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Relations between the consumption of antimicrobial growth promoters and the occurrence of resistance among *Enterococcus faecium* isolated from broilers

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SUMMARY

The present study investigates, at farm level, the effect of the time-span between sampling and the last time a particular antimicrobial growth promoter (AGP) was included in the feed on the probability of selecting an AGP-resistant *Enterococcus faecium* isolate from a broiler flock. The probability that a randomly selected *E. faecium* isolate was resistant to avilamycin, erythromycin or virginiamycin was 0·91, 0·92 and 0·84, respectively if the isolate originated from a broiler flock fed either avilamycin- or virginiamycin-supplemented feed. As the time-span between sampling and the last AGP consumption increased, the probability of isolating an *E. faecium* isolate resistant to a particular AGP decreased (probability $<0{\cdot}2$ within 3–5 years after last exposure to AGPs). The decrease in probability over time showed little farm-to-farm variation. The number of times a particular AGP was given to previous flocks reared in the same house had no effect on the probability of isolating a resistant isolate.

INTRODUCTION

The practice of adding antimicrobials to broiler feed to enhance growth was initiated in Denmark in the beginning of the 1960s. Since the beginning of the 1970s, antimicrobial growth promoters (AGPs) have been widely used in Danish broiler production. The Danish Plant Directorate has compiled data on the total yearly consumption of AGPs in Denmark. In 1995, the consumption of AGPs was estimated for each animal species. The most widely used growth promoter in Danish broiler production was avilamycin with 1400 kg, followed by avoparcin with 1100 kg and virginiamycin with 1090 kg while the consumption of bacitracin, spiramycin and flavophospholipol was 610, 507 and 48 kg, respectively [1]. In the same period 172 900 tons of poultry meat were produced [2]. Some of the antimicrobial agents used for growth promotion belonged to the same classes as antimicrobials used for human therapy. Consequently, resistance towards AGPs may result in resistance towards therapeutic human drugs. This cross-resistance is observed between avoparcin and vancomycin [3], virginiamycin and quinopristin/dalfopristin [4], tylosin; spiramycin and erythromycin [5] and avilamycin and evernimicins [6]. Already, in the report of the Swann Committee [7], concerns were raised that the use of antimicrobials for growth promotion could lead to the emergence of resistant human pathogenic bacteria (and ultimately, reduce the effect of important drugs such as penicillin and tetracycline for human therapy). This led to the adoption in the early 1970s of regulations within the European Union to restrict antimicrobials used for

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growth promotion to antimicrobial compounds not used in human therapy (Directive 70/524/EEU). In practice, it has proven almost impossible to maintain this distinction between antimicrobials used as AGPs for animals and those used as human therapeutics. Because of the increasingly limited therapies for humans, pharmaceutical companies have developed many old AGPs into drugs for human use. Some of these drugs (vancomycin, quinopristin/dalfopristin) are currently considered essential for the treatment of serious life-threatening infections in humans. In May 1995, the Danish Ministry of Food, Agriculture and Fisheries banned the AGP avoparcin because *E. faecium* isolates resistant to vancomycin and avoparcin were commonly found in faeces from pigs and poultry [3, 8]. Subsequently, avoparcin use was suspended in the European Union in 1997. In January 1998, the AGP virginiamycin also was banned in Denmark because of cross-resistance to quinopristin/dalfopristin [9]. In February 1998, the Danish poultry industry decided voluntarily to discontinue the use of all AGPs. In July 1999, four growth promoters were temporally suspended in the European Union: spiramycin, tylosin, bacitracin and virginiamycin.

Since 1995, the DANMAP programme (The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme) has monitored antimicrobial resistance among *E. faecium* isolated from broilers at slaughter. Resistance to avilamycin, vancomycin, virginiamycin and erythromycin was prevalent among *E. faecium* while broilers were fed AGP-supplemented feed. Results from DANMAP also showed that after the withdrawal of AGPs the proportion of *E. faecium* isolates resistant to AGPs decreased significantly [10, 11]. Similarly, a study from The Netherlands reported a significant decrease, from 80% in 1997 to 31% in 1999, in resistance to vancomycin among *E. faecium* from broilers after the suspension of the AGP avoparcin [12]. In a French study [13] faecal samples from broilers were collected in the first semester of 1999 and 2000, which was before and after the European Union suspended the AGPs spiramycin, tylosin, bacitracin and virginiamycin. From 1999 to 2000, resistance to erythromycin and virginiamycin decreased significantly from 59·0 and 49·2% to 45·7 and 18·8% respectively. In Norway, studies have been based on more sensitive isolation methods. Subsequently, vancomycin-resistant *E. faecium* could be isolated $1\frac{1}{2}$ years and 3 years after avoparcin was banned, in 96–98 and 99% of the farms where broilers were previously fed avoparcin-supplemented feed [14, 15].

The studies described above all focused on the effect of discontinuing the use of AGPs at a national level. The aim of the present study was to investigate this effect at farm level, notably the effect that (1) the time-span between sampling and the last AGP consumption at flock level and (2) the number of times AGPs were given to flocks reared in the same house had on the probability of selecting an AGP-resistant *E. faecium* isolate.

MATERIALS AND METHODS

Broiler production

In Denmark, broilers are produced on farms consisting of one or more houses, each house containing one flock of broilers. The houses have all-in/all-out production (i.e. houses are emptied, cleaned and disinfected before a new flock is introduced) with up to eight flocks produced per house per year. From 1993 to 2000, the number of broilers slaughtered in Denmark increased from 113 to 134 million birds. In the same period, the average flock size increased from 27 005 birds (range 1600–77 553) to 31 289 birds (range 700–62 900).

All broiler farms in Denmark are identified by a unique code (farm-id). Since 1992, state veterinary officers have performed ante-mortem inspection of all broiler flocks and completed a health certificate <48 h before the flock is slaughtered. For each flock, the health certificate provided a variety of informations. For this analysis, the following variables were included: farm-id, house-id, and type of AGP included in the feed. The slaughterhouses provided information about date of slaughter.

Collection of bacterial isolates

The *E. faecium* isolates from broilers originated from the DANMAP programme [11, 16]. The programme was initiated in the last quarter of 1995. All *E. faecium* isolates were recovered from cloacal swabs collected randomly from healthy broilers at slaughter by the meat inspection staff. The number of samples collected weekly from each slaughterhouse was proportional to the annual number of animals slaughtered and only a single isolate per farm per year was included [11]. This ensures as far as possible that the samples were representative of the Danish broiler population.

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Isolation, identification and susceptibility testing

The cloacal swabs were enriched in enterococcal broth (Becton Dickinson, Cockeysville, MD, USA) overnight at 42 °C, followed by subcultivation on Slanetz and Bartley agar (Merck, Darmstadt, Germany) at 37 °C for 18–24 h. Isolates resembling enterococci were subcultured and only isolates identified biochemically as *E. faecium* were included in this study [16]. During 1995 and 1996, susceptibility testing for erythromycin, vancomycin and virginiamycin was performed by tablet diffusion [16]. From 1997 onwards all susceptibility testing was done by determining the MIC using microwell broth dilution (Sensititre; Trek Diagnostic Systems, West Sussex, UK) in Mueller–Hinton broth (Merck) according to NCCLS guidelines [17]. Inhibition zone diameters corresponded with the break-point concentrations used in the MIC test. The two tests were validated against each other before the change of methods. All susceptibility testing for avilamycin between 1995 and 2000 was performed by determining MICs using agar dilution [16]. In categorizing the results the following break-points for resistance were used: for avilamycin, 16 μg/ml; for erythromycin, $\leqslant$22 mm and $\geqslant$8 μg/ml; for vancomycin, $\leqslant$10 mm and $\geqslant$32 μg/ml; and for virginiamycin, $\leqslant$22 mm and $\geqslant$8 μg/ml [16, 17].

The data sets

From the fourth quarter of 1995 to the end of 2000 a total of 854 *E. faecium* isolates were collected from broilers. Four data sets were generated containing the results of the susceptibility testing to avilamycin, erythromycin, vancomycin and virginiamycin, respectively. The four data sets containing the results of the susceptibility testing were each combined with data from the ante-mortem inspection.

For each *E. faecium* isolate the avilamycin data set contained the results of the avilamycin susceptibility testing (resistant/susceptible), sampling date, farm-id, house-id, whether the *E. faecium* isolate originated from a flock fed avilamycin-supplemented feed and if previous flocks reared in the same house were fed avilamycin-supplemented feed. We calculated the time-span (days) between the sampling date and the last time avilamycin was given to a flock reared in the same house. We also calculated how many times avilamycin was given to flocks reared in the same house between 1992 and 2000. In addition, time (days) was divided into the following categories (Time_cat): Cat_0days, the *E. faecium* isolate originated from a broiler flock, which had received avilamycin. Cat_<1year, the isolate originated from a flock that was not given avilamycin but avilamycin was given to a previous flock reared within 1 year in the same house. Cat_1–2years, avilamycin was given to a flock reared in the same house between 1 and 2 years before the time of sampling, Cat_2–3years, between 2 and 3 years before the time of sampling and finally Cat_>3years, more than 3 years before the time of sampling. A description of the variables is given in Table 1. In the validation of the data sets the exclusion criteria were: (1) records with no identifiable farm-id or house-id; (2) disagreement between the sampling date and the registered date of slaughter and (3) where *E. faecium* isolates originated from broiler flocks reared in a house with no records of AGP use before February 1998. The three other data sets vancomycin, virginiamycin and erythromycin were constructed in the same way as the avilamycin data set and the exclusion criteria were also the same.

The validated avilamycin and virginiamycin data sets contained 654 and 602 observations, respectively. Vancomycin is not used as a growth promoter in Denmark; therefore, the vancomycin data set contained the results of the vancomycin-susceptibility testing and the consumption of the glycopeptide AGP avoparcin. The validated vancomycin data contained 637 observations. Macrolides were not used as AGPs in any of the flocks in this study; therefore the erythromycin data set contains the results of the erythromycin susceptibility testing and consumption of the AGP virginiamycin. The validated erythromycin data had 600 observations.

Statistical analysis

The data had a strict hierarchical structure with a three-level hierarchy: farm–houses–flocks. Houses were clustered within the same farm and multiple measurements of resistance among *E. faecium* isolates collected from flocks reared in the same houses were clustered over time.

In all analyses, the dependent variables were the results of the susceptibility testing coded resistant *vs.* susceptible. Because the dependent variables were binary and the data had a strict hierarchical structure, a generalized linear mixed model (GLMM) with binomial family and logit link was chosen for the analysis. The macro glimmix from the statistical package SAS (version 8.0) and MLwiN (version 1.10.0006) were used for the analyses [18–20]. The results from

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Table 1. *Description of the variables used in the analyses*

Dependent variable	Independent variable	Description of variables and levels	No. of levels	Minimum	Median	Maximum
Avil_res		Result of susceptibility testing coded resistant/susceptible	2	—	—	—
	Farm_id	Farm identification number	205	—	—	—
	House_id	House identification number within farms	325	—	—	—
	Time	Days between sampling and the last time a flock was fed avilamycin	—	0	435	2752
	Time_cat	Time between sampling and the last time a flock was fed avilamycin: Cat_0days = 0 days; Cat_ < 1year = 1–365 days; Cat_1–2years = 366–730 days; Cat_2–3years = 731–1095; Cat_ > 3years = > 1095days	5	—	—	—
	No_avil	No. of times previous flocks reared in the same house were fed avilamycin-supplemented feed	—	1	12	29
Vanc_res		Result of susceptibility testing coded resistant/susceptible	2	—	—	—
	Farm_id	Farm identification number	204	—	—	—
	House_id	House identification number within farms	317	—	—	—
	Time	Days between sampling and the last time a flock was fed avoparcin	—	225	1172	2570
	Time_cat	Time between sampling and the last time a flock was fed avoparcin: Cat_ < 1–2years = 225–730 days; Cat_2–3years = 731–1095; Cat_ > 3years = > 1095days	3*	—	—	—
	No_avo	No. of times previous flocks reared in the same house were fed avoparcin-supplemented feed	—	1	12	22
Eryt_res		Result of susceptibility testing coded resistant/susceptible	2	—	—	—
	Farm_id	Farm identification number	203	—	—	—
	House_id	House identification number within farms	314	—	—	—
	Time	Days between sampling and the last time a flock was fed virginiamycin	—	0	352	2830
	Time_cat	Time between sampling and the last time a flock was fed virginiamycin: Cat_0days = 0 days; Cat_ < 1year = 1–365 days; Cat_1–2years = 366–730 days; Cat_2–3years = 731–1095; Cat_ > 3years = > 1095days	5	—	—	—
	No_virg	No. of times previous flocks reared in the same house were fed virginiamycin-supplemented feed	—	1	6	17
Virg_res		Result of susceptibility testing coded resistant/susceptible	2	—	—	—
	Farm_id	Farm identification number	203	—	—	—
	House_id	House identification number within farms	314	—	—	—
	Time	Days between sampling and the last time a flock was fed virginiamycin	—	0	350	2830
	Time_cat	Time between sampling and the last time a flock was fed virginiamycin: Cat_0days = 0 days; Cat_ < 1year = 1–365 days; Cat_1–2years = 366–730 days; Cat_2–3years = 731–1095; Cat_ > 3years = > 1095days	5	—	—	—
	No_virg	No. of times previous flocks reared in the same house were fed virginiamycin-supplemented feed	—	1	6	17

* Avoparcin was banned in May 1995 and the DANMAP programme was initiated in the fourth quarter of 1995. The smallest time difference between sampling a broiler flock and the last time a flock reared in the same house received avoparcin was 225 days, therefore the variable time has only 3 levels.

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Table 2. *The final models for avilamycin, vancomycin, erythromycin and virginiamycin*

Antibiotic	Effects	Level	Estimate	(95% CI)	χ^2	*P* value	Probability of resistant isolate
Avilamycin	Intercept	—	−2·355	(−3·011–−1·761)	55·38	<0·0001	—
	Time_cat	Cat_0days	4·670	(3·873–5·521)	125·11	<0·0001	0·91
		Cat_<1year	2·549	(1·861–3·298)	48·49	<0·0001	0·55
		Cat_1–2years	1·501	(0·810–2·270)	16·00	<0·0001	0·30
		Cat_2–3years	0·866	(0·101–1·667)	4·62	0·032	0·18
		Cat_>3years	0·000	(0·000–0·000)	—	—	0·09
Vancomycin	Intercept	—	−2·611	(−3·009–−2·165)	143·48	<0·0001	—
	Time_cat	Cat_<1–2years*	2·302	(1·718–2·837)	64·69	<0·0001	0·42
		Cat_2–3years	1·006	(0·456–1·564)	11·32	<0·0008	0·17
		Cat_>3years	0·000	(0·000–0·000)	—	—	0·07
Erythromycin	Intercept	—	−0·404	(−0·973–0·148)	2·14	0·14	—
	Time_cat	Cat_0days	2·858	(2·085–3·810)	44·91	<0·0001	0·92
		Cat_<1year	0·862	(0·229–1·552)	7·15	0·0075	0·62
		Cat_1–2years	0·108	(−0·573–0·813)	0·10	0·75	0·43
		Cat_2–3years	−1·498	(−2·341–−0·781)	14·17	0·0002	0·13
		Cat_>3years	0·000	(0·000–0·000)	—	—	0·40
Virginiamycin	Intercept	—	−0·345	(−0·880–0·162)	1·72	0·19	—
	Time_cat	Cat_0days	1·984	(1·315–2·696)	31·94	<0·0001	0·84
		Cat_<1year	0·550	(−0·037–1·173)	3·23	0·072	0·55
		Cat_1–2years	0·043	(−0·590–0·683)	0·02	0·90	0·42
		Cat_2–3years	−0·275	(−0·917–0·374)	0·70	0·40	0·35
		Cat_>3years	0·000	(0·000–0·000)	—	—	0·41

* Cat_<1–2: Since the use of avoparcin was banned before the DANMAP programme was initiated, the time period between date of sampling and when a flock reared in the same house received avoparcin was between 225 and 730 days.

the two programs were very similar but as expected the estimates differed slightly from program to program. Only the results from the MLwiN program are presented.

In the first analysis the variables farm-id and house-id nested within farm-id were included as random effects. For all four AGPs, none or only a minor variation was detected at house level; therefore, only farm-id was included as a random effect in the final model. The fixed effects were the time categories (Time_cat), number of times a particular AGP was fed to previous flocks reared in the same house and the interaction between these two variables. A full model was fitted for each dependent variable (each antimicrobial). Backward elimination of fixed effects was performed using *P* values. The criteria for keeping the fixed effect in the model was $P<0{\cdot}05$. The 95% confidence intervals for the fixed effect estimates were obtained by Markov Chain Monte Carlo methods.

In a second analysis a model was fitted for each antimicrobial containing the fixed effect time (days) and the random effect farm-id. This type of model allowed us to model, for each antimicrobial, the coherence between the probability of obtaining a resistant *E. faecium* isolate and time.

Finally, in a third analysis a model was fitted for each antimicrobial containing the fixed effect time (days) and the random effects farm-id and the interaction between farm-id and time. This model allowed us to model, for each farm-id, the coherence between the probability of obtaining a resistant *E. faecium* isolate and time.

RESULTS

In the first analysis backward elimination resulted in the models shown in Table 2. In all four models time_cat was the only variable that remained in the models ($P<0{\cdot}001$). This means that the number of times a particular AGP was given to previous flocks reared in the same house had no effect on the probability of isolating a resistant isolate. Figure 1 shows the coherence between time since a previous broiler flock reared in the same house was fed AGP-supplemented feed and the probability of collecting

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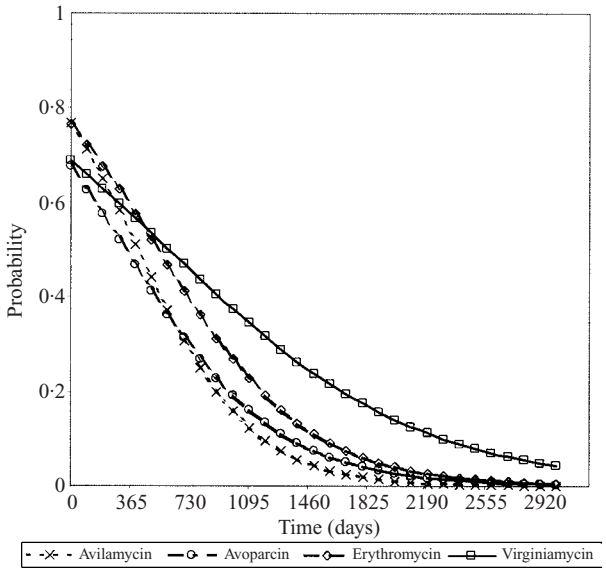

Fig. 1. The coherence between the predicted probability of isolating an AGP-resistant *E. faecium* isolate and the time (days) since a previous broiler flock reared in the same house was fed AGP-supplemented fed.

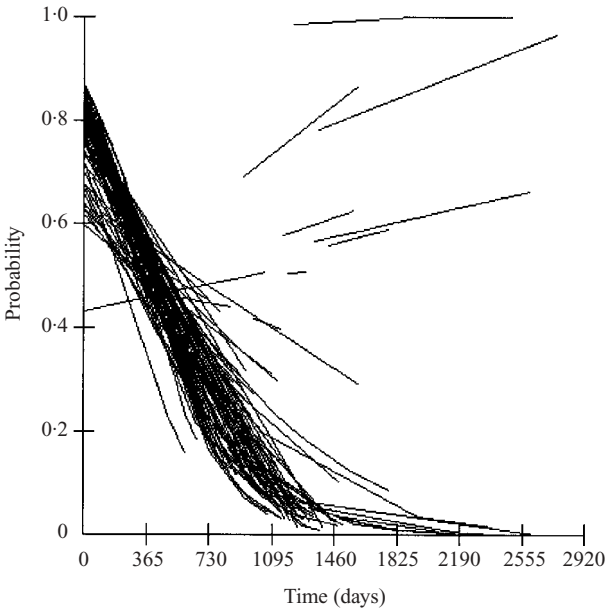

Fig. 2. The coherence between the predicted probability of isolating an avilamycin-resistant *E. faecium* isolate and the time (days) since a previous broiler flock reared in the same house was fed avilamycin-supplemented fed. Each line represents one farm.

an AGP-resistant *E. faecium* isolate (second analysis). The four curves were based on the following parameter estimates: avilamycin $\alpha = 1{\cdot}05$ (0·152), $\beta = -0{\cdot}00271$ (0·000313); avoparcin $\alpha = 0{\cdot}823$ (0·318), $\beta = -0{\cdot}00232$ (0·000327); erythromycin $\alpha = 1{\cdot}25$ (0·150), $\beta = -0{\cdot}00246$ (0·000267); virginiamycin $\alpha = 0{\cdot}884$ (0·132), $\beta = -0{\cdot}00174$ (0·000272). The figures given in parentheses are the standard error of the estimates. Figures 2–5 show, at farm level, the coherence between the predicted probabilities of isolating AGP-resistant *E. faecium* isolates and time after a broiler flock reared in the same house had AGP-supplemented feed discontinued. Each line in the Figures represents one farm (third analysis).

Avilamycin

The probability that a randomly selected *E. faecium* isolate was resistant to avilamycin was 0·91 when the isolate originated from a broiler flock fed avilamycin-supplemented feed (Table 2). An increase in time_cat from 0 days to more than 3 years after discontinuation of avilamycin resulted in a steady and significant decrease in the probability from 0·91 to 0·09. Figure 2 shows, for each farm, the coherence between the predicted probability of isolating an avilamycin-resistant *E. faecium* isolate and the time after a broiler flock reared in the same house had avilamycin-supplemented feed discontinued. An increase in time resulted in a decreased predicted probability of collecting an avilamycin-resistant *E. faecium* isolate, which corroborates the results in Table 2. In addition, almost all of the lines in Figure 2 lie within one dense line indicating only a minor variation in the coherence between the predicted probability and time between farms. Only very few farms did not follow the general pattern.

Vancomycin

Avoparcin was banned in May 1995 and the DANMAP programme was initiated in the last quarter of 1995, therefore no *E. faecium* isolates were collected from flocks fed avoparcin-supplemented feed. As a result, the first time category (Cat_<1–2years) represents the period 225–730 days after avoparcin use was discontinued. The analysis showed that the probability that an *E. faecium* isolate was resistant to vancomycin decreased significantly as the time_cat between date of sampling and when a previous broiler flock reared in the same house was fed avoparcin-supplemented feed increased (Table 2). Thus, the steady decrease in probabilities found in the vancomycin model was similar to the decrease in probabilities found in the avilamycin model (Table 2). Figure 3 shows, for each farm, that an increase in time following the withdrawal of avoparcin-supplemented

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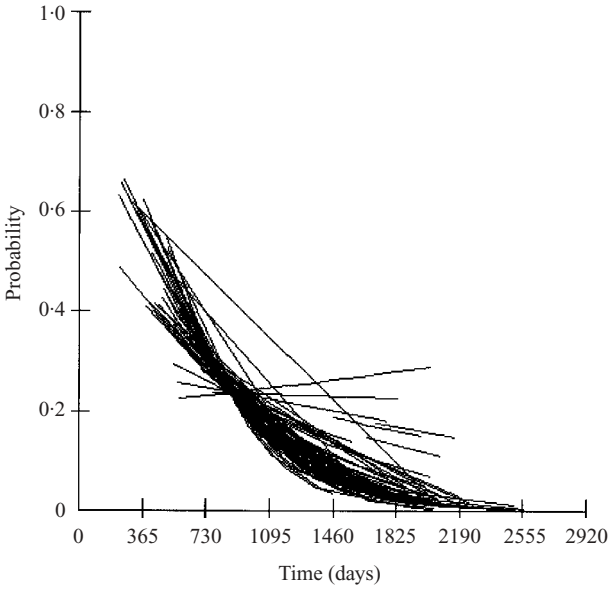

Fig. 3. The coherence between the predicted probability of isolating a vancomycin-resistant *E. faecium* isolate and the time (days) since a previous broiler flock reared in the same house was fed avoparcin-supplemented fed. Each line represents one farm.

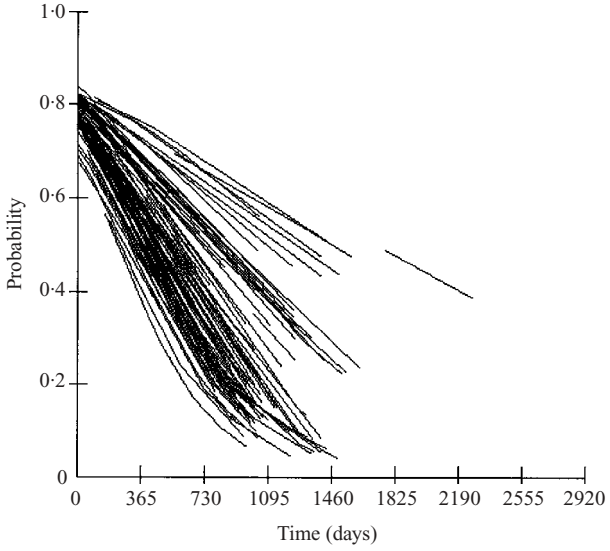

Fig. 4. The coherence between the predicted probability of isolating an erythromycin-resistant *E. faecium* isolate and the time (days) since a previous broiler flock reared in the same house was fed virginiamycin-supplemented fed. Each line represents one farm.

feed resulted in a decrease in the predicted probability of isolating vancomycin-resistant *E. faecium* (VRE). These findings substantiate the results from Table 2. As in the avilamycin model, the lines are grouped into a dense line indicating only a minor variation in the coherence between the predicted probability and the time between farms.

Erythromycin

The predicted probability of collecting an erythromycin-resistant *E. faecium* isolate declined steadily from 0·92 in the category 0 days to 0·13 in the category 2–3 years (Table 2). This decline was followed by an increase in probability from 0·13 to 0·40 between 2 and 3 years and more than 3 years, which indicates that after 3 years variables other than time_cat were important in explaining the probability of collecting an erythromycin-resistant isolate. Figure 4 shows for each farm the coherence between the predicted probability and the time since a flock reared in the same house was fed virginiamycin-supplemented feed. For a majority of the farms, the probability was below 0·2 within 1460 days (4 years). In the remaining farms, the decline in probability was reduced which is probably connected with the observed increase in probability from 0·13 in Cat_2–3years to 0·40 in Cat_>3years (Table 2).

Virginiamycin

The first analysis showed that the probability that a randomly selected *E. faecium* isolate was resistant to virginiamycin was 0·84 if the isolate originated from a broiler flock fed virginiamycin-supplemented feed (Table 2). For the time category <1 year (Cat_<1year) the probability was 0·55. Both findings are in accordance with the findings from the avilamycin and erythromycin models. The predicted probabilities for the categories less than 1 year to more than 3 years (Cat_<1year–Cat_>3years) were not significantly different indicating that no further decline in probabilities were observed. Figure 5 shows for each farm the coherence between the predicted probability of isolating a virginiamycin-resistant *E. faecium* isolate and time since a flock reared in the same house was fed virginiamycin-supplemented feed. The lines representing the farms form a fan-shaped structure, which was not seen for the other AGPs. As was observed for erythromycin resistance, the farms can be divided into two groups. A large group of farms follow a similar pattern with a probability below 0·2 within 1460 days (4 years). In the other group, there was either a slow or, in rare cases, no decrease in probability, which indicated that factors other than time influenced the probability of randomly selecting a virginiamycin-resistant *E. faecium* isolate.

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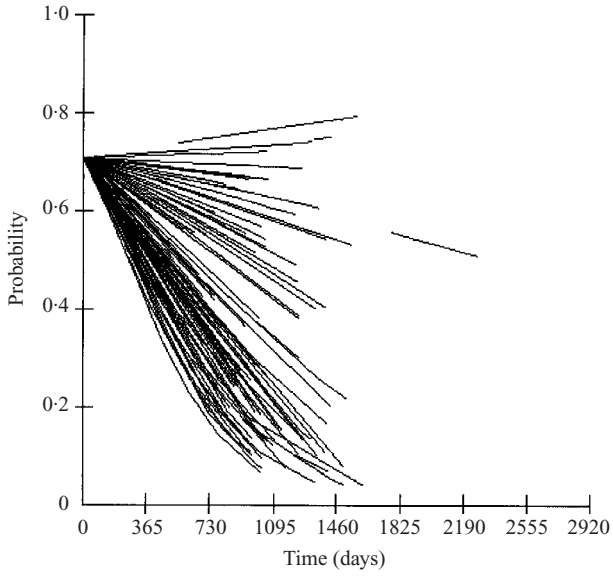

Fig. 5. The coherence between the predicted probability of isolating a virginiamycin-resistant *E. faecium* isolate and the time (days) since a previous broiler flock reared in the same house was fed virginiamycin-supplemented fed. Each line represents one farm.

DISCUSSION

To our knowledge this is the first time that (1) the time-span between sampling and the last AGP consumption at flock level and (2) the number of times AGPs have been given to previous flocks reared in the same house have been analysed to predict the probability of selecting an AGP-resistant *E. faecium* isolate. The results of the analyses showed that the probability that a randomly selected *E. faecium* isolate was resistant to a particular AGP decreased as the time between sampling and the last AGP consumption increased (Table 2 and Figs 1–5). For all four antimicrobials, only time-span (Time_cat) between sampling and the last AGP consumption was significant. This indicates that if time-span is taken into account when predicting the probability of collecting a resistant *E. faecium* isolate, the number of times a particular AGP is given to flocks reared in the same house is not significant. In addition, for a majority of the farms the decline over time (days) in predicted probability of selecting an AGP-resistant *E. faecium* isolate showed almost no variation from farm to farm (Figs 2–5). A possible explanation for the lack of variation between farms is the all-in/all-out approach applied in Danish broiler production, where houses are emptied, cleaned and disinfected and left empty for approximately 2 weeks before a new flock is introduced. This approach will inevitably result in a quantitative reduction in the number of bacteria transferred between consecutive flocks.

The probability that a randomly collected *E. faecium* isolate was resistant to avilamycin, erythromycin or virginiamycin was 0·91, 0·92 and 0·84, respectively, when the isolate originated from a flock fed either avilamycin- or virginiamycin-supplemented feed (Table 2). It seems reasonable that if a broiler flock was fed a particular AGP then a large proportion of the *E. faecium* isolates from that flock would be resistant. The observed decrease in probability of selecting a resistant *E. faecium* isolate indicates a shift in the ratio between sensitive and resistant isolates. It does not necessarily mean that the resistances to avilamycin, vancomycin, erythromycin and virginiamycin are being eliminated. A Danish study [21] showed that 5 years after avoparcin was banned the VRE prevalence was 0·12 (17/140) using a non-selective method. However, using a selective method the VRE prevalence was 0·74 (104/140). In our study VRE was isolated from a broiler flock 2164 days (5·9 years) after the last use of avoparcin in the feed. Borgen et al. [22] and Heuer et al. [23] isolated VRE from the environment of cleaned and disinfected broiler houses in the absence of a selective pressure from avoparcin. VRE was not isolated from the hatchery environment or from the feed mill samples in any of the studies. In addition, Heuer et al. [23] demonstrated the presence of identical or closely related clones of VRE in consecutive broiler flocks reared in the same house indicating that VRE from the broiler house environment may be transmitted between consecutive flocks. A comparison of the avilamycin, erythromycin and virginiamycin prevalence using a selective and a non-selective method has not been performed, and so far no studies have investigated the persistence of avilamycin, erythromycin and virginiamycin resistances among *E. faecium* isolated from the broiler houses. Based on experiences with VRE persistence, it is reasonable to assume that avilamycin-, erythromycin- and virginiamycin-resistant strains will also persist in the absence of a selective pressure.

In a French study, faecal samples from broilers were collected in the first semester of 1999 and 2000. In that period an increase in avilamycin resistance from 43·5 to 71·4% was observed [13]. The AGP avilamycin is not suspended in the European Union, the authors therefore suggested that the observed increase in avilamycin resistance was caused by

increased avilamycin consumption. The same trend has been observed in the DANMAP programme where an increase in both avilamycin and virginiamycin consumption led to an increase in resistance to these two AGPs among *E. faecium* from broilers [10, 11]. Our study showed that if the time-span increased between sampling and the last time a previous flock from the same house was fed avilamycin-supplemented feed, the probability of isolating an avilamycin-resistant *E. faecium* isolate would decrease (Table 2). Figure 2 shows that a small number of the farms did not follow the general decrease in the probability of isolating an avilamycin-resistant *E. faecium* isolate. In most of these farms, the *E. faecium* isolates were collected more than 700 days after the last avilamycin consumption and at the same time the avilamycin-resistant isolates made up a large proportion of the total number of *E. faecium* isolates. The result was a predicted probability of collecting an avilamycin-resistant *E. faecium* that was higher compared to most other farms in the study. Therefore, the lines fall far outside the general pattern. This result is consistent with a quantitative reduction and no elimination.

After 1995 very little if any tylosin or spiramycin was used in Danish broiler production. Therefore, the occurrence of erythromycin resistance among *E. faecium* from broilers was probably due to selection by the streptogramin-growth-promoter virginiamycin. Streptogramins consist of two components, streptogramin A and B, and streptogramin B is commonly selected together with resistance for macrolide and lincosamide (known as the MLS_B phenotype) [24].

The decline in the probability of isolating an erythromycin-resistant *E. faecium* isolate was very similar to the results for avilamycin except for the time category 3 or more years where 26 (42%) of a total of 62 *E. faecium* isolates were resistant to erythromycin. Sixteen (62%) of the 26 isolates were also resistant to avilamycin, of which 14 isolates originated from flocks that were fed avilamycin-supplemented feed. Therefore, co-selection for erythromycin resistance by avilamycin might to some extent explain the high probability of erythromycin resistance (0·40 in Cat_>3years). The increase in probability from 2–3 years to more than 3 years is also evident from Figure 4. However, the Figure also indicates that the increase only occurred in a minor number of the farms.

There was no further decrease in the probability of isolating an *E. faecium* isolate resistant to virginiamycin if the time-span between sampling and the last time a flock from the same house was fed virginiamycin-supplemented feed exceeded 1 year (Table 2). Figure 5 indicates that the decline in probability varied between farms. For a large part of the farms, there was a decline in probability similar to that observed for the other AGPs. In the remaining group of farms, the rate of the decline varied considerably. A thorough examination of the data from the categories 1–2 years to 3 or more years (Cat_1–2years to Cat_>3years) showed that 83 (71%) out of 117 virginiamycin-resistant *E. faecium* isolates were collected in 1999 and 2000. Virginiamycin was banned in early 1998, indicating that factors other than the consumption of virginiamycin selected for virginiamycin resistance. From 1995 to 1998, a total of 240 *E. faecium* isolates were resistant to virginiamycin, 214 (89%) out of the 240 isolates were also resistant to erythromycin. In 1999, 23 (48%) of 48 *E. faecium* isolates were resistant to both virginiamycin and erythromycin, and in 2000, it was 3 (8%) out of 38 isolates. In 1999 and 2000, the resistance combination erythromycin/virginiamycin declined and the resistance combination penicillin/virginiamycin emerged. In 1999, 24 (50%) of 48 isolates were resistant to virginiamycin and penicillin, but not erythromycin. In 2000, it was 32 (84%) of 38 isolates. After the ban of virginiamycin in Denmark in early 1998, the direct selection of the resistance combination erythromycin/virginiamycin disappeared while the therapeutic consumption of β-lactams might have selected for the penicillin/virginiamycin resistance combination. Further studies are needed to fully explain the emergence of the penicillin/virginiamycin resistance combination among *E. faecium* from broilers. Due to differences between farms and possible co-selection, Figures 2–5 give a more precise description of the coherence between the probability of collecting an AGP-resistance *E. faecium* isolate compared to Figure 1.

Due to cross-resistances between the AGPs and important therapeutic antimicrobials: avilamycin (evernimicins), avoparcin (vancomycin), virginiamycin (quinupristin/dalfopristin) and tylosin/spiramycin (erythromycin, azithromycin, etc.), the withdrawal of the AGPs was expected to result in a decline in resistant *E. faecium* isolates and thereby to reduce human exposure to these resistances. The results from our study showed that there was a time-dependent quantitative reduction in the proportion of *E. faecium* isolates resistant to these antimicrobial

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agents. It is therefore likely that humans are now exposed to fewer *E. faecium* isolates resistant to AGP via broiler meat. To what extent resistances to avilamycin, erythromycin or virginiamycin persist in low levels at the farms has not been examined, therefore further research is needed.

Conclusions

The analyses showed that the probability that a randomly collected *E. faecium* isolate was resistant to avilamycin, erythromycin or virginiamycin was 0·91, 0·92 and 0·84, respectively, when the isolate originated from a flock fed either avilamycin- or virginiamycin-supplemented feed. As the time-span between sampling and the last AGP consumption increased the probability that a randomly selected *E. faecium* isolate was resistant to a particular AGP decreased (probability $<0{\cdot}2$ within 3–5 years after last exposure to AGPs). In addition, the decrease in probability of selecting an AGP-resistant isolate was very similar from farm to farm. The number of times a particular AGP was given to previous flocks reared in the same house was not significant.

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