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

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From 1995 to 2000, a total of 673 *Enterococcus faecium* and 1,088 *Enterococcus faecalis* isolates from pigs together with 856 *E. faecium* isolates from broilers were isolated and tested for susceptibility to four classes of antimicrobial agents used for growth promotion as part of the Danish program of monitoring for antimicrobial resistance. The four antimicrobials were avilamycin, erythromycin, vancomycin, and virginiamycin. Major changes in the use of antimicrobial agents for growth promotion have occurred during the last 6 years in Denmark. The government banned the use of avoparcin in 1995 and of virginiamycin in 1998. Furthermore, the producers have voluntarily stopped all use beginning in 1999. The avoparcin ban in 1995 was followed by a decrease in the occurrence of glycopeptide-resistant *E. faecium* (GRE) in broilers, from 72.7% in 1995 to 5.8% in 2000. The occurrence of glycopeptide resistance among isolates from pigs remained constant at around 20% from 1995 to 1997. It was shown that, in GRE from pigs, the genes encoding macrolide and glycopeptide resistance were genetically linked and that, following the decrease in the use of tylosin during 1998 and 1999, the occurrence of GRE in pigs decreased to 6.0% in 2000. From 1995 to 1997 the occurrence of erythromycin resistance among *E. faecium* and *E. faecalis* isolates from pigs was almost 90%. Use of tylosin decreased considerably during 1998 and 1999, and this decrease was followed by decreases in the occurrence of resistance to 46.7 and 28.1% among *E. faecium* and *E. faecalis* isolates from pigs, respectively. Erythromycin resistance among *E. faecium* isolates from broilers reached a maximum of 76.3% in 1997 but decreased to 12.7% in 2000 concomitantly with more limited use of virginiamycin. Use of virginiamycin increased from 1995 to 1997 and was followed by an increased occurrence of virginiamycin resistance among *E. faecium* isolates in broilers, from 27.3% in 1995 to 66.2% in 1997. In January 1998 the use of virginiamycin was banned in Denmark, and the occurrence of virginiamycin resistance decreased to 33.9% in 2000. Use of avilamycin increased from 1995 to 1996 and was followed by an increase in avilamycin resistance among *E. faecium* isolates from broilers, from 63.6% in 1995 to 77.4% in 1996. Since 1996 avilamycin usage has decreased, followed by a decrease in resistance to 4.8% in 2000. Our observations show that it is possible to reduce the occurrence of antimicrobial resistance in a national population of food animals when the selective pressure is removed. Cases in which resistance to vancomycin was linked to resistance to erythromycin were exceptions. In such cases resistance did not decrease until the use of both avoparcin and tylosin was limited.

No acquired resistance genes were demonstrated for bacteria isolated from patients more than 60 years ago (18), but emergence of antimicrobial resistance has invariably followed the introduction of new antimicrobial compounds (25). Multiply resistant pathogenic bacteria have emerged worldwide during the last couple of decades, and multiple antimicrobial resistance is now one of the serious concerns of the new millennium (15, 23, 28). In particular the worldwide emergence of glycopeptide-resistant enterococci and the increase in their occurrence, pose a serious threat to the continued possibility of curing infections in humans (12, 14, 22, 31; D. F. Sahm, Program Abstr. 1st Int. ASM Conf. Enterococci, abstr. addendum, 2000). It is unlikely that new antimicrobial agents can be developed at a rate to adequately combat the increasing number of multiresistant bacteria.

As early as the 1960s, concern over the emergence of resistant bacteria in domestic animals led the Swann Committee

(32) to recommend that antimicrobials that were of value for treatment of humans should not be approved for growth promotion in food animals. These guidelines have since been implemented in most European countries. However, the guidelines did not take into account antimicrobials that were of little or no significance in human medicine at the time when they were approved for growth promotion in food animals. Because of the emergence of multiply resistant bacteria causing infections in humans, some of these classes of antimicrobials have become important last resort drugs in the treatment of such infections. Examples include avoparcin, virginiamycin, and avilamycin, which belong to the same classes as the human drugs vancomycin, quinupristin-dalfopristin (Synercid), and evernimicin (Ziracin), respectively. In 2000 the manufacturer of Ziracin suspended its development. European Union rules for approval of feed additives have recently been amended; they now require application for reapproval of all such additives, including antimicrobials used for growth promotion, at fixed intervals.

It has been demonstrated that food animals may serve as a reservoir of resistant bacteria and/or resistance genes that may

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TABLE 1. Use of AGPs in Denmark from 1994 through 2000[a]

Growth promoter	Antimicrobial class	Amt used (kg) in:						
		1994	1995	1996	1997	1998	1999	2000
Avilamycin	Oligosaccharide	433	1,665	2,740	670	7	91	0
Avoparcin[b]	Glycopeptide	24,117	5,690					
Bacitracin[c]	Polypeptide	13,689	7,910	8,399	8,544	3,945	63	
Carbadox[d]	Quinoxaline-di-N-oxide	10,012	1,181	1,985	4,153	1,803	293	
Flavomycin	Flavophospholipol	77	48	18	93	6	665	0
Monensin	Ionophor	4,755	5,007	4,741	3,008	935	0	0
Olaquindox[d]	Quinoxaline-di-N-oxide	22,483	16,213	13,486	17,595	28,445	9,344	
Salinomycin	Ionophor	213	850	759	460	113	0	0
Spiramycin[c]	Macrolide	95	507	15	3	0.3	0	
Tylosin[c]	Macrolide	37,111	52,275	68,350	62,009	13,148	1,827	
Virginiamycin[e]	Streptogramin	2,801	2,590	5,055	10,644	892	0	
Total		115,786	93,936	105,548	107,179	49,294	12,283	0

[a] See reference 8.
[b] Banned in Denmark in May 1995; approval suspended in the European Union in 1997.
[c] Approval suspended in the European Union from July 1999.
[d] Banned by the European Union in 1999 because of toxicity.
[e] Banned in Denmark in January 1998; approval suspended in the European Union from July 1999.

spread to the human population and thereby limit the medical value of these new antimicrobial classes (1, 3, 36). Thus, interventions reducing this reservoir of resistance genes among food animals may prolong the lifetime of these drugs for human use. For these reasons international public health organizations such as the World Health Organization have recommended the termination or rapid phasing out of the use of antimicrobial growth promoters (AGPs) that are also used for therapy or are under development for therapy (37).

Since 1995, major changes in the consumption of antimicrobial agents used for growth promotion have occurred in Denmark. Avoparcin was banned in May 1995, and virginiamycin was banned in January 1998. Furthermore, the food animal industries decided in 1998 to voluntarily stop all use of antimicrobial agents for growth promotion by the end of 1999. These steps were taken as precautionary measures, to prevent future problems with antimicrobial resistance in human medicine. The ban and voluntary withdrawal were based on the expectation that removal of the selective antimicrobial pressure in animals would reduce the exposure of humans, via food, to resistant bacteria from animals.

Several studies have shown that the occurrence of resistance is closely related to the medical use of a drug, even though the association may be variable (11, 15, 26). This association has also been demonstrated for antimicrobial agents used for growth promotion (4, 6, 9). In contrast, little information is available about the effects of terminating the use of an antimicrobial agent on the occurrence of antimicrobial resistance in large human or animal populations outside hospitals.

Danish food animal production is highly industrialized and well monitored, with the main emphasis on a few categories, such as porkers, broilers, layers, and milk cows, and with limited variation in animal breeds and production methods within each production category. Therefore, it provides a homogenous population suitable for studying changes in bacterial populations living in these reservoirs. A continuous national monitoring program for antimicrobial resistance was established in 1995 (7). This program monitors, for example, the occurrence of resistance among enterococci isolated from healthy animals

at slaughter and thereby gives an overview of the general trend of resistance in food animals.

In this study, changes in the occurrence of resistance to glycopeptides, macrolides, oligosaccharides, and streptogramins that have taken place following termination of the use of these antimicrobial agents for growth promotion are reported.

MATERIALS AND METHODS

Details of the sampling scheme and of the isolation, identification, and susceptibility testing of enterococci from broilers and pigs have been published previously (5, 10).

Collection of samples. All isolates were recovered from cecal samples (pigs) or cloacal swabs (broilers) from healthy animals, and only a single isolate per pig herd or broiler flock was included. The number of samples collected monthly from pigs and weekly from broilers from each slaughterhouse was proportional to the annual number of animals slaughtered. This ensures, as far as possible, that samples are representative of the entire country and that each sample represents a herd or flock.

Isolation, identification, and susceptibility testing. For broilers, cloacal swabs were enriched in enterococcal broth (Becton Dickinson) overnight at 42°C, followed by subcultivation on Slanetz and Bartley agar (Difco) at 37°C for 18 to 24 h. Cecal samples from pigs were streaked directly onto Slanetz and Bartley agar. Isolates resembling enterococci were subcultured and identified biochemically as *Enterococcus faecalis* and *Enterococcus faecium* (5). During 1995 and 1996, susceptibility testing for erythromycin, vancomycin, and virginiamycin was performed by tablet diffusion (5). From 1997 onwards all susceptibility testing was done by determining the MIC using microwell broth dilution (Sensititre; Trek Diagnostic Systems) in Mueller Hinton broth (Difco) according to NCCLS guidelines (27). All susceptibility testing for avilamycin from 1995 to 2000 was performed by determining MICs using agar dilution as previously described (5). In categorizing the results the following breakpoints (5, 27) for resistance were used: for avilamycin, ≥ 16 μg/ml; for erythromycin, ≥ 8 μg/ml; for vancomycin, ≥ 32 μg/ml; and for virginiamycin, ≥ 8 μg/ml.

Data handling. Test results were stored in an Oracle, version 7.14, database. Data processing and evaluation were carried out in PC SAS, versions 6.12 and 8.0, and EpiInfo, version 6.02. The 95% confidence interval for the estimated percent resistance was calculated by using a SAS macro according to the work of Blyth (13).

Use of antimicrobial agents for growth promotion. Manufacturers licensed to produce premixes are obliged to collect data on the use of antimicrobials for growth promotion. These data are requested by The Danish Plant Directorate and published annually. Avilamycin, avoparcin, and virginiamycin have never been used for therapy. Data on the use of antimicrobial agents for therapy are collected by the Danish Medicine Agency. All data are published annually in the Danish Integrated Antimicrobial Resistance Monitoring and Research Pro-

TABLE 2. Production of food animals in Denmark
from 1995 through 1999[a]

Yr	No. of animals slaughtered (in millions)		
	Poultry	Cattle	Pigs
1995	113.00		19.00
1996	111.50	0.79	20.53
1997	130.62	0.79	21.18
1998	129.33	0.73	22.87
1999	140.12	0.70	22.53

[a] See reference 16.

gramme (DANMAP) report (8). The use of antimicrobial agents for growth promotion in Denmark from 1994 through 2000 is shown in Table 1. The level of use of macrolides and lincosamides for treatment of production animals has been 7 to 8 tons annually in the period 1995 through 1999. Because antimicrobials for food animals are freely available—although therapeutics are available only by prescription—and are competitively priced compared with other European countries, it is generally considered that there is no black market, or only a negligible one, for antimicrobials in food animals. Therefore, the official figures closely represent actual consumption.

Size of the animal population. Statistics on the production of broilers, cattle, and pigs in Denmark are published annually by Danmarks Statistik (16). The levels of production from 1995 through 1999 are given in Table 2.

RESULTS AND DISCUSSION

The use of antimicrobial agents for growth promotion decreased drastically from 1995 to 2000 (Table 1). In the same period the production of food animals remained constant or even increased (Table 2). Thus, the decreased usage of AGPs does not correlate with major changes in food animal production.

The monitoring of antimicrobial resistance among food animals in Denmark is based on randomly selected bacterial isolates. The sampling strategy used will detect only the most prevalent flora. Thus, resistance may be present at a low level and not be detected by the monitoring system. However, the results can be compared over time and will show changes in the occurrence of resistance.

From 1995 to 2000, a total of 673 *E. faecium* and 1,088 *E. faecalis* isolates from pigs, together with 856 *E. faecium* isolates from broilers, were isolated and tested for antimicrobial susceptibility. The trends in the occurrence of resistance over time and the 95% confidence intervals are given in Table 3. Different numbers of isolates were examined in different years, and during 1995 only a very limited number of isolates were included. This gives very wide 95% confidence intervals for the 1995 results (Table 3).

Over the entire period only four (0.6%) *E. faecium* isolates from pigs were resistant to avilamycin. *E. faecalis* isolates have naturally decreased susceptibility to streptogramin, and thus no results for virginiamycin are given. All *E. faecalis* isolates were susceptible to vancomycin, and 21 (1.9%) of the isolates from pigs were resistant to avilamycin.

Resistance to antimicrobial agents used for growth promotion was more common in *E. faecium* than in *E. faecalis*. The only exception was macrolide resistance, where similar levels were observed. The trends in resistance and the consumption of antimicrobials as growth promoters from 1995 to 2000 are presented in Fig. 1.

The total level of consumption of each of the antimicrobial agents used for growth promotion in Denmark is well known, but it is very difficult to obtain exact data on the consumption of antimicrobial agents for growth promotion for the different food animal species. However, the macrolide tylosin is approved only for pigs, and avilamycin has been used primarily for broilers. After the ban on avoparcin in 1995, increased use

TABLE 3. Trends in resistance of *E. faecium* from broilers and pigs and *E. faecalis* from pigs in Denmark to
selected antimicrobial agents used for growth promotion

Bacterial species and origin	Yr	No. of isolates	% Resistance (95% confidence interval) to:			
			Avilamycin	Erythromycin	Vancomycin	Virginiamycin
Enterococcus faecium						
Broilers	1995	11	63.6 (30.8–89.1)	54.5 (23.4–83.3)	72.7 (39.0–94.0)	27.3 (6.0–61.0)
	1996	106	77.4 (68.2–84.9)	64.2 (54.3–73.2)	42.5 (32.9–52.4)	58.5 (48.5–68.0)
	1997	207	65.2 (58.3–71.7)	76.3 (69.9–81.9)	20.8 (15.5–26.9)	66.2 (59.3–72.6)
	1998	154	28.6 (21.6–36.4)	70.8 (62.9–77.8)	8.4 (4.6–14.0)	60.4 (52.2–68.2)
	1999	189	21.2 (15.6–27.7)	27.5 (21.3–34.5)	9.0 (5.3–14.0)	39.2 (32.2–46.5)
	2000	189	4.8 (2.2–8.8)	12.7 (8.3–18.3)	5.8 (2.9–10.2)	33.9 (27.2–41.1)
Pigs	1995	10	0.0 (0.0–30.9)	80.0 (44.4–97.5)	20.0 (2.5–55.6)	60.0 (26.2–87.8)
	1996	72	1.3 (0.0–7.5)	93.1 (84.5–97.7)	20.8 (12.2–32.0)	47.2 (35.3–59.3)
	1997	55	1.8 (0.1–9.7)	87.3 (75.5–94.7)	20.0 (10.4–33.0)	36.4 (23.8–50.4)
	1998	153	0.0 (0.0–2.4)	70.6 (62.7–77.7)	15.0 (9.8–21.7)	55.6 (47.3–63.6)
	1999	201	1.0 (0.1–3.5)	47.8 (40.7–54.9)	6.5 (3.5–10.8)	8.0 (4.6–12.6)
	2000	182	0.0 (0.0–2.0)	46.7 (39.3–54.2)	6.0 (3.1–10.6)	22.5 (16.7–29.3)
Enterococcus faecalis, pigs	1995	49	0.0 (0.0–7.3)	93.9 (83.1–98.7)	0.0 (0.0–7.3)	—[a]
	1996	259	1.9 (0.6–4.4)	90.7 (86.5–94.0)	0.0 (0.0–1.4)	—
	1997	248	3.6 (1.8–6.8)	91.1 (86.9–94.4)	0.0 (0.0–1.5)	—
	1998	136	0.7 (0.0–4.0)	78.7 (70.8–85.2)	0.0 (0.0–2.7)	—
	1999	200	2.5 (0.8–5.7)	48.0 (40.9–55.2)	0.0 (0.0–1.8)	—
	2000	196	0.5 (0.1–2.8)	28.1 (21.9–34.9)	0.0 (0.0–1.9)	—

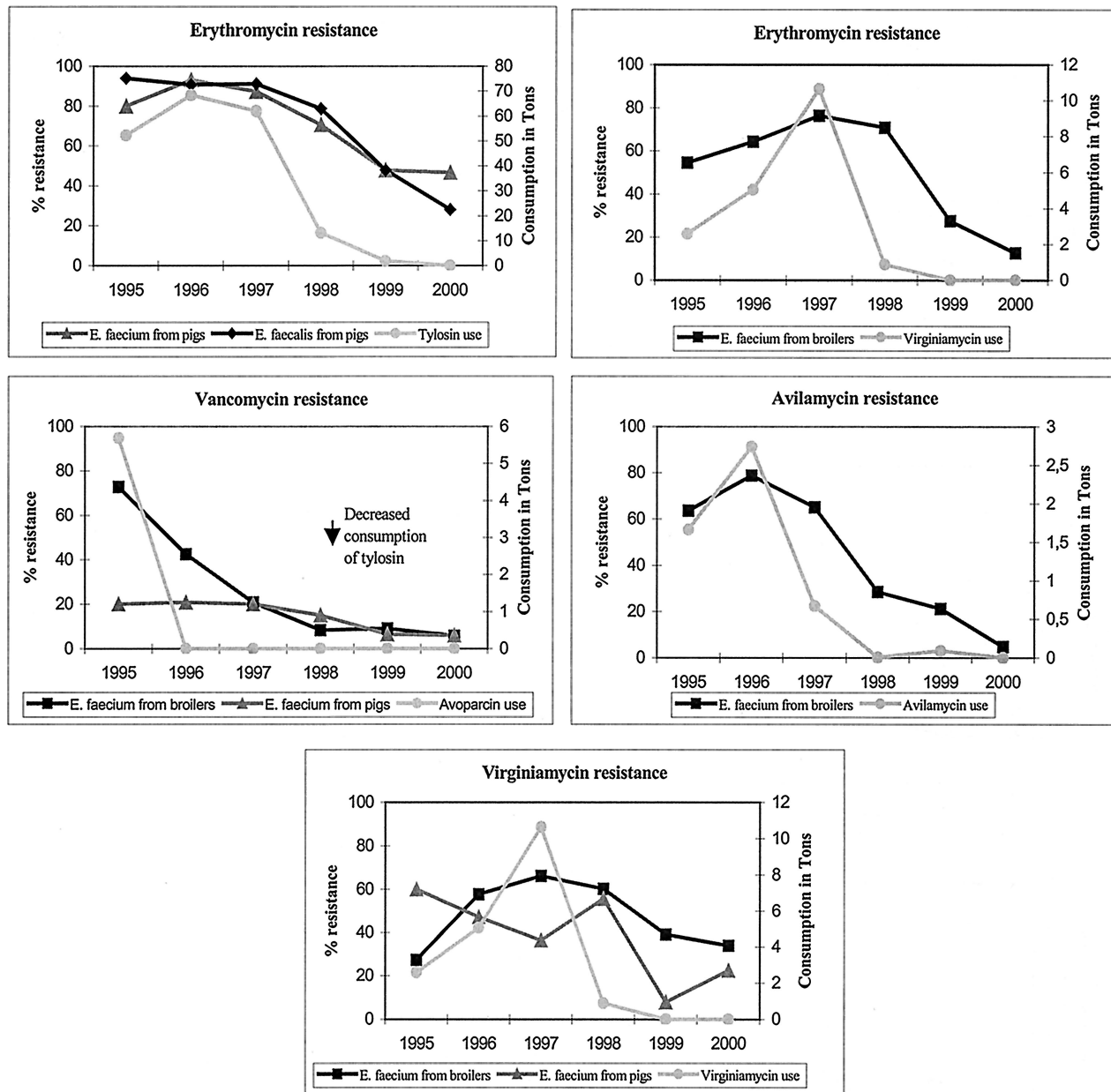[a] —, intrinsically resistant.

FIG. 1. Consumption of antimicrobial agents for growth promotion and occurrence of antimicrobial resistance in *E. faecium* and *E. faecalis* isolates from Danish pigs or broilers from 1995 to 2000.

of tylosin for pigs and of virginiamycin for broilers mainly replaced the use of avoparcin.

The avoparcin ban in 1995 was almost immediately followed by a decrease in the occurrence of glycopeptide-resistant *E. faecium* (GRE) in broilers (Table 3; Fig. 1). In 1995 72.7% of the isolates were resistant to vancomycin; this decreased to 5.8% in 2000. However, the occurrence of resistance among isolates from pigs remained relatively constant at around 20% during 1995 through 1996. It was shown that all GRE isolates from pigs belonged to the same clone and that genes encoding resistance to glycopeptides and macrolides were located close to each other on the same plasmid (2). Therefore, GRE in pigs

may well have been maintained as a consequence of coselection by use of the macrolide tylosin. Indeed, following the decrease in the use of tylosin during 1998 and 1999, the occurrence of GRE in pigs decreased significantly to 6.0% in 2000 (Fig. 1). Nonetheless, almost 6 years after the ban of avoparcin, resistant *E. faecium* can still be found among broilers and pigs in Denmark, showing that it can take a very long time for resistance to disappear.

For several years the macrolide tylosin was the most widely used AGP for pigs in Denmark. In 1995 the occurrence of resistance among *E. faecium* isolates from pigs was 80.0%, in 1996 it was 93.1%, and in 1997 it was 87.3%. Among *E. faecalis*

isolates resistance was 93.9% in 1995, 90.7% in 1996, and 91.1% in 1997. The use of tylosin decreased considerably during 1998 and 1999. This was almost immediately followed by a decrease in the occurrence of resistance among both *E. faecium* and *E. faecalis* isolates from pigs (Table 3; Fig. 1). Thus, in 1998 resistance among *E. faecium* isolates had decreased to 70.6%, and that among *E. faecalis* isolates had decreased to 78.7%. Resistance decreased further, to 47.8 and 48.0%, respectively, in 1999 and to 46.7 and 28.1%, respectively, in 2000. Macrolides are still widely used for treatment of infections in pigs, and thus future trends in the occurrence of resistance cannot yet be predicted.

The occurrence of macrolide resistance among *E. faecium* isolates from broilers has also been very high, reaching a maximum of 76.3% in 1997. It decreased to 27.5% during 1999 and to 12.7% during 2000, concomitantly with the more-limited use of virginiamycin (Tables 1 and 3; Fig. 1). Relatively minor amounts of the macrolide spiramycin have been used for growth promotion in broilers. However, virginiamycin is a natural combination of two structurally unrelated molecules (groups A and B) (30), and group B has the same mechanism of action as antimicrobials belonging to the macrolides (34). Enzymes methylating the target site of macrolides, lincosamides, and streptogramin B have been observed to be the most common cause of resistance in enterococci (19, 21). In addition, it has recently been shown that some of the genes encoding resistance to group A and group B, respectively, are genetically linked (20, 35). Thus, it seems likely that the use of virginiamycin as a growth promoter for broilers may have selected for macrolide resistance in *E. faecium*.

The use of virginiamycin increased between 1995 to 1997. The increase was seen mainly in broilers and was associated with an increase in the occurrence of resistance in *E. faecium*. Thus, virginiamycin resistance among *E. faecium* isolates from broilers increased from 27.3% in 1995 to 66.2% in 1997. In January 1998, the use of virginiamycin was banned in Denmark, and the occurrence of resistance subsequently decreased to 33.9% in 2000 (Fig. 1). More variation in the occurrence of virginiamycin resistance can be observed among *E. faecium* isolates from pigs. Thus, resistance decreased from 60.0% in 1995 to 36.4% in 1997, increased in 1998 to 55.6%, decreased again in 1999 to 8.0%, and increased again in 2000 to 22.5% (Table 3). The reason for this variation is not known. However, the increase in resistance during 2000 is mainly due to the emergence of isolates that are simultaneously resistant to erythromycin, kanamycin, penicillin, streptomycin, tetracycline, and virginiamycin (data not shown). These isolates will now be investigated further.

In Denmark, avilamycin has been used primarily for broilers. Use of this oligosaccharide increased from 1995 to 1996, followed by an increase in resistance among *E. faecium* isolates from 63.6% in 1995 to 77.4% in 1996. Since 1996, the level of use has decreased, almost immediately followed by a decrease in resistance to 4.8% in 2000 (Fig. 1).

The effects of the ban of growth promoters for food animals on the occurrence of antimicrobial resistance among humans have not been determined. However, the GRE carrier rate among healthy humans in Germany decreased from 13% in 1994 to 4% in 1997 (24) following the German ban on avoparcin in 1996. Similarly, a decrease in the occurrence of GRE has

been observed among poultry products in Italy during the 18 months following the ban of avoparcin (29) and among humans, broilers, and pigs in The Netherlands from 1997 to 1999 (33) following the ban here.

Our observations show that it is possible to reduce the occurrence of antimicrobial resistance in a national population of food animals when the selective pressure is removed. However, we have also demonstrated that under certain conditions resistance may persist, most likely as a consequence of coselection. It is not possible to foresee whether the occurrence of resistance will decrease to an undetectable level in the future. Vancomycin-resistant *E. faecium* can still be found among broilers and pigs in Denmark more than 5 years after the ban of avoparcin. In the future, resistant isolates may still persist in low numbers that are not detected by the monitoring methods used. In the case of antimicrobials used in animal production, it is of great importance to prevent increases in the resistance gene reservoir to the extent possible, in particular for agents belonging to classes that are or may become important in human medicine.

The results discussed above represent the first documented effects of large-scale interventions to reduce the occurrence of antimicrobial resistance. They demonstrate that the exposure of humans to bacteria resistant to antimicrobial drugs and to resistance genes through food can be reduced effectively by intervention.

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