Veterinary Microbiology 208 (2017) 1–7

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Effect of different oral oxytetracycline treatment regimes on selection of antimicrobial resistant coliforms in nursery pigs

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ARTICLE INFO

Keywords:
E. coli
Pig
Antimicrobial resistance
Oxytetracycline treatment regime
Co-selection

ABSTRACT

A major concern derived from using antimicrobials in pig production is the development of resistance. This study aimed to assess the impact of selected combinations of oral dose and duration of treatment with oxytetracycline (OTC) on selection of tetracycline resistant (TET-R) coliforms recovered from swine feces. The work encompassed two studies: 1) OTC 5 mg/kg and 20 mg/kg were administered to nursery pigs for 3 and 10 days, respectively, under controlled experimental conditions, and 2) 10 mg/kg, 20 mg/kg and 30 mg/kg OTC were given to a higher number of pigs for 6, 3 and 2 days, respectively, under field conditions. Statistical modeling was applied to analyze trends in the proportion of TET-R coliforms. In the experimental study, no statistical difference in proportion of TET-R coliforms was observed between treatments at the end of the trial (day 18) and compared to day 0. In the field study, treatment had a significant effect on the proportion of TET-R bacteria two days after the end of treatment (2dAT) with the regimes "low dose-six days" and "medium dose-three days" yielding the highest and lowest proportions of TET-R strains, respectively. No indication of co-selection for ampicillin- and sulphonamide -R bacteria was observed for any treatment at 2dAT. By the end of the nursery period, the proportion of TET-R bacteria was not significantly different between treatments and compared to day 0. Our results suggest that similar resistance levels might be obtained by using different treatment regimes regardless of the combinations of oral dose-duration of treatment.

1. Introduction

Antimicrobial resistance is recognized as a global health problem, and the World Health Organization considers it as one of the top health challenges facing the 21st century (FDA, 2000; CDC, 2014). The persistent increase in resistance is alarming, and the occurrence of high resistance levels continues to threaten the ability of both doctors and veterinarians to treat infections.

For many years, the association between antimicrobial resistant bacteria in humans and antimicrobial use in food animals has been debated (Jones and Ricke, 2003; Phillips et al., 2004; Chiller et al., 2004; Alpharma, 2007; Cox and Ricci, 2008; Falgenhauer et al., 2016). Based on a large amount of data, however, it is now evident that use of antimicrobials in food animals impacts human health through direct transfer of resistant bacteria, and more distantly through the food chain and the environment (Levy et al., 1976; Holmberg et al., 1984; Hummel et al., 1986; Fey et al., 2000). Since it is unrealistic and unethical for animal welfare reasons to completely avoid the use of antimicrobials in intensive livestock production, it is important to identify the antimicrobial applications in livestock that might have the highest impact on human health, and to minimize the development of resistance without compromising treatment efficacy.

Escherichia coli is a common facultative anaerobic bacterium in the intestinal microbiota of humans and animals (Karami et al., 2006), and it is therefore one of the commensal bacteria commonly used as an indicator in different types of studies in animals, humans, and food products (Karami et al., 2006; EFSA, 2012). Its ubiquitous presence in mammals and indications of resistance occurrence in the bacterium make it a good candidate for studies on antimicrobial selection pressure in the population (Vieira et al., 2011).

In Denmark, the swine sector accounted for ca. 80% of the veterinary use of antimicrobials in 2012 and tetracycline was the most frequently used drug in pig production (Apley et al., 1998; McDermott et al., 2002; DANMAP, 2013), commonly administered to treat

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http://dx.doi.org/10.1016/j.vetmic.2017.07.005
Received 21 September 2016; Received in revised form 8 June 2017; Accepted 6 July 2017

intestinal diseases in nursery pigs (Roberts, 1996; DANMAP, 2013). Approximately 36% of *E. coli* isolates were tetracycline resistant (TET-R) in the Danish antimicrobial resistance surveillance system of pigs in 2012 (DANMAP, 2013). Furthermore, this surveillance has shown high levels of TET-R *E. coli* (over 30%) over the past five years, suggesting that TET-R *E. coli* are endemic in the pig production. Tetracycline is also used in humans, accounting for 11% of the total consumption of antimicrobials in the Danish health care sector in 2012 (DANMAP, 2013), and resistance to this antimicrobial is very common in *E. coli* from humans (Calva et al., 1996; Karami et al., 2006; Marshall and Levy, 2011).

In previous studies, where a mathematical model was developed and used to simulate selection of tetracycline resistance following treatment (Græsbøll et al., 2014; Ahmad et al., 2015), it was predicted that all the doses tested led to a temporary advantage for TET-R strains compared to the sensitive ones in the intestine of nursery pigs. It was also predicted that the total amount of antibiotic used and the duration of treatment affected selection of resistance, as well as the time it took for the intestinal flora to get back to equilibrium. Based on these observations, the aim of the present work, encompassing two in vivo studies, was to analyze the effect of different tetracycline treatment regimes on emergence and selection of TET-R coliforms in the gut of nursery pigs. Also, co-selection for ampicillin (AMP) and sulphonamide (SUL) resistant bacteria was investigated.

2. Material and methods

2.1. Animals, experimental setup and ethical issues

Two studies, an experimental trial and a study under field conditions were performed.

For the experimental study, 24 seven-to-eight weeks old nursery cross-bred sex-mixed pigs (11–18 kg) were purchased from a specific-pathogen-free farm in Denmark. The animals were housed in a level 1 isolation unit at University of Copenhagen and weighed at least once a week. Animal experiments were carried out according to the Animals Scientific Act and after having obtained the license and approval of the Danish National Animal Experiment Inspectorate (license no. 2009/561-1675). At the end of the study, all pigs were euthanized by captive bolt pistol penetration followed by bleeding.

Pigs in the experimental trial were divided into five groups housed in five well-separated pens avoiding contact between them. After one week of acclimatization, four groups including five pigs each received a specific oxytetracyline (OTC) treatment as follows: groups 1 and 2; low dose of antibiotic (5 mg/kg) for three and 10 days (Do5.Dur3 and Do5.Dur10), groups 3 and 4; high dose of antibiotic (20 mg/kg) for three and 10 days (Do20.Dur3 and Do20.Dur10). Group 5, was not treated (Do0.Dur0) (Table 1). Terramycin®Vet. 20% solution (Orion Pharm, Copenhagen, Denmark) was orally and individually administered to all pigs at the specific dose in nutri-drink (Nutricia, Allerød, Denmark).

For the field study, 120 pigs were randomly selected at one specific farm in Denmark where pigs were housed under regular pig production conditions. Permission to perform these experiments was granted by the Danish Medicines Agency (license no. 2011090862/2012053751) and a written "Owner informed consent" was signed by the owner of the herd involved in the study.

The nursery pigs used in the field study were divided into six pens containing 20 pigs each. Treatment with OTC was started at week four after weaning. Pigs in different pens received the following OTC treatments (in duplicate): groups 1 and 2; low dose of antibiotic (10 mg/kg) for six days (Do10.Dur6), groups 3 and 4; medium dose (20 mg/kg) for three days (Do20.Dur3) and groups 5 and 6; high dose (30 mg/kg) for two days (Do30.Dur2). It was not possible to include a control group under field conditions. All treatments were implemented at the pen level, and were randomly allocated to pen by draw. Terramycin®Vet. 20% was administered orally through drinking water, through a dosing pump, and it was controlled that all the medicine was consumed within 24 h (Larsen et al., 2016).

2.2. Collection and microbiological analysis of fecal samples

Fecal samples (ca. 5 g) were collected from the rectum of all the pigs prior to antimicrobial treatment (day 0) and every second day over a period of 18 days (experimental study) and before starting the treatment (day 0), at two and 10 days after having finished the treatment (2dAT and 10dAT), as well as by the end of the nursery period (EN; 20 days after day 0) (field study). At every collection time CFU counts were performed. For this, serial 10-fold dilutions in PBS were prepared and inoculated on MacConkey agar (Oxoid, Thermo Scientific, Roskilde, Denmark) without antibiotic and on MacConkey agar supplemented with 16 μg/ml TET (both studies) and 16 μg/ml AMP or 250 μg/ml SUL (field study). Antibiotics were purchased from Sigma (Sigma-Aldrich, Copenhagen, Denmark). All counts were determined by the spot method (Cavaco et al., 2008). Briefly, 20 μl of each dilution was inoculated as a spot on two plates, followed by 24 h of incubation at 37 °C. Deep red colonies with a diameter of > 0.5 mm were counted. The species of one hundred such colonies had previously been tested by MaldiToff and all of them were shown to be *E. coli* (Katakweba et al., 2015). Such a control was not performed in the current study, and we will use the term coliforms, even though they are likely to be *E. coli*.

2.3. Statistical analysis

Average $\log_{10}$ transformed CFU of TET-R coliforms was compared between treatment groups (experimental study) using ANOVA with Turkey's multiple comparison test in GraphPad Prism, version 6.0 (GraphPad software, La Jolla, USA). Statistical modelling of CFU data was performed using a generalized linear model in the statistical software R (Version 3.2.5). The count data was assumed to be Poisson distributed, and in case of over dispersion this was relaxed to so-called quasi-poisson. The dilution was used as offset for each count. For the two distributional assumptions, ChiSq- and F-test with Pearson residuals (Venables and Ripley, 2002) were performed, respectively. In all

Table 1
Tetracycline treatment regimes used in experimental and field studies.

Study	Dose/duration (days)	Number of pigs (group)	Collection time (Day)[a]	Antibiotics used for selection
Experimental	Low (5 mg/kg)/3	5 (1)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	High (20 kg/kg)/3	5 (2)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	Low (5 mg/kg)/10	5 (3)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	High (20 kg/kg)/10	5 (4)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
	0/0	4 (5)	0,2,4,6,8,10,12,14,16,18	TET, no antibiotic
Field	Low (10 mg/kg)/6	40 (1 and 2)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic
	Medium (20 kg/kg)/3	40 (3 and 4)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic
	High (30 mg/kg)/2	40 (5 and 6)	SN,0,2dAT,10dAT,EN	AMP, TET, SUL, no antibiotic

[a] Day 0; day starting the treatment, day 2dAT and 10dAT: two and 10 days after having finished the treatment. SN; start of nursery period, EN; end of nursery period.

models interactions with the presence of antimicrobials were the focus. The CFU count in every sample was included as a nuisance parameter in all models. CFU counts were log transformed using the natural logarithm used as a link function. A *P*-value (P) < 0.05 was considered statistically significant.

The analysis was performed for tetracycline resistance (experimental and field study) as well as for co-selection of other antimicrobial resistances (field study). If three or more dilutions from the same sample on the same medium had positive counts, then, only the two least diluted counts were included to reduce over-dispersion. Only samples having counts both with and without antimicrobials in the media were included. For all individual samples an estimate of the proportion of resistant bacteria was made. If the proportion was above 100%, then the probability of being below 100% was calculated, and the sample was discarded if this probability was less than 0.1% (see Discussion).

The total CFU and CFU of resistant strains were also estimated for each treatment at each day in the experimental study. This was done using a generalized linear model with counts from individual pens on the individual days.

3. Results

3.1. Effect of different OTC treatment regimes on selection of TET-R coliforms under experimental conditions

An experimental study was first conducted in order to analyze which combinations of doses and duration of treatment might result in less development of resistance. Due to ethical reasons and cost of the experiments, only four (control group) or five pigs (groups subjected to treatment) were included in each treatment group.

Trends in CFU counts of total coliforms were analyzed over time for the four treatment groups (Fig. 1A). Slightly different trends in CFU counts of total coliforms were observed between the four treatments regimes, however, these were not statistically significant (Fig. 1A). The general trend was a decrease of the overall coliform population in all the groups regardless of the treatment over time (from day 0 to day 18). On the second day of treatment, a peak (not significant increase compared to day 0) in total number of coliforms was observed in three out of the four treatment groups (both groups treated with low doses and the group treated with high dose for a short period). In the group treated with a high dose for 10 days, the peak (not significant increase compared to day 0) was observed on day eight. In all treatment groups, the peak was followed by a decrease (not significant) on the following time point. At this time point, the CFU counts were not significantly different from those obtained at day 0 either. In the non-treated control group a peak like the one detected for the treated groups was not observed at any time point.

The number of TET-R coliforms showed a similar trend to the one detected for the total numbers of coliforms (Fig. 1B), indicating that the peak in total coliforms during the treatment period in all groups was caused by selective growth of TET-R coliforms. The average CFU count of TET-R coliforms was not significantly different between groups on day 0. On day four, where short duration treatments (three days) were over, only the average count of TET-R strains for the low dose for three days treatment group (Do5.Dur3) was not significantly different from the average count of the control group. On day 12, two days after the long treatments (10 days) ended, the average count of none of the groups was significantly different from the average count in the control group, and the low dose for three days treatment group (Do5.Dur3) showed a significantly lower average count than the high dose for 10 days group (Do20.Dur10). The highest numbers of TET-R coliforms by the end of the experiment (day 18) were detected in the two groups treated with high dose (significantly higher than the low dose treatment groups). However, at this time point, the average count of TET-R isolates of none of the treatment groups was statistically different from the average count in the control group. The group subjected to the low dose for three days regime showed a significantly lower average count than the three other treatment groups.

The proportion of TER-R coliforms over time was also analyzed (Fig. 1C). Before the start of the treatment, approximately half of the coliform population was TET-R. In the groups treated with high doses of TET, the proportion of TET-R coliforms approaches 100% shortly after treatment. On day four, a significant increase of the proportion of TET-

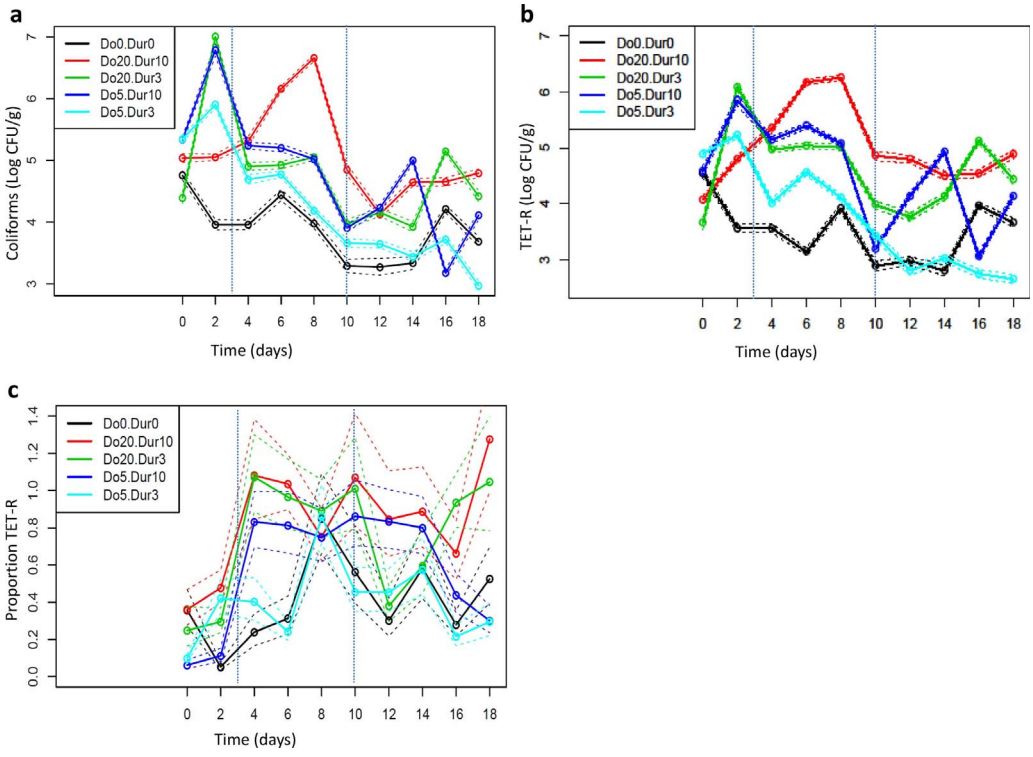

Fig. 1. Microbiological analysis of faecal samples from pigs treated in the experimental study with four OTC different treatment regimes. Log_{10}CFU counts of total coliforms (a), Log_{10}CFU counts of TET-R coliforms (b) and proportion of TET-R coliforms (c).
Do0.Dur0; no treatment administered; Do20.Dur10; high dose (20 m/kg)–10 days regime; Do20.Dur3; high dose (20 mg/kg)-three days regime; Do5.Dur10; low dose (5 mg/kg)–10 days regime; Do5.Dur3; low dose (20 mg/kg)-three days regime.
Discontinued vertical arrows indicate the end of the OTC treatment regime.
The dashed lines are 95% confidence intervals

R strains was observed compared to day 0 in all the groups, no matter the treatment. On day 18 (end of the experimental trial), no significant differences were observed for any of the groups compared to day 0.

Statistical modeling of the results showed that the proportion of TET-R strains did not vary significantly over time between groups ($P > 0,05$) but on the final day of the experiment (day 18) the two groups receiving the high dose showed the highest proportion of TET-R coliforms.

Contrary to this, the model showed that the CFU/g varied greatly between animals and over time ($P < 0.05$), which indicated that each pig responded individually to the treatment.

3.2. Effect of OTC treatment regimes on selection of TET-R coliforms under field conditions

While the results of the experimental study lacked clear results in terms of statistically significant results on the proportion of TET-R coliforms after treatment, the trend was that the group subjected to the regime low dose for a short period would have significantly fewer TET-R coliforms compared to the rest of the groups (Fig. 1C). Since we saw very little difference between the two groups that received high dose, we hypothesized that dose was more important than duration of treatment with regard to selection for resistance. In order to test this hypothesis, we performed a field study, where all pigs received the same total amount of OCT, but split over different number of treatment days. This allowed us to study the effect of dose without the confounding factor “total amount of antimicrobial”, since all pigs received the same total amount of OCT. The number of pigs and the conditions used were those used under regular pig production conditions in Denmark.

Also under field conditions, the trend in total numbers of coliforms was very similar in all the groups and with no significant difference between groups at any time-point (Fig. 2A). A steep significant decrease was observed in the first weeks after weaning (between the start of the nursery period and day 0) in all the three treatment groups. At 2dAT a small peak (not significant increase compared to day 0) was detected in the treatment groups; Do10.Du6 and Do20.Du3, followed by a decrease afterwards. At the end of the nursery period, the CFU counts of coliforms for all the groups were significantly lower from those detected at the start of the nursery period (SN) ($P < 0,05$) although not significantly different from the counts at day 0 (Fig. 2A).

A similar trend in CFU counts, with no significant difference between groups at any time point, was observed for TET-R coliforms (Fig. 2B). Also here, a small peak (not significant increase compared to day 0) was detected at 2dAT for Do10.Du6 and Do20.Du3, followed by a decrease.

As shown for the experimental trial, the proportion of TET-R coliforms was already between 20 and 40% of the population before the start of the treatment (day 0) (Fig. 2C).The proportion of TET-R increased as a response to treatment, however, at the end of the nursery period, there was not significant different in proportions between groups.

The changes in proportion of TET-R coliforms following tetracycline treatment was subjected to statistical modeling. A total of 2158 counts were made of which 142 were left out due to obvious mistakes in dilution series and another 36 counts were left out due to lack of completeness (lacking data for either total coliforms or TET-R coliforms) or infeasible proportion of resistance (see Material and methods). The remaining 1980 counts were included in the analysis. A three-way interaction with antimicrobial, time point and treatment was included in the model to describe the change in the proportion of TET-R coliforms. There was clear over-dispersion of data, and the dispersion parameter for the quasi-poisson model was estimated to be 7.61. The three-way interaction was significant ($P < 0.05$), meaning that evolution in proportion of TET-R coliforms was different over the tested time points between treatments.

To test the influence of the treatment on the proportion of TET-R bacteria at the end of the nursery period, the samples on day 0 and EN were merged and tested against the full model. It was found that treatment did not change the proportion of TET-R significantly ($P > 0.05$). Furthermore, it was tested if there was an immediate effect of the treatment by comparing day 0 and EN with 2dAT. Here a significant difference was found ($P < 0.05$), meaning that treatment increased the proportion of TET-R bacteria immediately after treatment.

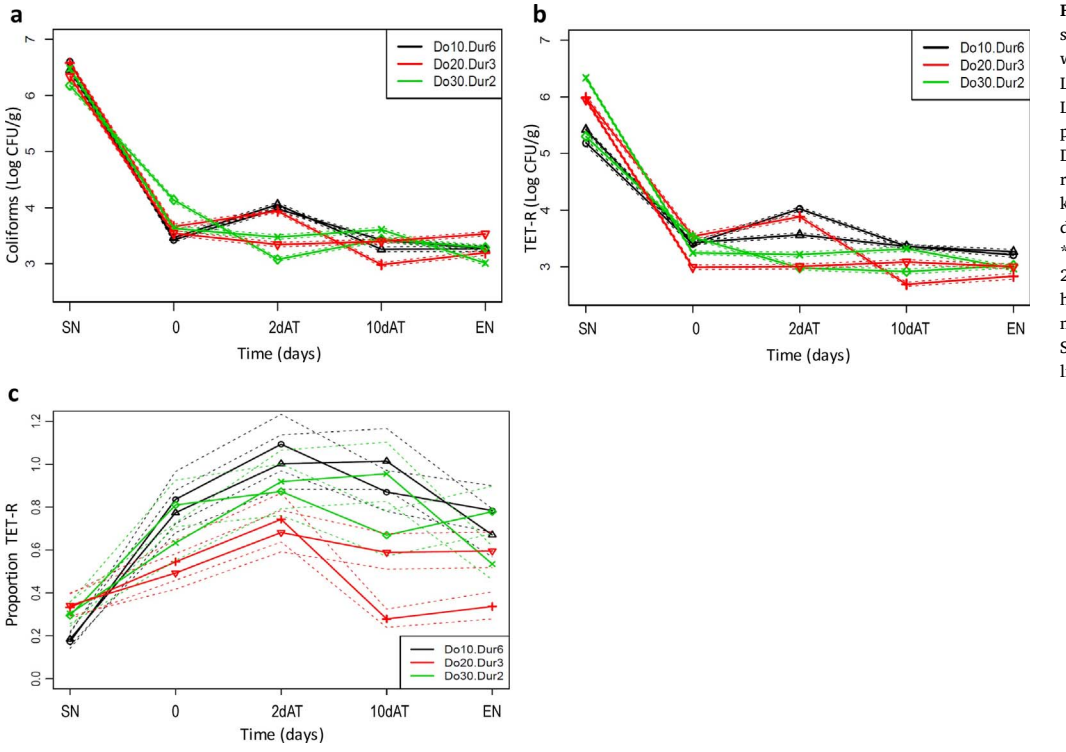

Fig. 2. Microbiological analysis of faecal samples from pigs treated in the field study with three different OTC treatment regimes. Log_{10}CFU counts of total coliforms (a), Log_{10}CFU counts of TET-R coliforms (b) and proportion of TET-R coliforms (c).
Do10.Dur6; low dose (10 mg/kg)-six days regime; Do20.Dur3; medium dose (20 mg/kg)-three days regime; Do30.Dur2; high dose (30 mg/kg)-two days regime.
*Day 0; day starting the treatment, day 2dAT and 10dAT: two and 10 days after having finished the treatment. SN; start of nursery period, EN; end of nursery period.
Six different symbols and six color codes are linked to each of the six treatment groups.

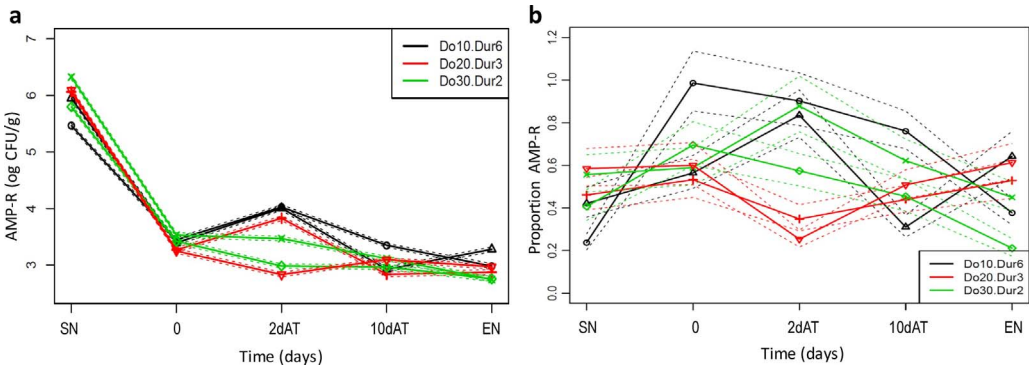

Fig. 3. Analysis of co-selection for AMP-R in the field study with three different OTC treatment regimes. Log_{10}CFU counts of AMP-R coliforms (a) and proportion of AMP-R coliforms (b).
Do10.Dur6; low dose (10 mg/kg)-six days regime; Do20.Dur3; medium dose (20 mg/kg)-three days regime; Do30.Dur2; high dose (30 mg/kg)-two days regime.
Same results were observed for co-selection of SUL-R coliforms.
*Day 0; day starting the treatment, day 2dAT and 10dAT: two and 10 days after having finished the treatment. SN; start of nursery period, EN; end of nursery period.
Six different symbols and six color codes are linked to each of the six treatment groups.

The OTC treatment Do20.Dur3 led to the lowest proportion of TET-R coliforms at this time point. By the end of the nursery period the proportion was back to the same level as before starting the treatment. This observation was independent of the treatment group (Fig. 2C).

3.3. Analysis of co-selection of AMP- and SUL −R bacteria

Selection with one antimicrobial can also promote selection for other resistances since they might be co-localized on the same genetic element (22). Therefore we also analyzed the effect of the OTC treatments regimes on co-selection for AMP- or SUL -R bacteria at 2dAT (time point where an effect of tetracycline treatment was observed) (Fig. 3).

For the analysis of co-resistance to AMP, 2154 counts were made of which 155 were left out due to mistakes in the dilution series and another 44 counts were left out due to lack of completeness or infeasible proportion of resistance. The remaining 1955 counts were included in the analysis. As for the TET model the counts expressed over-dispersion. The three-way interaction was not significant ($P > 0.05$). When comparing the proportion of AMP-R coliform at 2dAT to day 0, no significant difference was observed, contrary to the results obtained for TET-R bacteria ($P > 0.05$). Further, the proportion of AMP-R bacteria did not change significantly at later time points. The estimated proportion was 53% (95% CI [45%; 63%]). In the final model for AMP-R, the dispersion parameter was estimated to be 36.2.

For a similar analysis of SUL-R, 2143 counts were made of which 148 were left out as two less diluted counts were made and another 34 counts were left out due to lack of completeness or infeasible proportion of resistance. The remaining 1961 counts were included in the analysis. As for the TET model the counts expressed over-dispersion. As shown for AMP, no significant difference was observed for SUL-R proportions of coliforms at 2dAT compared to day 0 ($P > 0.05$) (not shown). As expected, the proportion of SUL-R bacteria did not change significantly at the rest of the time points tested as a consequence of tetracycline treatment, irrespective of the treatment regime (not shown). The estimated proportion of SUL-R in coliforms was 58% (95% CI [51%; 66%]). In the final model for SUL-R, the dispersion parameter was estimated to be 21.7.

For all three antimicrobials a full model was estimated, where each sample was allowed to have its own proportion of resistant bacteria – these models did not show sign of over-dispersion. For all three antimicrobials the full models were significantly better than the reduced models presented above. This means that there was a large and highly significant pig-to-pig variation in response to OTC treatment.

4. Discussion

Treatment with one or more antimicrobials is necessary for cure of diseases in livestock and therefore for animal welfare reasons (Levy and Marshall 2004; Marshall and Levy, 2011). Thus, careful investigations of dosing factors, such as treatment duration and drug concentration, are of relevance in order to provide optimal treatment protocols which may help keep the occurrence and development of resistance at the lowest possible level. In the present study the effect of different OTC treatment regimes with variation in oral dose and duration of treatment on the selection of TET-R strains was analyzed. Since resistance genes can be harbored on the same mobile genetic element, co-selection for AMP and SUL resistances was also investigated.

The work was divided in two in vivo experiments, a first one under experimental conditions followed by a second one carried out as a field study. Results showed that a high proportion of the coliforms present in the gut of the nursery pigs in Denmark are TET-R (data derived from both studies) (Figs. 1C, 2C), AMP-R and SUL-R (field study) (Fig. 3B). These data support previously observed selection of such resistant bacteria in pig production in Denmark and other countries due to the use of these antibiotics for several decades (Martinez and Baquero, 2000; Lipsitch et al., 2000; DANMAP, 2013; Jumbe et al., 2003; Levy and Marshall, 2004; Opatowski et al., 2011). This may be of relevance for the conclusions of the current study since there is less “room” for further selection of resistant coliforms when the starting level is high.

Over the time span of the experimental study (18 days), no statistical difference was observed in the proportion of TET-R coliforms between the OTC treatment regimes. Due to large pig-to-pig variation, the estimated proportion of TET-R bacteria remained statistically constant over time according to the model performed, despite the total number of TET-R coliforms was significantly different between groups at certain time points. Overall, our results are in disagreement with previous studies (Levy and Marshall 2004; Opatowski et al., 2011; Spicknall et al., 2013), however, also in our studies a trend was observed, with the high dose treatments (Do20.Dur10 and Do20.Dur3) leading to the highest proportion of TET-R coliforms by the end of the trial (day 18) (Fig. 1C), and differences with previous studies may partly be due to the rigorous statistical approach performed in the current study. Care should be taken not to over-interpret the trend observation with regard to the importance of dose versus duration of treatment, since results were not statistically significant. This may, however, be because only five pigs were included in each group. Furthermore, it should be noted that the step to the next dose level was large (from 20 mg/kg to 5 mg/kg). There may be an optimum of dose/duration combination for at given TET-MIC distribution in a population, and further studies are needed to establish that combination for the current population of TET-R coliforms in Danish pigs.

An important observation from this study was that pigs reacted differently to the same treatment, causing a variation, which tended to be larger than the variation caused by different treatments. We have previously shown that Danish nursery pigs contain up to 11 different *E. coli* strains when 50 colonies per pig were type by rep-PCR, but with large variation between pigs (Herrero-Fresno et al., 2015). The span in MICs for TET-R *E. coli* from Danish pigs, which make up the largest proportion of coliforms counted by our method, have been reported to

be between 0.25 and 512 mg/l with peaks at 0.5 mg/l for the sensitive part of the population and 32–68 mg/l for the resistant part (Ahmad et al., 2015). It is unknown how this span is represented in individual pigs, but large differences are bound to cause large differences in response to treatment with regard to selection of TET-R coliforms. Probably for this reason, in all statistical models a high over-dispersion was observed, which resulted in using the quasi-poisson family. Typical sources of over-dispersion are sources of variation that are not included in the model. In the present study it was assumed that all pigs in the same treatment group, the study units, had the same proportion of resistant bacteria at each time point.

Under field conditions, an statistical significant effect of treatment on development of TET-R *E. coli* was demonstrated at 2dAT compared to day 0, and the low dose-6 days (Do10.Dur6) treatment led to a higher proportion of TET-R bacteria compared to the other regimes at this time point (Fig. 2C). Even though there was not a significant effect of treatment on proportion of *E. coli* TET-R bacteria at time 10dAT and at end of the nursery period compared to day 0, the trend was that the treatment regime (Do10.Dur6) led to the highest proportion of TET-R coliforms at these time points (Fig. 2C). Do20.Dur3 regime (the one leading to the second highest proportion of TET-R coliforms on day 18 of the experimental trial) yielded the lowest proportion of TET-R bacteria at 2dAT in the field study (Fig. 2C). In general, one should put more emphasis on the results of the field study, since it encompasses the natural variation in dose between pigs that are flock treated with the antimicrobial.

In the field study, it was also demonstrated that selection with TET did not significantly affect development of AMP- and SUL −R bacteria at 2dAT (time point where an effect on treatment on selection of TET-R bacteria was detected). Therefore, at this time point, co-selection for AMP and SUL resistances is discarded.

Concerning duration of treatment, a mathematical simulation study concluded that short duration of TET administration should be preferable in order to reduce selection for TET-resistance, since less time under treatment would reduce the competitive growth of the R-strains (Ahmad et al., 2015). The authors concluded that more prolonged treatment resulted in an increased occurrence of resistance and therefore it took longer to return to equilibrium (Ahmad et al., 2015). It has previously been demonstrated that it might also be possible to reduce the duration of treatment with a higher daily dose level to achieve the desired efficacy and lower resistance levels (Geli et al., 2012). Our results are in accordance with these predictions, but only at the specific time point 2dAT. Thus, in the field study the medium-dose for three days treatment regime (Do20.Dur3) led to the lowest TET-R bacteria at 2dAT compared to day 0 followed by the high-dose regime (Do30.Dur2). By the end of the nursery period, all of the treatments can be assumed to be equal regarding TET-R development, and the number of TET-R coliforms was lower than when pigs entered the nursery period.

In the present work, we provide new insights into the association between tetracycline treatment regimes and development of resistance in food production animals. Our results suggest that similar resistance levels are obtained no matter the duration and concentration of TET, at least under the conditions used in this study, with the rigorous statistical approach applied and for the specific treatment regimes tested.

Conflict of interest

None to declare.

Acknowledgments

Authors would like to thank, P.R. Mortensen, A.B. Lind and M.Q. Pawlowski for technical assistance. The study was supported by the Danish Council (DFS) grant number 0603-00358B (project entitled “Minimizing Antibiotic Resistance Development-MINIRESIST”).

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