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Effect of tetracycline dose and treatment-mode on selection of resistant coliform bacteria in nursery pigs

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Running title: Selection for tetracycline resistant coliforms

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ABSTRACT

This study describes results of a randomized clinical trial investigating the effect of oxytetracycline treatment dose and mode of administration on selection of antibiotic resistant coliform bacteria in fecal samples from nursery pigs. Nursery pigs (pigs of 4-7 weeks of age) were treated with oxytetracycline against *Lawsonia intracellularis* induced diarrhea in five pig herds. Each group was randomly allocated to one of five treatment groups: oral flock treatment with (i) high (20 mg/kg), (ii) medium (10 mg/kg) and (iii) low (5 mg/kg) dosage, (iv) oral-pen-wise (small group) treatment (10 mg/kg), and (v) individual intramuscular injection treatment (10 mg/kg). All groups were treated once a day for five days. In all groups, treatment caused a rise in numbers and proportion of tetracycline resistant coliform bacteria right after treatment, followed by a significant drop by the time where pigs left the nursery unit. Counts and proportion of tetracycline-resistant coliforms did not vary significantly between treatment groups, except immediately after treatment, where the highest treatment dose resulted in the highest number of resistant coliforms. A control group treated with tiamuline did not show significant changes in number or proportion of tetracycline resistant coliforms. Selection for tetracycline-resistant coliforms was significantly correlated to selection for ampicillin- and sulfonamide-resistant, but not to cefotaxime-resistant strains. In conclusion, difference in dose of oxytetracycline and the way the drug was applied did not cause significantly different selection of tetracycline resistant coliform bacteria, under the conditions tested.
Antimicrobial resistance is a global treat to human health. Treatment of livestock with antimicrobials has a direct impact on this problem, and there is a need to improve the ways that we use antimicrobial in livestock production. We hypothesized that antibiotic resistance development following treatment of diarrhea in nursery pigs could be reduced by either lowering the dose of oxytetracycline or by replacing the commonly used practice of flock treatment with individual or small group treatments, since this would reduce the number of pigs treated. However, the study showed no significant difference between treatment-groups with respect to the number or proportion of tetracycline resistant coliforms selected. The most important conclusion is that under the practical field conditions, there will be no added value in terms of lowering resistance development by exchanging flock treatment with individual or small group treatment of nursery pigs. The reason for lack of effect of single animal treatment is probably that such animals share the environment with treated animals and take up resistant bacteria from the environment.
INTRODUCTION

Antibiotic resistant bacteria are a recognized threat to public health. They cause increased mortality of infectious diseases (1), higher cost of treatments due to prolonged recovery time and use of more expensive antibiotics, and they increase the need for, and thus the cost of, biosecurity in hospitals (2). The same is true in veterinary medicine, where resistant bacteria increase the cost of treatment and may lead to animal welfare problems due to unsuccessful treatments (3, 4). For these reasons, it is important to reduce selection of antibiotic resistant bacteria as far as possible.

Antibiotic resistance in the animal sector can reach humans through the food chain, the environment, and by direct and indirect contact to animals and animal products (5, 6). While antibiotic resistant pathogenic bacteria are the immediate threat, antibiotic resistance in commensal bacteria of food animals is considered a reservoir of antibiotic resistance genes that may aggravate the problem (7). For example, surveillance results show 36% tetracycline resistance in commensal E. coli from pigs in Denmark (8). Thus, minimizing resistance in the commensal flora of food animals may be important in order to reduce the risk to human health from use of antibiotics in the livestock industry.

Enteric disease is very common in industrial pig production, especially in the nursery period (9). As a consequence, the highest single indication for use of antibiotics in the Danish livestock industry is treatment of diarrhoea in pigs in this period, and 42% of total antibiotic use for pigs in Denmark is for this indication, with tetracycline as the most used drug class (8). In order to reduce the total amount of antibiotics used in the pig industry, it is important to find more intelligent ways to treat enteric diseases in the nursery period.
Treatment of nursery pigs against diarrhea is often carried out using oral flock-treatment, where a full section of pigs is treated with antibiotic in the feed or water, when disease is seen in a pre-fixed proportion of the population. The justification for this approach is that apparently healthy animals in close proximity to diseased individuals are likely to be sub-clinically infected and will progress to develop clinical disease (10, 11). This batch treatment regime exposes the commensal intestinal flora of all pigs to a selective pressure, which is presumed to increase the total amount of resistant bacteria in farms significantly, when compared to treatment of individual pigs (12, 13). However, to the authors’ knowledge, this has not been investigated under field conditions.

Apart from the treatment regime (flock versus individual treatment), selection of antibiotic resistant bacteria are influenced by factors such as treatment-dose (14, 15), number of animals housed together (16), and other management factors (17-21). Among these factors, mathematical modeling suggests that dose may play a particularly important role for selection of resistant coliform bacteria following tetracycline treatment (22). Such modeling predicts that consumption of high doses of antibiotics is positively correlated to a subsequent high proportion of resistant fecal coliforms and to a longer time required for the proportion of resistant bacteria to non-resistant bacteria to return to pre-treatment equilibrium.

The aim of the present study was to determine the effect of five different oxytetracycline (OTC) treatment regimens with varying doses and varying modes of treatment on occurrence of antibiotic resistant coliform bacteria in nursery pigs in a randomized clinical field trial.
MATERIAL AND METHODS

Clinical field trial

The set-up of the randomized clinical field trial has previously been described in two studies measuring the efficacy of varying OTC treatment doses and treatment regimes (administration routes) for *Lawsonia intracellularis* diarrhea (11, 23), and the reader is referred to those two studies for a comprehensive description and for calculation of sample size. In brief, five herds with history of *L. intracellularis* induced diarrhea were pre-selected. Each herd had between 2300 and 3600 pen places, and an all-in all-out batch production in sectioned compartments. The flooring consisted of 1/3 solid floor and 2/3 slatted floor. In each herd 15 batches were included in the study after being weaned. At clinical signs of diarrhoea they were treated as described below and followed until at least the end of the seven-week nursery period. Where possible, pigs were also re-sampled in the week prior to slaughter. A batch was defined as a group of nursery pigs weaned at the same time and housed in a number of pens within one stable. In each batch 15 animals, randomly distributed over pens, were selected as trial pigs. The allocated treatment regimen, however, was applied to all pigs in the section as previously described (23). All trial pigs were ear tagged with a unique ID.

When a new batch was weaned, it was monitored once a week for outbreak of diarrhea. When an outbreak was detected, defined as at least 25 % pigs showing clinical signs of enteritis (watery feces, scouring of the back and/or a poor body score), pigs were subjected to one of five treatment regimens: oral flock-treatment in water with a standard dose of 10 mg/kg OTC (Terramycin®Vet. 20 %, Orion Pharma) for five days (ND, normal dose), oral flock-treatment in water with 20 mg/kg OTC for five days (HD, high dose); oral flock-treatment in water with 5 mg/kg
OTC for five days (LD, low dose), oral pen-wise (small group) treatment in water with a standard dose of 10 mg/kg OTC for five days (PW) or individual intra muscular treatment (IM) of pigs with diarrhea with a standard dose of 10 mg/kg OTC for five days. Pen-wise treatment was initiated when more than 25% of pigs in a pen had clinical signs of enteritis, while intramuscular treatment was initiated in animals showing clinical signs of enteritis. Flock treatment was administered through the common water supply, whereas pen-wise treatment was administered in water in troughs to pigs also having access to medicine-free water through the common water supply. Each treatment was repeated three times in each herd, and the order of the treatments was chosen at random. The number of pigs included from each farm in the different groups can be seen in Table 1. Outbreaks of diarrhea, and thus initiation of treatment, occurred from 2 to 6 weeks after weaning.

In order to be able to estimate selection of tetracycline-resistant coliform bacteria in pigs not exposed to tetracycline treatment, 25 pigs in one additional batch in herd A, suffering from an outbreak of diarrhea, were treated by oral flock-treatment with a standard dose (8 mg/kg) of tiamuline (Denagard®Vet, Novartis, Copenhagen, Denmark) for three days.

All pigs in the trial received 2500 ppm zinc-oxide supplement in the feed the first 14 days after weaning. Farmers were asked to keep record on all antibiotic treatments carried out in the herd before and during the field trial. This allowed controlling for confounding due to additional antibiotics treatments. A total of 889 pigs received antibiotic treatment before T1, and 402 pigs received treatment during the trial period between T2 and T3 (Supplementary material, Table S1). The treatments were farm specific: At one farm, pigs did very rarely received additional
treatments, neither before nor after the study treatment protocol. On three of the farms, the farmer regularly treated pigs with colistin shortly after entering the nursery unit, i.e. shortly before the trial period. On two of these three farms, other treatments than this was rare, while the remaining farmer additionally treated some pigs with doxycycline between T2 and T3. Finally, on one farm, pigs were often treated with amoxicillin before T1, but with no other treatments between T2 and T3. Antibiotic treatments between T3 and T4 were not consistently recorded and were thus not taken into account in the analyses. When analyzing for the effect of pre- and post-treatment with antibiotics there were no significant effect of the three largest additional treatment groups (colistin treatment before T1, amoxicillin treatment before T1, and doxycycline treatment after T2) on absolute number of tetracycline resistant coliform bacteria, proportion of tetracycline resistant coliforms or change in proportion of tetracycline resistant coliforms, and we concluded that these treatments were not confounders in our study.

Sampling

Faecal samples were collected from all trial pigs between October 2011 and April 2013, either at defecation or per rectum. Samples were collected from all pigs at three time points: Time point 1 (T1) was the first day of treatment, immediately before antibiotic administration, Time point 2 (T2) was two days after the end of the five day treatment, and Time point 3 (T3) was when pigs were moved from the nursery stables to finisher stables, either in the same herd or in other herds. When possible (n=296), a fourth sample (T4) was collected from rectum 1-7 days before slaughter. Samples were stored in 40 ml containers and shipped to the laboratory in cooled boxes.

Bacterial quantification
$10^1$ w/v suspensions were made from approximately 1 g of fecal sample in PBS, and one ml of this suspension was used for preparation of 10-fold serial dilutions from $10^{-2}$ to $10^{-4}$. Twenty μl of each dilution was plated on four MacConkey agar plates (Oxoid Ltd, Thermo Scientific, Roskilde, Denmark), containing different antibiotics (16 mg/L tetracycline, 16 mg/L ampicillin, 256 mg/L sulfamethizole, or 2 mg/L cefotaxime), and on a MacConkey agar plate without added antibiotics, using the principle of the drop plate method (24) with a 4x4 grid. Antibiotics were purchased from Sigma (Sigma-Aldrich, Copenhagen, Denmark). Antibiotic concentrations were based on EUCAST epidemiological cutoffs for *E. coli*, as recommended in (25).

Plates were incubated overnight at 37 °C followed by enumeration of dark red colonies with a size >0.5 mm. To confirm that colonies counted were coliforms, 100 colonies were randomly picked and subjected to species identification using Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS RUO, bioMérieux, France). All colonies were shown to belong to the species *E. coli* (data not shown). For each plate, a count expressed as colony-forming units (CFU) per gram were determined using a weighed arithmetic mean based on the two highest dilutions showing the separation between colonies, and finally CFU/g was $\log_{10}$ transformed. The detection limit for the method used was 500 colony forming units per gram of feces, corresponding to $\log_{10} = 2.70$.

In order to validate that this method distinguished between tetracycline resistant and susceptible isolates, at representative collection of commensal *E. coli* from Danish pigs, previously used to model the growth response of *E. coli* to antimicrobials (26) were tested. They consisted of 32 isolates with MIC between 0.24 μg/ml and 2.0 μg/ml (sensitive isolates) and 16 isolates with MIC
between 16 and 512 μg/ml (resistant isolates). They were grown in LB broth (Oxoid Ltd, Thermo
Scientific, Roskilde, Denmark) at 37 °C overnight. Ten-fold dilutions were made in PBS, and
dilutions were plated on McConkey agar without tetracycline and McConkey agar containing 16
μg/ml tetracycline. CFU was counted after 20 hours of incubation at 37 °C and the difference
between CFU estimation on the two plates was determined for each strain.

Statistics
The clinical trial was set up as a five-treatment-trial, and the statistical analysis for differences
between groups with respect to selection for resistant coliform bacteria was therefore carried out
with all groups in one analysis. The effects of the different treatment protocols on the number of
antimicrobial resistant bacteria were analyzed using either Log₁₀ transformed counts of resistant
bacteria or testing for significant changes in the square root of the proportion or change of
proportion of resistant bacteria, i.e. $\sqrt{\frac{R_{T_x}}{C_{T_x}}} \text{ or } \sqrt{\frac{R_{T_y}}{C_{T_x}}}$ where $R$ is the CFU/g count on the
antibiotic $R$ plate at time $T_x$ or $T_y$; and $C$ is the total CFU coliforms at time $T_x$ or $T_y$. Due to the
uncertainty of CFU counts, proportions could be higher than one; however, proportions above two
were considered outliers and excluded. The square root transformation was selected to improve
the normality of the residuals of the tests. Pigs with drop out data (data missing at any of the time
points $T_1$-$T_3$) were removed from the study, while drop out of data for $T_4$ had to be accepted
because only a small fraction was available for sampling.

Analyses were performed by Linear Mixed-Effects Models to determine significant differences in
resistant coliform bacteria and fraction of resistant bacteria from $T_2$-$T_3$ using lmer from the
package lme4 in R version 3.2.2 (27). When testing for the effect of treatment, farm ID and the interaction between farms and treatment were included as fixed effects, while batch of pigs was included as a random effect. To identify the significant effects, back wise elimination was performed using the step function and AIC (Akaike information criterion). Confidence intervals (CI) were found by bootstrapping using bootMer from the lmerTest package. Test of differences of multiple groups at single time points was done using Kruskal-Wallis Rank Sum Test (kruskal.test), while test for differences in numbers or proportions of resistant bacteria between different time points within group was done using Student’s t-Test (t.test), and correlation was tested using Pearson’s product moment correlation coefficient (cor.test), all in R (27).

Ethical statement

The clinical trial was approved by the Danish Medicines Agency (License no. 2011090862 / 2012053751), and the participating herd owners signed a written “Owner informed consent” explaining the scope of the field trial.

RESULTS

Effect of treatment-dose and treatment-regimes with OTC on selection of tetracycline resistant coliform bacteria

In total, 224 pigs received high dose as flock-treatment (HD), 241 pigs received normal dose as flock-treatment (ND) and 224 pigs received low dose as flock-treatment (LD). 241 pigs belonged to the pen-wise treatment (PW) group and 221 pigs to the individual intra muscular treatment (IM) group. In total, samples from 1167 animals were analyzed (Table 1).
The method used to count consisted of McConkey agar with added tetracycline. In order to validate that this method distinguished between tetracycline resistant and tetracycline sensitive coliform bacteria, 49 coliform strains were plated on agar with and without antimicrobials. The CFUs of cultures of sensitive strains were $7.0 \pm 0.5 \log_{10}$ units lower on plates containing tetracycline than on plates without antibiotic, and only one strain showed colonies. The corresponding values for resistant strains were difference of $0.3 \pm 0.5 \log_{10}$ units, and all strains showed colonies (Supplementary material, Figure S1).

**Effect of OTC dose on selection of tetracycline resistant coliform bacteria**

As can be seen from figure 1, variation between pigs with respect to $\log_{10}$ CFU/g tetracycline-resistant coliform was large in all groups at all time-points. The average number of coliform bacteria and tetracycline resistant coliform bacteria did not differ significantly between groups before initiation of treatment (T1) (Supplementary material, Figure S2 and Figure 1). On average, pigs carried $6.0 \pm 0.8 \log_{10}$ CFU/g total coliform bacteria and $5.5 \pm 0.9 \log_{10}$ CFU/g tetracycline resistant coliform bacteria at T1. Treatment irrespective of dose caused a significant rise in the number of tetracycline-resistant coliforms at T2 followed by a significant drop towards the time where pigs left the nursery unit (T3) (paired one-sided t-test, p<0.0005). The rise from T1 to T2 was highest in the HD group. In all three dose-groups, the average $\log_{10}$ CFU/g tetracycline-resistant coliform bacteria at slaughter were significantly below the T1 value (paired t-test, one-sided P<0.05). The proportions of tetracycline-resistant coliforms also increased significantly in all groups following treatment (paired one-sided t-test, P<0.005), but dropped to below the starting
point at slaughter (T₄) (Figure 2). The differences between proportions at T₁ and T₄, however, were not significant.

We analyzed for the overall effect of treatment-dose on the change in proportion of tetracycline-resistant coliforms between T₁ and T₃ using a mixed linear model. In this analyses farm was included as a fixed effect and batch as a random effect. We found no significant effect of treatment-dose. The only significant effect in the model was the random effect of batch.

**Effect of treatment mode on selection of tetracycline-resistant coliform bacteria**

The use of PW or IM treatment strategies, with the aim to treat fewer pigs than by flock-treatment, did not significantly affect the number of tetracycline-resistant coliform bacteria selected or the proportion of resistant coliforms at different timepoints. As for oral batch-treatment, the number and proportion of resistant bacteria at slaughter (T₄) was lower than before treatment (Figure 1 and Figure 2). The only significant effect in the logistic model here, too, was the batch effect.

In both the PW and the IM groups some pigs did not receive treatment (n=26 and n=79) (Table 1). The mean Log₁₀ CFU/g tetracycline resistant coliforms in these groups at T₃ (5.0 Log₁₀ CFU/g and 5.2 Log₁₀ CFU/g) were lower than the mean Log₁₀ CFU/g tetracycline resistant coliforms in the treated pigs (5.4 Log₁₀ CFU/g and 5.3 Log₁₀ CFU/g). The difference was significant in the PW group but not the IM group (two-sided t-test, p=0.01 and p=0.39) (Supplementary material, Figure S2). At T₄ there were no significant differences between treated and untreated pigs in PW group (p=0.06).
Control treatment with tiamuline

For animal welfare reasons, the clinical trial did not contain a non-treated, control group. Instead, a control experiment, where pigs suffering from *Lawsonia intracellularis* induced diarrhea were treated with an unrelated antibiotic, tiamuline, was conducted. As shown in Figure 3, treatment with this drug did not result in a significant increase in the number of tetracycline-resistant coliforms. Similarly, the proportion of tetracycline-resistant coliform bacteria did not change as a result of treatment. This showed that the effects seen after OTC treatment were specifically related to the use of this drug, and did not represent normal development in the coliform flora of nursery pigs.

Co-selection for other antibiotics

In all treatment groups, there were no significant differences in number of AMP, SUL and CTX resistant coliforms before initiation of treatment (data not shown). The counts showed a close, highly significant correlation between the changes in proportion of tetracycline-resistant coliforms from T1 to T2 and changes in proportion of ampicillin and sulfonamide resistant coliforms between the same time points (Pearson’s product moment correlation coefficient, P<0.0001), indicating that these resistances were selected together. On the contrary, no significant correlation was observed between tetracycline- and cefotaxime-resistant coliforms (data not shown).

Nevertheless, 282 out of the 1167 pigs analyzed were found to carry cefotaxime-resistant coliforms at T1 (average Log10 CFU in positive pigs was 3.2 with a range from 2.7 (detection limit) to 7.0 Log10 CFU/g), and at least one pig in all farms were positive for cefotaxime-resistant coliforms.

Discussion
The purpose of this study was to estimate the effect of OTC treatment dose and treatment regimes on selection of tetracycline resistant coliforms in nursery pigs under field conditions. We used an easy agar-dilution counting method, based on including breakpoint concentrations of OTC to McConkey plates. This method has previously been validated for use with McConkey agar and added tetracycline (28), however, with 8 ug/ml as the added concentration of tetracycline. We performed our own method validation with 16 ug/ml tetracycline added to the plates, and found that this, too, gave 100 % ability to distinguish between tetracycline sensitive and resistant coliforms.

In accordance with a previous study (14), we observed a significantly higher number of tetracycline resistant *E. coli* right after the treatment in the group receiving the highest dose, but in contrast to the previous publication, the concentration and proportion returned to the starting level within 3-4 weeks. Proportions of resistant coliforms at T4, corresponding to shortly before slaughter and thus the time where the pigs enter the food chain, was significantly below the before treatment level. Thus, pigs receiving a high dose of tetracycline may shortly show higher level of resistant bacteria, but according to our results, they do not possess a higher risk of transfer of resistant bacteria to consumers.

Reports on proportion of tetracycline resistance in randomly collected *E. coli* from pigs in Denmark have been published since the 1970ties (29). Comparison between these old studies and results of the current surveillance program in Denmark (30) shows that the mean proportion of tetracycline resistant commensal *E. coli* has varied over the years, however, it seems never to clime above approximately 40 %. A possible reason for the minimal selective effect of dose in our study may be
the very high starting concentrations of resistant bacteria. While this is representative for proportions of tetracycline resistant commensal *E. coli* in pigs in Denmark (30), it is much higher than the 1-10 % chlortetracycline-resistant *E. coli* detected by Delsol et al (14) prior to their experiment. Our results may thus not be representative for farms with an initial lower concentration of tetracycline-resistant bacteria. Compared to previously published studies, a high number of pigs were included in the present study, and conclusions must be considered strong. Still, the trails were only conducted in five different herds with quite similar management practices. We cannot rule out that under very different management practices, results would have been different.

Previous studies on the effect of dose on selection of resistance have generally been concerned with differences between therapeutic and sub-therapeutic concentrations of antibiotics (see meta-analysis (31)). In contrast, we considered therapeutic doses. Putting results together, and including studies from poultry as well, there seems to be minimal effect of treatment dose on selection of resistant indicator bacteria (31, 32). This indicates that within quite broad ranges, veterinarians might change dose to achieve a better treatment efficacy, without changing the selection of resistant bacteria significantly. It should be noted that while 5 mg/kg, corresponding to the low dose used in the current study, is sufficient to reduce *L. intracellularis* below the threshold for pathological changes in the intestine of pigs, it takes 10 mg/kg to eliminate the bacterium to non-detectable levels (23).

On a population level, there is a direct association between the intensity of use of antibiotics and the proportion of bacteria resistant to such antibiotics. This has been demonstrated for clinical as
well as indicator bacteria and from both humans (33, 34) and farm animals (35), though the
relation is not always straight forward (36). As a consequence, there is a tendency to argue against
flock treatment of farm animals. A large proportion of the reduction in amount of antimicrobials
used in the Netherlands to treat farm animals has been reported to be due to restricted use of
flock treatment (37), and legal restrictions specifying certain pre-conditions on use of flock
medication have been gradually introduced in Denmark. Although phasing out oral flock-
treatment leads to less antibiotic usage, it has never been thoroughly investigated whether this
also leads to less resistance under field conditions, where untreated animals are housed in close
proximity to treated animals, and we tried to answer this question in the current study.

Surprisingly, we did not observe any significant differences in selection of tetracycline resistant
coliform bacteria when we compared oral flock to oral pen-wise (small group) and single animal
IM treatments. This is difficult to explain, given that the overall use of OTC was 15 % and 44 %
lower in the PW and IM treatment groups. A detailed analysis of our results showed that
untreated pigs in the PW group, but not in the IM group, had significantly lower counts of
tetracycline resistant coliforms than the treated pigs in the same groups. The most like
explanation for the lack of difference in in the individual treatment group is that they shared the
environment (the pen) with treated pigs, and thus were exposed to high number of tetracycline
resistant coliforms that were excreted from treated pigs. Contrary to this, untreated pigs in the
PW group always shared the pen with untreated pigs.

The lack of overall difference between PW and ND groups, we believe, is simply a matter of
numbers. The vast majority of pigs in the PW groups got treated, because the pen fulfilled the
criterion for treatment against diarrhoea. In that respect, our study confirms previous observations that once diarrhoea is observed in a fraction of the nursery pigs, there is a high risk that the remaining pigs are sub-clinically infected (10). Taken together, our results, nevertheless indicated that a form of treatment, where treated pigs are separated from untreated pigs, might be a better strategy for reducing antimicrobial resistance than individual treatment, where treated and untreated pigs share the same pen. PW and IM treatments with OTC have been shown to be ineffective compared to flock-treatment for treatment of *L. intracellularis* diarrhoea (11). When this observation is combined with our results, continued use of oral flock-treatment seems justified, at least as far as conditions are similar to those investigated in the current study. In the study of treatment efficacy (11), the authors argued that oral flock-treatment may be needed as long as there are no good, rapid and precise diagnostic methods for detection of individual pigs with intestinal disturbance, since pigs with intestinal disturbance may go unnoticed with current diagnostic procedures. This puts emphasis on improved diagnostics corresponding well to the WHO action plan against antimicrobial resistance, which emphasise the need for development of improved diagnostic tests in the fight against antibiotic resistance (38). The results of the current study might also indicate that measuring antibiotic consumption is not always a good surrogate for measuring antimicrobial resistance, even though this is currently one of the cornerstones in national surveillance programs on antibiotic resistance.

For animal welfare reasons, we could not leave pigs untreated when outbreaks of diarrhoea were present. To be able to control for natural development in the coliform flora, we chose instead to treat a batch with tiamuline, a drug belonging to the groups of pleuromutilins and used exclusively in veterinary medicine. As this drug does not select for tetracycline resistant coliforms, this group...
could be used to create a baseline for natural fluctuation in numbers and proportions of tetracycline resistant coliforms in nursery pigs. The results showed that the fluctuations we observed in tetracycline treated pigs in the clinical trials were associated with the OCT treatment and were different from the fluctuations in pigs treated with tiamuline.

Langlois et al. (39) showed that pigs in herds with a history of previous routine use of antibiotics developed higher numbers of tetracycline-resistant coliforms following chlortetracycline treatment than pigs from another herd without such a history. During and before the current clinical trial, farmers were allowed to treat pigs for other diseases, when needed. Treatments between birth and T1 may very well influence selection between T1 and T2 by having pre-selected for tetracycline resistant coliforms. However, we systematically collected data on consumption of antibiotics in the period from T1 to T3 and analysed for the effect of pre- and post-treatment with other antibiotics on selection for tetracycline resistant coliforms. The results showed no significant effect of the three most commonly additional treatments and we ruled out additional treatments as a confounding factor. After time point T3, pigs were distributed to different fattening units, and only a fraction of pigs were re-sampled at T4. No records on antibiotic use were available to us covering the time periods from birth to T1 and between T3 and T4. We cannot rule out that treatment between T3 and T4 may be the reason for lack of differences between groups at T4. However, in general, number of treatments in the fattening period are far below the number in the nursery period in Danish pig production (8), making this less critical for the current study.

The fact that flock and pen-wise (small group) treatment was carried out as water medication introduced an uncertainty with regard to dose obtained by the individual pig. We ensured that the
dose given to the flock and the pen was consumed (in total), but we could not ensure that all pigs received equal treatment. This means that dose in flock treated and pen-wise treated groups is an average of pigs, and there will be variation between pigs. Similarly, treatments (T₁) were initiated when the clinical inclusion criterion was fulfilled, while T₃ (end of the nursery period) was a fixed date for each pig. This introduced variation in the duration of the period between T₁ and T₃, and this too may be a factor in lack of significant differences between treatments. On the other hand, this is the situation in real life, and our results represent the naturally occurring variation in dosing and treatment time under field conditions.

Besides being tetracycline resistant, commensal E. coli from food animals in Denmark are commonly resistant to ampicillin and sulphonamides (8), indicating co-selection, and a study from the United States indicated that tetracycline treatment of calves could lead to co-selection for resistance genes encoding 3rd and 4th generation cephalosporin resistance (40). It has been reported that commensal tetracycline-resistant E. coli are often resistant to ampicillin and further they may carry class-1 integrons encoding sulfonamide resistance genes (41). To test whether tetracycline treatment resulted in specific increase of coliforms with other resistance markers, all samples were also cultured on MacConkey agar containing ampicillin, sulfonamide or cefotaxime. The latter drug was included to investigate possible selection of extended-spectrum beta-lactamase (ESBL)-producing bacteria, which constitute a growing health concern (42). In the current study, selection of tetracycline-resistant coliforms from T₁ to T₂ was significantly associated with selection for ampicillin- and sulphonamide-resistant coliforms. Since we have not characterized the bacteria counted in the current study, we cannot prove that this is co-selection caused by co-localization of the resistance genes, but the observation is hard to explain by any
other mechanisms. One of the most prominent antibiotic resistance threats to human health is the growing prevalence of ESBL producing Gram-negative bacteria (43). In the current study we found that ESBL producing coliforms could be identified in all farm and on average approximately 20 % of the pigs were shown to be carriers. However, there is currently no indication that pigs are and important reservoir for ESBL infection in humans in Denmark (8), and based on our results, the use of tetracycline can be ruled out as a (co)selection factor for such bacteria. The Danish pig industry does not use cephalosporin drugs, and due to this the prevalence of ESBL has decreased rapidly in recent years (35).

Several studies have been published recently, modelling development of tetracycline resistance in pigs following different treatment scenarios (22, 44-46). Such models have been fed with data on growth responses in *E. coli* to different concentrations of tetracycline. In relation to our study, the multi-strain, multi-pig model by Græsbøll et al. (22) is the most relevant. This model predicts, that high dose will results in a higher proportion of tetracycline resistant bacteria than low dose. In that sense our field study is in agreement with the results of the model. However, the modelling also predicts that the proportion will return to pre-treatment level in a dose dependent manner. This prediction was not confirmed by our field study. At T3 there was no significant difference between treatment groups.

Measuring resistance in coliform bacteria is a widely used method for studies of development of antibiotic resistance in bacterial populations, both in the society in general and in intervention studies (47), but it is a narrow approach. It is therefore indicated to make follow up studies where one looks at the changes in the microbiome in general, since not only coliform bacteria will be a
risk for transfer of resistance genes to human pathogenic bacteria through the food chain. Such studies should preferably be carried out using culture independent techniques.

In conclusion, the current study showed that dose of oxytetracycline during flock treatment and mode of application did not have a significant influence on the selection of coliform bacteria in the intestine of nursery pigs, under the conditions tested. This means that doses can be set putting emphasis on consideration to efficacy and prize of treatment, and that, from an antibiotic resistance point of view, there appears to be no benefits from using single animal treatment, unless treated animals are separated from non-treated pen-mates.

Acknowledgement

This study would not have been possible without the constructive collaboration from the owners and workers in the five participating pig herds. The authors acknowledge this valuable contribution. Pia Rønnow Mortensen, Tony Bønnelycke are thanked for skillful technical assistance. The study was supported by a grant from the Danish Innovation Fund (Grant number 0603-00385B).

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Anon. 2010. DANMAP 2010 - Consumption of antimicrobial agents and antimicrobial resistance in bacteria from food animals, food and humans in Denmark. DANMAP - The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme.


Table 1. Overview of number of pigs included in the study in each treatment group, as distributed on the five participating farms.

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T1 – T4 refer to the time points where samples were obtained. T1 was immediately before treatment, T2 was to days after end of treatment, T3 corresponded to the time where pigs left the nursery unit, and T4 was 1-4 days before slaughter.
Figure 1. Box plot illustrating Log_{10} CFU/g tetracycline resistant coliforms in fecal samples from pigs at different time points relative to treatment with different doses of OTC or given OTC by different modes of treatment. Normal, Low and High refer to groups of pigs subjected to five days of oral OTC batch treatment using 10 mg/kg (ND), 5 mg/kg (LD), and 20 mg/kg (HD) dosages, respectively. Injection (IM) and Pen (PW) refer to groups treated with 10 mg/kg OTC for five days individually by injection and pen-wise. T1-T4 refers to the time points where fecal samples were obtained: T1: immediately before treatment, T2: two days after end of treatment, T3: when pigs left the nursery unit, T4: 1-7 days before slaughter. The boxes indicate the interquartile range. The open circles indicate data points more than 1.5 times the interquartile range from the median.
Figure 2. Box plot illustrating the square root of proportions of tetracycline resistant coliforms in fecal samples from pigs at different time points relative to treatment with different doses of OTC or with different treatment modes. Normal (ND), Low (LD) and High (HD) refer to groups of pigs subjected to five days of oral OTC batch treatment using 10 mg/kg, 5 mg/kg, and 20 mg/kg dosages, respectively. Injection (IM) and Pen (PW) refer to groups treated with 10 mg/kg OTC for five days individually by injection and pen-wise treatment, respectively. T1-T4 refers to the time points where fecal samples were obtained: T1: immediately before treatment, T2: two days after end of treatment, T3: when pigs left the nursery unit, T4: 1-7 days before slaughter. The boxes indicate the interquartile range. The open circles indicate data points more than 1.5 times the interquartile range from the median.
Figure 3. Log$_{10}$CFU/g tetracycline-resistant coliforms (A) and proportion of tetracycline resistant coliforms (B) in fecal samples from pigs treated orally as batch-treatment with tiamuline for three days. T$_1$-T$_3$ refers to the time points where fecal samples were obtained: T$_1$: Immediately before treatment, T$_2$: Two days after end of treatment, T$_3$: When pigs left the nursery unit. The boxes indicate the interquartile range. The open circles indicate data points more than 1.5 times the interquartile range from the median.