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

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Received 14 November 2008; returned 12 December 2008; revised 29 January 2009; accepted 30 January 2009

**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 Tn1546 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 Tn1546 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. An increasing proportion of these infections are caused by vancomycin-resistant enterococci (VRE) first isolated in 1986.

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. 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. After the withdrawal of avoparcin, the prevalence of VRE in farm animals in Europe rapidly declined.

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.

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. 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.

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. 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,
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 Steinheim, Germany) and incubated at 37

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-Esculin agar (Oxoid) and incubated at 37°C for 24 h. Presumptive enterococci were identified to species level according to Devriese et al. Isolates were stored at −70°C for further investigations.

Investigation of resistance genotype

In a subset of isolates, the gene encoding vancomycin resistance was determined using PCR for the vanA gene. 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., 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 Tn1546 transposon was typed using an overlapping PCR method modified from Woodford et al. 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 Tn1546 transposon typing

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

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. 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-coccii panels (SVA, Upsala, 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.
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 $\chi^2$ test using Stata software (release 10, StatCorp, 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 ($\chi^2$ test, $P \leq 0.001$).

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 Tn1546 transposons (Figure 2).

Discussion

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

![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.](image)

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

<table>
<thead>
<tr>
<th>No. of isolates (%)</th>
<th>VAN</th>
<th>NAR</th>
<th>ERY</th>
<th>BAC</th>
<th>TET</th>
<th>AMP</th>
<th>VIR</th>
<th>STR</th>
<th>GEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>344 (89.6)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>14 (3.6)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>11 (2.8)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>9 (2.3)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>2 (0.5)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1 (0.3)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>1 (0.3)</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
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<td>S</td>
</tr>
<tr>
<td>1 (0.3)</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

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).
revealed that this is due to the spread of one major clone of *E. faecium* carrying the same Tn1546 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*. 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. 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. 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. 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. 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. 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. 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. 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. 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. 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.

Acknowledgements

Part of the data discussed in this paper has been published in the yearly reports from the SVARM programme and as a poster (P2124) at the Eighteenth European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 2008.

Funding

Surveillance data presented in this paper are a result of the routine work within the SVARM Programme at the National Veterinary Institute, Uppsala, Sweden. General research funding for O. N.’s PhD project, of which this paper is a part, has been received from The Swedish Farmers’ Foundation for Agricultural Research. The work with MLST was supported by a Short Term Mission from Med-Vet-Net.

Transparency declarations

None to declare.
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