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Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs

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Abstract

Mathematical and statistical modeling can be a very useful tool in understanding and fighting antimicrobial resistance (AMR). Here we present investigations of mixed effect models of varying complexity in order to identify and address possible management factors affecting the tetracycline AMR levels in Danish pig farms. Besides antimicrobial exposure during pigs life cycle, the type of production seems to also have an influence. The results concludes that not only fully integrated farms (CHR integrated) but also farms in a production network with a single ownership (CVR integrated) might have a preventive effect on levels of tetracycline AMR compared to more complex trading patterns.

Key words: Antimicrobial resistance, Tetracycline, Danish pig production, Mixed effect modeling

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1. Introduction

Antimicrobial resistance (AMR) is a continuing concern as it affects both animals and humans. AMR in livestock might be associated with a substantial economical burden and in addition, there might be a potential risk of spread of AMR from livestock to humans through the food chain (Barza, 2002; Hammerum and Heuer, 2009). In Denmark, 75% of the usage of veterinary antimicrobials is within the pig production (74.7 tonnes in 2018) and even though there has been a significant reduction in the tetracycline use the past years (DANMAP, 2019), tetracycline resistance genes are still widespread in slaughter pigs.

The use of antimicrobials increases the levels of AMR, however, it is also of great interest to investigate whether other factors have significant influence on the resistance level. Management factors such as size of slaughter pig farms, movements within pig production networks and antimicrobial administration practices have already gained interest when modeling AMR levels at pig farms (Birkegård et al., 2017; Sørensen et al., 2018; Varga et al., 2009; Vieira et al., 2009; Zuliani et al., 2019). In addition, differentiating between the antimicrobial exposure for the age groups of the pig incorporates the dynamic changes of the composition of microbiome during the pigs growth (Zhao et al., 2015) and may provide a need for a more detailed measure of the exposure (Turnidge and Christiansen, 2005). Modeling AMR levels in livestock can work as a tool to discover these factors in order to decide on optimal treatment and production strategies, thereby enhancing the effect with a reduced risk of AMR emergence and spread (Bjork et al., 2015; Opatowski
et al., 2011).

In this work, fecal samples collected in 2015, 2017 and 2019 from Danish slaughter pigs were analyzed and different mixed effect models were explored in order to investigate the association between tetracycline resistance and management factors. Data for tetracycline resistance was expressed as an overall level of resistance against tetracycline by summing over the twelve measured genes: \( \text{tet}(A) \), \( \text{tet}(B) \), \( \text{tet}(C) \), \( \text{tet}(L) \), \( \text{tet}(O)-1 \), \( \text{tet}(O)-2 \), \( \text{tet}(PA) \), \( \text{tet}(Q) \), \( \text{tet}(W) \), \( \text{tet}(X) \), and \( \text{tet}(32) \). The aim of the modeling process was to investigate how levels of tetracycline resistance at slaughter were affected by use of different antimicrobials for different age groups of pigs, and explore if management factors had a significant effect on the levels of tetracycline resistance in the Danish pig production.

2. Materials and methods

2.1. Data description

The data originates from four sources, the CHR register, the VetStat register, the pig movement register, and fecal samples from slaughter pigs in Denmark sampled over three periods in 2015, 2017 and 2019 as part of the Veterinærforlig II and III projects.

2.2. CHR register

The CHR register (det Centrale Husdyrbrugsregister) is a database administered by the Danish Veterinary and Food Administration. All livestock in Denmark are registered with a unique CHR number, which are associated with a specific geographical location of a farm. Information like type
of animal, age group of animals, number of animals, and geographical location are all linked to the CHR number and updated at least yearly (ongoing) (Fødevarestyrelsen, 2020a). In addition, all farms are registered under a CVR number which is a unique identification number linked to the owner. This means that multiple farms can be registered under the same CVR number, but only one farm can be registered within the CHR number. In the Danish pig industry, the life cycle of a slaughter pig consists of three rearing periods corresponding to their age (age group), thus piglets: birth-7 kg, weaners: 7-30 kg and finishers: 30-slaughter.

2.3. Movement register

Danish pig producers are required to register all movements of pigs between farms (Fødevarestyrelsen, 2020b). Usually the movements between or within farms happens when the pigs are entering a new rearing period or move to slaughter houses, as the latter are regarded as farms. The farmers are to report information on number of received pigs combined with CHR and CVR number of both sending and receiving farm. Since slaughter pigs are not registered individually, specific slaughter pigs are not traceable through the production chain.

2.4. VetStat

VetStat is a register for drugs prescribed for animals in Denmark, subsequently all drugs sold for animal purposes are reported (Stege et al., 2003). VetStat is monitoring how much and which antimicrobials are bought on a farm level. Information about the type of animal and age group for which the antimicrobials are intended are recorded in this system as well, thereby
revealing the antimicrobial use within the farms. In addition, The Defined Animal Daily Dose (ADD), which is the average dose for the main indication within each age group of the specific type of animal, is also registered in order to compare across antimicrobial classes and age groups in a standardized way.

2.5. Antimicrobial resistance samples

Over three periods in 2015, 2017 and 2019 fecal samples were collected from Danish finishers at the slaughter line in slaughterhouses. Samples from five individual slaughter pigs were pooled together based on previous findings describing the optimal way of pooling samples (Clasen et al., 2016). The pooled samples were analyzed using the qPCR method in order to get the most sensitive and accurate detection of resistance at farm level (Schmidt et al., 2015). In total data consists of samples from 673 unique farms during the three periods, from where 164 farms have been sampled multiple times. In 2015, 2017 and 2019 the number of unique farms represented in data are 390, 216 and 190, respectively. DNA from samples (10 ng/µl) were investigated using a Fluidigm HD Biomark System and Gene expression 48 x 48’ arrays for qPCR amplification according to the manufacturer’s instructions. The fluorescent dye EvaGreen was added to the reaction mixture to enable real-time quantification of amplification. The following amplification protocol was used: 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 60 s at 60°C for extension and annealing, where the fluorescence was measured after each cycle. A melting curve analysis was performed at the end of the qPCR to detect unspecific amplicons. Primers were synthesized at DNA Technology A/S (Aarhus Denmark). Tetracycline resistance gene levels were measured
as relative quantification ($\Delta C_q$) values that were determined for each of the samples as follows:

$$\Delta C_q = C_{q,\text{Reference gene}} - C_{q,\text{Gene of interest}}$$

$\Delta C_q$-values were calculated from all samples normalized against their respective 16S rDNA which was used as normalization standard. The primers used are listed in supplementary material. The $C_q$-values indicate the number of doubling cycles the qPCR machine ran until detection based on a prespecified cutoff value. This means that this data is naturally on a log$_2$-scale.

The normalization of the $C_q$-values yields the $\Delta C_q$-values which are used to estimate the proportion that has the investigated gene in the sample i.e. $2^{\Delta C_q}$ is an expression of the proportion of bacteria in the sample containing the gene. The twelve tetracycline resistance genes tested and considered are $tet(A)$, $tet(B)$, $tet(C)$, $tet(L)$, $tet(M)$, $tet(O)-1$, $tet(O)-2$, $tet(PA)$, $tet(Q)$, $tet(W)$, $tet(X)$, and $tet(32)$ (Johnson et al., 2016). In order to summarize a total tetracycline resistance level in the slaughter pigs, it was chosen to sum up all the $\Delta C_q$-values in a variable $R(tet) = \sum_k 2^{\Delta C_q(k)}$.

### 2.6. Average Lifetime Exposure (ALEX)

In order to estimate the Danish slaughter pigs exposure to antimicrobials a systematic algorithm was build. ALEX was developed based on the LEA algorithm (Birkegård et al., 2017) with the primary goal to track the slaughter pig movements and calculate the average antimicrobial exposure at the farms during the rearing periods.

The algorithm has two main steps. In the first step, Tracing, the movements based on the slaughter date is traced back to identify the locations
during the different rearing periods including internal movements on farms. The age group of the movements are estimated based on the CHR register and a specific time window for the movements, while the internal movements are based on the CHR registry in case of no identified movements within the specific time period. In the second step, Calculating Exposure, the weighted exposure in each rearing period is calculated based on a smoothing of antimicrobial purchases in VetStat during a specific time window. This weighted calculation is done for each rearing period for 12 antimicrobial classes.

Figure 1: Example of the Average Lifetime EXposure (ALEX) algorithm. In the algorithm the average lifetime exposure to antimicrobials of pigs in the Danish pig industry is estimated based on the CHR registry, movement registry and VetStat.

ALEX takes a slaughter date and the corresponding CHR number as input and traces the pigs movement patterns through the movement register, here it is important to notice, that the pig could come from a fully integrated (all age groups) farm or possibly come from different other farms depending on the individual farm production strategy. In Figure 1, an example of a finisher farm with internal production and movements from other farms is
illustrated. The time windows for the movements are chosen based on the average weight gain period of the pigs in the different rearing periods and the parameter analysis in LEA (Birkegård et al., 2017). The time window of the weaner to finisher moves was 64-99 days prior to slaughter and the time window of piglet to weaner moves was 104-160 days with the average time of the finisher rearing period being 75 days, weaner period being 53 days and piglet period being 30 days. A cutoff value of 7 farms within a production network was chosen as a reasonable maximum. When the trace is completed, VetStat data is used to calculate the average antimicrobial exposure for the traced farms in the periods where the pig potentially could have been there. The time windows for estimating the antimicrobial exposure were chosen rather broad in order to give a smoothed estimate. This means that the antimicrobial purchases were smoothed over a 180 days period prior to the movement of each rearing group. Since slaughter pigs are not registered individually they can only be traced through movements between farms leading to possibly more than one trace for a pig. In ALEX the antimicrobial exposure is weighted according to the number of pigs moved and the estimated internal movements which are not registered. The outcome of the algorithm is an estimate of antimicrobial exposure within each rearing period for the different antimicrobial classes calculated as $AMU_{kg}$ per pig ($(kg/pig \times day)$) i.e. the average dose for treatment of one kilogram pig.

2.7. Mixed effect models

The data consists of repeated measurements on slaughter pigs at Danish slaughterhouses. The data was not collected in a balanced way meaning that some farms were only sampled once, while others were sampled multiple
times. In total, 1,032 observations from 673 different farms were collected and analyzed during the years.

By applying mixed effect models, repeated measurements sampled at the same farm over time can be utilized, which means that all data collected can be included in the modeling process while avoiding bias in the models. It is assumed that each farm has a different background level of AMR due to factors such as the environment and management approaches which can be modeled by introducing different intercepts by including farms as a random effect in the model. Mixed effects models can be used to describe the variation between farms rather than the difference between them assuming that the farms represent a random selection from the population and that observations within the same farm are correlated.

The estimates in the model were derived by maximum likelihood (ML) rather than restricted maximum likelihood (REML) since the main interest was to estimate the fixed effects of the model rather than the random.

The estimates of the models were tested using Bonferroni correction at an overall level of $\alpha = 0.05$ to account for the multiple comparisons and the models were reduced by backwards elimination.

In all the tested models, variables based on the different antimicrobial classes presented in Table 1, within each of the three rearing periods piglet, weaner, and finisher were included.

However, before estimating the parameters in the mixed effect models, variables with less than five observations within the group of antimicrobial class and age group were removed from the data. In the DANMAP 2018 report the usage of the antimicrobial classes Cephalosporins and Fluoro-
Table 1: Antimicrobial classes considered in the modeling process.

<table>
<thead>
<tr>
<th>Antimicrobial classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
</tr>
<tr>
<td>Amphenicols</td>
</tr>
<tr>
<td>Lincosamides</td>
</tr>
<tr>
<td>Macrolides</td>
</tr>
<tr>
<td>Simple penicillins</td>
</tr>
<tr>
<td>Extended penicillins</td>
</tr>
<tr>
<td>Sulfonamides (incl. trimethoprim)</td>
</tr>
<tr>
<td>Tetracyclines</td>
</tr>
<tr>
<td>Pleuromutilins</td>
</tr>
<tr>
<td>Cephalosporins</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

*quinolones* are negligible which was also reflected in data where only four, five and none records of cephalosporins were observed within the piglet, weaner and finisher rearing period, respectively and no records of fluoroquinolones were observed in any of the rearing periods (DANMAP, 2019). In addition, there were not observed any antimicrobial use of the class *Other* during the finisher rearing period in data as expected from *DANMAP 2018*. Therefore, they were not considered in the following analysis. In addition to the exposure variables, three variables *Network size* (Number of farms in network), *Production type* (CHR/CVR integrated or other) and *Number of antimicrobial classes* were constructed. The *Network size* was measured based on the
back tracing in ALEX by counting how many different farms were part of the production network. If no movements were found in the given period and the farm had production of all three age groups according to the CHR register, it was categorized as 'size 1' (a fully CHR integrated farm). If movements from only one other farm (from piglet to weaner or weaner to finisher) were detected it was categorized as 'size 2' (partly CHR integrated farm) and so on. The maximum number of farms in a network was 7 as our cutoff in ALEX, which means that a more complex trading pattern with multiple piglet or weaner farms involved was found. *Production type* was also based on the results of the ALEX algorithm, however, this variable indicates if the production network is CHR integrated, CVR integrated or neither. The variable *Number of antimicrobial classes* was constructed as a counting variable counting how many different antimicrobial classes the pigs were exposed to within the three different age groups, piglet, weaner and finisher. This was again based on the output of ALEX.

3. Results

The distribution of estimated tetracycline exposure based on VetStat for the sampled farms during the three periods 2015, 2017 and 2019 is shown in Figure 2a. The distribution tendency of the average tetracycline exposure is moving towards a lower consumption over time which is in agreement with what is observed and reported by DANMAP (DANMAP, 2019). The decline in tetracycline exposure could be a reflection of the differentiated *yellow card* initiative which was implemented in 2016 in order to reduce the usage of certain antimicrobials based on weighted ADDs (DANMAP, 2019).
In Figure 2b the distribution of $R(tet)$ is shown, it is observed that there is a decreasing trend in the mean over time. Distribution plots for the individual tetracycline resistance genes can be found in supplementary material.

Figure 2: (a) Change in the distribution of antimicrobial exposure of tetracycline in Danish pig production based on antimicrobial purchases registered in VetStat estimated by ALEX. (b) The distribution of $R(tet)$ (sum of $2^{\Delta C_{q}}$ of all tetracycline resistance genes) over time.

In ALEX the cutoff value of 7 farms within a production network resulted in 1032 traceable observations from the 673 sampled farms. The traceability of ALEX was 91.0% which means that for over 90% of the sampled slaughter pig farms traced by ALEX, it was possible to detect one or more plausible routes from piglet period to slaughter. In 9.0% of the cases, however, the tracing failed due to missing detection of either possible piglet or weaner farms, or detection of too many farms in the trading network. A sensitivity analysis was performed on the cutoff value, decreasing the cutoff value to 5 resulted in 1,009 observations from 665 different farms while increasing the cutoff to 10 resulted in 1,035 observations from 676 different farms. However, neither of the cutoff values changed the overall conclusion of the models.

Different linear mixed effect models have been applied to data as part of the modeling process in order to investigate the relationship between the
level of tetracycline resistance and factors such as antimicrobial exposure and management features.

3.1. Model 1: Simple mixed effect model

The first mixed effect model tested was,

$$\log_2(R(tet)_{ij}) = \mu_i + \sum_{AM} (\beta^{(AM,p)} AMU^{(AM,p)}_{ij} + \beta^{(AM,w)} AMU^{(AM,w)}_{ij} + \beta^{(AM,f)} AMU^{(AM,f)}_{ij}) + \epsilon_{ij}$$

where $R(tet)_{ij}$ describes the total resistance towards tetracycline (presented as $\sum_k 2^{\Delta C_{q,k}}$ where $k$ symbolizes the 12 tetracycline genes tested) for farm $i = 1, ..., 673$ at sampling point $j = 1, ..., n_i$, $n_i$ is the number of observations for the $i$th farm, $\mu_i$ is the farm-specific mean ($\mu_i \sim N(0, \sigma_i^2)$), $AMU^{(AM,p)}$, $AMU^{(AM,w)}$ and $AMU^{(AM,f)}$ are the estimated exposure variables divided into the different antimicrobial classes ($AM$, see Table 1) within each of the three age groups (piglet, weaner, finisher), respectively, $\beta^{(AM,p)}$, $\beta^{(AM,w)}$ and $\beta^{(AM,f)}$ are the unknown coefficients and $\epsilon_{ij}$ are the residuals ($\epsilon_{ij} \sim N(0, \sigma^2)$). $R(tet)_{ij}$ was log$_2$ transformed due to the nature of the $C_q$ values.

This model is referred to as Model 1.

3.2. Model 2: Mixed effect model accounting for network size

In order to fit data better, Model 1 was expanded to include a term accounting for the size of the production network. After applying the model and testing for significance, it was clear that it was possible to reduce the levels of the variable Networksize due to the relative small presence in data and similar estimates of the coefficients of 'size 3'-'size 7', see Figure 3a. This was done by merging these types into a new level called 'size 3+'. However, reducing the levels even more to 'size 1' and 'size 2+' did worsen the fit of the model, see Table 2.
3.3. Model 3: Mixed effect model accounting for production type

Instead of only considering the size of production network, another way of distinguishing between the productions is to categorize the types by fully CHR integrated farms i.e. where the physical location of three rearing periods is the same, CVR integrated farms i.e. farms where the owner is the same, but the pigs might be moved between farms with different locations and other. As seen in Figure 3b there exists a fair amount of CHR and CVR integrated farms in Denmark. Therefore, a model with type levels 'CHR integrated', 'CVR integrated', 'other - 2' and 'other - 3+' was constructed.

![Distribution of production network size](image1)

![Distribution of production types](image2)

Figure 3: Distributions of production types associated with pig production in Denmark detected by ALEX. (a) Distribution of possible number of farms i.e. Network size. (b) Distribution of Production types: CHR integrated, CVR integrated and other.

3.4. Model 4: Mixed effect model also accounting for the diversity of antimicrobial exposure

A version of the mixed effect model including antimicrobial exposure within age group and antimicrobial class and production type as previous, however, expanded with a variable accounting for how many different antimicrobial classes were prescribed for the specific age groups in the rearing
periods (\textit{Number of antimicrobial classes}) was also applied. By considering how many different classes rather than only the amount of antimicrobials the pig potentially could have been exposed to, it can be seen as an expression of the overall health profile for the farm network. However, this did not have any significant impact on the measured tetracycline resistance levels in the slaughter pigs when testing and the variable was eliminated in the backwards selection, thus resulting in the same model as Model 3. Model checking plots were inspected and did not raise concern (not included in this article).

3.5. Final model

In Table 2, a comparison of the linear mixed effects models (\textit{Model 1-3}) is shown. Here it is seen that the best fit based on AIC, after reducing the models by backward selection, is \textit{Model 3} with the variable accounting for the production type i.e. whether the farm is CHR integrated, CVR integrated, partly CHR integrated with only two farms involved or if more than three farms are part of the production network.

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
<th>Loglik</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>1686.2</td>
<td>-836.11</td>
</tr>
<tr>
<td>Model 2 - size: n farms</td>
<td>1670.1</td>
<td>-823.05</td>
</tr>
<tr>
<td>Model 2 - size: 1,2,3,4+ farms</td>
<td>1666.9</td>
<td>-824.43</td>
</tr>
<tr>
<td>Model 2 - size: 1,2,3+ farms</td>
<td>1664.9</td>
<td>-824.47</td>
</tr>
<tr>
<td>Model 2 - size: 1,2+ farms</td>
<td>1675.1</td>
<td>-830.56</td>
</tr>
<tr>
<td>Model 3 - type: CHR int., CVR int., 2, 3+ farms</td>
<td>1653.7</td>
<td>-817.83</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the linear mixed effect models (\textit{Model 1-3}).
The estimates of the fixed effects of the significant variables after backward selection can be found in Table 3. Here it is noticed that CHR integrated farms does have the lowest estimated intercept i.e. the lowest background level of tetracycline resistance (all other types got a positive value added). The estimate of CVR integrated farms shows a positive effect (higher AMR) compared to the CHR integrated farms, however, the effect is lower than for farms that are neither CHR nor CVR integrated. There are no significant AMU variables in the model for the piglet rearing period, however, the tetracycline exposure in the weaner rearing period and the pleuromutilins exposure in finisher rearing period seems to have a positive effect, while tetracycline exposure in the finisher rearing period have a greater positive influence on the level of tetracycline resistance. This means that only tetracycline and pleuromutilins exposure for weaner and finisher rearing periods are having a significant influence on tetracycline resistance levels in slaughter pigs while the type of production has a highly significant effect.

The correlation between the observed and fitted $R(tet)$ can be seen in Figure 4. The Pearson correlation coefficient is 0.89 giving a sense of the accuracy of the predictions. The fitted values predict the observed values best for low values of $R(tet)$, while the variance of the predictions seems to increase for higher values as expected when fitting on a log$_2$ scale.

3.6. Biological interpretation of model results

Model 3 represents data best according to the calculated AIC. The biological interpretation of the main results of the model is:

- CHR integrated farms have the lowest background level of tetracycline resistance
<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>Std. error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept (i.e. CHR integrated)</td>
<td>−1.13</td>
<td>6.40 × 10^{-2}</td>
<td>&lt; 2 × 10^{-16}</td>
</tr>
<tr>
<td>Type (CVR integrated)</td>
<td>0.177</td>
<td>7.52 × 10^{-2}</td>
<td>1.8 × 10^{-2}</td>
</tr>
<tr>
<td>Type (Other: 2 farms)</td>
<td>0.271</td>
<td>7.17 × 10^{-2}</td>
<td>1.7 × 10^{-4}</td>
</tr>
<tr>
<td>Type (Other: 3+ farms)</td>
<td>0.428</td>
<td>6.95 × 10^{-2}</td>
<td>1.1 × 10^{-9}</td>
</tr>
<tr>
<td>Tetracyclines finisher ( $\beta^{(tet,f)}$ )</td>
<td>7.70 × 10^{-3}</td>
<td>6.79 × 10^{-4}</td>
<td>&lt; 2 × 10^{-16}</td>
</tr>
<tr>
<td>Pleuromutilins finisher ( $\beta^{(ple,f)}$ )</td>
<td>2.12 × 10^{-3}</td>
<td>5.61 × 10^{-4}</td>
<td>1.7 × 10^{-4}</td>
</tr>
<tr>
<td>Tetracyclines weaner ( $\beta^{(tet,w)}$ )</td>
<td>3.26 × 10^{-3}</td>
<td>7.46 × 10^{-4}</td>
<td>1.3 × 10^{-5}</td>
</tr>
</tbody>
</table>

Table 3: Estimates and standard error for the fixed effects parameters of the linear mixed effect model with production type included and farm as random effect (Model 3).

- CVR integrated farms have lower background level of tetracycline resistance compared to non-CHR and non-CVR integrated
- Tetracycline used within the weaner and finisher period together with pleuromutilins used within the finisher period leads to a significant elevation of tetracycline resistance levels
- Other investigated antimicrobials do not affect the tetracycline resistance levels
- The type of production, i.e. CHR-, CVR-, partly CHR integrated, or with more than 3 farms in the production network, has the most pronounced effect on tetracycline resistance levels compared to AMU.
4. Discussion and Conclusion

AMR is an ongoing challenge. In order to reduce the occurrence, mathematical and statistical modeling can be used to get a better understanding of the underlying mechanisms. By analyzing data obtained in the real world, interactions between resistance levels and influential factors can be exposed leading to potential strategies on how to reduce AMR in the pig production.

The data presented in this article is collected as a real world study, where Danish slaughter pigs were sampled during three periods. The samples were analyzed by qPCR measuring 11 unique tetracycline resistance genes. The probes \textit{tet}(O)-1 and \textit{tet}(O)-2 detects different regions of the \textit{tet}(O) gene and are therefore not completely independent in distribution. The final model was performed for a scenario excluding \textit{tet}(O)-2 and a scenario where a mean...
value of tet(O)-1 and tet(O)-2 was included instead of the individual measurements, however, this did not change the overall conclusions of the modeling.

In order to give an easily comparable measurement of resistance the sum of the genes (R(tet)) was used as an expression for the overall tetracycline level in the slaughter pigs. The sum was primarily driven by tet(O)-1, tet(O)-2, tet(W) tet(Q), and tet(32) which have very few non-detections and high ∆Cq mean values (see supplementary material). Out of the total count of tetracycline resistance genes in the samples, the number of copies of measured tet(W) genes constitutes 45% on average with a standard deviation of 8%. As a comparison tet(Q) and tet(O)-2 constitute 18 ± 8% and 17 ± 6%, respectively, reflecting the considerable dominance of tet(W). The resistance genes explored in this study are recovered from pigs, however, it would be interesting to investigate whether the genes are found in zoonotic bacteria since this would pose a risk for humans as well (Afema et al., 2014; Pan et al., 2018, 2019; Mather et al., 2013)

Data on influential factors such as antimicrobial purchases and size of trading network was retrospectively collected from Danish registries. The antimicrobial exposure was estimated through the ALEX algorithm in order to calculate the average exposure based on the purchases by the farms during the different rearing periods oppose to calculating the antimicrobial exposure as a lifetime exposure (Dalhoff Andersen et al., 2020). When working with registries it is important to remember, that the data is not collected for research or scientific purposes. Missing and faulty (incorrect) data are well known issues when working with data registries, however, the Danish registries are updated often thereby reducing reporting delays. Estimating
the antimicrobial exposure based on registries can be done in different ways. Most important sources of uncertainties are that the exact moving pattern of the slaughter pigs sampled are not available and the antimicrobial purchases are registered on an age group level within each farm not distinguishing between the specific pigs or the piglet/sow age group.

The ALEX algorithm was capable of tracing 91.0% of the sampled slaughter pig farms which is comparable to the traceability of the LEA algorithm (Birkegård et al., 2017). The untraceable cases were mostly due to faulty registration in the CHR and the movement registers leading to incomplete paths of weaner and piglet trading networks. However, the data still gives an idea of how the trends of AMR is affected by the antimicrobial exposure in Danish pig production.

The overall fit of the final model captured the trend of the data well, however, the variance of the prediction seemed to increase for higher values. The final model was extended to include a first order autoregressive covariance structure in order to check for autocorrelation in the errors, however, this did not change the fit of the model. Using mixed effect modeling the main influential factors on tetracycline resistance were found to be type of production, tetracycline exposure in the weaner and finisher rearing periods and pleuromutilins exposure in the finisher rearing period. The association between tetracycline exposure and tetracycline resistance in Danish slaughter pigs has been suggested by previous modeling work, where also different management factors such as number of produced pigs and route of administration were considered (Birkegård et al., 2017; Dalhoff Andersen et al., 2020; Vieira et al., 2009). Management factors have gained a growing interest within the field.
of antibiotic resistance research, however, the herd size of livestock farms appears to be the most significant one causing many of the other factors such as biosecurity, number of animal suppliers, human traffic in the herd etc. (Bokma et al., 2020; Jiang et al., 2019; Raasch et al., 2018; Sørensen et al., 2018; Xu et al., 2020; Zuliani et al., 2019). The results presented here suggest that there is a strong association between the levels of tetracycline resistance in slaughter pigs and the type of production. This indicates that complicated moving patterns involving multiple farms and owners yield increased tetracycline resistance compared to fully (CHR) integrated farms where pigs stay on the same geographical location during their entire life cycle but also CVR integrated farms where pigs are moved between farms with a single ownership. This is in agreement with similar results obtained by Sørensen et al. (2018). The findings indicate that CHR but also CVR integrated farms work as a preventive tool against higher tetracycline resistance.

5. Funding

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6. Acknowledgments

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References


Clasen, J., Mellerup, A., Olsen, J., Angen, Ø., Folkesson, S., Hisham Beshara
Halasa, T., Toft, N., Birkegård, A., 2016. Determining the optimal number
of individual samples to pool for quantification of average herd levels of
antimicrobial resistance genes in danish pig herds using high-throughput

Dalhoff Andersen, V., Møller Aarestrup, F., Munk, P., Stengaard Jensen, M.,
de Knecht, L., Bortolaia, V., nudsen, B., Lukjancenko, O., Birkegård, A.,
Vigre, H., 2020. Predicting effects of changed antimicrobial usage on the
abundance of antimicrobial resistance genes in finisher’ gut microbiomes.

DANMAP, 2019. DANMAP 2018 - Use of antimicrobial agents and occur-
rence of antimicrobial resistance in bacteria from food animals, food and
humans in Denmark. Technical Report. Statens Serum Institut and Na-
tional Food Institute, DTU.

https://chr.fvst.dk. URL: https://chr.fvst.dk. 2020-14-08.

URL: https://svineflyt.fvst.dk. 2020-14-08.

48, 916–921. doi:https://doi.org/10.1086/597292.

Jiang, Z., Paudyal, N., Xu, Y., Deng, T., Li, F., Pan, H., Peng, X., He, Q.,
Yue, M., 2019. Antibiotic resistance profiles of salmonella recovered from


Varga, C., Rajic, A., McFall, M.E., Reid-Smith, R.J., Deckert, A.E., Checkley, S.L., McEwena, S.A., 2009. Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic


Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs

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Abstract

Mathematical and statistical modeling can be a very useful tool in understanding and fighting antimicrobial resistance (AMR). Here we present investigations of mixed effect models of varying complexity in order to identify and address possible management factors affecting the tetracycline AMR levels in Danish pig farms. Besides antimicrobial exposure during pigs life cycle, the type of production seems to also have an influence. The results concludes that not only fully integrated farms (CHR integrated) but also farms in a production network with a single ownership (CVR integrated) might have a preventive effect on levels of tetracycline AMR compared to more complex trading patterns.

Keywords: Antimicrobial resistance, Tetracycline, Danish pig production, Mixed effect modeling

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1. Introduction

Antimicrobial resistance (AMR) is a continuing concern as it affects both animals and humans. AMR in livestock might be associated with a substantial economical burden and in addition, there might be a potential risk of spread of AMR from livestock to humans through the food chain (Barza, 2002; Hammerum and Heuer, 2009). In Denmark, 75% of the usage of veterinary antimicrobials is within the pig production (74.7 tonnes in 2018) and even though there has been a significant reduction in the tetracycline use the past years (DANMAP, 2019), tetracycline resistance genes are still widespread in slaughter pigs.

The use of antimicrobials increases the levels of AMR, however, it is also of great interest to investigate whether other factors have significant influence on the resistance level. Management factors such as size of slaughter pig farms, movements within pig production networks and antimicrobial administration practices have already gained interest when modeling AMR levels at pig farms (Birkegård et al., 2017; Sørensen et al., 2018; Varga et al., 2009; Vieira et al., 2009; Zuliani et al., 2019). In addition, differentiating between the antimicrobial exposure for the age groups of the pig incorporates the dynamic changes of the composition of microbiome during the pigs growth (Zhao et al., 2015) and may provide a need for a more detailed measure of the exposure (Turnidge and Christiansen, 2005). Modeling AMR levels in livestock can work as a tool to discover these factors in order to decide on optimal treatment and production strategies, thereby enhancing the effect with a reduced risk of AMR emergence and spread (Bjork et al.,
In this work, fecal samples collected in 2015, 2017 and 2019 from Danish slaughter pigs were analyzed and different mixed effect models were explored in order to investigate the association between tetracycline resistance and management factors. Data for tetracycline resistance was expressed as an overall level of resistance against tetracycline by summing over the twelve measured genes: \( tet(A) \), \( tet(B) \), \( tet(C) \), \( tet(L) \), \( tet(M) \), \( tet(O)-1 \), \( tet(O)-2 \), \( tet(PA) \), \( tet(Q) \), \( tet(W) \), \( tet(X) \), and \( tet(32) \). The aim of the modeling process was to investigate how levels of tetracycline resistance at slaughter were affected by use of different antimicrobials for different age groups of pigs, and explore if management factors had a significant effect on the levels of tetracycline resistance in the Danish pig production.

2. Materials and methods

2.1. Data description

The data originates from four sources, the CHR register, the VetStat register, the pig movement register, and fecal samples from slaughter pigs in Denmark sampled over three periods in 2015, 2017 and 2019 as part of the Veterinarførlig II and III projects.

2.2. CHR register

The CHR register (det Centrale Husdyrbrugsregister) is a database administered by the Danish Veterinary and Food Administration. All livestock in Denmark are registered with a unique CHR number, which are associated with a specific geographical location of a farm. Information like type
of animal, age group of animals, number of animals, and geographical location are all linked to the CHR number and updated at least yearly (ongoing) (Fødevarestyrelsen, 2020a). In addition, all farms are registered under a CVR number which is a unique identification number linked to the owner. This means that multiple farms can be registered under the same CVR number, but only one farm can be registered within the CHR number. In the Danish pig industry, the life cycle of a slaughter pig consists of three rearing periods corresponding to their age (age group), thus piglets: birth-7 kg, weaners: 7-30 kg and finishers: 30-slaughter.

2.3. Movement register

Danish pig producers are required to register all movements of pigs between farms (Fødevarestyrelsen, 2020b). Usually the movements between or within farms happens when the pigs are entering a new rearing period or move to slaughter houses, as the latter are regarded as farms. The farmers are to report information on number of received pigs combined with CHR and CVR number of both sending and receiving farm. Since slaughter pigs are not registered individually, specific slaughter pigs are not traceable through the production chain.

2.4. VetStat

VetStat is a register for drugs prescribed for animals in Denmark, subsequently all drugs sold for animal purposes are reported (Stege et al., 2003). VetStat is monitoring how much and which antimicrobials are bought on a farm level. Information about the type of animal and age group for which the antimicrobials are intended are recorded in this system as well, thereby
revealing the antimicrobial use within the farms. In addition, The Defined Animal Daily Dose (ADD), which is the average dose for the main indication within each age group of the specific type of animal, is also registered in order to compare across antimicrobial classes and age groups in a standardized way.

2.5. Antimicrobial resistance samples

Over three periods in 2015, 2017 and 2019 fecal samples were collected from Danish finishers at the slaughter line in slaughterhouses. Samples from five individual slaughter pigs were pooled together based on previous findings describing the optimal way of pooling samples (Clasen et al., 2016). The pooled samples were analyzed using the qPCR method in order to get the most sensitive and accurate detection of resistance at farm level (Schmidt et al., 2015). In total data consists of samples from 673 unique farms during the three periods, from where 164 farms have been sampled multiple times. In 2015, 2017 and 2019 the number of unique farms represented in data are 390, 216 and 190, respectively. DNA from samples (10 ng/µl) were investigated using a Fluidigm HD Biomark System and Gene expression 48 x 48’ arrays for qPCR amplification according to the manufacturer’s instructions. The fluorescent dye EvaGreen was added to the reaction mixture to enable real time quantification of amplification. The following amplification protocol was used: 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 60 s at 60°C for extension and annealing, where the fluorescence was measured after each cycle. A melting curve analysis was performed at the end of the qPCR to detect unspecific amplicons. Primers were synthesized at DNA Technology
A/S (Aarhus Denmark). Tetracycline resistance gene levels were measured as relative quantification ($\Delta C_q$) values that were determined for each of the samples as follows:

$$\Delta C_q = C_{q, \text{Reference gene}} - C_{q, \text{Gene of interest}}$$

$\Delta C_q$-values were calculated from all samples normalized against their respective 16S rDNA which was used as normalization standard. The primers used are listed in supplementary material. The $C_q$-values indicate the number of doubling cycles the qPCR machine ran until detection based on a prespecified cutoff value. This means that this data is naturally on a $\log_2$-scale. The normalization of the $C_q$-values yields the $\Delta C_q$-values which are used to estimate the proportion that has the investigated gene in the sample i.e. $2^{\Delta C_q}$ is an expression of the proportion of bacteria in the sample containing the gene.

The twelve tetracycline resistance genes tested and considered are $tet(A)$, $tet(B)$, $tet(C)$, $tet(L)$, $tet(M)$, $tet(O)-1$, $tet(O)-2$, $tet(\text{PA})$, $tet(Q)$, $tet(W)$, $tet(X)$, and $tet(32)$ (Johnson et al., 2016). In order to summarize a total tetracycline resistance level in the slaughter pigs, it was chosen to sum up all the $\Delta C_q$-values in a variable $R(tet) = \sum_k 2^{\Delta C_q(k)}$.

2.6. Average Lifetime EXposure (ALEX)

In order to estimate the Danish slaughter pigs exposure to antimicrobials a systematic algorithm was build. ALEX was developed based on the LEA algorithm (Birkegård et al., 2017) with the primary goal to track the slaughter pig movements and calculate the average antimicrobial exposure at the farms during the rearing periods.
The algorithm has two main steps. In the first step, *Tracing*, the movements based on the slaughter date is traced back to identify the locations during the different rearing periods including internal movements on farms. The age group of the movements are estimated based on the CHR register and a specific time window for the movements, while the internal movements are based on the CHR registry in case of no identified movements within the specific time period. In the second step, *Calculating Exposure*, the weighted exposure in each rearing period is calculated based on a smoothing of antimicrobial purchases in VetStat during a specific time window. This weighted calculation is done for each rearing period for 12 antimicrobial classes.

![Diagram of the ALEX algorithm](image)

Figure 1: Example of the Average Lifetime EXposure (ALEX) algorithm. In the algorithm the average lifetime exposure to antimicrobials of pigs in the Danish pig industry is estimated based on the CHR registry, movement registry and VetStat.

ALEX takes a slaughter date and the corresponding CHR number as input and traces the pigs movement patterns through the movement register, here it is important to notice, that the pig could come from a fully integrated (all age groups) farm or possibly come from different other farms depending
on the individual farm production strategy. In Figure 1, an example of a finisher farm with internal production and movements from other farms is illustrated. The time windows for the movements are chosen based on the average weight gain period of the pigs in the different rearing periods and the parameter analysis in LEA (Birkegård et al., 2017). The time window of the weaner to finisher moves was 64-99 days prior to slaughter and the time window of piglet to weaner moves was 104-160 days with the average time of the finisher rearing period being 75 days, weaner period being 53 days and piglet period being 30 days. A cutoff value of 7 farms within a production network was chosen as a reasonable maximum. When the trace is completed, VetStat data is used to calculate the average antimicrobial exposure for the traced farms in the periods where the pig potentially could have been there. The time windows for estimating the antimicrobial exposure were chosen rather broad in order to give a smoothed estimate. This means that the antimicrobial purchases were smoothed over a 180 days period prior to the movement of each rearing group. Since slaughter pigs are not registered individually they can only be traced through movements between farms leading to possibly more than one trace for a pig. In ALEX the antimicrobial exposure is weighted according to the number of pigs moved and the estimated internal movements which are not registered. The outcome of the algorithm is an estimate of antimicrobial exposure within each rearing period for the different antimicrobial classes calculated as $AMU_{kg}$ per pig ($[kg_{pig} \times day]$) i.e. the average dose for treatment of one kilogram pig.
2.7. Mixed effect models

The data consists of repeated measurements on slaughter pigs at Danish slaughterhouses. The data was not collected in a balanced way meaning that some farms were only sampled once, while others were sampled multiple times. In total, 1,032 observations from 673 different farms were collected and analyzed during the years.

By applying mixed effect models, repeated measurements sampled at the same farm over time can be utilized, which means that all data collected can be included in the modeling process while avoiding bias in the models. It is assumed that each farm has a different background level of AMR due to factors such as the environment and management approaches which can be modeled by introducing different intercepts by including farms as a random effect in the model. Mixed effects models can be used to describe the variation between farms rather than the difference between them assuming that the farms represent a random selection from the population and that observations within the same farm are correlated.

The estimates in the model were derived by maximum likelihood (ML) rather than restricted maximum likelihood (REML) since the main interest was to estimate the fixed effects of the model rather than the random.

The estimates of the models were tested using Bonferroni correction at an overall level of $\alpha = 0.05$ to account for the multiple comparisons and the models were reduced by backwards elimination.

In all the tested models, variables based on the different antimicrobial classes presented in Table 1, within each of the three rearing periods piglet, weaner, and finisher were included.
<table>
<thead>
<tr>
<th>Antimicrobial classes</th>
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</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
</tr>
<tr>
<td>Amphenicols</td>
</tr>
<tr>
<td>Lincosamides</td>
</tr>
<tr>
<td>Macrolides</td>
</tr>
<tr>
<td>Simple penicillins</td>
</tr>
<tr>
<td>Extended penicillins</td>
</tr>
<tr>
<td>Sulfonamides (incl. trimethoprim)</td>
</tr>
<tr>
<td>Tetracyclines</td>
</tr>
<tr>
<td>Pleuromutilins</td>
</tr>
<tr>
<td>Cephalosporins</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

Table 1: Antimicrobial classes considered in the modeling process.

However, before estimating the parameters in the mixed effect models, variables with less than five observations within the group of antimicrobial class and age group were removed from the data. In the *DANMAP 2018* report the usage of the antimicrobial classes *Cephalosporins* and *Fluoroquinolones* are negligible which was also reflected in data where only four, five and none records of cephalosporins were observed within the piglet, weaner and finisher rearing period, respectively and no records of fluoroquinolons were observed in any of the rearing periods (*DANMAP, 2019*). In addition, there were not observed any antimicrobial use of the class *Other* during the finisher rearing period in data as expected from *DANMAP 2018*. Therefore,
they were not considered in the following analysis. In addition to the exposure variables, three variables *Network size* (Number of farms in network), *Production type* (CHR/CVR integrated or other) and *Number of antimicrobial classes* were constructed. The *Network size* was measured based on the back tracing in ALEX by counting how many different farms were part of the production network. If no movements were found in the given period and the farm had production of all three age groups according to the CHR register, it was categorized as ‘size 1’ (a fully CHR integrated farm). If movements from only one other farm (from piglet to weaner or weaner to finisher) were detected it was categorized as ‘size 2’ (partly CHR integrated farm) and so on. The maximum number of farms in a network was 7 as our cutoff in ALEX, which means that a more complex trading pattern with multiple piglet or weaner farms involved was found. *Production type* was also based on the results of the ALEX algorithm, however, this variable indicates if the production network is CHR integrated, CVR integrated or neither. The variable *Number of antimicrobial classes* was constructed as a counting variable counting how many different antimicrobial classes the pigs were exposed to within the three different age groups, piglet, weaner and finisher. This was again based on the output of ALEX.

3. Results

The distribution of estimated tetracycline exposure based on VetStat for the sampled farms during the three periods 2015, 2017 and 2019 is shown in Figure 2a. The distribution tendency of the average tetracycline exposure is moving towards a lower consumption over time which is in agreement
with what is observed and reported by DANMAP (DANMAP, 2019). The decline in tetracycline exposure could be a reflection of the differentiated yellow card initiative which was implemented in 2016 in order to reduce the usage of certain antimicrobials based on weighted ADDs (DANMAP, 2019).

In Figure 2b the distribution of $R(tet)$ is shown, it is observed that there is a decreasing trend in the mean over time. Distribution plots for the individual tetracycline resistance genes can be found in supplementary material.

![Antibiotic exposure per pig (AMU kg [kg pig * day])](image)

![Distribution of total resistance against tetracycline](image)

**Figure 2:** (a) Change in the distribution of antimicrobial exposure of tetracycline in Danish pig production based on antimicrobial purchases registered in VetStat estimated by ALEX. (b) The distribution of $R(tet)$ (sum of $2^{ΔCq}$ of all tetracycline resistance genes) over time.

In ALEX the cutoff value of 7 farms within a production network resulted in 1032 traceable observations from the 673 sampled farms. The traceability of ALEX was 91.0% which means that for over 90% of the sampled slaughter pig farms traced by ALEX, it was possible to detect one or more plausible routes from piglet period to slaughter. In 9.0% of the cases, however, the tracing failed due to missing detection of either possible piglet or weaner farms, or detection of too many farms in the trading network. A sensitivity analysis was performed on the cutoff value, decreasing the cutoff value to 5 resulted in 1,009 observations from 665 different farms while increasing the...
cutoff to 10 resulted in 1,035 observations from 676 different farms. However, neither of the cutoff values changed the overall conclusion of the models.

Different linear mixed effect models have been applied to data as part of the modeling process in order to investigate the relationship between the level of tetracycline resistance and factors such as antimicrobial exposure and management features.

3.1. Model 1: Simple mixed effect model

The first mixed effect model tested was,

$$
\log_2(R(tet)_{ij}) = \mu_i + \sum_{AM} \left( \beta^{(AM,p)} AMU^{(AM,p)}_{ij} + \beta^{(AM,w)} AMU^{(AM,w)}_{ij} + \beta^{(AM,f)} AMU^{(AM,f)}_{ij} \right) + \epsilon_{ij}
$$

where $R(tet)_{ij}$ describes the total resistance towards tetracycline (presented as $\sum_k 2^{\Delta C_q,k}$ where $k$ symbolizes the 12 tetracycline genes tested) for farm $i = 1, ..., 673$ at sampling point $j = 1, ..., n_i$, $n_i$ is the number of observations for the $i$th farm, $\mu_i$ is the farm-specific mean ($\mu_i \sim N(0, \sigma_i^2)$), $AMU^{(AM,p)}$, $AMU^{(AM,w)}$ and $AMU^{(AM,f)}$ are the estimated exposure variables divided into the different antimicrobial classes ($AM$, see Table 1) within each of the three age groups (piglet, weaner, finisher), respectively, $\beta^{(AM,p)}$, $\beta^{(AM,w)}$ and $\beta^{(AM,f)}$ are the unknown coefficients and $\epsilon_{ij}$ are the residuals ($\epsilon_{ij} \sim N(0, \sigma^2)$). $R(tet)_{ij}$ was log$_2$ transformed due to the nature of the $C_q$ values.

This model is referred to as Model 1.

3.2. Model 2: Mixed effect model accounting for network size

In order to fit data better, Model 1 was expanded to include a term accounting for the size of the production network. After applying the model and testing for significance, it was clear that it was possible to reduce the levels of the variable Networksize due to the relative small presence in data.
and similar estimates of the coefficients of ’size 3’-’size 7’, see Figure 3a. This was done by merging these types into a new level called ’size 3+’. However, reducing the levels even more to ’size 1’ and ’size 2+’ did worsen the fit of the model, see Table 2.

3.3. Model 3: Mixed effect model accounting for production type

Instead of only considering the size of production network, another way of distinguishing between the productions is to categorize the types by fully CHR integrated farms i.e. where the physical location of three rearing periods is the same, CVR integrated farms i.e. farms where the owner is the same, but the pigs might be moved between farms with different locations and other. As seen in Figure 3b there exists a fair amount of CHR and CVR integrated farms in Denmark. Therefore, a model with type levels ’CHR integrated’, ’CVR integrated’, ’other - 2’ and ’other - 3+’ was constructed.

Figure 3: Distributions of production types associated with pig production in Denmark detected by ALEX. (a) Distribution of possible number of farms i.e. Network size. (b) Distribution of Production types: CHR integrated, CVR integrated and other.
3.4. Model 4: Mixed effect model also accounting for the diversity of antimicrobial exposure

A version of the mixed effect model including antimicrobial exposure within age group and antimicrobial class and production type as previous, however, expanded with a variable accounting for how many different antimicrobial classes were prescribed for the specific age groups in the rearing periods (Number of antimicrobial classes) was also applied. By considering how many different classes rather than only the amount of antimicrobials the pig potentially could have been exposed to, it can be seen as an expression of the overall health profile for the farm network. However, this did not have any significant impact on the measured tetracycline resistance levels in the slaughter pigs when testing and the variable was eliminated in the backwards selection, thus resulting in the same model as Model 3. Model checking plots were inspected and did not raise concern (not included in this article).

3.5. Final model

In Table 2, a comparison of the linear mixed effects models (Model 1-3) is shown. Here it is seen that the best fit based on AIC, after reducing the models by backward selection, is Model 3 with the variable accounting for the production type i.e. whether the farm is CHR integrated, CVR integrated, partly CHR integrated with only two farms involved or if more than three farms are part of the production network.

The estimates of the fixed effects of the significant variables after backward selection can be found in Table 3. Here it is noticed that CHR integrated farms does have the lowest estimated intercept i.e. the lowest background level of tetracycline resistance (all other types got a positive value added).
<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
<th>Loglik</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>1686.2</td>
<td>-836.11</td>
</tr>
<tr>
<td>Model 2 - size: n farms</td>
<td>1670.1</td>
<td>-823.05</td>
</tr>
<tr>
<td>Model 2 - size: 1,2,3,4+ farms</td>
<td>1666.9</td>
<td>-824.43</td>
</tr>
<tr>
<td>Model 2 - size: 1,2,3+ farms</td>
<td>1664.9</td>
<td>-824.47</td>
</tr>
<tr>
<td>Model 2 - size: 1,2+ farms</td>
<td>1675.1</td>
<td>-830.56</td>
</tr>
<tr>
<td>Model 3 - type: CHR int., CVR int., 2, 3+ farms</td>
<td>1653.7</td>
<td>-817.83</td>
</tr>
</tbody>
</table>

Table 2: Comparison of the linear mixed effect models (Model 1-3).

The estimate of CVR integrated farms shows a positive effect (higher AMR) compared to the CHR integrated farms, however, the effect is lower than for farms that are neither CHR nor CVR integrated. There are no significant AMU variables in the model for the piglet rearing period, however, the tetracycline exposure in the weaner rearing period and the pleuromutilins exposure in finisher rearing period seems to have a positive effect, while tetracycline exposure in the finisher rearing period have a greater positive influence on the level of tetracycline resistance. This means that only tetracycline and pleuromutilins exposure for weaner and finisher rearing periods are having a significant influence on tetracycline resistance levels in slaughter pigs while the type of production has a highly significant effect.

The correlation between the observed and fitted $R(tet)$ can be seen in Figure 4. The Pearson correlation coefficient is 0.89 giving a sense of the accuracy of the predictions. The fitted values predict the observed values best for low values of $R(tet)$, while the variance of the predictions seems to increase for higher values as expected when fitting on a log₂ scale.
Variable & Estimate & Std. error & P-value \\
\hline
Intercept (i.e. CHR integrated) & -1.13 & $6.40 \times 10^{-2}$ & $< 2 \times 10^{-16}$ \\
Type (CVR integrated) & 0.177 & $7.52 \times 10^{-2}$ & $1.8 \times 10^{-2}$ \\
Type (Other: 2 farms) & 0.271 & $7.17 \times 10^{-2}$ & $1.7 \times 10^{-4}$ \\
Type (Other: 3+ farms) & 0.428 & $6.95 \times 10^{-2}$ & $1.1 \times 10^{-9}$ \\
Tetracyclines finisher ($\beta^{(tet,f)}$) & $7.70 \times 10^{-3}$ & $6.79 \times 10^{-4}$ & $< 2 \times 10^{-16}$ \\
Pleuromutilins finisher ($\beta^{(ple,f)}$) & $2.12 \times 10^{-3}$ & $5.61 \times 10^{-4}$ & $1.7 \times 10^{-4}$ \\
Tetracyclines weaner ($\beta^{(tet,w)}$) & $3.26 \times 10^{-3}$ & $7.46 \times 10^{-4}$ & $1.3 \times 10^{-5}$ \\
\hline

Table 3: Estimates and standard error for the fixed effects parameters of the linear mixed effect model with production type included and farm as random effect (Model 3).

3.6. Biological interpretation of model results

Model 3 represents data best according to the calculated AIC. The biological interpretation of the main results of the model is:

- CHR integrated farms have the lowest background level of tetracycline resistance

- CVR integrated farms have lower background level of tetracycline resistance compared to non-CHR and non-CVR integrated

- Tetracycline used within the weaner and finisher period together with pleuromutilins used within the finisher period leads to a significant elevation of tetracycline resistance levels
Figure 4: Correlation between the observed $R(tet)$ and the fitted $R(tet)$ by the linear mixed effect model also accounting for the production type (Model 3). The Pearson correlation coefficient is 0.89.

- Other investigated antimicrobials do not affect the tetracycline resistance levels
- The type of production, i.e. $CHR_-$, $CVR_-$, partly $CHR$ integrated, or with more than 3 farms in the production network, has the most pronounced effect on tetracycline resistance levels compared to AMU.

4. Discussion and Conclusion

AMR is an ongoing challenge. In order to reduce the occurrence, mathematical and statistical modeling can be used to get a better understanding of the underlying mechanisms. By analyzing data obtained in the real world,
interactions between resistance levels and influential factors can be exposed leading to potential strategies on how to reduce AMR in the pig production.

The data presented in this article is collected as a real world study, where Danish slaughter pigs were sampled during three periods. The samples were analyzed by qPCR measuring 11 unique tetracycline resistance genes. The probes \textit{tet}(O)-1 and \textit{tet}(O)-2 detects different regions of the \textit{tet}(O) gene and are therefore not completely independent in distribution. The final model was performed for a scenario excluding \textit{tet}(O)-2 and a scenario where a mean value of \textit{tet}(O)-1 and \textit{tet}(O)-2 was included instead of the individual measurements, however, this did not change the overall conclusions of the modeling.

In order to give an easily comparable measurement of resistance the sum of the genes (\(R(tet)\)) was used as an expression for the overall tetracycline level in the slaughter pigs. The sum was primarily driven by \textit{tet}(O)-1, \textit{tet}(O)-2, \textit{tet}(W) \textit{tet}(Q), and \textit{tet}(32) which have very few non-detections and high \(\Delta C_q\) mean values (see supplementary material). Out of the total count of tetracycline resistance genes in the samples, the number of copies of measured \textit{tet}(W) genes constitutes 45% on average with a standard deviation of 8%.

As a comparison \textit{tet}(Q) and \textit{tet}(O)-2 constitute 18 ± 8% and 17 ± 6%, respectively, reflecting the considerable dominance of \textit{tet}(W). The resistance genes explored in this study are recovered from pigs, however, it would be interesting to investigate whether the genes are found in zoonotic bacteria since this would pose a risk for humans as well (Afema et al., 2014; Pan et al., 2018, 2019; Mather et al., 2013)

Data on influential factors such as antimicrobial purchases and size of trading network was retrospectively collected from Danish registries. The
antimicrobial exposure was estimated through the ALEX algorithm in order to calculate the average exposure based on the purchases by the farms during the different rearing periods oppose to calculating the antimicrobial exposure as a lifetime exposure (Dalhoff Andersen et al., 2020). When working with registries it is important to remember, that the data is not collected for research or scientific purposes. Missing and faulty (incorrect) data are well known issues when working with data registries, however, the Danish registries are updated often thereby reducing reporting delays. Estimating the antimicrobial exposure based on registries can be done in different ways. Most important sources of uncertainties are that the exact moving pattern of the slaughter pigs sampled are not available and the antimicrobial purchases are registered on an age group level within each farm not distinguishing between the specific pigs or the piglet/sow age group.

The ALEX algorithm was capable of tracing 91.0% of the sampled slaughter pig farms which is comparable to the traceability of the LEA algorithm (Birkegård et al., 2017). The untraceable cases were mostly due to faulty registration in the CHR and the movement registers leading to incomplete paths of weaner and piglet trading networks. However, the data still gives an idea of how the trends of AMR is affected by the antimicrobial exposure in Danish pig production.

The overall fit of the final model captured the trend of the data well, however, the variance of the prediction seemed to increase for higher values. The final model was extended to include a first order autoregressive covariance structure in order to check for autocorrelation in the errors, however, this did not change the fit of the model. Using mixed effect modeling the
main influential factors on tetracycline resistance were found to be type of production, tetracycline exposure in the weaner and finisher rearing periods and pleuromutilins exposure in the finisher rearing period. The association between tetracycline exposure and tetracycline resistance in Danish slaughter pigs has been suggested by previous modeling work, where also different management factors such as number of produced pigs and route of administration were considered (Birkegård et al., 2017; Dalhoff Andersen et al., 2020; Vieira et al., 2009). Management factors have gained a growing interest within the field of antibiotic resistance research, however, the herd size of livestock farms appears to be the most significant one causing many of the other factors such as biosecurity, number of animal suppliers, human traffic in the herd etc. (Bokma et al., 2020; Jiang et al., 2019; Raasch et al., 2018; Sørensen et al., 2018; Xu et al., 2020; Zuliani et al., 2019). The results presented here suggest that there is a strong association between the levels of tetracycline resistance in slaughter pigs and the type of production. This indicates that complicated moving patterns involving multiple farms and owners yield increased tetracycline resistance compared to fully (CHR) integrated farms where pigs stay on the same geographical location during their entire life cycle but also CVR integrated farms where pigs are moved between farms with a single ownership. This is in agreement with similar results obtained by Sørensen et al. (2018). The findings indicate that CHR but also CVR integrated farms work as a preventive tool against higher tetracycline resistance.
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References


DANMAP, 2019. DANMAP 2018 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Technical Report. Statens Serum Institut and National Food Institute, DTU.


