APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2005, p. 5383–5390 Vol. 71, No. 9
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.9.5383–5390.2005

Occurrence and Relatedness of Vancomycin-Resistant Enterococci in Animals, Humans, and the Environment in Different European Regions

Inger Kühn,[1]* Aina Iversen,[1] Maria Finn,[2] Christina Greko,[2] Lars G. Burman,[3] Anicet R. Blanch,[4] Xavier Vilanova,[4] Albert Manero,[4] Huw Taylor,[5] Jonathan Caplin,[5] Lucas Domínguez,[6] Inmaculada A. Herrero,[6] Miguel A. Moreno,[6] and Roland Möllby[1]

Microbiology and Tumor Biology Center, Karolinska Institutet, SE-171 77 Stockholm, Sweden[1]; National Veterinary Institute (SVA), Department of Antibiotics, SE-750 07 Uppsala, Sweden[2]; Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden[3]; Department of Microbiology, University of Barcelona, Diagonal 645, 08028 Barcelona, Spain[4]; Environment & Public Health Research Unit, School of the Environment, University of Brighton, Lewes Road, Brighton BN2 4GJ, United Kingdom[5]; and Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain[6]

Received 19 October 2004/Accepted 23 March 2005

Vancomycin-resistant enterococcci (VRE) in Europe are thought to have emerged partly due to the use of the glycopeptide avoparcin in animal husbandry. We compared the occurrence of VRE in geographical regions of Europe in which until 1997 large amounts of avoparcin were used (Spain, United Kingdom, and Denmark) with the occurrence of VRE in Sweden, where avoparcin was banned in 1986. We also studied the relatedness between VRE strains from different regions and habitats. In total, 2,580 samples were collected from humans, animals, and the environment (soil, sewage, recipient water). VRE resistant to 20 μg/ml vancomycin were identified in 8.2% of the samples and were found most frequently in raw and treated urban sewage samples (means, 71% and 36% of the samples, respectively), pig manure (17%), and hospital sewage (16%). The proportions of VRE-positive sewage samples were similar in Sweden, Spain, and the United Kingdom, whereas pig feces and manure were more often positive in Spain than in Sweden (30% versus 1%). Most VRE were *Enterococcus faecium* carrying *vanA*, and computerized biochemical phenotyping of the isolates of different ecological origins showed a high degree of polyclonality. In conclusion, it seems that animal-associated VRE probably reflect the former use of avoparcin in animal production, whereas VRE in human-associated samples may be a result of antibiotic use in hospitals. Since there seems to be a reservoir of the resistance genes in all countries studied, precautions must be taken to limit the use of antibiotics and antibiotic-like feed additives.

Enterococci are members of the normal intestinal microflora in humans and animals, and they are common in environments affected by animal and human fecal material. These organisms are not considered primary pathogens, but because of their ability to acquire high-level resistance to antimicrobial agents enterococci have emerged as nosocomial pathogens worldwide (30). Concern has especially been focused on enterococci that show high-level resistance to the glycopeptide antibiotic vancomycin (vancomycin-resistant enterococci [VRE]), which until recently has been the drug of last resort against multiresistant enterococci and against methicillin-resistant *Staphylococcus aureus*.

In the United States, the incidence of bloodstream infection due to VRE increased from 0.4% in 1989 to 25.2% in 1999 (35), and between 15 and 24% of the hospitalized patients were reported to be colonized with VRE in 1998 (33). A later report indicated that 15% of all clinically relevant isolates of gram-positive cocci were VRE (17), whereas VRE of the VanA and VanB types have not been reported in animals so far. Thus, in the United States VRE are thought to have emerged and spread entirely as a result of the antibiotic use in hospitals. In Europe, infections caused by VRE remain a moderate problem in most countries. A European surveillance study found VRE in 7% of enterococcal isolates from blood cultures (13). On the other hand, healthy carriers of VRE in the community are relatively common in certain areas. In The Netherlands, 5 to 10% of healthy people were colonized with VRE (12, 41), and a study in a cattle-rearing region in France revealed that as many as 12% of nonhospitalized citizens and 37% of hospitalized patients were carriers of VRE (14). The relatively large community reservoir of VRE in Europe has been linked to the use of avoparcin in livestock. This compound is a glycopeptide that is closely related to vancomycin and has been used as a growth promoter in livestock in Europe for many years. In Denmark, for example, about 24,000 kg of avoparcin was used for growth promotion in animals in 1994, while only 24 kg of vancomycin was used to treat humans (45). The use of avoparcin is associated with a high prevalence of VRE both in feces of exposed animals and in meat products (2, 3, 46). A link between avoparcin use and the community reservoir of VRE in Europe is further supported by the presence of genotypically indistinguishable *vanA* gene clusters in isolates of VRE from human and nonhuman sources (21, 36, 37) and by a decreased VRE colonization rate in healthy Europeans after avoparcin was banned in 1997 (22, 42).

* Corresponding author. Mailing address: Microbiology and Tumor Biology Center, Karolinska Institutet, Box 280, SE-171 77 Stockholm, Sweden. Phone: 46 8 5248 71 55. Fax: 46 8 5248 79 08. E-mail: inger.kuhn@mtc.ki.se.

TABLE 1. Samples studied and proportions with VRE20 (enterococci growing in the presence of 20 μg/ml vancomycin)

Sample source	No. of samples (% with VRE20)				
	Sweden	Denmark	Spain	United Kingdom	All countries
Human origin					
Urban sewage (raw)	35 (57)		49 (90)	21 (52)	105 (71)
Urban sewage (treated)	32 (19)		50 (54)	27 (22)	109 (36)
Hospital sewage	14 (36)		29 (17)	26 (4)	69 (16)
Healthy humans (fecal)	24 (0)			39 (0)	63 (0)
Hospitalized patients (fecal)	18 (0)			0	18 (0)
Clinical isolates	97 (0)		55 (2)	6 (0)	158 (1)
Animal origin					
Pig (fecal)[a]	64 (0)		68 (26)		132 (14)
Pig (manure)[a,b]	54 (2)		47 (34)		101 (17)
Farmland with pig manure[a,b]	70 (0)		46 (0)		116 (0)
Crop from farmland with manure[a]	4 (0)		13 (0)		17 (0)
Pig (cecal)	306 (0.3)	134 (5)	242 (8)		682 (4)
Broiler chicken (cecal)	150 (3)	137 (2)	100 (0)		387 (2)
Cattle (cecal)	194 (0)	134 (1)			328 (1)
Mixed or unknown origin					
Farmland or crop with no manure[a]	24 (0)		66 (3)		90 (2)
Pig feed[a]	21 (0)		35 (6)		56 (4)
Surface water	37 (3)		75 (12)	37 (0)	149 (7)
All samples					2,580 (8.2)

[a] Longitudinal data from one farm per country.
[b] Data from two sampling occasions at 12 Swedish and 11 Spanish farms.

If the assumptions that usage of avoparcin has selected for VRE and that the VRE strains or their resistance genes have spread to humans via the food chain were true, VRE would be found less frequently in Sweden than in other European countries. In Sweden, the use of antibiotics as feed additives was prohibited in 1986, whereas in the rest of the European Union avoparcin was not banned until in January 1997 (Commission Directive 97/6 EC) and most other antibiotics were banned in December 1998 (Commission regulations 2788/98 and 2821/98). Indeed, an investigation in Sweden revealed a low prevalence of VRE in feces both from hospitalized patients in 1998 (9 of 841 patients studied, all from the same hospital, carried VanB strains with similar pulsed-field gel electrophoresis patterns) and from healthy individuals (of 670 individuals studied, 1 person who had recently returned from Africa carried a VanA VRE) (39). The number of patients colonized with VRE has also remained low; only 20 to 40 cases per year were reported in 2002 and 2003, and the first five cases of VRE among Swedish bloodstream infections were reported in 2003 (7a).

Within the framework of a European study, enterococcal populations in samples obtained from various human, animal, and environmental sources in four European countries (Sweden, United Kingdom, Denmark, and Spain) have been studied (5, 26). One objective of the present study was to compare the occurrence of VRE in geographical regions of Europe in which large amounts of avoparcin and other growth promoters were used previously with the occurrence of VRE in a region in which avoparcin was not used (Sweden). A second objective was to study possible epidemiological relationships between VRE isolates obtained from different parts of the food chain in these regions.

MATERIALS AND METHODS

Samples. Sampling sites were selected to be representative of enterococcal populations in various links of the food chain (Table 1), and samples were collected from April 1998 to December 2000 (5, 26). Human specimens were obtained from healthy and hospitalized individuals (feces) in Sweden and from patients with enterococcal infections in Sweden, Spain, and the United Kingdom. Hospital sewage, urban sewage, and surface water receiving treated sewage were sampled at two to eight sites in Sweden, Spain, and the United Kingdom. Cecal contents of randomly selected healthy animals (pigs, cattle, and broilers) were obtained from slaughterhouses in Sweden, Denmark, and Spain. Pig manure and samples of farmland with pig manure were collected twice from 12 farms in Sweden and from 11 farms in Spain. In addition, a longitudinal study of one pig farm in Sweden and one pig farm in Spain was performed. In this study, samples were collected from pig feed, pig feces, manure, farmland, and crops with and without manure eight times during 2 years in order to identify any flow of VRE between different links of the food chain.

Isolation of enterococci. The sampling and isolation techniques used have been described previously (26). Briefly, environmental samples with presumed high counts of enterococci were serially 10-fold diluted in phosphate-buffered saline (PBS) and then membrane filtered, whereas samples with presumed low counts of enterococci were filtered undiluted. Filters were then transferred to m-*Enterococcus* agar (MEA) (Becton Dickinson, Sparks, Md.) containing vancomycin (8 μg/ml). Fecal and cecal samples were diluted with PBS, and 10- to 100-μl portions of the solutions were spread on MEA with vancomycin (8 μg/ml).

Enrichment for VRE in broth (final vancomycin concentration, 8 μg/ml) was also performed with all samples, undiluted liquid samples, and PBS suspensions of solid samples in order to detect low numbers of VRE, as described previously (19). After incubation for 24 h at 37°C, 10 μl of each enrichment culture was spread on MEA with vancomycin (8 μg/ml).

All MEA plates with vancomycin were incubated for 48 h at 37°C. *Enterococcus*-like colonies were typed using the PhP-RF system (see below), and one isolate representing each PhP-RF type in the sample was subcultured on bile esculin agar plates (Becton Dickinson) for 24 h at 44°C. Esculin-positive and catalase-negative colonies were defined as VRE8 and saved in glycerol broth at −70°C. These isolates were also subcultured on MEA supplemented with 20 μg vancomycin per ml, and isolates showing growth were defined as VRE20. VRE8 and VRE20 are collectively referred to as VRE below.

Typing of enterococci using the PhenePlate system. A rapid semiautomated and computerized typing method for enterococci, based on measurements of the

TABLE 2. Distribution of the major species *E. faecalis, E. faecium,* and *E. hirae* among VRE8 (enterococci able to grow in the presence of 8 μg/ml vancomycin) and among normal enterococci (isolates from media without any antibiotic)[a]

Enterococci	Origin		No. of isolates	%			
	Country	Animal		*E. faecalis*	*E. faecium*	*E. hirae*	Other
VRE8	Sweden	Animal	33	12	38	8	42
	Sweden	Human	99	22	57	0	21
	Spain	Animal	99	4	64	2	30
	Spain	Human	282	5	80	1	13
	United Kingdom	Human	86	8	84	0	8
	Total		599	8	71	2	20
Normal		Animal	8,483	22	27	34	17
		Human	6,100	38	34	14	14

[a] See references 26 and 32.

kinetics of 11 biochemical reactions (PhP-RF plates) (27), one variant of the PhenePlate typing system (PhPlate Stockholm AB; www.phplate.se), was used for typing at least one pure colony and one streak (pooled colonies) from MEA supplemented with 8 μg/ml of vancomycin. If the pure and pooled colonies yielded different results, this was regarded as an indication of the presence of several types of VRE, and then several isolates were selected from the same sample for additional typing. One isolate representing each PhP-RF type in the sample was tested to confirm that it was an enterococcus and was saved for further analysis (see above). Isolates with vancomycin MICs of ≥32 μg/ml were further phenotyped using a PhP-FS plate, which is a more discriminatory phenotyping system in which 24 different reactions are used (19, 25).

Species verification, determination of vancomycin MIC, and screening for vancomycin resistance genotype. Species were verified by comparing the PhP typing data with a reference database containing the PhP patterns of 178 isolates representing 17 species (31). The vancomycin MICs of VRE8 isolates were determined by broth microdilution using Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom) and commercially available microdilution panels (VetMIC; National Veterinary Institute, Uppsala, Sweden). The tests were performed according to the recommendations of the National Committee for Clinical Laboratory Standards (approved standard M 7-A6) (34). *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* BM 4147 (high-level vancomycin resistance, VanA type), and *Enterococcus gallinarum* UB-CET970 (low-level vancomycin resistance, VanC type) were used as controls. For a subset of isolates with MICs of ≥32 μg/ml, further confirmation of the species *E. faecalis* and *E. faecium* and identification of the resistance genes *vanA* and *vanB* were performed by PCR (10, 11).

RESULTS

Occurrence of vancomycin-resistant enterococci. Of 2,580 samples analyzed, 10.9% contained VRE8, and 8.2% contained VRE20 (Table 1). For about one-third of the VRE20-positive samples, enterococci could be isolated directly from MEA plates without enrichment (data not shown), indicating that VRE made up quite a large part of the enterococcal populations in such samples. In the other two-thirds of the samples, VRE20 were found only after enrichment in vancomycin broth.

Samples from urban sewage, hospital sewage, and pig manure can be considered pooled fecal samples from a large number of humans in the community, from a large number of humans in hospitals, and from many pigs, respectively. VRE20 were most common in raw urban sewage in all three countries studied (52 to 90% of the samples), followed by treated urban sewage (19 to 54%) (Table 1). VRE20 were also relatively common in samples from hospital sewage in Sweden (36%), mainly because one large hospital was positive on almost all sampling occasions (19). VRE20 were more prevalent in samples related to pig farms (manure and feces) in Spain (mean, 30%; range, 26 to 34%) than in Sweden (1 of 118 samples [0.8%]). After data from the longitudinal study in Spain were excluded, VRE20 were found in 35% of the samples from pig manure from 6 of the 11 pig farms studied (data not shown). Some samples from slaughtered pigs in Denmark and Spain also yielded VRE20 (5 to 8%), whereas only 1 of 306 samples from slaughtered pigs in Sweden was positive (0.3%) (Table 1). VRE20 were found only in a few samples from slaughtered chickens (0 to 3%) and cattle (0 to 1%) in Sweden, Denmark, and Spain (Table 1). Despite the many VRE20-positive samples from pig manure in Spain, none of the corresponding samples collected from crops and farmland fertilized with pig manure was positive (Table 1). Interestingly, VRE20 grew from Spanish samples from farmland or crops without manure, from pig feed, and from surface water (3 to 12%) and from one of the surface water samples from Sweden (Table 1).

Distribution of species and types among VRE8. In all countries *E. faecium* was more common among the VRE8 isolates (mean, 71%; range, 38 to 84%) than among enterococci isolated from the same samples on media without antibiotics (mean, 30%) (Table 2). The reverse was true for *E. faecalis* and *E. hirae*. The dominance of *E. faecium* among VRE8 was most pronounced among isolates of human origin. VRE8 belonging to *E. faecalis* were most often found in urban sewage in Sweden (22%). A minor proportion of isolates belonged to unknown species. Such VRE8 isolates were usually not VRE20 and were excluded from further analysis.

All 599 VRE8 isolates were typed using the PhP-RF screening system. Their diversity, as measured by Simpson's diversity index (D_i) (18), was 0.94, and it was almost as high as that for "normal" unrelated enterococci obtained in the present study (D_i, 0.95 to 0.97) (26, 32). Taken together, these data indicated that vancomycin resistance was widespread among enterococci and thus not confined to certain clones.

Resistance to vancomycin. When available, one VRE8 per sample, representing a distinct PhP-RF type (see below), was subjected to determination of the vancomycin MIC. A total of 178 such isolates from 140 samples were available. Of these 178 isolates, 33 had MICs of 8 to 16 μg/ml, 5 had MICs of 32 to 64 μg/ml, and 140 had MICs of >128 μg/ml. Thus, 81% of the susceptibility-tested VRE8 were resistant to ≥32 μg/ml and were possibly VanA or VanB VRE.

Extended PhP typing and screening for vancomycin resistance genes in selected VRE. A total of 127 isolates that were

confirmed to be enterococci, which had vancomycin MICs of ≥32 μg/ml and represented separate PhP-RF types (only one isolate per PhP-RF type and sample was included), were phenotyped further using the more discriminatory PhP-FS system (Fig. 1). Of these 127 isolates, 111 belonged to the *E. faecium* group (including *E. hirae*) and 16 were *E. faecalis*. Notably, 12 *E. faecalis* isolates were from Sweden (all but one from urban sewage), whereas the other four were from the United Kingdom (three isolates of human origin) and Spain (one isolate from a broiler); all of these organisms were *vanA* positive (data not shown). The D_i for these VRE was 0.99 as determined by PhP-FS, which indicated that most VRE belonged to unique PhP types, further supporting the notion that they were members of different clonal lineages. A total of 16 common types, each comprising between 2 and 11 isolates, that could indicate a common origin or clonal spread of certain VRE strains were found (Fig. 1). Most of these types were obtained from only animals or humans, but two types included isolates from both animals and humans (Fig. 1).

The PhP-FS data for animal-related ($n = 36$) and human-related ($n = 72$) vancomycin-resistant *E. faecium* and *E. hirae* were also subjected to separate cluster analyses (Fig. 2 and 3). The diversity was lower among the animal isolates (D_i, 0.92) than among the human isolates (D_i, 0.99). All animal isolates possessed the *vanA* gene, and one major cluster of 11 *E. faecium* strains was found among the animal isolates, mainly from pigs in Denmark and Spain (Fig. 2). Among the human isolates, eight small clusters of two to five identical isolates were identified, and the isolates were usually from the same country (Fig. 3). One cluster of 10 *E. faecium* isolates that had identical or similar PhP fingerprints was identified among Swedish and United Kingdom isolates of human origin (Fig. 3). Their PhP-FS fingerprints were similar to those of clone FMSE1, which is a nationwide *E. faecium* clone resistant to ampicillin and ciprofloxacin found in Sweden in 1997 (40). Of the 67 isolates of human origin that were screened for vancomycin resistance genes, 21% were *vanB* positive and 79% contained *vanA*. All 14 isolates with the *vanB* genotype were from Sweden or the United Kingdom, and notably, eight of these isolates were hospital associated (clinical isolates or isolates from hospital sewage) (Fig. 3).

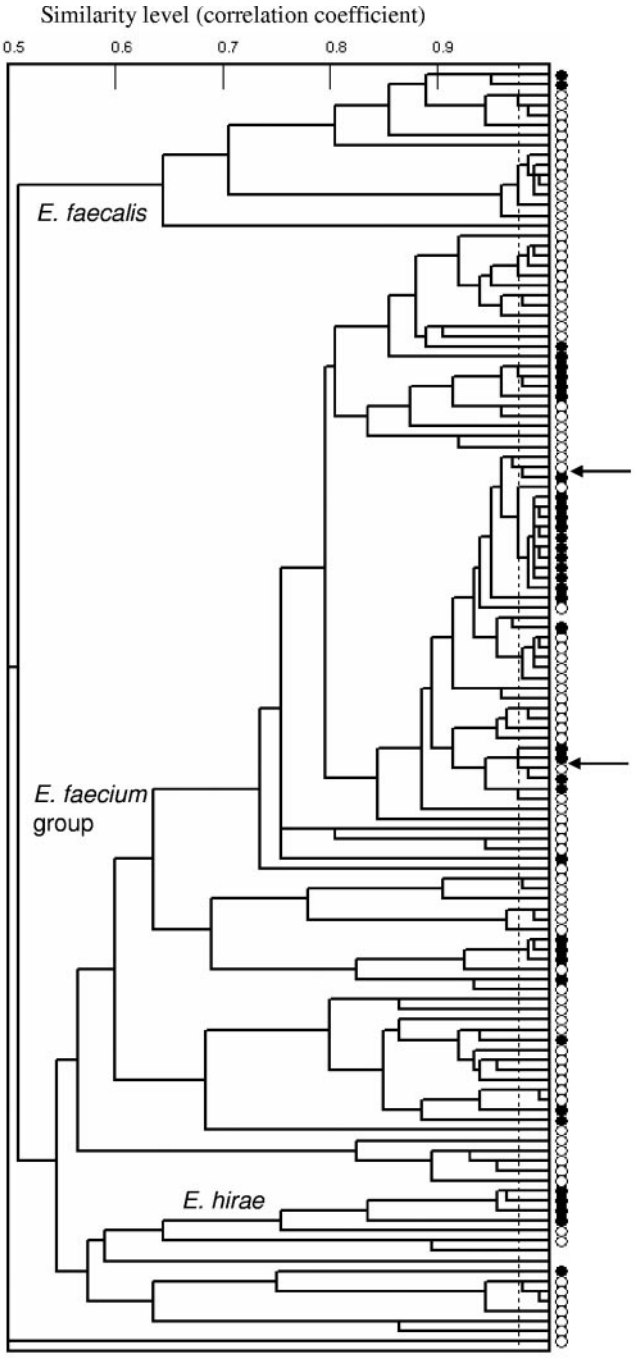
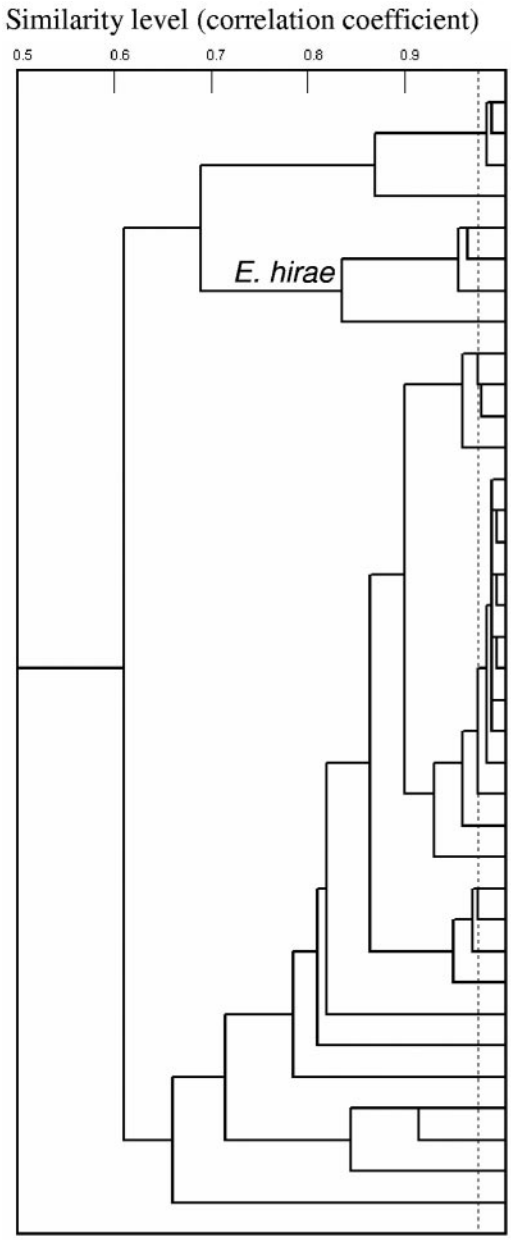


FIG. 1. Dendrogram showing the results of a cluster analysis (unweighted-pair group method using average linkages) of PhP-FS typing data for 127 isolates confirmed to be enterococci, having vancomycin MICs of ≥32 μg/ml, and representing diverse clusters (only one isolate per PhP-RF type and sample was included). The dotted line indicates the identity level that was determined by the reproducibility of the typing method (25). Isolates that show similarity to each other higher than this level were assigned to the same PhP type. Solid circles indicate isolates of animal origin, open circles indicate isolates of human origin, and no mark indicates isolates having other or mixed origins. The arrows indicate the only two PhP types that comprise isolates of both human origin and animal origin.

DISCUSSION

We studied the occurrence of VRE in 2,580 samples collected in different geographical regions in Europe, both from different links of the food chain and from humans, after the ban of avoparcin use in animal feed. A major question was whether VRE were more common in countries where large amounts of avoparcin were recently used (Denmark, Spain, and United Kingdom) than in Sweden, where avoparcin has not been used since 1986. Indeed, VRE20 were isolated more often from Spanish pig-related samples (manure and feces [30%] and ceca [8%]) than from Swedish samples (1% and 0.3%). In contrast, high proportions of VRE-positive urban sewage samples were found in Sweden, Spain, and the United Kingdom (52 to 90%), apparently reflecting a human source rather than an animal source and despite the fact that the consumption of antibiotics in hospitals in Sweden (population, 9 million) is among the lowest in Europe (8, 15), which applies

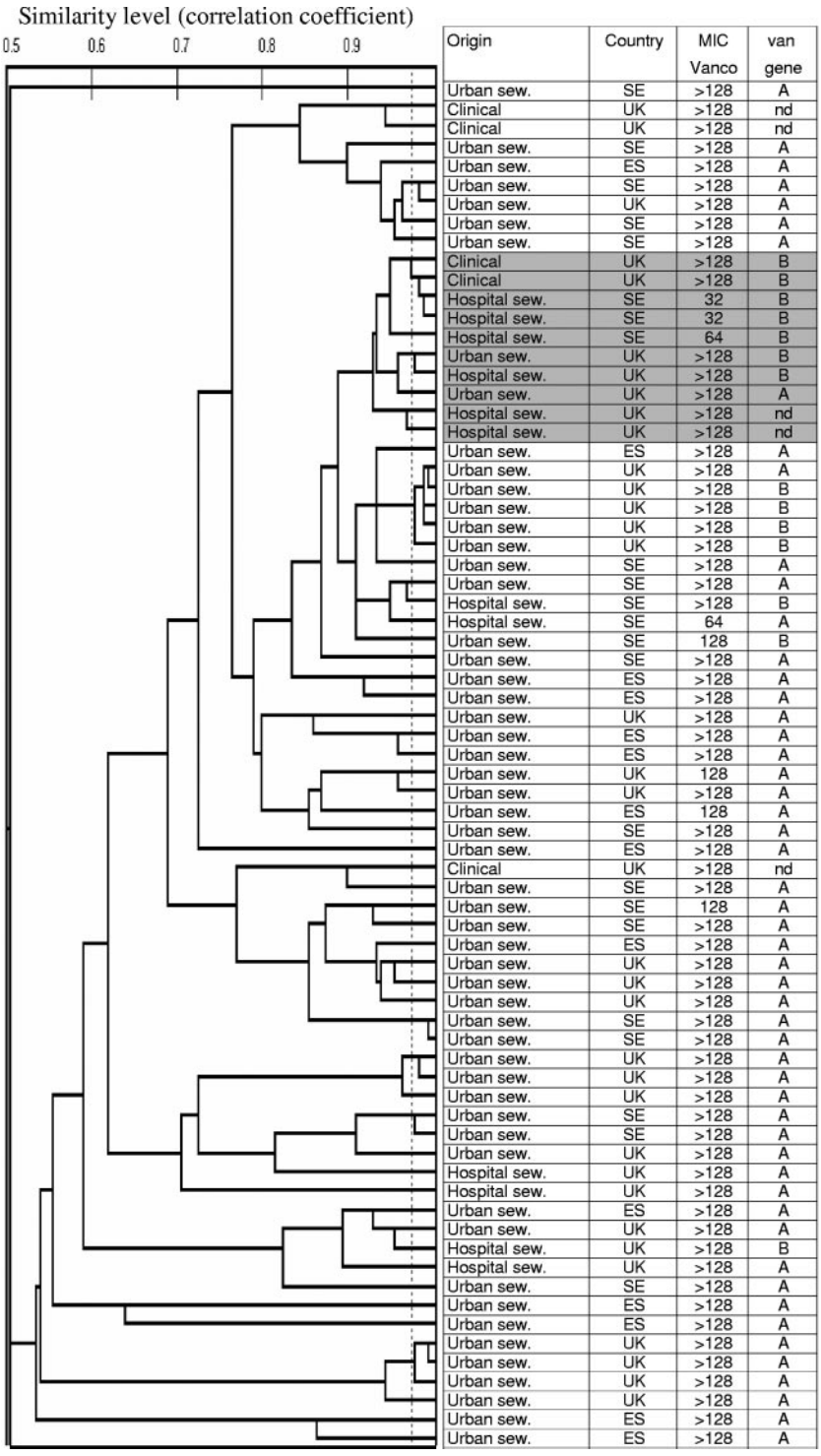


Origin		MIC	Van
Animal	Country	Vanco	gene
SB	SE	128	A
SB	SE	>128	A
SB	SE	>128	A
SB	SE	>128	A
SB	DK	128	A
SB	DK	>128	A
SB	DK	128	A
SP	ES	>128	A
SP	SE	>128	A
SP	ES	>128	A
SP	ES	>128	A
SC	DK	>128	A
SC	DK	>128	A
SP	ES	>128	A
SP	ES	>128	A
SP	DK	>128	A
SP	ES	>128	A
SP	DK	>128	A
SP	DK	>128	A
SP	ES	>128	A
SP	ES	>128	A
SP	DK	>128	A
SP	ES	>128	A
SP	DK	>128	A
SP	ES	>128	A
PF	ES	>128	A
PM	ES	>128	A
SP	ES	>128	A
PM	ES	>128	A
SP	DK	>128	A
SP	ES	>128	A
SP	ES	>128	A
SP	DK	128	A
SP	DK	>128	A
SP	ES	>128	A
PF	ES	>128	A

FIG. 2. Dendrogram showing the results of a cluster analysis (unweighted-pair group method using average linkages) of PhP-FS typing data for 32 *E. faecium* isolates and four *E. hirae* isolates of animal origin having vancomycin MICs of ≥32 μg/ml. The isolates included isolates from broilers from slaughterhouses (SB), from cattle from slaughterhouses (SC), from pigs from slaughterhouses (SP), from pig feces (PF), and from pig manure (PM). SE, Sweden; DK, Denmark; ES, Spain. Shading indicates a cluster of identical *E. faecium* isolates having different origins.

particularly to vancomycin as its only oral use (against *Clostridium difficile* diarrhea) is strongly discouraged in order to avoid selection of VRE. Thus, vancomycin usage in Swedish hospitals is about one-half that in the United Kingdom, which in turn uses 5- to 10-fold less than the United States. Any difference between the three countries with regard to the use of vancomycin and other antimicrobials in human medicine was probably neutralized by other factors influencing the occurrence of VRE, such as import via foreign travel (10 to 20% of the Swedish population per year) and meat products and further transmission within each country. All animal isolates tested carried the *vanA* gene, whereas 42% of the hospital-related isolates and 9% of other human-related isolates were the VanB type. Similarly, other workers found the VanB type in occasional clinical VRE isolates (11%) and fecal VRE from hospitalized patients (6%) in Europe (16), whereas the VanB type was rare among animal isolates (9, 29).

Other workers have also reported the presence of VRE in sewage (44) and treated sewage (43). In another recent study we found enterococci belonging to an ampicillin- and ciprofloxacin-resistant clonal group previously found to colonize hospitalized patients throughout Sweden (40) not only in hos-



Origin	Country	MIC Vanco	van gene
Urban sew.	SE	>128	A
Clinical	UK	>128	nd
Clinical	UK	>128	nd
Urban sew.	SE	>128	A
Urban sew.	ES	>128	A
Urban sew.	SE	>128	A
Urban sew.	UK	>128	A
Urban sew.	SE	>128	A
Urban sew.	SE	>128	A
Clinical	UK	>128	B
Clinical	UK	>128	B
Hospital sew.	SE	32	B
Hospital sew.	SE	32	B
Hospital sew.	SE	64	B
Urban sew.	UK	>128	B
Hospital sew.	UK	>128	B
Urban sew.	UK	>128	A
Hospital sew.	UK	>128	nd
Hospital sew.	UK	>128	nd
Urban sew.	ES	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	B
Urban sew.	UK	>128	B
Urban sew.	UK	>128	B
Urban sew.	UK	>128	B
Urban sew.	SE	>128	A
Urban sew.	SE	>128	A
Hospital sew.	SE	>128	B
Hospital sew.	SE	64	A
Urban sew.	SE	128	B
Urban sew.	SE	>128	A
Urban sew.	ES	>128	A
Urban sew.	ES	>128	A
Urban sew.	UK	>128	A
Urban sew.	ES	>128	A
Urban sew.	ES	>128	A
Urban sew.	UK	128	A
Urban sew.	UK	>128	A
Urban sew.	ES	128	A
Urban sew.	SE	>128	A
Urban sew.	ES	>128	A
Clinical	UK	>128	nd
Urban sew.	SE	>128	A
Urban sew.	SE	128	A
Urban sew.	SE	>128	A
Urban sew.	ES	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	SE	>128	A
Urban sew.	SE	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	SE	>128	A
Urban sew.	SE	>128	A
Urban sew.	UK	>128	A
Hospital sew.	UK	>128	A
Hospital sew.	UK	>128	A
Urban sew.	ES	>128	A
Urban sew.	UK	>128	A
Hospital sew.	UK	>128	B
Hospital sew.	UK	>128	A
Urban sew.	SE	>128	A
Urban sew.	ES	>128	A
Urban sew.	ES	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	UK	>128	A
Urban sew.	ES	>128	A
Urban sew.	ES	>128	A

FIG. 3. Dendrogram showing the results of a cluster analysis (unweighted-pair group method using average linkages) of PhP-FS typing data for 72 *E. faecium* isolates of presumed human origin (urban and hospital sewage isolates, clinical isolates) having vancomycin MICs of ≥32 μg/ml. Shading indicates isolates showing high similarity to the ampicillin- and ciprofloxacin-resistant clone FMSE1 previously found nationwide in Sweden. SE, Sweden; ES, Spain; UK, United Kingdom; sew., sewage.

pital sewage but also in raw and treated urban sewage (20). Thus, it is possible that antibiotic-resistant enterococci present in the human population are enriched in hospitals, released from there into municipal sewers to become established, and then further released via sewage treatment plants into recipient water and transferred back into humans. Furthermore, as most VRE8 isolates from sewage were also resistant to macrolides, tetracyclines, and other antibiotics (unpublished data),

selection of such strains by a wide range of agents in hospitalized and other humans receiving antibiotic treatment and the release of these strains into sewage are likely. Coselection of resistance to vancomycin and macrolides has been demonstrated for *E. faecium* in pigs (1) and in poultry (6, 7) and has been suggested to contribute to the persistence of VRE in Danish pigs after the avoparcin ban.

It has been suggested that VRE can reach humans through the food chain by contaminated meat products (23). Therefore, a second aim of this study was to investigate common sources and transmission routes of VRE strains between animals, food of animal origin, humans, sewage, and possibly countries, including the epidemiology of *vanA* and *vanB*. A related mechanism of spread of VRE could be the use of manure containing VRE as a fertilizer of crops to be consumed by humans. In the present study 34% of the pig manure samples in Spain contained VRE. The enterococcal counts were generally 10^4 to 10^5 CFU per gram, but in farmland that had received the manure and in crops growing on such farmland a 3- to 5-log reduction had occurred (26) and VRE were not isolated from any sample. It thus seems that low levels of VRE in manure represent a negligible risk for further spread to crops and humans.

Most isolates from the countries studied belonged to unique PhP types, which is a strong indication that they represented different clones (27). This supported the hypothesis that vancomycin resistance has emerged more by horizontal spread of the *vanA* gene cluster, and to some extent the *vanB* cluster, among a wide range of enterococci of various origins than by transmission of a few major clones (4, 21, 28). In this study about 16 mostly small clusters, indicating a common reservoir or clonal spread of some VRE strains, were found in animals of the same species (pigs in Denmark and Spain) and in human-related samples. Only in two cases did a human isolate and an animal isolate have the same PhP type, and in these cases there was no known epidemiological relationship. This finding is in line with reports showing that VRE from animals and humans for the most part belong to separate genotypes (7, 47). Thus, our study supported the concept that clonal spread of VRE strains between animals and humans is rare in the absence of close contact, like that between animals and their farmers (7, 37). Instead, as proposed also by other workers, a possible connection between different animal VRE reservoirs and human enterococci is horizontal spread of the *vanA* gene cluster between distinct enterococcal strains (24, 48).

An interesting finding was that 10 vancomycin-resistant *E. faecium* isolates from Sweden and the United Kingdom, mainly of hospital origin and of the VanB type, had PhP types that were identical or similar to those of the multiresistant clonal group FMSE1 mentioned above, which was previously found in Sweden (Fig. 3). It seems that this hospital-associated cluster, which was consistently vancomycin susceptible in 1997 (39), is international and has acquired *vanA* and *vanB*. Further evidence for this theory is that the FMSE1-like VRE generally were resistant to ampicillin (MIC, $\geq$32 μg/ml) in the present study.

In conclusion, it seems that VRE among animals and humans in Europe have evolved in largely two different ways. VRE associated with animal production were rare in Sweden but were common in the other countries studied, particularly in samples from pig production, and probably remain as a memory of times when avoparcin and other antimicrobial drugs were used as growth promoters. Human-associated VRE were common (in sewage) and belonged mostly to nonanimal strains, having acquired either *vanA* or *vanB* resistance genes. This was also true for Sweden, despite the absence of a large animal reservoir, and might reflect "silent" carriage in the human population, possibly due to import of VRE via animal products and tourism, as well as selection for enterococci carrying *vanA* and *vanB* by antibiotic use in human medicine. No exchange of VRE strains between humans and animals could be demonstrated. Instead, spread of the v*anA* gene cluster from animal VRE to human enterococcal strains may contribute to the current prevalence of VRE in clinics. Considering the many reservoirs of VRE in all of the countries studied, the use of antibiotics in both the human and animal populations must be minimized in order to limit continuing expansion of VRE.

ACKNOWLEDGMENTS

This work was supported by grants FAIR5-CT97-3709 and EVK1-CT-2000-00080 from the European Commission and by grants 2001-3847, 2001-913, 2002-1775, and 2003-1343 from The Swedish Research Council for Environment, Agricultural Sciences, and Spatial Planning (Formas).

REFERENCES

1. **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:**2774–2777.
2. **Aarestrup, F. M.** 1995. Occurrence of glycopeptide resistance among *Enterococcus faecium* isolates from conventional and ecological poultry farms. Microb. Drug Resist. **1:**255–257.
3. **Bager, F., M. Madsen, J. Christensen, and F. M. Aarestrup.** 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:**95–112.
4. **Bingen, E. H., E. Denamur, N. Y. Lambert-Zechovsky, and J. Elion.** 1991. Evidence for the genetic unrelatedness of nosocomial vancomycin-resistant *Enterococcus faecium* strains in a pediatric hospital. J. Clin. Microbiol. **29:** 1888–1892.
5. **Blanch, A. R., J. L. Caplin, A. Iversen, I. Kuhn, A. Manero, H. D. Taylor, and X. Vilanova.** 2003. Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. J. Appl. Microbiol. **94:**994–1002.
6. **Borgen, K., M. Sorum, Y. Wasteson, H. Kruse, and H. Oppegaard.** 2002. Genetic linkage between *erm*(*B*) and *vanA* in *Enterococcus hirae* of poultry origin. Microb. Drug Resist. **8:**363–368.
7. **Borgen, K., Y. Wasteson, H. Kruse, and R. J. Willems.** 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:**3133–3137.

7a. **Cars, O., and K. Ekdahl (ed.).** 2003. SWEDRES 2002: a report on Swedish antibiotic utilisation and resistance in medicine. The Swedish Strategic Programme for the Rational Use of Antimicrobial Agents and the Swedish Institute for Infectious Disease Control, Solna, Sweden.

8. **Cars, O., S. Molstad, and A. Melander.** 2001. Variation in antibiotic use in the European Union. Lancet **357:**1851–1853.
9. **Del Grosso, M., A. Caprioli, P. Chinzari, M. C. Fontana, G. Pezzotti, A. Manfrin, E. D. Giannatale, E. Goffredo, and A. Pantosti.** 2000. Detection and characterization of vancomycin-resistant enterococci in farm animals and raw meat products in Italy. Microb. Drug Resist. **6:**313–318.
10. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33:**24–27.
11. **Dutka-Malen, S., S. Evers, and P. Courvalin.** 1995. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J. Clin. Microbiol. **33:**1434.
12. **Endtz, H. P., N. van den Braak, A. van Belkum, J. A. Kluytmans, J. G. Koeleman, L. Spanjaard, A. Voss, A. J. Weersink, C. M. Vandenbroucke-Grauls, A. G. Buiting, A. van Duin, and H. A. Verbrugh.** 1997. Fecal carriage of vancomycin-resistant enterococci in hospitalized patients and those living in the community in The Netherlands. J. Clin. Microbiol. **35:**3026–3031.

13. **European Antimicrobial Resistance Surveillance System.** Accessed 15 April 2004. EARSS annual report 2002. [Online.] http://www.earss.rivm.nl/PAGINA/DOC/rep2002/annual-report-2002.pdf.
14. **Gambarotto, K., M. C. Ploy, P. Turlure, C. Grelaud, C. Martin, D. Bordessoule, and F. Denis.** 2000. Prevalence of vancomycin-resistant enterococci in fecal samples from hospitalized patients and nonhospitalized controls in a cattle-rearing area of France. J. Clin. Microbiol. **38:**620–624.
15. **Goosens, H., M. Ferech, R. Vander Stichele, and M. Elseviers.** 2005. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet **365:**579–587.
16. **Goossens, H., D. Jabes, R. Rossi, C. Lammens, G. Privitera, and P. Courvalin.** 2003. European survey of vancomycin-resistant enterococci in at-risk hospital wards and in vitro susceptibility testing of ramoplanin against these isolates. J. Antimicrob. Chemother. **51**(Suppl. 3)**:**iii5–iii12.
17. **Harbarth, S., W. Albrich, D. A. Goldmann, and J. Huebner.** 2001. Control of multiply resistant cocci: do international comparisons help? Lancet Infect. Dis. **1:**251–261.
18. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
19. **Iversen, A., I. Kühn, A. Franklin, and R. Möllby.** 2002. High prevalence of vancomycin-resistant enterococci in Swedish sewage. Appl. Environ. Microbiol. **68:**2838–2842.
20. **Iversen, A., I. Kühn, M. Rahman, A. Franklin, L. G. Burman, B. Olsson-Liljequist, E. Torell, and R. Möllby.** 2004. Evidence for transmission between humans and the environment of a nosocomial strain of *Enterococcus faecium*. Environ. Microbiol. **6:**55–59.
21. **Jensen, L. B., P. Ahrens, L. Dons, R. N. Jones, A. M. Hammerum, and F. M. Aarestrup.** 1998. Molecular analysis of Tn*1546* in *Enterococcus faecium* isolated from animals and humans. J. Clin. Microbiol. **36:**437–442.
22. **Klare, I., D. Badstubner, C. Konstabel, G. Bohme, H. Claus, and W. Witte.** 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:**45–52.
23. **Klare, I., H. Heier, H. Claus, G. Bohme, S. Marin, G. Seltmann, R. Hakenbeck, V. Antanassova, and W. Witte.** 1995. *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:**265–272.
24. **Klare, I., C. Konstabel, D. Badstubner, G. Werner, and W. Witte.** 2003. Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. Int. J. Food Microbiol. **88:**269–290.
25. **Kühn, I., L. G. Burman, S. Haeggman, K. Tullus, and B. E. Murray.** 1995. Biochemical fingerprinting compared with ribotyping and pulsed-field gel electrophoresis of DNA for epidemiological typing of enterococci. J. Clin. Microbiol. **33:**2812–2817.
26. **Kühn, I., A. Iversen, L. G. Burman, B. Olsson-Liljequist, A. Franklin, M. Finn, F. Aarestrup, A. M. Seyfarth, A. R. Blanch, X. Vilanova, H. Taylor, J. Caplin, M. A. Moreno, L. Dominguez, I. A. Herrero, and R. Möllby.** 2003. Comparison of enterococcal populations in animals, humans, and the environment—a European study. Int. J. Food Microbiol. **88:**133–145.
27. **Kühn, I., A. Iversen, and R. Möllby.** 2003. The PhenePlate™ system for studies of the diversity of enterococcal populations from the food chain and the environment. Int. J. Food Microbiol. **88:**189–196.
28. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319:**157–161.
29. **Lemcke, R., and M. Bulte.** 2000. Occurrence of the vancomycin-resistant genes *vanA*, *vanB*, *vanCl*, *vanC2* and *vanC3* in *Enterococcus* strains isolated from poultry and pork. Int. J. Food Microbiol. **60:**185–194.
30. **Linden, P. K., and C. B. Miller.** 1999. Vancomycin-resistant enterococci: the clinical effect of a common nosocomial pathogen. Diagn. Microbiol. Infect. Dis. **33:**113–120.
31. **Manero, A., and A. R. Blanch.** 1999. Identification of *Enterococcus* spp. with a biochemical key. Appl. Environ Microbiol. **65:**4425–4430.
32. **Manero, A., X. Vilanova, M. Cerda-Cuellar, and A. R. Blanch.** 2002. Characterization of sewage waters by biochemical fingerprinting of enterococci. Water Res. **36:**2831–2835.
33. **Martone, W. J.** 1998. Spread of vancomycin-resistant enterococci: why did it happen in the United States? Infect. Control Hosp. Epidemiol. **19:**539–545.
34. **National Committee for Clinical Laboratory Standards.** 2000. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, PA.
35. **National Nosocomial Infections Surveillance.** 1999. System report, data summary from January 1990-May 1999, issued June 1999. Am. J. Infect. Control **27:**520–532.
36. **Simonsen, G. S., L. Smabrekke, D. L. Monnet, T. L. Sorensen, J. K. Moller, K. G. Kristinsson, A. Lagerqvist-Widh, E. Torell, A. Digranes, S. Harthug, and A. Sundsfjord.** 2003. Prevalence of resistance to ampicillin, gentamicin and vancomycin in *Enterococcus faecalis* and *Enterococcus faecium* isolates from clinical specimens and use of antimicrobials in five Nordic hospitals. J. Antimicrob. Chemother. **51:**323–331.
37. **Stobberingh, E., A. van den Bogaard, N. London, C. Driessen, J. Top, and R. Willems.** 1999. Enterococci with glycopeptide resistance in turkeys, turkey farmers, turkey slaughterers, and (sub)urban residents in the south of The Netherlands: evidence for transmission of vancomycin resistance from animals to humans? Antimicrob. Agents Chemother. **43:**2215–2221.
38. Reference deleted.
39. **Torell, E., O. Cars, B. Olsson-Liljequist, B. M. Hoffman, J. Lindback, and L. G. Burman.** 1999. Near absence of vancomycin-resistant enterococci but high carriage rates of quinolone-resistant ampicillin-resistant enterococci among hospitalized patients and nonhospitalized individuals in Sweden. J. Clin. Microbiol. **37:**3509–3513.
40. **Torell, E., I. Kühn, B. Olsson-Liljequist, S. Haeggman, B. M. Hoffman, C. Lindahl, and L. G. Burman.** 2003. Clonality among ampicillin-resistant *Enterococcus faecium* isolates in Sweden and relation to ciprofloxacin resistance. Clin. Microbiol. Infect. **9:**1011–1019.
41. **van den Bogaard, A. E., P. Mertens, N. H. London, and E. E. Stobberingh.** 1997. High prevalence of colonization with vancomycin- and pristinamycin-resistant enterococci in healthy humans and pigs in The Netherlands: is the addition of antibiotics to animal feeds to blame? J. Antimicrob. Chemother. **40:**454–456.
42. **van den Bogaard, A. E., and E. E. Stobberingh.** 2000. Epidemiology of resistance to antibiotics. Links between animals and humans. Int. J. Antimicrob. Agents **14:**327–335.
43. **Vilanova, X., A. Manero, M. Cerda-Cuellar, and A. R. Blanch.** 2004. The composition and persistence of faecal coliforms and enterococcal populations in sewage treatment plants. J. Appl. Microbiol. **96:**279–288.
44. **Volkmann, H., T. Schwartz, P. Bischoff, S. Kirchen, and U. Obst.** 2004. Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). J. Microbiol. Methods **56:**277–286.
45. **Wegener, H. C., F. M. Aarestrup, L. B. Jensen, A. M. Hammerum, and F. Bager.** 1998. The association between the use of antimicrobial growth promoters and development of resistance in pathogenic bacteria towards growth promoting and therapeutic antimicrobials. J. Anim. Feed Sci. **7:**7–14.
46. **Wegener, H. C., M. Madsen, N. Nielsen, and F. M. Aarestrup.** 1997. Isolation of vancomycin resistant *Enterococcus faecium* from food. Int. J. Food Microbiol. **35:**57–66.
47. **Willems, R. J., J. Top, N. van Den Braak, A. van Belkum, H. Endtz, D. Mevius, E. Stobberingh, A. van Den Bogaard, and J. D. van Embden.** 2000. Host specificity of vancomycin-resistant *Enterococcus faecium*. J. Infect. Dis. **182:**816–823.
48. **Willems, R. J., J. Top, N. van den Braak, A. van Belkum, D. J. Mevius, G. Hendriks, M. van Santen-Verheuvel, and J. D. van Embden.** 1999. Molecular diversity and evolutionary relationships of Tn*1546*-like elements in enterococci from humans and animals. Antimicrob. Agents Chemother. **43:**483–491.