococcus*. [online] Available from: http://www.bacterio.cict.fr/e/enterococcus.html. [Accessed 2011-04-26].

Fisher, K. & Phillips, C. (2009). The ecology, epidemiology and virulence of Enterococcus. *Microbiology* 155(Pt 6), 1749-57.

Franklin, A., Greko, C., Pringle, M., Kuhn, I., Rehbinder, V., Finn, M. & Bengtsson, B. Vancomycin-resistant enterococci (VRE) in Swedish broiler chickens. In: *Proceedings of 2nd International ASM-FEMS Conference on Enterococci*, Helsingör, Denmark, August 28-31, 2005 2005. p. 94.

Freitas, A.R., Coque, T.M., Novais, C., Hammerum, A.M., Lester, C.H., Zervos, M.J., Donabedian, S., Jensen, L.B., Francia, M.V., Baquero, F. & Peixe, L. (2011). Human and Swine Hosts Share Vancomycin-Resistant Enterococcus faecium CC17 and CC5 and Enterococcus faecalis CC2 Clonal Clusters Harboring Tn1546 on Indistinguishable Plasmids. *J Clin Microbiol* 49(3), 925-31.

French, G.L. (1998). Enterococci and vancomycin resistance. *Clin Infect Dis* 27 Suppl 1, S75-83.

Garbutt, J.M., Ventrapragada, M., Littenberg, B. & Mundy, L.M. (2000). Association between resistance to vancomycin and death in cases of Enterococcus faecium bacteremia. *Clin Infect Dis* 30(3), 466-72.

Garcia-Migura, L., Liebana, E., Jensen, L.B., Barnes, S. & Pleydell, E. (2007). A longitudinal study to assess the persistence of vancomycin-resistant Enterococcus faecium (VREF) on an intensive broiler farm in the United Kingdom. *FEMS Microbiol Lett* 275(2), 319-25.

Garcia-Migura, L., Pleydell, E., Barnes, S., Davies, R.H. & Liebana, E. (2005). Characterization of vancomycin-resistant Enterococcus faecium isolates from broiler poultry and pig farms in England and Wales. *J Clin Microbiol* 43(7), 3283-9.

Ghidan, A., Dobay, O., Kaszanyitzky, E.J., Samu, P., Amyes, S.G., Nagy, K. & Rozgonyi, F. (2008). Vancomycin resistant enterococci (VRE) still persist in slaughtered poultry in hungary 8 years after the ban on avoparcin. *Acta Microbiol Immunol Hung* 55(4), 409-17.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiol Rev* 26(2), 163-71.

Gradel, K.O., Jorgensen, J.C., Andersen, J.S. & Corry, J.E. (2004a). Monitoring the efficacy of steam and formaldehyde treatment of naturally Salmonella-infected layer houses. *J Appl Microbiol* 96(3), 613-22.

Gradel, K.O., Sayers, A.R. & Davies, R.H. (2004b). Surface disinfection tests with Salmonella and a putative indicator bacterium, mimicking worst-case scenarios in poultry houses. *Poult Sci* 83(10), 1636-43.

Hammerum, A.M., Jensen, L.B. & Aarestrup, F.M. (1998). Detection of the satA gene and transferability of virginiamycin resistance in Enterococcus faecium from food-animals. *FEMS Microbiol Lett* 168(1), 145-51.

Hammerum, A.M., Lester, C.H. & Heuer, O.E. (2010). Antimicrobial-resistant enterococci in animals and meat: a human health hazard? *Foodborne Pathog Dis* 7(10), 1137-46.

Hansson, I., Engvall, E.O., Lindblad, J., Gunnarsson, A. & Vagsholm, I. (2004). Surveillance programme for Campylobacter species in Swedish broilers, July 2001 to June 2002. *Vet Rec* 155(7), 193-6.

Hasman, H. & Aarestrup, F.M. (2005). Relationship between copper, glycopeptide, and macrolide resistance among Enterococcus faecium strains isolated from pigs in Denmark between 1997 and 2003. *Antimicrob Agents Chemother* 49(1), 454-6.

Hegstad, K., Mikalsen, T., Coque, T.M., Werner, G. & Sundsfjord, A. (2010). Mobile genetic elements and their contribution to the emergence of antimicrobial resistant Enterococcus faecalis and Enterococcus faecium. *Clin Microbiol Infect* 16(6), 541-554.

Heuer, O.E., Pedersen, K., Andersen, J.S. & Madsen, M. (2002a). Vancomycin-resistant enterococci (VRE) in broiler flocks 5 years after the avoparcin ban. *Microb Drug Resist* 8(2), 133-8.

Heuer, O.E., Pedersen, K., Jensen, L.B., Madsen, M. & Olsen, J.E. (2002b). Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban. *Microb Drug Resist* 8(4), 355-61.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., Van Embden, J.D. & Willems, R.J. (2002). Multilocus sequence typing scheme for Enterococcus faecium. *J Clin Microbiol* 40(6), 1963-71.

Hummel, R., Tschape, H. & Witte, W. (1986). Spread of plasmid-mediated nourseothricin resistance due to antibiotic use in animal husbandry. *J Basic Microbiol* 26(8), 461-6.

Idris, U., Lu, J., Maier, M., Sanchez, S., Hofacre, C.L., Harmon, B.G., Maurer, J.J. & Lee, M.D. (2006). Dissemination of fluoroquinolone-resistant Campylobacter spp. within an integrated commercial poultry production system. *Appl Environ Microbiol* 72(5), 3441-7.

Iversen, A., Kuhn, I., Franklin, A. & Mollby, R. (2002). High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl Environ Microbiol* 68(6), 2838-42.

Jansson, D., Greko, C. & Bengtsson, B. Preliminary study of the effect of probiotics on colonization by vancomycin-resistant *Enterococcus faecium* in a chicken experimental model. In: *Proceedings of XVI Congress of the World Veterinary Poultry Association*, Marrakesh, Morocco 2009a.

Jansson, D., Nilsson, O., Greko, C., Lindblad, J. & Bengtsson, B. Inter-batch contamination of broiler farms and equipment by a vancomycin-resistant clone of *Enterococcus faecium*. In: *Proceedings of XVI Congress of the World Veterinary Poultry Association*, Marrakesh, Morocco 2009b.

Jensen, L.B., Garcia-Migura, L., Valenzuela, A.J., Lohr, M., Hasman, H. & Aarestrup, F.M. (2010). A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 80(1), 25-43.

Johnsen, P.J., Osterhus, J.I., Sletvold, H., Sorum, M., Kruse, H., Nielsen, K., Simonsen, G.S. & Sundsfjord, A. (2005). Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal Enterococcus faecium population. *Appl Environ Microbiol* 71(1), 159-68.

Johnsen, P.J., Townsend, J.P., Bohn, T., Simonsen, G.S., Sundsfjord, A. & Nielsen, K.M. (2011). Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures. *J Antimicrob Chemother.* 66(3): 608-610.

Johnson, P.D., Ballard, S.A., Grabsch, E.A., Stinear, T.P., Seemann, T., Young, H.L., Grayson, M.L. & Howden, B.P. (2010). A sustained hospital outbreak of vancomycin-resistant Enterococcus faecium bacteremia due to emergence of vanB E. faecium sequence type 203. *J Infect Dis* 202(8), 1278-86.

Kirst, H.A., Thompson, D.G. & Nicas, T.I. (1998). Historical yearly usage of vancomycin. *Antimicrob Agents Chemother* 42(5), 1303-4.

Klare, I., Badstubner, D., Konstabel, C., Bohme, G., Claus, H. & Witte, W. (1999). Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from

poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. *Microb Drug Resist* 5(1), 45-52.

Klare, I., Heier, H., Claus, H., Bohme, G., Marin, S., Seltmann, G., Hakenbeck, R., Antanassova, V. & Witte, W. (1995a). Enterococcus faecium strains with vanA-mediated high-level glycopeptide resistance isolated from animal foodstuffs and fecal samples of humans in the community. *Microb Drug Resist* 1(3), 265-72.

Klare, I., Heier, H., Claus, H., Reissbrodt, R. & Witte, W. (1995b). vanA-mediated high-level glycopeptide resistance in Enterococcus faecium from animal husbandry. *FEMS Microbiol Lett* 125(2-3), 165-71.

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol* 88(2-3), 123-31.

Kramer, A., Schwebke, I. & Kampf, G. (2006). How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 6, 130.

Kuhn, I., Iversen, A., Finn, M., Greko, C., Burman, L.G., Blanch, A.R., Vilanova, X., Manero, A., Taylor, H., Caplin, J., Dominguez, L., Herrero, I.A., Moreno, M.A. & Mollby, R. (2005). Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. *Appl Environ Microbiol* 71(9), 5383-90.

Lauderdale, T.L., Shiau, Y.R., Wang, H.Y., Lai, J.F., Huang, I.W., Chen, P.C., Chen, H.Y., Lai, S.S., Liu, Y.F. & Ho, M. (2007). Effect of banning vancomycin analogue avoparcin on vancomycin-resistant enterococci in chicken farms in Taiwan. *Environ Microbiol* 9(3), 819-23.

Lebreton, F., Leclercq, R., Courvalin, P. & al., e. (2010). VanN, a D-Ala-D-Ser type of transferable vancomycin resistance in E. faecium 08-174. *Poster 41A: 3rd ASM on Enterococci*.

Leclercq, R., Derlot, E., Duval, J. & Courvalin, P. (1988). Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *N Engl J Med* 319(3), 157-61.

Lester, C.H., Frimodt-Moller, N., Sorensen, T.L., Monnet, D.L. & Hammerum, A.M. (2006). In vivo transfer of the vanA resistance gene from an Enterococcus faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers. *Antimicrob Agents Chemother* 50(2), 596-9.

Lester, C.H. & Hammerum, A.M. (2010). Transfer of vanA from an Enterococcus faecium isolate of chicken origin to a CC17 E. faecium isolate in the intestine of cephalosporin-treated mice. *J Antimicrob Chemother* 65(7), 1534-6.

Lim, S.K., Kim, T.S., Lee, H.S., Nam, H.M., Joo, Y.S. & Koh, H.B. (2006). Persistence of vanA-type Enterococcus faecium in Korean livestock after ban on avoparcin. *Microb Drug Resist* 12(2), 136-9.

Läkemedelsverket *www.mpa.se*. [online] Available from: www.mpa.se. [Accessed 2011-03-16].

Martin, J.D. & Mundt, J.O. (1972). Enterococci in insects. *Appl Microbiol* 24(4), 575-80.

Martins da Costa, P., Oliveira, M., Bica, A., Vaz-Pires, P. & Bernardo, F. (2007). Antimicrobial resistance in Enterococcus spp. and Escherichia coli isolated from poultry feed and feed ingredients. *Vet Microbiol* 120(1-2), 122-31.

McCormick, M.H., McGuire, J.M., Pittenger, G.E., Pittenger, R.C. & Stark, W.M. (1955). Vancomycin, a new antibiotic. I. Chemical and biologic properties. *Antibiotics annual* 3, 606-11.

McDonald, L.C., Kuehnert, M.J., Tenover, F.C. & Jarvis, W.R. (1997). Vancomycin-resistant enterococci outside the health-care setting: prevalence, sources, and public health implications. *Emerg Infect Dis* 3(3), 311-7.

Mead, G.C., Hudson, W.R. & Hinton, M.H. (1994). Use of a marker organism in poultry processing to identify sites of cross-contamination and evaluate possible control measures. *Br Poult Sci* 35(3), 345-54.

Morrison, D., Woodford, N., Barrett, S.P., Sisson, P. & Cookson, B.D. (1999). DNA banding pattern polymorphism in vancomycin-resistant Enterococcus faecium and criteria for defining strains. *J Clin Microbiol* 37(4), 1084-91.

Mundt, J.O. (1963). Occurrence of enterococci in animals in a wild environment. *Appl Microbiol* 11, 136-40.

Nilsson, O., Greko, C., Franklin, A. & Bengtsson, B. Investigating potential sources of vancomycin resistant enterococci (VRE) for broilers in Sweden. In: *Proceedings of 4th Med-Vet-Net Annual Scientific Meeting*, St. Malo, France, 11-14 June 2008.

Novais, C., Coque, T.M., Costa, M.J., Sousa, J.C., Baquero, F. & Peixe, L.V. (2005). High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. *J Antimicrob Chemother* 56(6), 1139-43.

Pantosti, A., Del Grosso, M., Tagliabue, S., Macri, A. & Caprioli, A. (1999). Decrease of vancomycin-resistant enterococci in poultry meat after avoparcin ban. *Lancet* 354(9180), 741-2.

Patel, R. (2003). Clinical impact of vancomycin-resistant enterococci. *J Antimicrob Chemother* 51 Suppl 3, iii13-21.

Patel, R., Piper, K., Cockerill, F.R., 3rd, Steckelberg, J.M. & Yousten, A.A. (2000). The biopesticide Paenibacillus popilliae has a vancomycin resistance gene cluster homologous to the enterococcal VanA vancomycin resistance gene cluster. *Antimicrob Agents Chemother* 44(3), 705-9.

Quednau, M., Ahrne, S., Petersson, A.C. & Molin, G. (1998). Antibiotic-resistant strains of Enterococcus isolated from Swedish and Danish retailed chicken and pork. *J Appl Microbiol* 84(6), 1163-70.

Reybrouck, G. (2004). Antifungal activity of disinfectants. In: Fraise, A.P.*, et al.* (Eds.) *Principles and Practice of Disinfection, Preservation & Sterilization*. 4th. ed. Oxford: Blackwell Publishing Ltd. ISBN 1-4051-0199-7.

Reynolds, P.E. (1989). Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur J Clin Microbiol Infect Dis* 8(11), 943-50.

Rosvoll, T.C., Pedersen, T., Sletvold, H., Johnsen, P.J., Sollid, J.E., Simonsen, G.S., Jensen, L.B., Nielsen, K.M. & Sundsfjord, A. (2010). PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* 58(2), 254-68.

Sahlstrom, L., Rehbinder, V., Albihn, A., Aspan, A. & Bengtsson, B. (2009). Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. *Acta Vet Scand* 51, 24.

Sakai, Y., Tsukahara, T., Bukawa, W., Matsubara, N. & Ushida, K. (2006). Cell preparation of Enterococcus faecalis strain EC-12 prevents vancomycin-resistant enterococci colonization in the cecum of newly hatched chicks. *Poult Sci* 85(2), 273-7.

Sambrook, J. & Russell, D.W. (Eds.) (2001). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

SCENHIR (2009) *Assessment of the Antibiotic Resistance Effects of Biocides*. [online] Available from: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf. [Accessed 2011-04-26].

Schjorring, S. & Krogfelt, K.A. (2011). Assessment of bacterial antibiotic resistance transfer in the gut. *Int J Microbiol*. 2011, 312956.

Schleifer, K.H. & Kilpper-Bälz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom.rev. as *Enterococcus faecalis* comb. nov. and *Enterococcus faecium* comb. nov. *Int J Syst Bacteriol* 34(1), 31-34.

Sherman, J.M. (1937). The Streptococci. *Bacteriol Rev* 1(1), 3-97.

Slader, J., Domingue, G., Jorgensen, F., McAlpine, K., Owen, R.J., Bolton, F.J. & Humphrey, T.J. (2002). Impact of transport crate reuse and of catching and processing on Campylobacter and Salmonella contamination of broiler chickens. *Appl Environ Microbiol* 68(2), 713-9.

Slanetz, L.W. & Bartley, C.H. (1957). Numbers of enterococci in water, sewage, and feces determined by the membrane filter technique with an improved medium. *J Bacteriol* 74(5), 591-5.

Sletvold, H., Johnsen, P.J., Hamre, I., Simonsen, G.S., Sundsfjord, A. & Nielsen, K.M. (2008). Complete sequence of Enterococcus faecium pVEF3 and the detection of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter. *Plasmid* 60(1), 75-85.

Smittskyddsinstitutet *Vancomycin-resistant Enterococc infection (VRE)*. [online] Available from: http://www.smittskyddsinstitutet.se/in-english/statistics/vancomycin-resistant-enterococc-infection-vre/. [Accessed 2011-03-17].

Soderblom, T., Aspevall, O., Erntell, M., Hedin, G., Heimer, D., Hokeberg, I., Kidd-Ljunggren, K., Melhus, A., Olsson-Liljequist, B., Sjogren, I., Smedjegard, J., Struwe, J., Sylvan, S., Tegmark-Wisell, K. & Thore, M. (2010). Alarming spread of vancomycin resistant enterococci in Sweden since 2007. *Euro Surveill* 15(29).

Sorensen, T.L., Blom, M., Monnet, D.L., Frimodt-Moller, N., Poulsen, R.L. & Espersen, F. (2001). Transient intestinal carriage after ingestion of antibiotic-resistant Enterococcus faecium from chicken and pork. *N Engl J Med* 345(16), 1161-6.

Sorum, M., Johnsen, P.J., Aasnes, B., Rosvoll, T., Kruse, H., Sundsfjord, A. & Simonsen, G.S. (2006). Prevalence, persistence, and molecular characterization of glycopeptide-resistant enterococci in Norwegian poultry and poultry farmers 3 to 8 years after the ban on avoparcin. *Appl Environ Microbiol* 72(1), 516-21.

Stokstad, E.L. & Jukes, T.H. (1950). Further observations on the " animal protein factor ". *Proc Soc Exp Biol Med* 78, 523-528.

Stokstad, E.L., Jukes, T.H., Pierce, J., Page, A.C. & Franklin, A.L. (1949). The multiple nature of the animal protein factor. *J Biol Chem* 180(2), 647-54.

SVARM (2011). *Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) 2010*. Uppsala, Sweden: The National Veterinary Institute (SVA). ISSN 1650-6332. To be released 9th June 2011.

SWEDRES (2010). *SWEDRES 2009*. Solna, Sweden: Swedish Strategic Programme for the Rational Use of Antimicrobial Agents & Swedish Institute for Infectious Disease Control. ISSN 1400-3473.

SWEDRES (2011). *SWEDRES 2010*. Solna, Sweden: Swedish Institute for Infectious Disease Control. ISSN 1400-3473. To be released 9th June 2011.

Tamarin, R.H. (2004). *Principles of Genetics*. 7th. ed. Singapore: McGraw-Hill Education.

Thiercelin, M. (1899). Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogene. *C R Seances Soc Biol Fil* 51, 269-271.

Toomey, N., Monaghan, A., Fanning, S. & Bolton, D.J. (2009). Assessment of horizontal gene transfer in Lactic acid bacteria--a comparison of mating techniques with a view to optimising conjugation conditions. *J Microbiol Methods* 77(1), 23-8.

Torell, E., Fredlund, H., Tornquist, E., Myhre, E.B., Sjoberg, L. & Sundsfjord, A. (1997). Intrahospital spread of vancomycin-resistant Enterococcus faecium in Sweden. *Scand J Infect Dis* 29(3), 259-63.

Trobos, M., Lester, C.H., Olsen, J.E., Frimodt-Moller, N. & Hammerum, A.M. (2009). Natural transfer of sulphonamide and ampicillin resistance between Escherichia coli residing in the human intestine. *J Antimicrob Chemother* 63(1), 80-6.

Tschape, H. (1994). The spread of plasmids as a function of bacterial adaptability. *FEMS Microbiology Ecology* 15, 23-32.

Uttley, A.H., Collins, C.H., Naidoo, J. & George, R.C. (1988). Vancomycin-resistant enterococci. *Lancet* 1(8575-6), 57-8.

Walsh, C. (2003). *Antibiotics: actions, origins, resistance*. Washington, DC: ASM Press.

van Belkum, A., Tassios, P.T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N.K., Fussing, V., Green, J., Feil, E., Gerner-Smidt, P., Brisse, S. & Struelens, M. (2007). Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin Microbiol Infec* 13 Suppl 3, 1-46.

van den Bogaard, A.E., Bruinsma, N. & Stobberingh, E.E. (2000). The effect of banning avoparcin on VRE carriage in The Netherlands. *J Antimicrob Chemother* 46(1), 146-8.

van den Braak, N., van Belkum, A., van Keulen, M., Vliegenthart, J., Verbrugh, H.A. & Endtz, H.P. (1998). Molecular characterization of vancomycin-resistant enterococci from hospitalized patients and poultry products in The Netherlands. *J Clin Microbiol* 36(7), 1927-32.

Wegener, H.C. (1998). Historical yearly usage of glycopeptides for animals and humans: the American-European paradox revisited. *Antimicrob Agents Chemother* 42(11), 3049.

Werner, G., Coque, T.M., Hammerum, A.M., Hope, R., Hryniewicz, W., Johnson, A., Klare, I., Kristinsson, K.G., Leclercq, R., Lester, C.H., Lillie, M., Novais, C., Olsson-Liljequist, B., Peixe, L.V., Sadowy, E., Simonsen, G.S., Top, J., Vuopio-Varkila, J., Willems, R.J., Witte, W. & Woodford, N. (2008). Emergence and spread of vancomycin resistance among enterococci in Europe. *Euro Surveill* 13(47).

WHO (2009). *WHO list of critically important antimicrobials for human medicine*. Geneva: WHO.

WHO *WHO, Programmes and projects, Zoonoses and veterinary public health*. [online] [Accessed 2011-03-16].

Vidal, M., Forestier, C., Charbonnel, N., Henard, S., Rabaud, C. & Lesens, O. (2010). Probiotics and intestinal colonization by vancomycin-resistant enterococci in mice and humans. *J Clin Microbiol* 48(7), 2595-8.

Wierup, M., Lowenhielm, C., Wold-Troell, M. & Agenas, I. (1987). Animal consumption of antibiotics and chemotherapeutic drugs in Sweden during 1980, 1982 and 1984. *Vet Res Commun* 11(5), 397-405.

Willems, R.J., Top, J., van Den Braak, N., van Belkum, A., Endtz, H., Mevius, D., Stobberingh, E., van Den Bogaard, A. & van Embden, J.D. (2000). Host specificity of vancomycin-resistant Enterococcus faecium. *J Infect Dis* 182(3), 816-23.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. *Science* 279(5353), 996-7.

Witte, W. (2000). Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents* 16 Suppl 1, S19-24.

Woodford, N., Adebiyi, A.M., Palepou, M.F. & Cookson, B.D. (1998). Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. *Antimicrob Agents Chemother* 42(3), 502-8.

Xu, X., Lin, D., Yan, G., Ye, X., Wu, S., Guo, Y., Zhu, D., Hu, F., Zhang, Y., Wang, F., Jacoby, G.A. & Wang, M. (2010). vanM, a new glycopeptide resistance gene cluster found in Enterococcus faecium. *Antimicrob Agents Chemother* 54(11), 4643-7.

Yoshimura, H., Ishimaru, M., Endoh, Y.S., Suginaka, M. & Yamatani, S. (1998). Isolation of glycopeptide-resistant enterococci from chicken in Japan. *Antimicrob Agents Chemother* 42(12), 3333.

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Spread without known selective pressure of a vancomycin-resistant clone of *Enterococcus faecium* among broilers

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***Objectives*: The aim of this paper was to describe an increased occurrence of vancomycin-resistant enterococci (VRE) in Swedish broilers since 2000 and to investigate the genetic relatedness of isolates.**

***Methods*: Caecal content from slaughtered broilers was cultured for VRE on medium supplemented with vancomycin (16 mg/L). Species identification, antibiotic susceptibility determination, vancomycin resistance genotyping, multilocus sequence typing (MLST) and characterization of Tn*1546* were performed.**

***Results*: The proportion of VRE-positive samples increased gradually from <1% in 2000 to slightly over 40% in 2005. Between 2005 and 2006, the proportion of VRE-positive samples decreased and between 2006 and 2007, it was stable at just below 30%. All isolates tested were *Enterococcus faecium* and carried the *vanA* gene. A majority of the isolates had similar antibiograms, the same MLST sequence type and Tn*1546* transposon.**

***Conclusions*: The proportion of VRE-positive samples from broilers has increased since 2000, and this is due to the spread of one major clone. Moreover, this has taken place in an environment without any obvious selective pressure.**

Keywords: epidemiology, MLST, *vanA*

Introduction

The glycopeptide vancomycin is an antimicrobial that, in human medicine, is primarily used to treat infections with Gram-positive bacteria resistant to other antimicrobials. Enterococci are ubiquitous microorganisms and are a part of the normal intestinal flora of man and many other animals. Traditionally, enterococci were considered to be low-grade pathogens with little clinical relevance. During the last two decades, this has changed and they are now increasingly important as opportunistic pathogens causing nosocomial infections in immunocompromised patients.[1] An increasing proportion of these infections are caused by vancomycin-resistant enterococci (VRE) first isolated in 1986.[1,2]

In the 1990s, *Enterococcus faecium* carrying the *vanA* gene, i.e. VRE, were common in the intestinal flora of farm animals in Europe, mainly due to the extensive use of the glycopeptide growth promoter avoparcin.[3] When the association between avoparcin and VRE was confirmed, its use was discontinued, first in Denmark, Finland and Norway and eventually, in 1997, in the whole of the European Union (Commission Directive 97/6 EC). The main reason for this was to evade a pool of VRE that could potentially spread into the community and further into hospital settings.[1] After the withdrawal of avoparcin, the prevalence of VRE in farm animals in Europe rapidly declined.[4]

In Sweden, no growth promoters have been used since 1986, and the use of avoparcin was discontinued even before that. Avoparcin was only used for some years in the late 1970s and early 1980s at quantities of about eight tons per year.[5] Consequently, the situation in Sweden differed from that in other European countries, and VRE were not isolated in samples from Swedish broilers in the middle of the 1990s.[6] Using medium supplemented with vancomycin, VRE were isolated from the intestinal contents from 4 of 150 broilers and 1 of 306 pigs in a survey conducted from 1998 to 2000.[7]

Since 2000, the presence of VRE in the intestinal contents from broilers and other farm animals has been regularly monitored as part of the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) programme.[8] In this programme, VRE have not been isolated from pigs or cattle and only rarely in samples from broilers when medium without vancomycin was used for bacteriological culture. However,

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since 2000, VRE have been isolated from an increasing proportion of samples from broilers when medium supplemented with vancomycin was used. The aim of this paper was to describe this increase and to investigate the genetic relatedness of isolates.

Materials and methods

Sampling

From 2000 to 2002 and from 2004 to 2007, caeca from healthy broilers were collected at slaughterhouses. Samples were collected throughout the year except for 2005 and 2006, when samples were only collected during one spring and one autumn month. All samples were from unique broiler flocks, but not from unique production sites. In 2000 and 2001, samples were collected at four slaughterhouses representing 40% of the total annual slaughter of broilers in Sweden. Additional slaughterhouses were included so that in 2002 ~70% and from 2004 onwards over 90%, of the total annual slaughter was covered. Initially, mainly flocks from the southwest and southeast of Sweden were sampled. As additional slaughterhouses were included, the remaining parts of the country where broiler chickens are produced were also covered.

Bacterial isolation and identification

Caecal content (0.5 g) was suspended in 4.5 mL of saline from which 0.1 mL was streaked on Slanetz–Bartley agar (Oxoid, Basingstoke, UK) supplemented with vancomycin (Sigma-Aldrich, Steinheim, Germany) and incubated at 37°C for 48 h. The vancomycin concentration in the Slanetz–Bartley agar was 8 mg/L during 2000–02. From 2004 onwards, the concentration was 16 mg/L. Moreover, between 2000 and 2002, an enrichment step, i.e. incubation in Enterococcosel (Merck, Darmstadt, Germany) supplemented with vancomycin (final concentration of 8 mg/L) at 37°C for 24 h, was included before culture on solid medium.

From plates with growth of colonies typical for enterococci, at least one colony was subcultured on blood agar (Oxoid) and Bile-Esculine agar (Oxoid) and incubated at 37°C for 24 h. Presumptive enterococci were identified to species level according to Devriese *et al.*[9] Isolates were stored at −70°C for further investigations.

Susceptibility testing

Susceptibility against a panel of antimicrobials (Table 2) was tested by the determination of MIC using microdilution methods according to the standards of the CLSI.[10] The *Enterococcus faecalis* reference strains ATCC 29212 and ATCC 33186 were used for quality control. The tests were performed using cation-adjusted Mueller–Hinton broth (Difco, Sparks, USA) using VetMIC™ E-cocci panels (SVA, Uppsala, Sweden). Susceptibility data were interpreted according to epidemiological cut-off values suggested by EUCAST (http://www.eucast.org), except for narasin where a cut-off of >2 mg/L was used instead of the recommended value of >4 mg/L. Isolates with MICs above the wild-type cut-off value were considered resistant.

Investigation of resistance genotype

In a subset of isolates, the gene encoding vancomycin resistance was determined using PCR for the *vanA* gene.[11] During 2000 and 2001, all VRE isolates obtained were investigated and from 2002 through 2005 every fourth, in 2006 every third and in 2007 every fifth consecutive isolate was investigated. In total, 117 out of 384 isolates obtained were investigated.

Multilocus sequence typing (MLST)

To determine the genetic relatedness of VRE isolates, MLST was performed as described previously by Homan *et al.*,[12] with modifications as described on the MLST web site (http://www.mlst.net). In total, 48 VRE isolates from Swedish broilers were analysed, including the first 2 isolates from 2000 and 46 isolates selected at random from the total of 338 isolates obtained from January 2001 until June 2007.

Tn1546 transposon typing

For the 48 VRE isolates typed by MLST, the *vanA*-containing Tn*1546* transposon was typed using an overlapping PCR method modified from Woodford *et al.*[13] Briefly, the primers were adjusted (Table 1), and a touchdown PCR strategy was employed using HotStarTaq master mix (Qiagen GmbH, Germany). The initial denaturation step of 15 min at 95°C was followed by 10 cycles consisting of 30 s at 94°C, 30 s at 61°C down to 51°C and 1 min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 51°C and 1 min at 72°C,

Table 1. The 10 primer pairs used for Tn*1546* transposon typing

	Sequences (5′ → 3′)	
	forward primer	reverse primer
Primer pair 1	GGA TTT ACA ACG CTA AGC C	GCC TTT ATC AGA TGC TAC C
Primer pair 2	GGT TTT CGA TTA TTG GAA G	TAA AAA TAA TAG AAC GCA TCG AAT AC
Primer pair 3	CTT GAA AGT CAC GGA ATG	GGT TAA CAC CAG CCA TTA C
Primer pair 4	GGA TGG ACT AAC ACC AAT C	GTA TAA TTC AAC CAA ATC GG
Primer pair 5	GTG AAG GGA TTG AAT TGG	CCA ATC CCC AAG TTT CC
Primer pair 6	CGA CTA TTC CAA ACT AGA ACG A	CAT AGT ATA ATC GGC AAC GC
Primer pair 7	CTT CTT GCG CTG AAG AG	CTA TTT CCA TGC TTA TCA CC
Primer pair 8	CAG GAG CAT GAA TAG AAT AAA AG	GGA TTT ACT ATT ATC ACC AAT GTA G
Primer pair 9	CAC TTA TGA AAA TTC ATC TAC ATT G	CCA AGA AAG CCT CCA ACA
Primer pair 10	GCT ATT GGA GCG ACA GAC A	GCG GAT TTA CAA CGT TAA G

with a final extension step of 7 min at 72°C. BM4147 was included as a positive control. The result was visualized by running the PCR products on a 1% agarose gel with 0.01% ethidium bromide.

Statistical analysis

Differences in the proportion of VRE-positive samples between years were tested with Pearson's χ^2 test using Stata software (release 10, Stata, College Station, TX, USA).

Results

VRE isolation and identification

VRE were isolated from 384 of the 1853 samples cultured between 2000 and 2007 (Figure 1). All VRE were *E. faecium* with MICs of vancomycin of >64 mg/L. The proportion of VRE-positive samples increased gradually from <1% in 2000 to slightly over 40% in 2005. Between 2005 and 2006, the proportion of VRE-positive samples decreased and from 2006 to 2007, it was stable at just below 30%. The difference in the proportion of VRE-positive samples between years was statistically significant (χ^2 test, $P \leq 0.001$).

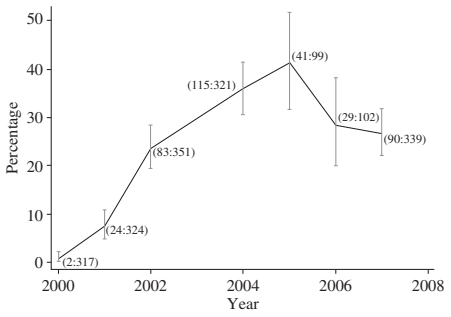

Figure 1. Proportion of VRE-positive samples from broilers from 2000 to 2007; 95% confidence intervals are indicated. The numbers of positive and cultured samples are given in brackets. No samples were analysed in 2003.

Resistance phenotype and genotype

All VRE were high-level vancomycin-resistant (MIC > 64 mg/L), and all isolates investigated by PCR ($n = 117$) carried the *vanA* gene. In addition, the majority of isolates (89.6%) had a phenotype also including resistance to narasin (MIC 4–16 mg/L) and erythromycin (MIC 8–16 mg/L) (Table 2). A minority of isolates had other resistance phenotypes (Table 2), including two isolates with MICs of ampicillin of 8 mg/L, i.e. just above the cut-off value.

MLST

MLST analysis of 48 VRE isolates revealed three different sequence types (STs). The 46 randomly selected isolates from 2001 to 2007 appeared to have ST310, whereas the two isolates from 2000 had ST13 and ST370.

Tn1546 transposon typing

All investigated isolates rendered PCR products for all 10 primer pairs, and the products for each primer pair were of the same size for all the isolates. The profile of the investigated strains was the same as that of BM4147, indicating identical Tn*1546* transposons (Figure 2).

Discussion

Since 2000, the proportion of VRE-positive samples from Swedish broilers has increased considerably. MLST analysis

Table 2. Antibiograms of VRE from Swedish broilers ($n = 384$) from 2000 until 2007

	Resistance pattern								
No. of isolates (%)	VAN	NAR	ERY	BAC	TET	AMP	VIR	STR	GEN
344 (89.6)	R	R	R	S	S	S	S	S	S
14 (3.6)	R	R	S	S	S	S	S	S	S
11 (2.8)	R	R	S	R	S	S	S	S	S
9 (2.3)	R	R	R	S	S	S	R	S	S
2 (0.5)	R	R	S	S	R	S	S	S	S
1 (0.3)	R	R	S	S	S	R	S	S	S
1 (0.3)	R	R	S	S	S	S	R	S	S
1 (0.3)	R	R	R	S	R	S	S	S	S
1 (0.3)	R	R	R	R	S	R	S	S	S

R, resistant; S, susceptible.
Resistance corresponds to MICs above the epidemiological cut-off values suggested by EUCAST (http://www.eucast.org). However, for narasin, a cut-off of >2 mg/L was used instead of the recommended value of >4 mg/L, and for streptomycin and gentamicin, only high-level resistance (MIC > 256 mg/L) was evaluated.
Antimicrobials included and cut-off values (mg/L) used are vancomycin (VAN, >4), narasin (NAR, >2), erythromycin (ERY, >4), bacitracin (BAC, >32), tetracycline (TET, >2), ampicillin (AMP, >4), virginamycin (VIR, >4), streptomycin (STR, >256) and gentamicin (GEN, >256).

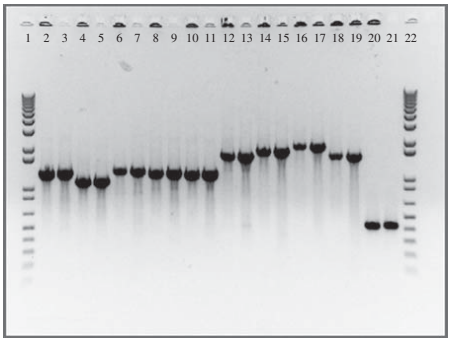

Figure 2. Electrophoresis gel comparing the Tn*1546* transposon patterns of a representative strain (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20) and reference strain BM4147 (lanes 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21). Lanes 1 and 22 contain 1 kb ladder (Promega, Benelux, B.V.).

revealed that this is due to the spread of one major clone of *E. faecium* carrying the same Tn*1546* type. The increase is evident when vancomycin-supplemented medium is used, but there is no apparent change in the prevalence of vancomycin resistance among randomly selected *E. faecium*.[8] This indicates that only a small proportion of enterococci in the intestinal flora of broilers are VRE. This is in contrast to the course of events in other parts of Europe. When avoparcin was still in use during the 1990s, up to 80% of randomly selected *E. faecium* from broilers were vancomycin-resistant.[14] After the withdrawal of avoparcin in Europe, there was a sharp reduction in the proportion of VRE among randomly selected *E. faecium* from intestinal contents.[15] This indicates a decline in actual numbers. However, a high prevalence of VRE is still found in broilers when medium supplemented with vancomycin is used.[16] In Sweden, avoparcin has not been used since the early 1980s, and the prevalence of VRE among Swedish broilers and their possible clonality at that time are not known in detail. In studies from the late 1990s, only occasional isolates of VRE were found.[6,7] Also the diversity of the VRE population among broilers is different between Sweden and the rest of Europe. In Europe, several different strains of VRE have been isolated, even on the same farm and sampling occasion.[17] This is in contrast to the apparent clonality of isolates from Swedish broilers shown by MLST in this study.

In Sweden, there is currently no obvious selection pressure for VRE in broiler production. Avoparcin has, as previously mentioned, not been used since the early 1980s, indicating that vancomycin resistance *per se* is not the selective advantage of the clone. Ionophores are commonly used for prophylaxis against coccidiosis, but selection for VRE by this use is unlikely since ionophore resistance is widespread also among vancomycin-susceptible enterococci from Swedish broilers.[8] Moreover, therapeutic use of antimicrobials is rare in Swedish broiler production. Instead, the emphasis is on disease control by biosecurity, including hygiene barriers and a strict all-in all-out procedure.

The increase in the proportion of VRE-positive samples since 2000 could be explained by the introduction of VRE or the emergence of a very competitive strain, in the late 1990s. Alternatively, there could have been changes in management at that time favouring persistence and transmission of VRE. Yet another scenario is that the explanation lies in a combination of these factors. The fact that only three different MLST STs have been documented indicates that VRE in Swedish broilers did not emerge due to selective pressure by avoparcin, i.e. there has probably been an introduction at some point after the withdrawal of avoparcin. That the two STs found in 2000 (ST13 and ST370) were not re-isolated indicates that glycopeptide resistance is not the selective advantage of the dominating clone (ST310).

The reason for discontinuing the use of avoparcin in the EU was to avoid a pool of resistance genes among farm animals that could, via the food chain, influence the situation in human medicine. The potential for such an influence was indirectly indicated by the reduced prevalence of VRE in healthy humans observed after the withdrawal of avoparcin.[15] Since only a small proportion of enterococci in the intestinal flora of broilers are VRE, and since chicken meat is normally thoroughly heat-treated before consumption, the risk of such a spillover in Sweden is low. Infections caused by VRE are still uncommon in Sweden and, in addition, the majority of human cases are caused by *E. faecium* carrying the *vanB* gene.[18] Therefore, the presence of VRE among Swedish broilers does not seem to have affected the situation within Swedish healthcare. Nor is it clear if the situation in the community is affected. Two separate studies on sewage, as an indicator of community carriage, gave contradictive results with 5% and 54% of VRE, respectively, being *E. faecium* carrying the *vanA* gene.[19,20] Nevertheless, a pool of resistance genes in production animals is unwanted. Therefore, studies aimed at counteracting the spread and persistence of ST310 are warranted. This includes identifying routes of spread and possible selective pressures, as well as selective advantages of ST310.

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Transparency declarations

None to declare.

References

1. Bonten MJ, Willems R, Weinstein RA. Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infect Dis* 2001; **1**: 314–25.

2. Leclercq R, Derlot E, Duval J *et al.* Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. *N Engl J Med* 1988; **319**: 157–61.

3. Bager F, Madsen M, Christensen J *et al.* Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prev Vet Med* 1997; **31**: 95–112.

4. Aarestrup FM, Seyfarth AM, Emborg HD *et al.* Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrob Agents Chemother* 2001; **45**: 2054–9.

5. Wierup M, Lowenhielm C, Wold-Troell M *et al.* Animal consumption of antibiotics and chemotherapeutic drugs in Sweden during 1980, 1982 and 1984. *Vet Res Commun* 1987; **11**: 397–405.

6. Quednau M, Ahrne S, Petersson AC *et al.* Antibiotic-resistant strains of *Enterococcus* isolated from Swedish and Danish retailed chicken and pork. *J Appl Microbiol* 1998; **84**: 1163–70.

7. Kuhn I, Iversen A, Finn M *et al.* Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. *Appl Environ Microbiol* 2005; **71**: 5383–90.

8. SVARM 2007. In: Bengtsson B, Greko C, Grönlund-Andersson U, eds. Swedish Veterinary Antimicrobial Resistance Monitoring. *ISSN 1650-6332*. The National Veterinary Institute (SVA), Uppsala, Sweden, 2008.

9. Devriese LA, Pot B, Collins MD. Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* 1993; **75**: 399–408.

10. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria Isolated from Animals—Third Edition: Approved Standard M31-A3*. CLSI, Wayne, PA, USA, 2008.

11. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 1995; **33**: 24–7.

12. Homan WL, Tribe D, Poznanski S *et al.* Multilocus sequence typing scheme for *Enterococcus faecium*. *J Clin Microbiol* 2002; **40**: 1963–71.

13. Woodford N, Adebiyi AM, Palepou MF *et al.* Diversity of VanA glycopeptide resistance elements in enterococci from humans and non-human sources. *Antimicrob Agents Chemother* 1998; **42**: 502–8.

14. Aarestrup FM. Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. *Microb Drug Resist* 1995; **1**: 255–7.

15. Klare I, Badstubner D, Konstabel C *et al.* Decreased incidence of VanA-type vancomycin-resistant enterococci isolated from poultry meat and from fecal samples of humans in the community after discontinuation of avoparcin usage in animal husbandry. *Microb Drug Resist* 1999; **5**: 45–52.

16. Borgen K, Simonsen GS, Sundsfjord A *et al.* Continuing high prevalence of VanA-type vancomycin-resistant enterococci on Norwegian poultry farms three years after avoparcin was banned. *J Appl Microbiol* 2000; **89**: 478–85.

17. Garcia-Migura L, Liebana E, Jensen LB *et al.* A longitudinal study to assess the persistence of vancomycin-resistant *Enterococcus faecium* (VREF) on an intensive broiler farm in the United Kingdom. *FEMS Microbiol Lett* 2007; **275**: 319–25.

18. SWEDRES 2007. In: Struwe J, Olson-Liljequist B, eds. Swedish Strategic Programme for the Rational Use of Antimicrobial Agents & Swedish Institute for Disease Control. *ISSN 1400-3473*. The Swedish Institute for Infectious Disease Control, Solna, Sweden, 2008.

19. Iversen A, Kuhn I, Franklin A *et al.* High prevalence of vancomycin-resistant enterococci in Swedish sewage. *Appl Environ Microbiol* 2002; **68**: 2838–42.

20. Sahlström L. *Recycled Biowaste as a Source of Infection*. Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, 2006. http://diss-epsilon.slu.se/archive/00001170/01/Avhandling_nr_070.2006_Tryckfil.pdf (08 January 2009, date last accessed).

II

Genetic diversity among VRE isolates from Swedish broilers with the coincidental finding of transferrable decreased susceptibility to narasin

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Keywords: VRE, broiler, transfer, narasin, plasmid addiction system

Abstract

One clone of vancomycin resistant *Enterococcus faecium* with the *vanA* gene (VRE) is predominant among Swedish broilers but other clones do exist. In this study, the molecular diversity among the clones is investigated. The aims of the study were to better understand why one clone of VRE is predominant in Swedish broiler production and to better assess the potential for zoonotic gene transfer from the different clones.

A total of 26 isolates were separated into 11 clones. Vancomycin resistance was transferrable from the predominant and five of the minority clones. Decreased susceptibility to narasin was co-transferred with the vancomycin resistance in four of the clones, including the predominant one. The plasmid addiction system axe-txe was not detected, and the ω-ε-ζ system was detected in one of the minority clones but it was not co-transferred with the vancomycin resistance.

The results of this study do not explain why one clone is predominant among VRE in Swedish broiler production but the results confirm the potential for zoonotic spread of vancomycin resistance genes. The near absence of investigated plasmid addiction systems indicates that they do not play an important role in the epidemiology of VRE in Swedish broiler production. On the other hand, the coincidental finding that decreased susceptibility to narasin can be co-transferred with the *vanA* gene indicates that the use of narasin might play a role in the persistence of vancomycin resistance in enterococci in Swedish broiler production.

Introduction

Vancomycin resistant enterococci (VRE) of the *vanA* genotype were found in abundance among production animals in the 1990's due to use of the glycopeptide avoparcin as growth promoter (Hammerum *et al.*, 2010). The common occurrence was thought to be of importance for the epidemiology of VRE in human healthcare through dissemination of the *vanA* gene to enterococci adapted to humans (Hammerum *et al.*, 2010). Such transfer has been documented to occur *in vivo* (Lester *et al.*, 2006). The perceived risk of a reservoir of resistance genes in production animals lead to a discontinuation of the use of avoparcin with a subsequent reduction in prevalence of VRE in food producing animals (Sundsfjord *et al.*, 2001; Anonymous, 1997).

The situation regarding VRE in Swedish broiler production differs from that in other parts of Europe in several ways. For example, one clone of *vanA* carrying *E. faecium* is predominant even if other minority clones are also found (Nilsson *et al.*, 2009b). It is known that VRE persists at Swedish broiler farms between batches of birds but attempts to explain the epidemiology by differences in management routines of farms have not been successful (Jansson *et al.*, 2009; Nilsson *et al.*, 2009a). Furthermore, there is currently no known selective pressure favouring VRE in Swedish broiler production. Growth promoting antimicrobials have not been used since 1986 and therapeutic use of antimicrobials is rare although the ionophore narasin is routinely used for prophylaxis of coccidiosis (SVARM, 2010).

Persistence of VRE in Norwegian broiler production after discontinued avoparcin use has been attributed to linkage between the vancomycin resistance and the ω-ε-ζ plasmid addiction system (Johnsen *et al.*, 2005). Similarly, presence of the ω-ε-ζ or other plasmid addiction systems could be an explanation for the persistence of vancomycin resistance in enterococci also in Swedish broiler production.

Understanding the epidemiology of VRE in Swedish broiler production is important in order to underpin intervention strategies. Increased knowledge regarding the genetic diversity among VRE from Swedish broilers could provide pieces in the epidemiological puzzle. Traits that could be of importance are the presence of common genetic elements like specific plasmids or variants of the Tn*1546* transposon. Another factor contributing to the situation with one predominant clone is if the vancomycin resistance is not transferrable. The possible transferability of the *vanA* gene is also one

factor in properly assessing the potential for zoonotic gene transfer from the clones of VRE in Swedish broiler production.

The aims of this study were to explore why one clone of VRE is predominant in Swedish broiler production and to better assess the potential for zoonotic gene transfer from the clones. Therefore, clones of VRE from Swedish broilers were identified and molecularly characterized.

Material and Methods

Description of isolates

Isolates were chosen from a collection of VRE isolated from Swedish broilers between 1998 and 2009 through the Swedish Veterinary Antimicrobial Resistance Monitoring programme (SVARM) or the European research programme for Agriculture and Fisheries (FAIR) (Kuhn *et al.*, 2005; SVARM). From this collection, one isolate of the VRE clone dominating among Swedish broilers was selected. Further, isolates with resistance phenotypes different from the phenotype of the dominating clone were selected (Table 1). Three of the isolates have been previously described (Nilsson *et al.*, 2009b) and the remaining 23 isolates were characterized with MLST (Anonymous, 2008; Homan *et al.*, 2002). Then from the 26 isolates, 1 isolate representing each combination of ST and resistance pattern (n=11, hereafter called clone representatives) was chosen at random for further characterization (Table 2).

Transferability of vancomycin resistance

The ability of the clone representatives to transfer vancomycin resistance was investigated by filter mating as previously described by Hammerum et al. (Hammerum *et al.*, 1998). The plasmid free *Enterococcus faecium* 64/3 (Werner *et al.*, 2003) was used as recipient and *E. faecium* F9730129-1 as positive control.

The susceptibility of the transconjugants to various antimicrobials (Table 1) was tested by determination of MIC using a microdilution method according to standards of the CLSI (2008). The *E. faecalis* reference strains ATCC 29212 was used for quality control. The tests were performed using cation-adjusted Mueller–Hinton broth (Difco, Sparks, USA) and VetMIC™ E-cocci panels (SVA, Uppsala, Sweden). Results were interpreted according to epidemiological cut-off values issued by EUCAST (2011). The absence of the *vanA* gene in the recipient and the presence of *vanA* in clone

representatives and in transconjugants was confirmed by PCR (Dutka-Malen *et al.*, 1995).

To confirm that isolates obtained via filter mating were true transconjugants their genetic relationship to the clone representatives and the recipient strain was investigated by pulsed-field gel electrophoresis (PFGE). Total DNA in agarose gel plugs were treated with *Sma*I (New England BioLabs, Ipswich, UK) before separation by PFGE using a CHEF Mapper apparatus (BioRad Laboratories, Richmond California, USA) with settings as previously described (Rosvoll *et al.*, 2010; Sahlstrom *et al.*, 2009). Band patterns were analyzed using BioNumerics 5.10 (Applied Maths, Belgium) with settings according to Werner et al. (2007).

Tn*1546* transposon typing

The Tn*1546* transposon in the clone representatives and the transconjugants was typed using an overlapping PCR as previously described (Nilsson *et al.*, 2009b; Woodford *et al.*, 1998), with the modification that a standard and not a touchdown PCR strategy was used.

Plasmid analyses

The number of plasmids and their size in the clone representatives, the recipient strain and the transconjugants were analyzed by digestion with S1 nuclease (Sigma-Aldrich, Stockholm, Sweden) as previously described (Barton *et al.*, 1995) before separation by PFGE as described above.

The presence of five enterococcal plasmid replicon types (pRE25, pEF418, pIP501, pRUM and pHTβ) and two plasmid addiction systems (axe-txe and ω-ε-ζ) in the clone representatives and the transconjugants were investigated by PCR as previously described (Jensen *et al.*, 2010; Rosvoll *et al.*, 2010; Sletvold *et al.*, 2008).

To study if vancomycin resistance is plasmid mediated in clone representatives and transconjugants, plasmid DNA was isolated with QIAprep (Qiagen AB, Solna, Sweden). Then, remaining chromosomal DNA was removed by digestion with Plasmid-Safe ATP-Dependent DNase (Epicentre Biotchnologies, Wisconsin, USA) according to manufacturers' instructions and as previously described (Moritz & Hergenrother, 2007). Finally, PCR for the *vanA* gene was performed as for the Tn*1546* transposon typing described above. The absence of chromosomal DNA was

confirmed by PCR for the *atpA* gene as for the MLST scheme described above.

Results

Description of isolates

The isolates belonged to eight different STs, including one previously not described (assigned ST587) and had six different resistance patterns (Table 1). They separated into eleven different combinations of ST and resistance pattern, and from each of the combinations one clone representative (n=11) was further characterized (Table 2).

Transferability of vancomycin resistance

Six of the clone representatives transferred vancomycin resistance at frequencies varying from 10^{-4} – 10^{-8} per donor cell (mean varying from 10^{-4} – 10^{-7}), compared to a frequency of 10^{-7} – 10^{-8} for the positive control (Table 2).

The *Sma*I PFGE digestion patterns for the recipient and the transconjugants had more than 90% similarity but less than 65% similarity to the clone representatives from which vancomycin resistance was transferred. This confirms that the transconjugants originate from the recipient strain (Figure 1). The overall similarity among the different clone representatives was 72%.

The MIC of vancomycin for the transconjugants was >128 mg/L whereas the MIC of the recipient was ≤1 mg/L. From four of the isolates, decreased susceptibility to narasin was co-transferred with vancomycin resistance (Table 2). The MIC of narasin for these transconjugants were 2 or 4 mg/L compared to 4 or 8 mg/L for their respective clone representatives and 0.25 mg/L for the recipient. Co-transfer of resistance to bacitracin, erythromycin, tetracycline or virginamycin was not observed.

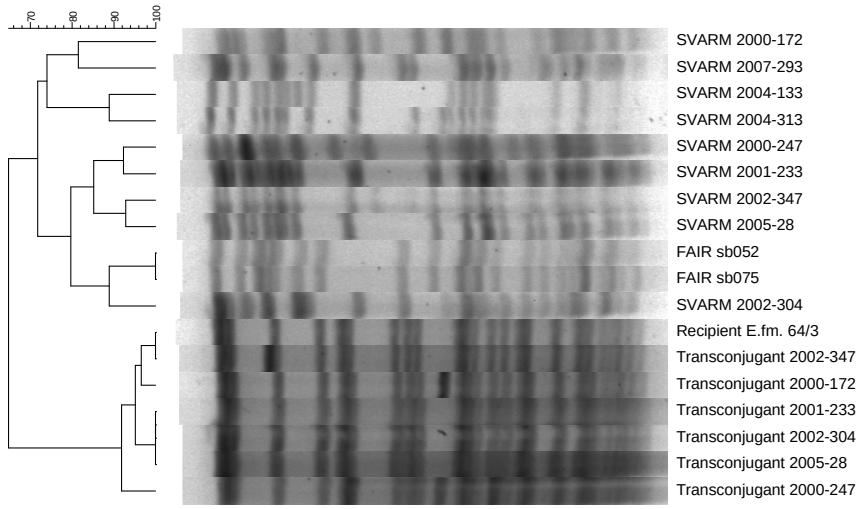

Figure 1. Dendrogram and *Sma*I digestion patterns of the clone representatives, the recipient strain and the transconjugants.

Tn1546 transposon typing

Among the clone representatives, all but one isolate had a Tn*1546* transposon of the *E. faecium* BM4147 prototype variant, i.e. Type A variant according to the classification of Woodford et al. (Table 2) (1998). The remaining isolate (FAIR sb075) was of Type P according to the same classification. All transconjugants had the prototype variant of the Tn*1546* transposon.

Plasmid analyses

Using S1 nuclease and PFGE, between one and four plasmids in the range of 25 to 200 kilobases (kb) were seen in the clone representatives (Figure 2 and Table 2). In the recipient no plasmid, and in each of the transconjugants only one plasmid was seen. For all transconjugants, the size of the plasmid corresponded to a plasmid in their respective clone representative.

The plasmid replicon type pRE25 was detected among the clone representatives and the transconjugants but not pEF418, pIP501, pRUM or pHTβ (Table 2). Despite repeated attempts, none of the tested plasmid replicon types was detected in nine of the isolates (five clone representatives and four transconjugants). Of the two plasmid addiction systems, only ω-ε-ζ was detected and only in one of the clone representatives (Table 2).

Analyses with PCR on plasmid DNA were negative for the *atpA* gene confirming that any remnants of chromosomal DNA were removed by the DNase. Contrary, plasmid DNA from all clone representatives and transconjugants were positive for the *vanA* gene.

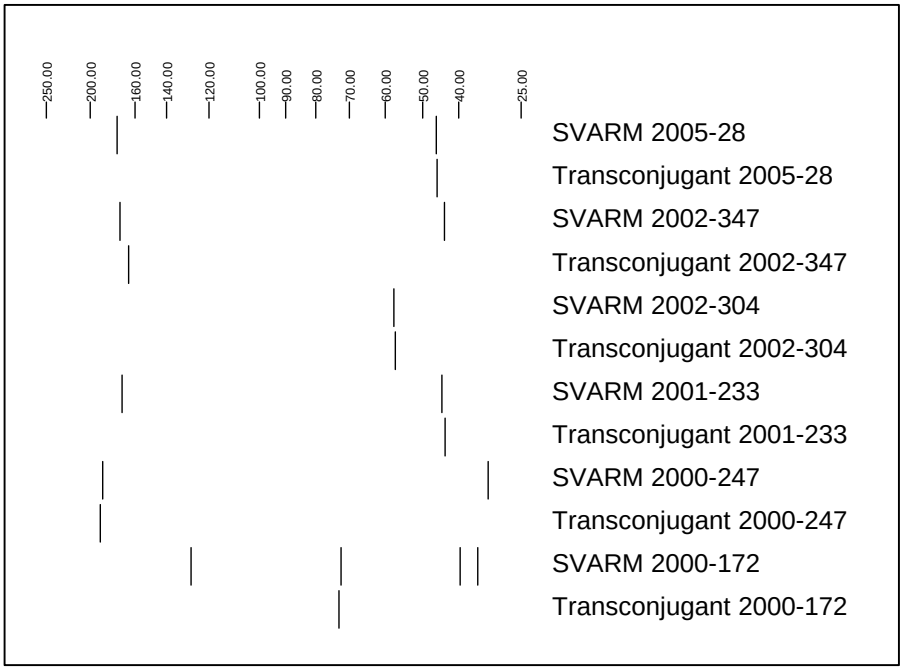

Figure 2. Plasmids detected by S1 nuclease analyses in the transconjugants and their respective clone representatives. The scale indicates size of the plasmids in kilobases.

Table 1. *Sequence types and resistance patterns of the included VRE.*

		Resistance pattern								
Isolate	MLST	Va	Na	Em	Ba	Te	Ap	Vm	Sm	Gm
SVARM 2001-233¤★	310	>128	8	32	2	1	2	2	64	16
SVARM 2005-028★	8	>128	8	4	≤1	≤0.5	2	2	32	8
FAIR sb075★	9	>128	8	1	32	≤0.5	4	1	64	8
SVARM 2002-082	9	>128	4	1	8	≤0.5	4	2	64	8
SVARM 2007-293★	10	>128	4	≤0.5	≤1	≤0.5	4	2	32	4
SVARM 2002-347★	310	>128	8	4	≤1	≤0.5	2	4	32	8
SVARM 2005-043	310	>128	8	4	≤1	≤0.5	2	2	64	8
SVARM 2006-054	310	>128	8	4	≤1	1	2	4	32	4
SVARM 2004-033	248	>128	8	1	>128	≤0.5	≤0.25	4	32	4
SVARM 2004-076	248	>128	4	1	>128	≤0.5	≤0.25	4	32	4
SVARM 2004-079	248	>128	4	1	>128	≤0.5	≤0.25	4	32	4
SVARM 2004-262	248	>128	4	1	128	≤0.5	≤0.25	4	32	8
SVARM 2004-312	248	>128	8	1	128	≤0.5	≤0.25	4	32	4
SVARM 2004-313★	248	>128	4	1	>128	≤0.5	≤0.25	2	32	4
SVARM 2005-053	248	>128	4	≤0.5	>128	1	≤0.25	2	32	4
SVARM 2007-333	248	>128	4	1	128	≤0.5	≤0.25	4	32	8
SVARM 2009-091	248	>128	4	1	>128	≤0.5	≤0.25	2	32	8
SVARM 2004-133★	310	>128	8	1	>128	≤0.5	≤0.25	2	32	4
SVARM 2002-073	587	>128	4	≤0.5	128	≤0.5	4	4	32	4
SVARM 2002-304★	587	>128	4	≤0.5	128	≤0.5	4	4	32	4
FAIR sb024	9	>128	8	≤0.5	16	64	2	2	64	8
FAIR sb036	9	>128	8	1	16	>64	4	2	64	8
FAIR sb052★	9	>128	8	1	16	>64	4	2	64	8
SVARM 2002-137	9	>128	4	1	8	64	2	2	64	8
SVARM 2000-172★	370	>128	8	1	64	64	2	1	64	8
SVARM 2000-247★	13	>128	4	>64	≤1	≤0.5	≤0.25	64	64	8

¤ = the representative of the clone that dominates among VRE from Swedish broilers.

★ = isolates chosen as clone representatives

Shaded fields indicate MIC above the epidemiological cut off values issued by EUCAST (2011).

Antimicrobials included and cut of values (mg/L) used are vancomycin (Va, >4), narasin (Na, >2), erythromycin (Em, >4), bacitracin (Ba, >32U), tetracycline (Te, >2), ampicillin (Ap, >4), virginamycin (Vm, >4), streptomycin (Sm, >512) and gentamicin (Gm, >32).

Table 2. *Characteristics of clone representatives and transconjugants.*

Isolate	Resistance pattern[a]	Conjugation[b]		Transposon type[c]	No. of Plasmids[d]	repA[e]	PAS[f]
		Va	Na				
SVARM 2007-293	Va, Na	-	-	A	2	-	-
SVARM 2005-028	Va, Na	1.2×10^{-4}	+	A	2	pRE25	-
SVARM 2004-313	Va, Na, Ba	-	-	A	2	-	-
SVARM 2004-133	Va, Na, Ba	-	-	A	2	-	-
SVARM 2002-347	Va, Na	4.8×10^{-6}	-	A	2	-	-
SVARM 2002-304	Va, Na, Ba	5.3×10^{-6}	+	A	1	pRE25	-
SVARM 2001-233¤	Va, Na, Er	2.3×10^{-5}	+	A	2	-	-
SVARM 2000-247	Va, Na, Em, Vm	1.0×10^{-6}	+	A	2	pRE25	-
SVARM 2000-172	Va, Na, Ba, Te	1.9×10^{-7}	-	A	4	pRE25	ω-ε-ζ
FAIR sb075	Va, Na	-	-	P	3	pRE25	-
FAIR sb052	Va, Na, Te	-	-	A	2	pRE25	-
Transconjugant 2005-028	Va, Na	na	Na	A	1	-	-
Transconjugant 2002-347	Va	na	Na	A	1	-	-
Transconjugant 2002-304	Va, Na	na	Na	A	1	pRE25	-
Transconjugant 2001-233	Va, Na	na	na	A	1	-	-
Transconjugant 2000-247	Va, Na	na	na	A	1	pRE25	-
Transconjugant 2000-172	Va	na	na	A	1	-	-

¤ = the representative of the clone of VRE dominating in Swedish broiler production.

a) Va = vancomycin, Na = Narasin, Em = Erythromycin, Ba = Bacitracin, Te = Tetracycline and Vm = virginamycin. See Table 1 for definition of resistance. b) Transfer frequency of vancomycin resistance per donor cell, - = no transfer/co-transfer of resistance, + = co-transfer of decreased susceptibility to narasin, na = not applicable. c) Transposon type according to Woodford et al (1998). d) Number of plasmids seen with S1 and PFGE analysis. e) Plasmid replicon types detected by PCR analysis, - = no plasmid replicon types detected. f) Plasmid addiction aystems detected by PCR analysis, - = no plasmid addiction system detected.

Discussion

Vancomycin resistance was transferred from the predominant as well as from five of the minority clones and at frequencies similar to or lower than reported elsewhere (Rosvoll *et al.*, 2010; Jung *et al.*, 2006; Lester *et al.*, 2006; Sundsfjord *et al.*, 2001). Thus, the predominance of one clone among VRE in Swedish broiler production is not due to that clones' inability to transfer the vancomycin resistance. Furthermore, as the *vanA* gene was transferrable from the predominant clone it is likely that any given VRE from Swedish broilers will have vancomycin resistance that is transferrable to other enterococci. Thereby they constitute a reservoir with the potential for zoonotic spread of vancomycin resistance, as described previously for VRE of broiler origin (Lester & Hammerum, 2010; Lester *et al.*, 2006). However, VRE in Swedish healthcare are mainly *E. faecium* with the *vanB* gene (SWEDRES, 2010). Hence, neither the abundance of VRE in production animals in other parts of Europe in the 1990's nor the presence of VRE in Swedish broiler production seems to have affected the situation in Swedish healthcare to a greater extent.

Even if presence of several clones of VRE in Swedish broilers is in accordance with reports from other parts of Europe, the predominance of one clone is not (Garcia-Migura *et al.*, 2007b; Novais *et al.*, 2005; Sorum *et al.*, 2004; Heuer *et al.*, 2002). The predominant and the minority clones are similar in that vancomycin resistance is plasmid mediated and that all but one of the minority clones carries the BM4147 prototype variant of the Tn*1546* transposon. The plasmid localization of the *vanA* gene and to some extent also the predominance of the prototype variant of the Tn*1546* transposon are in accordance with previous knowledge (Biavasco *et al.*, 2007; Courvalin, 2006; Jung *et al.*, 2006; Woodford *et al.*, 1998).

Between one and four plasmids were seen in the S1 analysis and the only plasmid replicon type detected was pRE25 but only in six of the eleven clones. Both the presence of pRE25 and the number of plasmids are in accordance with what has been described elsewhere in *E. faecium* from animals (Rosvoll *et al.*, 2010). Furthermore, the pRE25 plasmid has been associated with transferable vancomycin resistance in *E. faecium* (Garcia-Migura *et al.*, 2007a). In two of the transconjugants the pRE25 plasmid replicon type was also detected, indicating a connection to the vancomycin resistance in these clones. The indication was especially strong for isolate

SVARM 2002-304 and its respective transconjugant where the pRE25 replicon type was detected and only one plasmid (of about 57.5 kb) was seen in the S1 analysis. Even if not confirmed, it is probable that the visible plasmid is indeed of the pRE25 plasmid replicon type and mediating vancomycin resistance.

Presence of plasmid addiction systems has been described in both vancomycin susceptible and resistant enterococci of human as well as animal origin (Rosvoll *et al.*, 2010; Moritz & Hergenrother, 2007; Johnsen *et al.*, 2005; Grady & Hayes, 2003). Persistence of VRE in the absence of selective pressure has been suggested to be associated with linkage of the *vanA* gene and the ω-ε-ζ system (Johnsen *et al.*, 2005). But this does not seem to be the situation in Sweden since the axe-txe system was not detected in any, and the ω-ε-ζ system in only one of the minority clones. Besides, the ω-ε-ζ system was not co-transferred with vancomycin resistance indicating that they are not located on the same plasmid. It is therefore unlikely that the investigated plasmid addiction systems play such an important role in VRE epidemiology among Swedish broilers as has been suggested for ω-ε-ζ in Norway (Johnsen *et al.*, 2005).

Decreased susceptibility to narasin was co-transferred with vancomycin resistance from the predominant and three of the minority clones. To our knowledge, transferrable resistance for an ionophore has not been previously described. Furthermore, decreased susceptibility to narasin is widespread among vancomycin susceptible as well as resistant enterococci from Swedish broilers (SVARM, 2010). Hence, use of narasin is not expected to select for VRE (Nilsson *et al.*, 2009b). However, co-transfer of the *vanA* gene and decreased susceptibility to narasin indicates that use of narasin may play a role in persistence of vancomycin resistance among enterococci in Swedish broilers. The mechanism of the decreased susceptibility to narasin and its connection to the vancomycin resistance gene remains to be clarified but perhaps the traits are closely linked to each other. Thus, when retaining the decreased susceptibility to narasin the enterococci also retain the vancomycin resistance.

In conclusion, the predominance of one clone among VRE in Swedish broiler production is not due to inability of that, or other clones to transfer the *vanA* gene. Nor is the predominance explained by other molecular similarities or dissimilarities among the clones because there does not seem to be any correlations regarding the differences. That the vancomycin

resistance is transferrable from the predominant clone and several of the minority clones confirms that VRE among Swedish broilers constitutes a pool of resistance genes with the potential to disseminate to other enterococci, including those colonizing humans. But so far the presence of VRE among Swedish broilers does not seems to have affected the situation in Swedish health care where the majority of VRE are *E. faecium, vanB* (SWEDRES, 2010). Finally, the coincidental finding that decreased susceptibility to narasin can be co-transferred with the *vanA* gene raises questions on the role of narasin in the persistence of vancomycin resistance in enterococci in Swedish broilers. To completely clarify the role of narasin, experimental studies as well as genetic investigations are probably needed.

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Conflict of interest

None to declare.

References

Anonymous (1997). Commission directive 97/6/EC. *Official journal of the European Communities* L35 5.2.97, 11-13.

Anonymous *Multi Locus Sequence Typing home page, E. faecium*. [online] Available from: http://efaecium.mlst.net/. [Accessed 2010-08-14].

Barton, B.M., Harding, G.P. & Zuccarelli, A.J. (1995). A general method for detecting and sizing large plasmids. *Anal Biochem* 226(2), 235-40.

Biavasco, F., Foglia, G., Paoletti, C., Zandri, G., Magi, G., Guaglianone, E., Sundsfjord, A., Pruzzo, C., Donelli, G. & Facinelli, B. (2007). VanA-type enterococci from humans, animals, and food: species distribution, population structure, Tn1546 typing and location, and virulence determinants. *Appl Environ Microbiol* 73(10), 3307-19.

CLSI (2008). Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria Isoalted from Animals; Approved Standard - Third Edition. Wayne, PA: Clinical and Laboratory Standards Institute. CLSI document M31-A3.

Courvalin, P. (2006). Vancomycin resistance in gram-positive cocci. *Clin Infect Dis* 42 Suppl 1, S25-34.

Dutka-Malen, S., Evers, S. & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *J Clin Microbiol* 33(1), 24-7.

EUCAST *EUCAST, Clinical Breakpoints*. [online] Available from: http://www.srga.org/eucastwt/MICTAB/index.html. [Accessed 2011-02-21].

Garcia-Migura, L., Liebana, E. & Jensen, L.B. (2007a). Transposon characterization of vancomycin-resistant Enterococcus faecium (VREF) and dissemination of resistance associated with transferable plasmids. *J Antimicrob Chemother* 60(2), 263-8.

Garcia-Migura, L., Liebana, E., Jensen, L.B., Barnes, S. & Pleydell, E. (2007b). A longitudinal study to assess the persistence of vancomycin-resistant Enterococcus faecium (VREF) on an intensive broiler farm in the United Kingdom. *FEMS Microbiol Lett* 275(2), 319-25.

Grady, R. & Hayes, F. (2003). Axe-Txe, a broad-spectrum proteic toxin-antitoxin system specified by a multidrug-resistant, clinical isolate of Enterococcus faecium. *Mol Microbiol* 47(5), 1419-32.

Hammerum, A.M., Jensen, L.B. & Aarestrup, F.M. (1998). Detection of the satA gene and transferability of virginiamycin resistance in Enterococcus faecium from food-animals. *FEMS Microbiol Lett* 168(1), 145-51.

Hammerum, A.M., Lester, C.H. & Heuer, O.E. (2010). Antimicrobial-resistant enterococci in animals and meat: a human health hazard? *Foodborne Pathog Dis* 7(10), 1137-46.

Heuer, O.E., Pedersen, K., Jensen, L.B., Madsen, M. & Olsen, J.E. (2002). Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban. *Microb Drug Resist* 8(4), 355-61.

Homan, W.L., Tribe, D., Poznanski, S., Li, M., Hogg, G., Spalburg, E., Van Embden, J.D. & Willems, R.J. (2002). Multilocus sequence typing scheme for Enterococcus faecium. *J Clin Microbiol* 40(6), 1963-71.

Jansson, D., Nilsson, O., Greko, C., Lindblad, J. & Bengtsson, B. Inter-batch contamination of broiler farms and equipment by a vancomycin-resistant clone of *Enterococcus faecium*. In: *Proceedings of XVI Congress of the World Veterinary Poultry Association*, Marrakesh, Morocco 2009.

Jensen, L.B., Garcia-Migura, L., Valenzuela, A.J., Lohr, M., Hasman, H. & Aarestrup, F.M. (2010). A classification system for plasmids from enterococci and other Gram-positive bacteria. *J Microbiol Methods* 80(1), 25-43.

Johnsen, P.J., Osterhus, J.I., Sletvold, H., Sorum, M., Kruse, H., Nielsen, K., Simonsen, G.S. & Sundsfjord, A. (2005). Persistence of animal and human glycopeptide-resistant enterococci on two Norwegian poultry farms formerly exposed to avoparcin is associated with a widespread plasmid-mediated vanA element within a polyclonal Enterococcus faecium population. *Appl Environ Microbiol* 71(1), 159-68.

Jung, W.K., Hong, S.K., Lim, J.Y., Lim, S.K., Kwon, N.H., Kim, J.M., Koo, H.C., Kim, S.H., Seo, K.S., Ike, Y., Tanimoto, K. & Park, Y.H. (2006). Phenotypic and genetic characterization of vancomycin-resistant enterococci from hospitalized humans and from poultry in Korea. *FEMS Microbiol Lett* 260(2), 193-200.

Kuhn, I., Iversen, A., Finn, M., Greko, C., Burman, L.G., Blanch, A.R., Vilanova, X., Manero, A., Taylor, H., Caplin, J., Dominguez, L., Herrero, I.A., Moreno, M.A. & Mollby, R. (2005). Occurrence and relatedness of vancomycin-resistant enterococci in animals, humans, and the environment in different European regions. *Appl Environ Microbiol* 71(9), 5383-90.

Lester, C.H., Frimodt-Moller, N., Sorensen, T.L., Monnet, D.L. & Hammerum, A.M. (2006). In vivo transfer of the vanA resistance gene from an Enterococcus faecium isolate of animal origin to an E. faecium isolate of human origin in the intestines of human volunteers. *Antimicrob Agents Chemother* 50(2), 596-9.

Lester, C.H. & Hammerum, A.M. (2010). Transfer of vanA from an Enterococcus faecium isolate of chicken origin to a CC17 E. faecium isolate in the intestine of cephalosporin-treated mice. *J Antimicrob Chemother* 65(7), 1534-6.

Moritz, E.M. & Hergenrother, P.J. (2007). Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. *Proc Natl Acad Sci U S A* 104(1), 311-6.

Nilsson, O., Greko, C. & Bengtsson, B. (2009a). Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production. *Acta Vet Scand* 51, 49.

Nilsson, O., Greko, C., Top, J., Franklin, A. & Bengtsson, B. (2009b). Spread without known selective pressure of a vancomycin-resistant clone of Enterococcus faecium among broilers. *J Antimicrob Chemother* 63(5), 868-72.

Novais, C., Coque, T.M., Costa, M.J., Sousa, J.C., Baquero, F. & Peixe, L.V. (2005). High occurrence and persistence of antibiotic-resistant enterococci in poultry food samples in Portugal. *J Antimicrob Chemother* 56(6), 1139-43.

Rosvoll, T.C., Pedersen, T., Sletvold, H., Johnsen, P.J., Sollid, J.E., Simonsen, G.S., Jensen, L.B., Nielsen, K.M. & Sundsfjord, A. (2010). PCR-based plasmid typing in Enterococcus faecium strains reveals widely distributed pRE25-, pRUM-, pIP501- and pHTbeta-

related replicons associated with glycopeptide resistance and stabilizing toxin-antitoxin systems. *FEMS Immunol Med Microbiol* 58(2), 254-68.

Sahlstrom, L., Rehbinder, V., Albihn, A., Aspan, A. & Bengtsson, B. (2009). Vancomycin resistant enterococci (VRE) in Swedish sewage sludge. *Acta Vet Scand* 51, 24.

Sletvold, H., Johnsen, P.J., Hamre, I., Simonsen, G.S., Sundsfjord, A. & Nielsen, K.M. (2008). Complete sequence of Enterococcus faecium pVEF3 and the detection of an omega-epsilon-zeta toxin-antitoxin module and an ABC transporter. *Plasmid* 60(1), 75-85.

Sorum, M., Holstad, G., Lillehaug, A. & Kruse, H. (2004). Prevalence of vancomycin resistant enterococci on poultry farms established after the ban of avoparcin. *Avian Dis* 48(4), 823-8.

Sundsfjord, A., Simonsen, G.S. & Courvalin, P. (2001). Human infections caused by glycopeptide-resistant Enterococcus spp: are they a zoonosis? *Clin Microbiol Infect* 7 Suppl 4, 16-33.

SVARM Swedish Veterinary Antimicrobial Resistance Monitoring. ISSN 1650-6332. http://www.sva.se/en/Target-navigation/Animal-health/Antibiotic-Resistance/Monitoring-of-antimicrobial-resistance/SVARM-reports/ (all SVARM reports). [online] [Accessed 2011-04-26].

SVARM (2010). *Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) 2009*. Uppsala, Sweden: The National Veterinary Institute (SVA). ISSN 1650-6332.

SWEDRES (2010). *SWEDRES 2009*. Solna, Sweden: Swedish Strategic Programme for the Rational Use of Antimicrobial Agents & Swedish Institute for Infectious Disease Control. ISSN 1400-3473.

Werner, G., Klare, I. & Witte, W. (2007). The current MLVA typing scheme for Enterococcus faecium is less discriminatory than MLST and PFGE for epidemic-virulent, hospital-adapted clonal types. *BMC microbiology* 7, 28.

Werner, G., Willems, R.J., Hildebrandt, B., Klare, I. & Witte, W. (2003). Influence of transferable genetic determinants on the outcome of typing methods commonly used for Enterococcus faecium. *J Clin Microbiol* 41(4), 1499-506.

Woodford, N., Adebiyi, A.M., Palepou, M.F. & Cookson, B.D. (1998). Diversity of VanA glycopeptide resistance elements in enterococci from humans and nonhuman sources. *Antimicrob Agents Chemother* 42(3), 502-8.

III

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Research

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Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production

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Abstract

Background: Vancomycin resistant enterococci are a frequent cause of nosocomial infections and their presence among farm animals is unwanted. Using media supplemented with vancomycin an increase in the proportion of samples from Swedish broilers positive for vancomycin resistant enterococci has been detected. The situation at farm level is largely unknown. The aims of this study were to obtain baseline knowledge about environmental contamination with vancomycin resistant enterococci in Swedish broiler production and the association between environmental contamination and colonisation of birds.

Methods: Environmental samples were taken before, during and after a batch of broilers at three farms. Samples were cultured both qualitatively and semi-quantitatively for vancomycin resistant enterococci. In addition, caecal content from birds in the batch following at each farm was cultured qualitatively for vancomycin resistant enterococci.

Results: The number of samples positive for vancomycin resistant enterococci varied among the farms. Also the amount of vancomycin resistant enterococci in the positive samples and the proportion of caecal samples containing vancomycin resistant enterococci varied among the farms. Still, the temporal changes in environmental contamination followed a similar pattern in all farms.

Conclusion: Vancomycin resistant enterococci persist in the compartments even after cleaning and the temporal changes in environmental contamination were similar among farms. There were however differences among farms regarding both degree of contamination and proportion of birds colonized with vancomycin resistant enterococci. The proportion of colonized birds and the amount of vancomycin resistant enterococci in the compartments seems to be associated. If the factor(s) causing the differences among farms could be identified, it might be possible to reduce both the risk for colonisation by vancomycin resistant enterococci of the subsequent flock and the risk for spread of vancomycin resistant enterococci via the food chain to humans.

Background

Vancomycin resistant enterococci (VRE) were first isolated in 1986 [1,2]. Since then, VRE have become endemic at many hospitals and are now considered a significant cause of nosocomial infections, mainly in immunocompromised patients [3]. In the early 1990s many farm ani-

mals in Europe were colonized with VRE. This was associated with extensive use of the glycopeptide avoparcin as a growth promoter [4], a use that was discontinued in the European Union in 1997 (Commission Directive 97/6 EC). In Sweden, avoparcin was only used for some years in the late 1970s and early 1980s [5,6] which could explain why VRE were not isolated from Swedish farm animals in the mid 1990s [7,8]. Later all use of growth promoters in Sweden was discontinued in 1986.

Vancomycin resistance is still rare among randomly selected enterococci isolated from farm animals in Sweden. However, using media supplemented with vancomycin an increase in the proportion of VRE-positive samples from Swedish broilers has been detected since 2000 [9]. It was shown that the increase is due to the spread of one clone of *vanA*-carrying *Enterococcus faecium* which has taken place in an apparently non-selective environment. In Swedish broiler production therapeutic use of antimicrobials is rare and instead the emphasis is on disease control by biosecurity. A farm to fork concept is applied to the control of food borne pathogens. Since VRE constitute a pool of resistance genes with possible implications for human healthcare, their occurrence in broiler production should if possible be contained. To this end, knowledge about colonisation of birds and environmental contamination at farm level is imperative.

Both VRE colonisation of broilers and contamination of farm environments has been studied elsewhere [10-12]. However, the almost monoclonal situation and low-level colonisation by VRE indicate a distinct epidemiological situation in Swedish broiler production. Therefore, the aims of this study were to obtain baseline knowledge about environmental contamination with VRE in Swedish broiler production and the association between environmental contamination and colonisation of birds.

Methods

Sampling

Three conveniently located broiler farms were chosen out of farms that previously had had broilers colonized with *van*A-carrying *E. faecium* (unpublished data). The three farms were chosen because they were similar in structure and size (i.e. number of houses and amount of broilers produced) and because the farmers were willing to participate. Each farm had four compartments and a total floor surface area between 5 200 and 7 000 m^2. Within farms, hygiene barriers, including changing of shoes, were in place and each compartment had separate ventilation. During the study period, no flock was given any antibiotic treatment apart from the anticoccidial agent narasin which was used in feed until 5 days prior to slaughter.

Environmental samples

Environmental samples for culture of VRE were taken at 7 occasions (S1-S7) and on each sampling occasion, 2-5 samples from each compartment were taken (Table 1). All samples were collected from the end of March until the beginning of July 2007. At S1 and S7 the compartments had been cleaned and were ready for the subsequent batch of birds except that the bedding was not in place. The samplings S2-S4 took place approximately 1, 2 and 3 weeks after arrival of birds, S5 took place 2-4 days before slaughter and S6 after loading the birds for slaughter but before cleaning of the compartments. Birds were slaughtered when they were 36 to 43 days old. Exact day of sampling was chosen to minimize time of sample transport. Initial sampling (S1) at each farm was made by one of the researchers (ON) and thereafter by the farmers according to oral and written instructions. Briefly, floor samples were obtained with "Sterisocks humid" (SodiBox, Névez, France) by walking back and forth two times in the compartment, covering a distance of approximately 300 - 400 meters. The socks were made of jersey material that was factory pre-moistened with 15 mL distilled water. They were used outside sterile boot-covers and covered the entire sole of the boots. Other environmental samples were taken with sterile cloths (Sterile cloth, SodiBox), factory pre-impregnated with buffered peptone solution with 10% neutralising agent (lecithin, Tween 80, L-histidine, and sodium thiosulfate). Samples from air inlet and air outlet were obtained by wiping a surface area of approximately 0.04 and 0.2 m^2 respectively. Samples from the water- and feedline were obtained by wiping 5 meters of the line and the adjacent nipples. After sampling, each sock and cloth was placed in a separate plastic sampling bag and sent to the laboratory by mail, no later than the following day. Until mailing, samples were stored at 6 °C.

Caecal samples

From the batches of broilers following the environmental sampling period, 10 caecas per group of birds slaughtered (slaughter group) were sampled. Caecas were collected at the slaughterhouse before the birds were scalded and sent to the laboratory by mail on the same day.

Bacterial isolation, identification and counting

Environmental samples

Samples arrived at the laboratory the day after mailing and were analysed on the day of arrival or at the latest the following day. Samples were cultured both for qualitative and semi-quantitative detection of VRE. First, Enterococcosel (Merck, Darmstadt, Germany) was added to the samples (25 mL to cloths and 50 mL to socks) which were then placed in a Stomacher (Stomacher®-80 Biomaster lab system, Seward Ltd., Worthing, United Kingdom) and treated for 1 minute. Thereafter, 10 mL of the solution was removed and divided in two aliquots. For semi-quantita-

Table 1: Results of bacteriological culture for vancomycin resistant enterococci of environmental samples.

		Farm A				Farm B				Farm C			
		1	2	3	4	1	2	3	4	1	2	3	4
	Floor	+ (1.7)	+ (1.8)#	+ (2.3)	+ (1.0)	-	-	+*	-	-	-	-	-
Before arrival	Air inlet	+ (1.4)	+ (1.3)#	+ (3.3)	+ (1.7)	-	+ (0.9)	+ (0.3)	+	-	+*	-	+
of birds	Air outlet	+ (1.5)	+ (0.8)	+ (1.4)	+ (2.1)	+ (1.3)*	+ (0.5)	+ (1.4)	+ (0.5)	-	-	-	-
(S1)	Feed line	+ (2.1)	+ (1.9)	+ (3.0)*	+ (3.5)	-	+ (1.3)	+ (0.0)	+ (0.7)*	+ (1.6)*	+ (0.6)	-	+ (0.5)*
	Water line	+ (1.3)*	+ (2.3)	+ (2.0)	+ (1.6)#	+ (1.1)	+*	+	-	-	-	-	-
6-8 days after	Floor	-	+	-	-	-	-	-	-	-	-	-	-
arrival of birds	Air inlet	+ (3.3)#	+ (3.2)*	+ (3.9)#	+ (3.1)	+ (1.7)	+ (1.4)	+*	+	-	+ (0.0)#	-	-
(S2)	Air outlet	+ (2.8)	+ (3.0)	+ (3.2)	+ (2.9)*	+ (1.3)*	+ (0.6)*	+ (1.9)	+ (0.6)*	+ (0.0)*	+#	-	-
13-15 days after	Floor	+ (3.3)*	+ (3.7)*	-	+ (4.2)	-	-	-	-	-	-	-	-
arrival of birds	Air inlet	+ (4.1)	-	-	+ (4.2)#	+ (2.6)#	+ (0.5)#	-	-	-	+ (0.6)*	-	-
(S3)	Air outlet	+ (4.1)	+ (4.2)	+ (4.2)*	+ (4.2)	+ (2.3)	-	+ (0.7)*	+ (0.6)*	+ (0.8)#	-	-	-
20-22 days after	Floor	+ (3.9)	+ (4.0)	+ (4.5)	+ (4.1)	-	-	+ (2.0)	+ (0.9)*	+ (2.2)*	+ (1.3)	-	-
arrival of birds	Air inlet	+ (4.1)#	+ (4.0)*	+ (4.3)	+ (4.2)	+ (2.4)*	+ (0.3)	+ (0.3)	+ (0.6)	+ (0.5)	+ (0.3)	-	-
(S4)	Air outlet	+ (3.8)	+ (4.1)	+ (4.1)#	+ (4.0)*	+ (2.6)	+ (2.9)*	+ (0.9)*	+ (1.5)	+ (0.8)	+ (0.8)*	-	-
2-4 days	Floor	+ (4.6)	+ (4.7)*	+ (4.9)	+ (4.6)#	+ (3.1)*	+ (3.0)*	+ (0.6)	+ (3.1)#	+ (2.6)	+ (2.1)#	-	+*
before slaughter	Air inlet	+ (4.5)	+ (4.6)	+ (4.6)#	+ (4.3)	-	-	+ (0.5)	-	+ (2.9)*	+ (0.0)	+ (0.6)#	+ (1.1)
(S5)	Air outlet	+ (4.6)*	+ (4.5)	+ (4.5)	+ (4.3)	-	+ (3.5)	+ (0.9)*	+ (3.4)	-	-	-	-
After loading for	Air inlet	+ (4.6)	+ (4.6)*	+ (4.6)	+ (4.4)	-	-	+ (0.9)	+ (0.8)#	+*	+ (2.5)	-	+ (0.0)*
slaughter (S6)	Air outlet	+ (4.5)*	+ (4.5)	+ (4.3)*	+ (4.4)*	+ (0.3)*	-	+ (0.9)*	+ (1.9)	+ (0.0)	+ (3.5)*	-	-
	Floor	+ (3.6)	+ (2.8)#	+ (3.7)	+ (3.5)	-	-	+#	-	+ (0.5)	+ (0.9)	+ (1.1)*	+ (1.1)
Before arrival	Air inlet	+ (3.5)*	+ (3.2)#	+ (3.8)	+ (2.9)	ns	+ (1.3)#	-	+*	-	-	+ (0.5)	-
of new birds	Air outlet	+ (3.6)	+ (3.1)	+ (3.9)	+ (3.9)	ns	+ (0.0)	+	+ (0.0)	-	+ (1.6)	-	+ (0.3)*
(S7)	Feed line	+ (3.1)	+ (2.9)	+ (2.9)*	+ (2.8)#	ns	+ (1.5)	-	+ (2.1)	+ (1.7)*	+ (1.2)	-	-
	Water line	+ (2.6)	+ (2.3)	+ (3.1)	+ (1.9)	ns	+ (1.2)	-	-	+	+ (1.2)*	-	-

+ = positive sample, - = negative sample, ns = sample not taken. Numbers in brackets indicate the amount of VRE (log number of colony forming units/plate, adjusted for dilution) in samples positive on direct plating.
* = isolates identified to species, # = isolates identified to species and analysed with MLST.

tive detection (degree of contamination), 0.1 mL from one aliquot was streaked on Slanetz-Bartley agar (Oxoid, Basingstoke, UK) supplemented with vancomycin (16 mg/L) (Sigma-Aldrich, Steinheim, Germany). For qualitative detection (presence of VRE), the other aliquot was pre-enriched at 37°C for 3-4 hours with the primary aim of resuscitating injured bacteria. Next, 0.1 mL was streaked on Slanetz-Bartley agar (Oxoid) supplemented with vancomycin (16 mg/L) (Sigma-Aldrich). The plates were then incubated at 37°C for 48 hours. The number of colonies with morphology consistent with enterococci from the non pre-enriched aliquot was recorded. If the number of colonies was too high for accurate counting the aliquot was diluted 1:10 and 1:100 and re-cultured as above. From the pre-enriched aliquot only growth or non-growth of colonies with morphology consistent with enterococci was recorded. From all positive samples at least one colony was sub-cultured on blood agar (Oxoid) and Bile-Esculine agar (Oxoid) and incubated at 37°C for 24 hours. Colonies with morphological appearance typical for enterococci on all media and positive reaction on Bile-Esculine agar were considered as *Enterococcus* sp. Isolates were stored at -70°C for further investigations.

Caecal samples

Caecal samples were cultured as previously described [9]. Briefly, caecal content (0.5 grams) was suspended in 4.5 mL saline from which 0.1 mL was streaked on Slanetz-Bartley agar (Oxoid) supplemented with vancomycin (16 mg/L) (Sigma-Aldrich) and incubated at 37°C for 48 hours. Samples with growth of colonies with morphology consistent with enterococci were handled as above.

Species identification

Species identification was done according to Devriese et al [13]. Environmental isolates chosen for multilocus sequence typing (MLST) analysis (see below) were included along with additional isolates so that at least one isolate, if existing, from each compartment and sampling occasion was included (n = 77). In addition, two caecal isolates per slaughter group were included (n = 8). Both additional environmental isolates and caecal isolates were selected at random within compartments and slaughter groups. The reference strain *Enterococcus faecalis* ATCC 29212 was used for quality control.

Susceptibility testing

All stored environmental and caecal isolates (n = 214) were tested for susceptibility to vancomycin by determination of MIC using micro dilution in broth according to the standards of the Clinical and Laboratory Standards Institute [14]. Tests were performed in cation adjusted Mueller-Hinton broth (Difco, Sparks, USA) using VetMIC™ panels (SVA, Uppsala, Sweden). The reference strain *Enterococcus faecalis* ATCC 29212 was used for quality control.

Multilocus sequence typing (MLST)

Among the stored environmental isolates (n = 189) 24 were selected at random and analysed with MLST as described by Homan et al [15], with modifications according to the MLST web site [16].

Statistical analysis

Absolute numbers of colonies from semi-quantitative detection (degree of contamination) in environmental samples were transformed to logarithmic values before statistical analysis. All analyses for environmental and caecal samples were done by Pearson's χ^2 test using Stata software (release 10, Stata, College Station, TX, USA). Statistical significance was set as $p = 0.05$.

Results

Sampling, bacterial isolation and counting

Environmental samples

The number of VRE-positive samples differed among the farms (Table 1). For each farm, the proportions of VRE-positive samples in total and on direct plating were: Farm A 94% and 93%; Farm B 64% and 54%; and Farm C 42% and 34%. Also the degree of contamination measured by semi-quantitative detection differed among the farms (Table 1).

At the first sampling (S1) VRE were present in the environment at all farms, but the number of positive samples and the degree of contamination varied among farms. At Farm A, VRE were detected on direct plating in all 20 samples taken initially; whereas at Farm C, VRE were only detected in 5 of the samples, of which only 3 were positive on direct plating (Table 1).

The amount of time before VRE were detected in the floor samples taken during the batch (S2-S5) varied both among farms and among compartments at the same farm. At Farm A, VRE were detected in floor samples from 1 of 4 compartments 1 week after arrival of birds, and in 3 of 4 compartments 2 weeks after arrival of birds. In contrast, at Farm B and Farm C, VRE were not detected in floor samples until 3 weeks after arrival of birds. Even though VRE were detected in floor samples from all but 1 compartment 2-4 days before slaughter, the degree of contamination varied between farms (Table 1).

At the first and the last sampling (S1 and S7) the number of positive samples was equal in 7 of the 11 compartments where sampling was completed according to schedule. However, in all of these 7 compartments more samples were positive on direct plating or the degree of contamination measured by semi-quantitative detection was higher, after the batch compared to before. Of the remaining compartments, 3 (all on Farm C) had more

VRE-positive samples and 1 (on Farm B) had fewer VRE-positive samples after the batch compared to before.

Among samples taken from cleaned compartments (S1 and S7), the feed line was the only sample, that with statistical significance predicted whether VRE could be detected in any sample from the compartment at that sampling occasion (χ^2 test, $p = 0.05$).

Caecal samples

At all three farms, birds from compartments 1 and 2 were slaughtered in one slaughter group and compartments 3 and 4 in another. The numbers of VRE-positive caecal samples were: from Farm A, 6 and 8 samples (70%); and from Farm B, 4 and 7 samples (55%). From Farm C VRE could not be isolated from any of the 20 caecal samples analysed. The differences between Farm C versus Farm A or Farm B was statistically significant (χ^2 test, $p < 0.001$).

Species identification, susceptibility testing and MLST

All identified isolates (n = 85) were *E. faecium*, all susceptibility tested isolates (n = 214) had MIC for vancomycin of ≥128 mg/L, and all isolates (n = 24) investigated with MLST were of ST310.

Discussion

The result of the species identification, susceptibility testing and MLST indicate that the VRE isolated from the study farms belong to the *vanA*-carrying *E. faecium* clone previously described to dominate among Swedish broilers [9].

Even though VRE were isolated in all compartments at all farms we found that environmental contamination with VRE at the three farms differed. Not only did the proportion of VRE-positive samples vary among the farms but also the degree of contamination. Differences among the farms were also seen in samples from individual chickens. VRE could not be detected in caecal samples from the farm with the lowest proportion of VRE-positive samples and the lowest degree of environmental contamination (Farm C) whereas from the other two farms 70% and 55% of the caecal samples were VRE-positive. This indicates an association between the degree of environmental contamination and colonisation of birds.

Although the degree of environmental contamination varied, the temporal changes in contamination followed a similar pattern in all farms. At the start of the study, when cleaned and empty compartments were sampled (S1), VRE were present in all but one compartment. That VRE persist even after cleaning and disinfection is in agreement with previous studies [10-12]. At all farms the degree of contamination increased during the batch and then decreased when the compartments were again cleaned after the batch. However, in floor samples taken when birds were present in the compartments (S2-S5), bedding and faeces stuck to the socks and were included in the samples. In such cases, the sample volume was larger than from empty floors, which could partly explain the apparent reduction of VRE in floor samples from S5 to S7. For samples from Air inlet and Air outlet the difference in the amount of material was negligible. Still, VRE were not eliminated from any of the compartments. In addition, two of the farms had a higher degree of VRE contamination after the studied batch, indicating that the cleaning routines are not sufficient, which could lead to a build-up of VRE within the compartments. However, it cannot be excluded that the higher degree of VRE contamination after, as compared to before the batch (S7 to S1) was influenced by climate factors. In empty compartments the ventilation is turned down and temperature and humidity could be affected by the outside climate. The study period was in the spring to early summer and the temperature in the empty compartments was probably lower at S1 than at S7 which could influence the degree of VRE contamination detected.

It has been suggested that VRE persisting in the compartments subsequently colonize the following batch of broilers [11]. Our study indicates that even the low degree of VRE contamination seen on Farm C at the start of the study (S1), is sufficient for amplification and spread. As soon as birds are put in to the compartments they would start to become colonized with the persisting VRE. Borgen et al [11] isolated VRE from faecal samples in 3 of 5 study units already after 1 week and after 3 weeks all study units were VRE-positive. In our study, only 1 of 12 compartments had a VRE-positive floor sample one week after arrival of birds (S2). On the other hand, at that time the bedding mainly comprises of shavings and therefore only a small proportion of the floor samples were actually faeces which would have decreased the sensitivity. Nevertheless, in both studies the time before VRE colonisation could be detected varied among study units.

As time proceeds, more and more birds would become colonized with VRE leading to increased contamination, both in the bedding and in the rest of the environment. Accordingly, there was an increase in the degree of VRE contamination on the floors during the first weeks of the rearing period. Garcia-Migura et al [10] describes a similar increase until the broilers were three weeks old, but the percentage of VRE-positive faecal samples decreased in the end of the rearing period. Also studies by Devriese et al and Kaukas et al [17,18] indicate a decreased proportion of *E. faecium* in the intestinal flora of chickens with increasing age. As mentioned, the floor samples in our study should be regarded as environmental samples from the floors rather than actual faecal samples. Therefore the

degree of colonisation of the birds in our study could have decreased without being reflected in the contamination of the floors. Still, even if the amount of VRE in the intestines of the birds is diminishing the VRE in the environment constitute a risk for later contamination of the carcasses. The skin and feathers of the birds will likely be contaminated by VRE from the environment, as indicated by a study finding elevated rates of enterococci in air samples taken behind running vehicles transporting poultry [19]. Furthermore, Rule et al [20] found enterococci in water samples from various places within poultry slaughter houses (e.g. scald tank and plucking facilities) implying that VRE on skin and feathers of the birds could contaminate the whole carcass is not unlikely.

Conclusion

In conclusion, the main findings of this study are that VRE persist in the compartments even after cleaning and that the temporal changes in environmental contamination is similar among studied units. There were however differences among the farms regarding both degree of contamination and proportion of birds colonized with VRE. Furthermore, the proportion of colonized birds and the amount of vancomycin resistant enterococci in the compartments seems to be associated. If the factor(s) causing the differences in degree of contamination and proportion of birds colonized with VRE among farms could be identified, it might be possible to reduce the amount of VRE both at the farms and in the birds. Thereby, both the risk for VRE-colonization of the subsequent flock and the risk for spread of VRE to humans via the food chain by contaminated broiler carcasses would be reduced.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The study was designed by all authors. ON did the field work and the laboratory work. ON drafted the manuscript and all authors revised, read and approved the final manuscript.

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References

1. Leclercq R, Derlot E, Duval J, Courvalin P: **Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium.** *N Engl J Med* 1988, **319**:157-161.
2. Uttley AH, Collins CH, Naidoo J, George RC: **Vancomycin-resistant enterococci.** *Lancet* 1988, **1**:57-58.
3. Werner G, Coque TM, Hammerum AM, Hope R, Hryniewicz W, Johnson A, Klare I, Kristinsson KG, Leclercq R, Lester CH, Lillie M, Novais C, Olsson-Liljequist B, Peixe LV, Sadowy E, Simonsen GS, Top J, Vuopio-Varkila J, Willems RJ, Witte W, Woodford N: **Emergence and spread of vancomycin resistance among enterococci in Europe.** *Euro Surveill* 2008, **13**:.
4. Bager F, Madsen M, Christensen J, Aarestrup FM: **Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant Enterococcus faecium on Danish poultry and pig farms.** *Prev Vet Med* 1997, **31**:95-112.
5. Anonymous: **SVARM 2000.** In *Swedish Veterinary Antimicrobial Resistance Monitoring* Edited by: Bengtsson B, Wallén C. Uppsala, Sweden: National Veterinary Institute (SVA); 2001:13-15.
6. Wierup M, Lowenhielm C, Wold-Troell M, Agenas I: **Animal consumption of antibiotics and chemotherapeutic drugs in Sweden during 1982 and 1984.** *Vet Res Commun* 1980, **11**:397-405.
7. Anonymous: **Antimicrobial Feed Additives; SOU 1997:32.** *Agriculture Mo, ed* 1997:264-266.
8. Quednau M, Ahrne S, Petersson AC, Molin G: **Antibiotic-resistant strains of Enterococcus isolated from Swedish and Danish retailed chicken and pork.** *J Appl Microbiol* 1998, **84**:1163-1170.
9. Nilsson O, Greko C, Top J, Franklin A, Bengtsson B: **Spread without known selective pressure of a vancomycin-resistant clone of Enterococcus faecium among broilers.** *J Antimicrob Chemother* 2009, **63**:868-872.
10. Garcia-Migura L, Liebana E, Jensen LB, Barnes S, Pleydell E: **A longitudinal study to assess the persistence of vancomycin-resistant Enterococcus faecium (VREF) on an intensive broiler farm in the United Kingdom.** *FEMS Microbiol Lett* 2007, **275**:319-325.
11. Borgen K, Sorum M, Kruse H, Wasteson Y: **Persistence of vancomycin-resistant enterococci (VRE) on Norwegian broiler farms.** *FEMS Microbiol Lett* 2000, **191**:255-258.
12. Heuer OE, Pedersen K, Jensen LB, Madsen M, Olsen JE: **Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban.** *Microb Drug Resist* 2002, **8**:355-361.
13. Devriese LA, Pot B, Collins MD: **Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups.** *J Appl Bacteriol* 1993, **75**:399-408.
14. Anonymous: **Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria Isoalted from Animals; Approved Standard - Third Edition.** In *CLSI document M31-A3* Wayne, PA: Clinical and Laboratory Standards Institute; 2008.
15. Homan WL, Tribe D, Poznanski S, Li M, Hogg G, Spalburg E, Van Embden JD, Willems RJ: **Multilocus sequence typing scheme for Enterococcus faecium.** *J Clin Microbiol* 2002, **40**:1963-1971.
16. **Multilocus Sequence Typing home page, *E. faecium*** [http://efaecium.mlst.net/]
17. Devriese LA, Hommez J, Wijfels R, Haesebrouck F: **Composition of the enterococcal and streptococcal intestinal flora of poultry.** *J Appl Bacteriol* 1991, **71**:46-50.
18. Kaukas A, Hinton M, Linton AH: **The effect of ampicillin and tylosin on the faecal enterococci of healthy young chickens.** *J Appl Bacteriol* 1987, **62**:441-447.
19. Barros LS, Amaral LA, Lorenzon CS, Junior JL, Neto JG: **Potential microbiological contamination of effluents in poultry and swine abattoirs.** *Epidemiol Infect* 2007, **135**:505-518.
20. Rule AM, Evans SL, Silbergeld EK: **Food animal transport: a potential source of community exposures to health hazards from industrial farming (CAFOs).** *Journal of Infection and Public Health* 2008, **1**:33-39.

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IV

Survival of vancomycin resistant *Enterococcus faecium* under laboratory and field conditions – a pilot study to investigate if eradication from broiler farms is possible?

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Keywords: VRE, growth, survival, disinfection, fitness

Abstract

The proportion of broilers in Sweden colonized with vancomycin resistant enterococci (VRE) increased rapidly from 2000 to 2005 but has since then stabilized. Presence of VRE among Swedish broilers is unwanted since they constitute a pool of resistance that could affect the situation in human healthcare. Enterococci are robust bacteria that can survive adverse conditions and VRE in Swedish broiler production has been shown to persist at farms between batches. The aim of this study was therefore to (i) determine the ability of VRE from Swedish broilers to withstand various physical conditions including exposure to disinfectants under laboratory conditions and, (ii) test the applicability of the laboratory findings in a pilot study on disinfection of farms.

The laboratory studies confirmed that VRE from Swedish broilers can withstand harsh conditions, grow in various temperatures and survive for long periods. The tested isolates did however seem fully susceptible to the tested disinfectants. This indicates that a reduction of VRE contamination in the broiler houses is possible, provided that cleaning and disinfection can be adequately performed.

In the field disinfection pilot study, two variants of a disinfection protocol combining steam and formaldehyde was tested at two different farms. After the disinfection, VRE could not be detected in environmental samples from the test compartments but was detected in environmental samples from the control compartments. The proportion of broilers colonized with VRE did however decrease in both the test and the control compartments making the effect of the disinfection on the proportion of broilers colonized with VRE inconclusive. Still, the results are promising and indicate that the occurrence of VRE can be reduced by adequate cleaning and disinfection.

Introduction

Prevalence of vancomycin resistant enterococci (VRE) among randomly selected enterococci from Swedish broilers is very low (SVARM, 2010). However, the proportion of Swedish broilers colonized with VRE increased rapidly from 2000 to 2005 (Nilsson *et al.*, 2009b). The increase was due to the spread of one major clone of *vanA* carrying *Enterococcus faecium* with multi locus sequence type 310 (MLST ST310). The factor(s) that caused this spread of one clone is not known but use of antimicrobials is limited in Swedish broiler production and the glycopeptide growth promoter avoparcin was only used during the late 1970s and early 1980s (Bager *et al.*, 1997; Wierup *et al.*, 1987). As VRE are important causes of nosocomial infections (Werner *et al.*, 2008) presence of VRE in farm animals is unwanted since they constitute a pool of resistance that could affect the situation in human healthcare. The risk of such a transmission was the justification for discontinuing the use of avoparcin in the European Union in 1997 (Commission Directive 97/6 EC).

Within the Swedish broiler production, an all-in all-out procedure with thorough cleaning and disinfection between all batches is practiced. However, enterococci are robust bacteria that can survive adverse conditions (Barnes, 1959) and the clone of VRE dominating in Swedish broiler production (ST310) has been shown to persist at farms (Nilsson *et al.*, 2009a). It has also been shown that there is no constant introduction of VRE, neither from the hatcheries nor from the feed (Nilsson *et al.*, 2008). Together this indicates that if VRE could be eliminated from the farms, the proportion of broilers colonized with VRE would probably also decrease. However, no clear differences in management routines between Swedish farms contaminated and not contaminated with VRE have been found (Jansson *et al.*, 2009). This could indicate that none of the cleaning and disinfection routines normally used is sufficiently effective, perhaps due to properties of the bacteria (e.g. resistance to disinfectants) or to practical difficulties in the application of disinfection protocols (e.g. assuring that the disinfectant reach into all cracks and all equipment). The aim of this study was therefore to (i) determine the ability of VRE from Swedish broilers to withstand various physical conditions including exposure to disinfectants under laboratory conditions and, (ii) test the applicability of the laboratory findings in a field disinfection pilot study.

Materials and Methods

Laboratory studies

Three VRE from caecal samples of healthy broilers (VRE:2000-172, VRE:2000-247, and VRE:2001-233), obtained and typed through the Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) programme (SVARM, 2010) were investigated. All three had previously been shown to be of different MLST types, including the dominating ST310 (in order as above: ST370, ST13 and ST310) (Nilsson *et al.*, 2009b). In all tests, either 4-h or 15-h pre-cultures of each isolate were used. Pre-cultures were prepared by suspending material from 3-5 fresh overnight colonies in 5 mL Mueller-Hinton broth (Difco, Sparks, USA) and incubating at 37°C for 4 or 15 h. All tests were performed in triplicates.

To test growth at different physical conditions (pH and temperature), a 4-h pre-culture was stepwise diluted $1{:}10^6$ in Mueller-Hinton broth (Difco) and incubated for 18 h, either at 37°C or at the test temperature (i.e. 45, 47, 49, or 50°C). For tests regarding pH; the broth was adjusted to pH 4, 5 or 6 with HCl and pH 9 or 10 with NaOH. Growth was determined as visible turbidity.

To test survival at different temperatures, a 15-h pre-culture was stepwise diluted $1{:}10^6$ in Mueller-Hinton broth (Difco), pipetted to empty Petri dishes (0.1 mL per dish), and left to dry at room temperature in a ventilation hood. The number of surviving bacteria in the dishes was determined after various times of storage at 6, 21.5 or 31°C (Figure 2a-c). Briefly, bacteria were dissolved with 0.1 mL saline solution before Mueller-Hinton-agar (Oxoid, Basingstoke, UK) was poured into the dishes. Plates were then incubated at 37°C over night before the number of colony forming units (CFU) was recorded. Survival curves were plotted as mean logarithmic number of CFU versus time in weeks.

To test tolerance of high temperatures, 50 µL from a 4-h pre-culture was inoculated in 5 mL of pre-heated Mueller-Hinton broth (Difco), incubated for 4 hours at the test temperature (i.e. 55, 60 or 65°C), cooled and incubated for 18 h at 37°C before growth was determined as visible turbidity.

The susceptibility to three commercial disinfectants: a glutaraldehyde (GLU-CID; Brenntag Nordic, Ballerup, Denmark), a combination of peroxygen

and an organic acid (Virkon-S®; Antec International, Suffolk, England), and a formaldehyde (Formalin; Merck KGaA, Darmstadt, Germany) was tested. The disinfectants were chosen since they are commonly used in Swedish broiler production. Susceptibility to GLU-CID and Virkon-S® was tested with a quantitative suspension test as described by Reybrouck (2004) whereas the susceptibility to Formalin was tested by exposing bacteria to gaseous formaldehyde. As "test suspension" for both tests, a 15-h pre-culture was mixed 1:1 with a soiling solution containing bovine serum albumin (10 g/L, Sigma-Aldrich) and yeast extract (10 g/L, Difco). For the quantitative suspension test, the test suspension was exposed to the disinfectants, neutralized with a quenching agent [0,1% Pepton (Difco), 0,5% Tween 80 (Merck, Darmstadt, Germany), 0,1% Natriumthiosulfat (Fluka, Steinheim, Germany) and 0,07% Lecitin (Sigma-Aldrich) in Super Q water (Johnston & Jones, 1995)], serially diluted and cultured on blood agar (Oxoid) to determine the number of colonyforming units per milliliter (CFU/mL). Before the tests, the efficacy of the quenching agent to the two disinfectants had been verified (data not shown). For the exposure to gaseous formaldehyde, 0.1 mL of test suspension was pipetted to empty Petri dishes and left to dry at room temperature in a ventilation hood before test plates were placed in a room fumigated over night with 5.33 mL of Formalin (40%) per cubic meter. The number of surviving bacteria in test and control dishes was determined in the same way as for survival in different temperatures.

Field disinfection experiments

Two farms previously having had broilers colonized with VRE were chosen. After the broilers had been sent to slaughter, the houses were either cleaned and disinfected according to the farm's normal routines (control compartments) or cleaned according to the farm's normal routines and disinfected with a combination of steam and formaldehyde (test compartments), as outlined by Gradel et al. (2004a).

At Farm A; two compartments in one house were used as test compartments and disinfected at a temperature of 50°C for 4h and two compartments in an identical house were used as control. At Farm B; a single compartment house was used as test compartment and disinfected at a temperature of 60°C for 4h whereas two compartments in one house (each of the same size as the test compartment) was used as control. The size of the test compartments were approximately 1900 square meters (5700 cubic meters)

at Farm A and 1600 square meters (4800 cubic meters) at Farm B. At both farms was 90 g of Formalin per cubic meter used.

From one (Farm A) and two (Farm B) batches of broilers sent for slaughter preceding the disinfection and the two batches' after the disinfection, caecal samples from ten birds per house or compartment were analyzed for the presence of VRE as previously described (Nilsson *et al.*, 2009a; Nilsson *et al.*, 2009b). In addition, environmental samples (air inlet, feed line and water line) from each compartment were collected before and after disinfection in the test compartments and at corresponding time points in the control compartments. All environmental samples were taken in duplicate except samples from the water line after disinfection at Farm A. Environmental samples were collected with sterile cloths and cultured for qualitative detection of VRE as previously described (Nilsson *et al.*, 2009a). From each sampling occasion, one isolate was selected at random for species identification and susceptibility testing as previously described (Nilsson *et al.*, 2009b; Devriese *et al.*, 1993). Caecal samples from broilers raised at Farm A could only be identified by house and not by compartment and therefore all samples were grouped accordingly. Furthermore, boxes in which the chickens for the batch directly following the disinfection were delivered were sampled and cultured as the environmental samples. At Farm A, ten boxes from each house were sampled whereas at Farm B only nine boxes in total were sampled since the day old chickens for both test and control compartments were of the same consignment.

Results

Laboratory studies

All three isolates grew at 45 and 47°C but none grew at 49 or 50°C. All three isolates grew in pH 6 and 9, VRE:2001-233 and VRE:2000-172 grew in pH 5 but not VRE:2000-247 and none of the isolates grew in pH 4 or 10. All isolates survived for 20 weeks in all temperatures (Figure 1a-c). Furthermore, only for VRE:2000-247 stored at 6°C did the mean reduction in viable CFU exceed 1 log during the period studied (Figure 1a). All isolates tolerated 55°C for 4h; both VRE:2000-172 and VRE:2000-247 tolerated 60°C for 4h but only in one of the triplicates; and only VRE:2000-247 tolerated 65°C for 4h, also then only in one of the triplicates.

No differences in susceptibility towards the tested disinfectants were detected among the isolates. Bacteria could not be reisolated from any of the samples treated with disinfectant. In the control samples, the amount of bacteria varied between 4.8 and 5.6 log CFU/mL (means 5.0 to 5.4 log CFU/mL) for the quantitative suspension test and 2.1 and 2.6 log CFU/mL (means 2.1 to 2.5 log CFU/mL) for the exposure to gaseous formaldehyde.

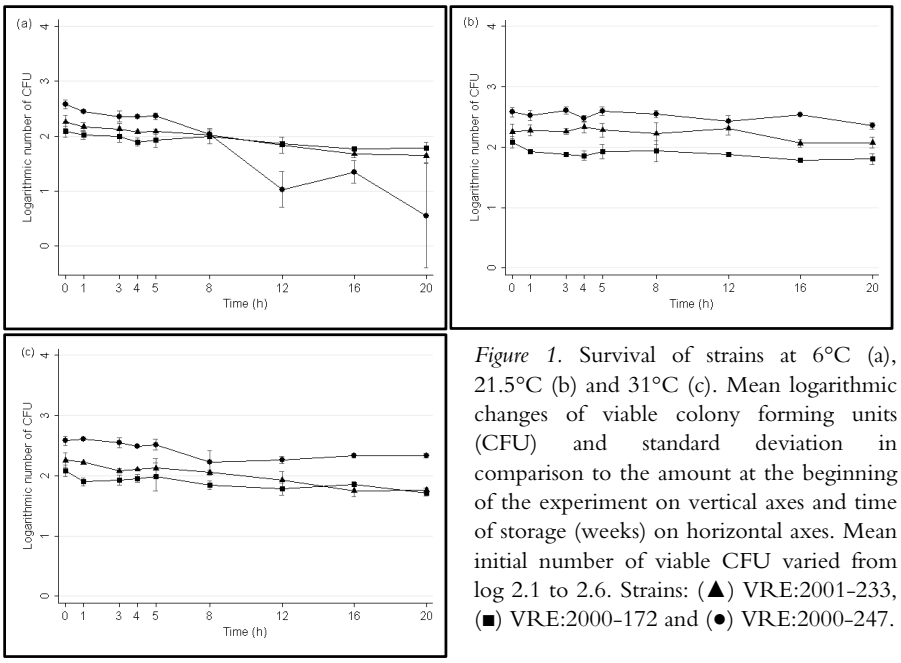

Figure 1. Survival of strains at 6°C (a), 21.5°C (b) and 31°C (c). Mean logarithmic changes of viable colony forming units (CFU) and standard deviation in comparison to the amount at the beginning of the experiment on vertical axes and time of storage (weeks) on horizontal axes. Mean initial number of viable CFU varied from log 2.1 to 2.6. Strains: (▲) VRE:2001-233, (■) VRE:2000-172 and (●) VRE:2000-247.

Field disinfection experiments

Isolation of VRE

The proportion of caecas positive for VRE varied among sampling occasions (Table 1). Notably, one of the caecas from broilers raised in the test compartments after disinfection at Farm A was positive for VRE. Also the proportion of environmental samples from which VRE was isolated varied but none of the samples taken from the test compartments after the disinfection were positive (Table 1). VRE was not isolated from any of the samples taken from the boxes in which the chickens were delivered. All isolates chosen for species identification and susceptibility testing were *E. faecium* and the MIC of vancomycin for these isolates was >128 mg/L.

Table 1. *Proportion of VRE positive samples of caecas from different batches of broilers and of the environment from different compartments (number positive/number cultured).*

	Farm A		Farm B	
	Test compartments	Control compartments	Test compartment	Control compartments
Caecas two batches before disinfection	na	na	1/10	5/20
Caecas one batch before disinfection	4/10	3/10	2/10	8/20
Environmental samples before disinfection	10/12	6/12	6/6	4/12
Environmental samples after disinfection	0/11	2/11	0/6	6/12
Caecas one batch after disinfection	1/10	0/10	0/10	1/20⋆
Caecas two batches after disinfection	0/10	2/10	0/10	1/20⋆

At Farm A the temperature in the test compartment was raised to 50°C for 4 hours and at Farm B to 60°C. At both farms the control compartment(s) were cleaned and disinfected according to the respective farms normal routines.
na = samples not taken.
⋆ Both the caecas positive for VRE were from broilers raised in one of the control compartments whereas all caecas from both batches' from the other control compartment were negative for VRE.

Discussion

The overall aim of this study was to find ways to decrease the level of VRE contamination in the environment at broiler farms and thereby also lower the proportion of broilers colonized with VRE at slaughter. All together, the results are promising and indicate that occurrence of VRE can be reduced by adequate cleaning and disinfection.

The laboratory studies detected no substantial differences among the investigated isolates, even if there were some variations in the fitness. For example, only the isolate of the dominating clone (VRE:2001-233) showed visible growth in Mueller-Hinton broth with 6% NaCl, but that isolate was the least tolerant to high temperatures. All isolates survived storage at all temperatures for the whole period of 20 weeks and generally showed less than 0.5 log reductions in CFU during storage. Hence, it is likely that they survive longer. The length of survival was comparable to or longer than in other studies (Ramadhan & Hegedus, 2005; Neely & Maley, 2000; Wendt *et al.*, 1998; Bale *et al.*, 1993). However, in some of those studies, as in our, some isolates survived for the full length of the experiment. Hence, definite conclusions on the full potential for survival of different enterococci cannot be drawn.

Decreased susceptibility to disinfectants has been described in various bacteria and sometimes also in association with antibiotic resistance (Cerf *et al.*, 2010; SCENHIR, 2009; EFSA, 2008). We detected no decreased susceptibility to the disinfectants. Thus, failure to reduce contamination with VRE at farm level probably lies in difficulties to fully apply to the disinfection protocols rather than in the protocols *per se* (Cerf *et al.*, 2010; Weber & Rutala, 2006). For example, practical difficulties in cleaning the houses adequately can lead to quenching of the disinfectant by remaining biological substances. Also difficulties in applying the disinfectant at various locations within the houses could contribute to the unsuccessful disinfection. Therefore we decided to test a new disinfection method rather than a new disinfectant in the field disinfection pilot study.

The efficacy of the chosen method towards enterococci had been shown under both laboratory and field conditions (Gradel *et al.*, 2004a; Gradel *et al.*, 2004b). Furthermore, the financial cost of the method was acceptable for practical use. For example, the cost of the disinfection in this study was

approximately 4000 euro per farm. Both temperatures, i.e. 50 and 60°C, tested in the pilot study reduced the amount of VRE after disinfection below the detection limit of the methodology for environmental samples. In contrast, VRE were still isolated after disinfection by the farms' normal routines. It has recently been suggested that it will take decades for VRE to disappear from farms even without the selective pressure of glycopeptides (Johnsen *et al.*, 2011). No interventions were however included in that prediction but the present study indicates that VRE persisting in the broiler houses can be removed. A reduction of VRE contamination in the broiler houses could lead to fewer birds colonized and consequently to further reduction of VRE occurrence in the broiler houses. Furthermore, that the effectiveness of tested disinfection method against VRE indicates that it can also be used for other agents since enterococci are known to be robust bacteria.

At Farm B, the reduction of VRE contamination in the compartments was reflected by the culture of samples of caecal content from broilers raised after disinfection. In the test house, VRE was not isolated from the two batches of broilers raised after disinfection. In contrast, from the two control compartments, VRE was isolated both from both the first and the second batch from one of the compartments but not in the other compartment.

However, at Farm A VRE was isolated from one sample from the first batch of broilers raised in the test compartments after disinfection but in no sample from the second batch. Notably, VRE was not isolated in samples from the first batch raised in the control compartments after disinfection, but from one csample from the second batch. The difference in proportion of samples positive for VRE between the farms indicates that the disinfection was more efficient at Farm B. This could possibly be attributed to the higher temperature used at Farm B but could also be due to other differences between the farms. The varying results regarding proportion of caecas positive for VRE between test and control compartments at Farm A can be due to lack of sensitivity in the sampling and culturing procedure as well as to the fact that the field disinfection experiments only involved two trials. However, cross contamination from other houses at the farm is another plausible explanation. Similar inconsistencies were also experienced by Gradel et al. when developing the disinfection method (Gradel *et al.*, 2004a; Gradel *et al.*, 2004b).

In conclusion, no substantial differences in the ability to withstand various physical conditions *in vitro* among the investigated isolates were detected and resistance to disinfectants does not seem to be the reason for persistence of VRE at farms. The field experiment was a pilot study to assess whether a new disinfection protocol can enable the elimination of VRE at farm level. The result of the field experiment was not conclusive since the proportion of chickens colonized with VRE diminished also in the control compartments. The environmental sampling did however show that disinfection with a combination of steam and formaldehyde is more efficient in reducing the level of VRE contamination than the farms' normal disinfection routines, thereby indicating that occurrence of VRE can be reduced by adequate disinfection.

Further work to elucidate the possibility to control VRE in Swedish broiler production could include large scale disinfection studies were all of the houses at farms are disinfected. In addition, better knowledge regarding the molecular characteristics (e.g. location and transferability) of the vancomycin resistance of VRE from Swedish broilers would be valuable to understand why a clone is dominating and to evaluate the risk of transfer and spread of the resistance genes.

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Conflict of interest

None to declare.

References

Bager, F., Madsen, M., Christensen, J. & Aarestrup, F.M. (1997). Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant Enterococcus faecium on Danish poultry and pig farms. *Prev Vet Med* 31(1-2), 95-112.

Bale, M.J., Bennett, P.M., Beringer, J.E. & Hinton, M. (1993). The survival of bacteria exposed to desiccation on surfaces associated with farm buildings. *J Appl Bacteriol* 75(6), 519-28.

Barnes, E.M. (1959). Differential and selective media for the faecal streptococci. *J Sci Food Agric* 10(12), 656-662.

Cerf, O., Carpentier, B. & Sanders, P. (2010). Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "resistance" has different meanings. *Int J Food Microbiol* 136(3), 247-54.

Devriese, L.A., Pot, B. & Collins, M.D. (1993). Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol* 75(5), 399-408.

EFSA (2008). Scientific opinion of the Panel on Biological Hazards on a request from DG SANCO on the assessment of the possible effect of the four antimicrobial treatment substances on the emergence of antimicrobial resistance. *EFSA Journal* 659, 1-26.

Gradel, K.O., Jorgensen, J.C., Andersen, J.S. & Corry, J.E. (2004a). Monitoring the efficacy of steam and formaldehyde treatment of naturally Salmonella-infected layer houses. *J Appl Microbiol* 96(3), 613-22.

Gradel, K.O., Sayers, A.R. & Davies, R.H. (2004b). Surface disinfection tests with Salmonella and a putative indicator bacterium, mimicking worst-case scenarios in poultry houses. *Poult Sci* 83(10), 1636-43.

Jansson, D., Nilsson, O., Greko, C., Lindblad, J. & Bengtsson, B. Inter-batch contamination of broiler farms and equipment by a vancomycin-resistant clone of *Enterococcus faecium*. In: *Proceedings of XVI Congress of the World Veterinary Poultry Association*, Marrakesh, Morocco 2009.

Johnsen, P.J., Townsend, J.P., Bohn, T., Simonsen, G.S., Sundsfjord, A. & Nielsen, K.M. (2011). Retrospective evidence for a biological cost of vancomycin resistance determinants in the absence of glycopeptide selective pressures. *J Antimicrob Chemother.* 66(3): 608-610.

Johnston, M.D. & Jones, M.V. (1995). Disinfection tests with intact biofilms: combined use of the Modified Robbins Device with impedance detection. *J Microbiol Methods* 21(1), 15-26.

Neely, A.N. & Maley, M.P. (2000). Survival of enterococci and staphylococci on hospital fabrics and plastic. *J Clin Microbiol* 38(2), 724-6.

Nilsson, O., Greko, C. & Bengtsson, B. (2009a). Environmental contamination by vancomycin resistant enterococci (VRE) in Swedish broiler production. *Acta Vet Scand* 51, 49.

Nilsson, O., Greko, C., Franklin, A. & Bengtsson, B. Investigating potential sources of vancomycin resistant enterococci (VRE) for broilers in Sweden. In: *Proceedings of 4th Med-Vet-Net Annual Scientific Meeting*, St. Malo, France, 11-14 June 2008.

Nilsson, O., Greko, C., Top, J., Franklin, A. & Bengtsson, B. (2009b). Spread without known selective pressure of a vancomycin-resistant clone of Enterococcus faecium among broilers. *J Antimicrob Chemother* 63(5), 868-72.

Ramadhan, A.A. & Hegedus, E. (2005). Survivability of vancomycin resistant enterococci and fitness cost of vancomycin resistance acquisition. *J Clin Pathol* 58(7), 744-6.

Reybrouck, G. (2004). Evaluation of the antibacterial and antifungal activity of desinfectants. In: Fraise, A.P.*, et al*. (Eds.) *Priciples and Practice of Disinfection, Preservation & Sterilisation*. 4th. ed. Oxford, UK: Blackwell Publishing. ISBN 1-4051-0199-7.

SCENHIR (2009) *Assessment of the Antibiotic Resistance Effects of Biocides*. [online] Available from: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_021.pdf [Accessed 2010-05-20].

SVARM (2010). *Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM) 2009*. Uppsala, Sweden: The National Veterinary Institute (SVA). ISSN 1650-6332.

Weber, D.J. & Rutala, W.A. (2006). Use of germicides in the home and the healthcare setting: is there a relationship between germicide use and antibiotic resistance? *Infect Control Hosp Epidemiol* 27(10), 1107-19.

Wendt, C., Wiesenthal, B., Dietz, E. & Ruden, H. (1998). Survival of vancomycin-resistant and vancomycin-susceptible enterococci on dry surfaces. *J Clin Microbiol* 36(12), 3734-6.

Werner, G., Coque, T.M., Hammerum, A.M., Hope, R., Hryniewicz, W., Johnson, A., Klare, I., Kristinsson, K.G., Leclercq, R., Lester, C.H., Lillie, M., Novais, C., Olsson-Liljequist, B., Peixe, L.V., Sadowy, E., Simonsen, G.S., Top, J., Vuopio-Varkila, J., Willems, R.J., Witte, W. & Woodford, N. (2008). Emergence and spread of vancomycin resistance among enterococci in Europe. *Euro Surveill* 13(47).

Wierup, M., Lowenhielm, C., Wold-Troell, M. & Agenas, I. (1987). Animal consumption of antibiotics and chemotherapeutic drugs in Sweden during 1980, 1982 and 1984. *Vet Res Commun* 11(5), 397-405.