Predicting effects of changed antimicrobial usage on the abundance of antimicrobial resistance genes in finisher' gut microbiomes

Dalhoff Andersen, Vibe; Møller Aarestrup, Frank; Munk, Patrick; Stengaard Jensen, Marie; de Knegt, Leonardo; Bortolaia, Valeria; nudsen, B. E.; Lukjancenko, Oksana; Birkegård, A. C.; Vigre, Håkan

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Predicting effects of changed antimicrobial usage on the abundance of antimicrobial resistance genes in finisher’s gut microbiomes


The National Food Institute, Technical University of Denmark, Lyngby, Denmark

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ABSTRACT

It is accepted that usage of antimicrobials (AMs) in food animals causes the emergence and spread of antimicrobial resistance (AMR) in this sector, while also contributing to the burden of AMR in humans. Curbing the increasing occurrence of AMR in food animals requires in-depth knowledge of the quantitative relationship between antimicrobial usage (AMU) and AMR to achieve desired resistance reductions from interventions targeting AMU. In the observational study, the relationships between lifetime AMU in 83 finisher batches from Danish farms and the AMR gene abundances of seven antimicrobial classes in their gut microbiomes were quantified using multi-variable linear regression models. These relationships and the national lifetime AMU in pigs were included in the predictive modelling that allowed for testing of scenarios with changed lifetime AMU for finishers produced in Denmark in 2014. A total of 50 farms from the observational study were included in validating the observational study and the predictive modelling. The results from the observational study showed that the relationship was linear, and that the parenteral usage of AMs had a high effect on specific AM classes of resistance, whereas the peroral usage had a lower but broader effect on several classes. Three different scenarios of changed lifetime AMU were simulated in the predictive modelling. When all tetracycline usage ceased, the predicted interval reductions of aminoglycoside, lincosamide and tetracycline resistance were 4–42%, 0–8% and 9–18%, respectively. When the peroral tetracycline usage of the 10% highest users was replaced with peroral macrolide usage, the tetracycline resistance fell by 1–2% and the macrolide and MLSb resistance increased by 5–8%. When all extended-spectrum penicillin usage was replaced with parenteral lincosamide usage, the beta-lactam resistance fell by 2–7%, but the lincosamide usage and resistance increased by 194% and 10–45%, respectively. The external validation provided results within the 95% CI of the predictive modelling outcome at national level, while the external validation at farm level was less accurate. In conclusion, interventions targeting AMU will reduce AMR abundance, though differently depending on the targeted AM-class and provided the reduction of one AM-class usage is not replaced with usage of another AM-class. Predicting several classes of AMR gene abundance simultaneously will support stakeholders when deciding on interventions targeting AMU in the finisher production to avoid adverse and unforeseen effects on the AMR abundance. This study provides a sound predictive modelling framework for further development, including the dynamics of AMU on AMR in finishers at national level.

1. Introduction

AMR is considered one of the most harmful threats to global health. AMU is accepted as the main cause (WHO, 2012; Friedman et al., 2016). In an attempt to reduce the occurrence of AMR, several AMU stewardship programs have been implemented. However, these programs have encountered obstacles to introducing major declining effects on the occurrence of AMR, and none successfully predicted any
changes (Jensen and Hayes, 2014; Tang et al., 2017).

Although production animals are generally acknowledged to contribute to the burden of AMR among humans (Marshall and Levy, 2011; Aarestrup, 2015), less is known about the causes of the emergence and spread of AMR in the food chain and the risk posed to humans (Aarestrup, 2015). The contribution of foodborne AMR to human infections is the subject of intense debate (Marshall and Levy, 2011; Aarestrup, 2015; Holmes et al., 2016). Consequently, the focus on AMU in production animals has grown immensely during the past decade (Marshall and Levy, 2011). In several countries, surveillance systems have been established to monitor trends and changes in AMU and AMR in animals (EMA, 2016). These monitoring systems have, in turn been efficiently applied to facilitate interventions and guidelines for improved antimicrobial (AM) stewardship (Jensen and Hayes, 2014; Wielinga et al., 2014; Tang et al., 2017).

Epidemiological studies have established that AMU and AMR in production animals are closely related (Rao et al., 2010; Aarestrup, 2015; Callens et al., 2015). Similarly, halting usage of e.g. vancomycin and third-generation cephalosporin has proven to reduce the occurrence of AMR to these compounds (Aarestrup et al., 2001; Agersø, and Aarestrup, 2013). However, less light has been shed on the quantitative relationship between AMU and AMR, as neither the quantification of AMU nor the characteristics of the AMs, e.g. route of administration, dose, duration of treatment, and concurrent interrelation between AM compounds, have been fully determined in terms of importance for the selection for AMR (Collineau et al., 2017). In addition, most studies conducted to date have focused on only one or a few indicator bacteria, whereas the bulk of relevant AMR genes might be present in an entire gut microbiome. The recent developments in next-generation sequencing allow complete quantification of the abundance of AMR genes in an entire gut microbiome (Munk et al., 2017).

Denmark is one of the largest exporters of pork products globally (SEGES, 2016), and the total AMU within Danish pig production is considerable; in 2016, it represented 75 % of the total amount of kilograms of active substance for animals and was 57 % higher than human usage (DANMAP, 2016). From a public-health perspective, the overall AMU in pig production in Denmark is still substantial although the Danish authorities have launched several initiatives to reduce the usage.

To provide the means for estimating an association between specific AMU and AMR at national level, two previously conducted studies developed and validated a method based on register data. This method quantified the associations between the AMUs of six AM-classes used in Danish pig production and the AMR abundances of these classes in ten finisher batches close to slaughter. The method was subsequently used to measure AMUs in finisher batches throughout the finishers’ lifetimes by combining usage in the piglet, weaner and finisher rearing period, independent of rearing site. The AMR abundances were measured using shotgun metagenomic sequencing, which gave a proportional content of AMR abundance independent of bacteria species (Andersen et al., 2017; Munk et al., 2017; Andersen et al., 2018). Spicknall et al. (2013) reviewed studies focused on predicting the effect of AMU changes on the occurrence of AMR. Most of these studies were based on theoretical data and, to the best of the authors’ knowledge, none concerning AMR gene abundances in the gut microbiomes of animals. More recently, Arepyeva et al. (2017) constructed a mathematical model describing the dependency between AMR and AMU. Moreover, a review has been published with recommendations for the building of models predicting the occurrence of resistance (Arepyeva et al., 2015). Improved knowledge of the effect of AMU of individual AM-classes on all AMR gene abundances combined with insight into either AMU or AMR for larger parts of a population allow for the prediction of the overall effect of an intervention targeting AMU in general or for individual AM-classes in a certain population, for instance a country (Andersen et al., 2017; Birkegård et al., 2017a,b; Andersen et al., 2018). Additionally, a modelling framework of the epidemiology of AMR abundance used to describe differences in the gut microbiomes of finishers under the influence of AM pressure can also be used to support knowledgeable guidance on AMU practices at farm level.

The objectives of this study were i) to perform an observational study on batches of finishers close to slaughter from Danish farms to estimate the quantitative relationships between AMU during the rearing period (lifetime AMU) and AMR gene abundances in their gut microbiomes, ii) to integrate these relationships into the predictive modelling of the abundances of AMR genes in finishers given the current lifetime AMU, as well as given changed lifetime AMU obtained from potential interventions in every finisher batch in Denmark, thereby enabling assessment of effects of potential AMU interventions on AMR at national level, and iii) to validate the results from the observational study and the predictive modelling.

2. Material and methods

The study included three parts: an observational study (Observational study), a predictive modelling (Predictive modelling), and a validation (Validation) of both the observational study and the predictive modelling.

2.1. Study design

The source population was restricted to Danish pig farms delivering more than 800 pigs annually and receiving pigs from a maximum of four suppliers annually in 2013. These farms were stratified based on the farm characteristics; production type (conventional or organic), annual number of pigs delivered for slaughter, and annual number of pig suppliers. Subsequently, of the 19.3 million pigs slaughtered that year, the selected source population comprised 90 % of the total number of slaughtered pigs (SEGES, 2014), and 57 % of the total number of pig farms (Table 1).

Based on data from the Central Husbandry Register (CHR) and the

<table>
<thead>
<tr>
<th>Strata</th>
<th>Source population</th>
<th>Total slaughtered</th>
<th>Study sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Farms</td>
<td>Conventional</td>
<td>Organic</td>
</tr>
<tr>
<td></td>
<td># Supplier annually</td>
<td># Slaughtered annually</td>
<td>Conventional</td>
</tr>
<tr>
<td>0 - 1</td>
<td>800–4999</td>
<td>1,816</td>
<td>24</td>
</tr>
<tr>
<td>2 - 4</td>
<td>800–4999</td>
<td>685</td>
<td>–</td>
</tr>
<tr>
<td>0 - 1</td>
<td>≥5000</td>
<td>938</td>
<td>–</td>
</tr>
<tr>
<td>2 - 4</td>
<td>≥5000</td>
<td>420</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>3,859</td>
<td>24</td>
<td>17,332,541</td>
</tr>
</tbody>
</table>
Pig Movement Database (PMD), the source population was separated into two sub-populations; i) the current most common farm size delivering pigs to the pork industry in Denmark and ii) larger-scale farms delivering more than 5000 pigs for slaughter annually, which is expected to be the predominant farm size for delivering pigs for slaughter in the near future (Jensen et al., 2011). The two sub-populations were subsequently separated based on the annual number of suppliers specifically to signify ownership complexity in the context of the rearing pathways of finisher batches. Thus, a rearing pathway with 0–1 supplier annually was assumed to cover farms owned by one farmer, and a rearing pathway with 2–4 suppliers was assumed to cover farms owned by different farmers (Table 1). The stratification was implemented to obtain a representative study sample within each stratum for comparison against farms producing the majority of pigs slaughtered annually in Denmark with the predominant supplier paths. For the purpose of comparison, the organic farms were chosen in the strata with 0–1 supplier and individual productions of 800–4999 slaughtered pigs per year only (Table 1).

The collection and laboratory analysis of samples from about 80 farms aligned with the resources in the project, thereby encompassing approximately 20 farms in each group – 80 conventional and 5 organic farms.

The list of potential farms was randomized within each stratum, and initially, letters of invitation were sent to 20 farms in each group. Farms were then contacted by telephone in the following weeks to determine if they were interested in participating and if so, to plan the visits. Hereafter, 20 additional farms across the groups were invited, until the appropriate number of farms to visit in the study was obtained.

For validation purposes, fifty of the conventional farms were re-visited and re-sampled at least six months after the first visit. The resources available covered the collection and laboratory analysis of samples from 50 farms in the project. The selection of these farms was not strata specific.

The predictive modelling study included every finisher batch delivered for slaughter during a specified period in Denmark.

### 2.2. Sampling

At each visited farm, samples were collected from finishers > 80 kg. With the help of the farmers, all sections containing these finishers were identified. The number of pens was counted. Then, using a randomized list of all pens counted obtained from random.org/lists, the first 30 pens on the list were sampled. If the farm had less than 30 pens containing finishers > 80 kg, random pens were sampled 2–3 times in order for the total number of samples to reach 24–30 pens. One person, accompanied by an assistant, carried out the sampling at all the farms. The sampling material was fecal material collected directly from the pigs as they defecated or immediately after from the pen floor. If collected from the floor, only the top of an undisturbed stool was collected. In the laboratory, each sample was mixed thoroughly and an equal amount from each sample was weighed out and pooled into one. This composite sample was then used for further analyses. Sampling took place from December 2014 to August 2016.

The samples were transported to the laboratory in a thick-walled polystyrene box containing cooling elements. Within 6 h of collection the samples were placed in 4 °C storage and were subsequently processed in the laboratory within 24 h of collection.

### 2.3. Data sources

Data on AMU were obtained from the Danish Veterinary Medicine Statistic Program database (VetStat), which contains records on purchased medicines prescribed by veterinarians for animals. Each record has information on the product name, substance, dispensing type, amount, target species, age-group, diagnosis group and farm code (ID) (Stege et al., 2003). In order to produce comparable data across records, substances were converted into a unit for measuring how many kilograms of pig were treated per day, known as the Animal Defined Daily Doses per kilogram (standard doses ADD (mg/kg)) (Jensen et al., 2004).

Demographic data on farms were obtained from the CHR and data on movements of pigs between farms, or from farms to slaughterhouses were obtained from the PMD (Stege et al., 2003; Houe et al., 2011). By combining data from the CHR and PMD, the movements of pigs between farms and the annual number of pigs moved out of a farm either to another farm or to a slaughterhouse could be obtained (Stege et al., 2003; Houe et al., 2011). As an adjustment factor for farm size, a proxy measure was calculated. First, the annual production of sold and/or slaughtered pigs on a farm was multiplied by the 2015 national average production durations for piglets, weaners and finishers, thus 30, 55, and 85 days, respectively. These intervals were then divided by 365 days in order to calculate the number of piglets, weaners and/or finisher in a farm on any given day (SEGES, 2016).

#### 2.3.1. Estimation of lifetime AMU

Daily AMUs were calculated using the interval between the date of the initial VetStat record and the date of the subsequent record (days) for the i’th active compound in mg (ACij) at the j’th dispensing level (parenteral/peroral) (ACij) of each AM product purchased during a specified period, in the k’th age-group (piglets/weaners/finishers) (ACik), on the l’th farm (ACijkl) and adjusted in accordance with the number of pigs at risk in the k’th age-group on the l’th farm (pigijkl) using the formula [1]:

\[
\text{ADD}_{ijkl} = \frac{AC_{ijkl}}{\text{days} \times \text{standard dose} \times \text{ADD}_{ijkl} \times \text{pig}_{ijkl}}
\]

(1)

The days was estimated for peroral as; days between VetStat records at farm, age-group and dispensing-type levels, and for parenteral as; days between VetStat records at farm, age-group, dispensing-type and AM-class levels (Andersen et al., 2018). In addition, the calculation of days was based on three assumptions. First, if the number of days was less than eight, the subsequent record date was used instead. Second, if no subsequent date was found, the mean of the former intervals in days was applied. If no prior number of days was available, 90 and 365 was utilised for peroral and parenteral dispensing-types, respectively. Third, all numbers of days exceeding 90 days for peroral dispensing and 365 days for parenteral dispensing-type were replaced with 90 and 365 days, respectively (Andersen et al., 2018). The \( \text{ADD}_{ijkl} \) measures the number of ADD doses for one kilogram pig per pig each day in a specified period of time of an active compound at dispensing level in an age-group on a farm.

The number of \( \text{ADD}_{ijkl} \) was then summarised per day at AM-class level (c) of aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, narrow-spectrum penicillins, pleuromutilins, polyoxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim, at the j’th dispensing level, in the k’th age-group, on the l’th farm (\( \text{ADD}_{ijkl} \)) using the formula [2]:

\[
\text{ADD}_{ijkl} = \sum_{c} \text{ADD}_{ijkl}^c
\]

(2)

The lifetime AMU of the batches of finisher was calculated for the c’th AM-class of the j’th dispensing type by summarizing the r’th rearing period of the the k’th age-group, and adjusted to suit the proportion (P) of animals being moved from a farm, and then summarized given the rearing pathways of the batches of finishers’ (b) using the formula [3]:

\[
\text{Lifetime AMU}_{ijkl}^b = \sum_{r,b} \text{ADD}_{ijkl}^b \times P
\]

(3)

The lifetime AMU is an average estimate related to usage during the entire rearing period independent of rearing site (Andersen et al., 2017, 2018). The finisher batches pathways were established by following them through rearing sites from the sampling farm back to the farm of birth. The Danish national average production durations for 2015 were
applied for the rearing periods per unit (in days) \cite{SEGES2016}, resulting in 30, 55 and 85 days in the farrowing (piglet), weaning (weaner) and finisher units (finisher), respectively. The lifetime AMU_{ab,j} quantifies the total number of kg doses per pig for each AM-class at dispensing level during the rearing period of 170 days [ADDkg/pig]. The AMU for sow was included in the usage for piglets, as previous studies have shown that this affects the occurrence of AMR in the gut microbiomes of piglets. Thus, it was assumed that the doses used to treat sows were equivalent to the doses used to treat piglets \cite{Callens2015}.

### 2.3.2. Estimation of AMR gene abundance

The methods used to extract DNA from the pooled fecal samples and obtain AMR gene abundances in the microbiome of finisher batches \((b)\) have been described in previous studies \cite{Munk2017}. In brief, all known genes encoding to the \(a^{th}\) class of AMR (AMR_{ab,0}) to of aminoglycosides, beta-lactams, lincosamides, macrolides, macrolides lincomamide streptogramin B (MLSb), sulfonamides and tetracyclines, were quantified using shotgun metagenomic sequencing \cite{Munk2017}. Resistance was quantified using the MGmapper tool against the ResFinder database, April 2017 \cite{Zankari2012}. As the database contains several highly homologous genes, when reads were mapped to identical parts of homologous gene variants unspecified mapping occurs. Read counts from variants of the same gene were aggregated to gene levels according to common gene names, resulting in the final resistance gene abundance matrix. For each AM-class, the raw read counts were normalized to the length of each gene and sequencing depth of each sample, and were thus measured as Fragments per Kilobase reference per Million fragments [FPKM] using the formula \cite{4}:

\[
AMR_{ab} = \frac{n \times (N - (l + 2 \times m))}{2 \times 10^3 R \times 10^6 bp} \tag{4}
\]

where \(n\) = number of mapped reads, \(N\) = total number of reads, \(l\) = gene length, \(i\) = insert size, \(m\) = minimum mapping length, \(R\) = reads and \(bp\) = base pair.

### 2.4. Data analyses

#### 2.4.1. Observational study

Data analyses focused on the quantitative association between lifetime AMU and AMR gene abundances of seven AM-classes; aminoglycosides, beta-lactams, lincosamides, macrolides, MLSb, sulfonamides and tetracyclines. From here, the lifetime AMUs at class level refers to the AM-classes; aminoglycosides, extended-spectrum penicillins, lincosamides, macrolides, narrow-spectrum penicillins, pleuromutilins, polymyxins, spectinomycin, sulfonamides, tetracyclines and trimethoprim.

Lifetime AMUs were plotted against AMR gene abundances at AM-class level in scatterplots. Next, a LOESS local non-parametric regression model, in which least squares regressions are performed in localized subsets of the dataset, i.e. 0.75 of the observations per regression (span width), was added to evaluate the relationships between lifetime AMUs and AMR gene abundances. Based on the LOESS regression lines, it was decided to model the effect of AMU on AMR as linear. Afterwards, the estimated uni-variable linear regression models with 95% CI of the respective AM-class were overlaid on the observations of each scatterplots. The scatterplots were finalized by implementing bi-square robust regression lines.

For each farm, the study design variables used to select farms were updated to the status at sampling i.e. the farms production types, yearly number of suppliers and slaughtered pigs were estimated 1 year prior to sampling. Then, additional simple linear regression analyses were performed to determine potential confounding effects of the updated design variables and dispensing type.

Multi-variable regression models were performed that included the potential direct and confounding effects of lifetime AMUs at AM-class and dispensing levels as well as updated design variables on each class of AMR gene abundance. Intermediate models were obtained using automated stepwise regression, with the lowest AIC value as the criteria for variable selection. Then, final models were obtained by removing insignificant variables using manual stepwise exclusion. The comparisons between models were performed using the ANOVA; Chi-square test. When these were not significantly different \((p \text{-value} > 0.05)\), the simpler model was preferred. The explanatory variables kept in the models were checked for potential correlation. At gene level, uni-variable regression models were used to estimate the effect of lifetime AMU at class and dispensing level on abundance of each resistance gene. Genes that were found in less than 10 samples were not included. To account for multiple regressions, p-values above 0.01 were assumed to be non-significant.

Diagnostic plots were applied to assess whether the assumptions of linear regression of the estimated models were fulfilled. Furthermore, bi-square robust regression was used to assess all models for the influence of potential outlying observations on the estimated effects using bi-square robust regression.

#### 2.4.2. Predictive modelling

The estimated effects of the multi-variable linear regression models in the observational study, combined with the data on AMU at pig farms and all movements of pigs between farms and to slaughterhouses in Denmark, provided the opportunity to predict the overall AMR gene abundances in the majority of Danish finishers at slaughter at national level \cite{Birkehagard2017}. Consequently, this framework also made it possible to predict the relative effect of different AMUs in all or a subset of pig farms on the overall AMR gene abundance in Danish finishers close to slaughter. In these predictions, it was assumed that the estimated effects of lifetime AMU on AMR gene abundance in the observational study could be generalized across all conventional finishers in Denmark.

Initially, the mean AMR gene abundance for each AM-class in each finisher batch delivered from finisher farms to slaughterhouses in Denmark \((AMR_{ab})\) was predicted using the \(\hat{\beta}\)-coefficients from the observational study’s multi-variable regression models and batch specific lifetime AMU \cite{Eq. 3} using the formula \cite{5}:

\[
AMR_{ab} = \beta_0 + \text{Lifetime AMU}_{ab,j} \hat{\beta}_j + \ldots \tag{5}
\]

The “…” refers to the additional \(\hat{\beta}\)-coefficients describing the effects of lifetime AMUs at class and dispensing level that had a significant effect on AMR gene abundances \cite{Table S5}.

Secondly, to obtain the baseline resistance gene abundance for each AM-class at national level \((AMR\text{-national level},)\), the predicted means of AMR gene abundances for each AM-class in each batch were summarised across all batches in Denmark, weighted with the number of finishers delivered for slaughter during the previous 6 months from the corresponding farm \((N)\). The sum constitutes the baseline AMR gene abundance measure for each AM-class \cite{Eq. 5} using the formula \cite{6}:

\[
AMR\text{-national level}_{ab} = \sum_j AMR_{ab,j} \times N_j \tag{6}
\]

Simultaneously, the same calculation was performed using data on lifetime AMUs mimicking alternative usage in either all or a subset of the batches as input. The relative effect on the AMR gene abundances of the different AMU scenarios was obtained by comparing the AMR gene abundance to the baseline gene abundance at national level.

To include the uncertainty in the estimated regression parameters, the above calculations of AMR gene abundances and the relative change were carried out 100,000 times (iterations), and in each iteration, random values of the effect parameters were selected from Gaussian probability distributions. These distributions were defined using the
3. Results

In the study the following main results were found: i) the observational study showed that the effect of lifetime AMU on AMR gene abundance was linear for most AM-classes. Furthermore, the parenteral usage of AM had a higher effect on specific AM-classes of resistance, whereas the peroral usage had a lower but broader effect on several AM-classes of resistance; ii) the predictive modelling showed that it is possible to predict the relative change in AMR gene abundance of several AM-classes simultaneously from changed usage of one or more AM(s); and iii) the validation provided results within the range of the predictive modelling outcome at national level, which supported the results of the predictive modelling, though the external validation at farm level was less accurate.

3.1. Study design

A total of 83 farm owners agreed to participate in the observational study; 78 conventional and 5 organic distributed across 4 strata (Table 1). Eight rounds of invitation letters were sent with a response rate of 66%. The finisher batches varied in rearing pathways, however, the pathway of the majority of batches were simple, i.e. pigs in a unit originated from the same farm or from one supplier only (Figure S1). The parenteral and peroral lifetime AMUs of the finisher batches are presented in Figure S3. The distribution of parenteral and peroral lifetime AMUs per rearing unit revealed that parenteral dispensing was mainly used for piglets, including sows, while peroral dispensing was more common for weaners and finishers.

The validation study consisted of 50 farms from the observational study, which were revisited and resampled six to eight months after the first visits. A total of 3079 finisher batches were delivered to slaughterhouses from April 2014 to June 2014 and these batches were all included in the predictive modelling.

3.2. Data analyses

3.2.1. Observational study

For the uni-variable regression models, the lifetime AMUs demonstrated a significant effect on the AMR gene abundance in six of the seven resistance AM-classes (Fig. 1). Though, for lincosamides, one observation with a Cook’s distance higher than one was identified, and when the impact of excluding this observation on the model was assessed, the lifetime usage of lincosamides remained significant. Therefore, the observation was not excluded from the subsequent analyses. The proportion of observed variations (adjusted $R^2$) in AMR gene abundances of the significant results that could be explained by the lifetime usages ranged from 6 % to 49 % (Fig. 1). The estimated models of bi-square robust regressions added to the scatterplots indicated that the data included no notable influential observations (Fig. 1).

Only the production-type was significant in determining the effect of the updated variables in the simple regression models. However, due to the few organic farms the remaining regression analyses were performed for the conventional farms only (Table S4). In contrast, the effect of parenteral and peroral usage in the simple regression models displayed substantial differences for all AM-classes of lifetime AMUs on the individual AMR gene abundances (Table S4).

From the multi-variable regression models, the significant results of the resistance gene abundances of the seven AM-classes demonstrated two noticeable outcomes; the overall difference between parenteral and peroral dispensing and the effect of peroral macrolides and both parenteral and peroral tetracyclines on several AM-class resistances (Fig. 2 and Table S5). The obtained estimates of the fitted bi-square robust regression models changed the significant $\beta$-coefficients by 7 % or less, indicating that the estimated effects are robust against outliers (Table S5). The annual numbers of suppliers and slaughtered pigs were included in each of the seven models, but were excluded during the automated model reduction.

The assessment of the effect of lifetime AMUs at class and dispensing levels on each gene of the seven AM-classes of resistance demonstrated that the specific AM-class resistance genes were affected primarily by the same AM-class usage (Figure S6). Conversely, the abundance of aminoglycoside resistance genes was affected by several AM-class usages, which is consistent with the results of the multi-variable regression model of aminoglycoside resistance (Table S5). In addition, the abundance of MLSb resistance genes, $erm(B)$, $erm(F)$ and $erm(G)$, seemed to be affected by the parenteral aminoglycoside usage, which was in alignment with the multi-variable regression model. Although most of the tetracycline resistance genes were affected by peroral tetracycline usage, several tetracycline resistance genes were affected by other AM-classes (Figure S6).

3.2.2. Predictive modelling

Table 2 shows the predicted effect of different lifetime AMU scenarios on the overall resistance gene abundance of different AM-classes
in the Danish pig production. In the scenario where the entire tetracycline usage ceased, the tetracycline resistance gene abundance can be expected to be reduced by 9–18 %. In addition, with the lincosamides and aminoglycosides resistance gene abundances also being reduced, even though the size of these reductions is less certain (Table 2).

In the scenario where the top 10 % highest users of peroral tetracycline usage replaced this AM-class with peroral usage of macrolides, at national level, the tetracycline usages can be expected to fall within the 10 % highest users by 38 % and the total usage by 16 %, while the total usage of macrolides can be expected to increase by 24 %. In total, a 16 % reduction of tetracycline usage in Danish pig production would be expected to reduce the tetracycline resistance gene abundance by 1–2 %. At the same time, the resistance gene abundances of beta-lactams, macrolides and MLSb can be expected to increase relatively more compared to the reduction of tetracycline resistance gene abundance, due to the increased macrolide usage (Table 2).

In the scenario where parenteral and peroral usage of extended-spectrum penicillins were replaced with parenteral lincosamide usage,
the beta-lactam resistance can be expected to be reduced by 2–7 %, but the lincosamide usage and resistance gene abundance can be expected to increase by 194 % and 10–45 %, respectively (Table 2).

3.2.3. Validation

Comparison of the uni-variable regression models of the observational study with the validation study models showed that the $\beta$-coefficients of the validation study overlapped the $\beta$-coefficients 95 % CI of the observational study of each AM-class resistance (Table S7 and Figure S8). When the intercepts of the AM-classes of the two studies were compared, only extended-spectrum penicillin and tetracycline did not have similar intercepts (Figure S8).

When compared to the observed adjusted $R^2$ values, the predicted $R^2$ values of the multi-variable regression models of the observational study were underestimated by between 4 % and 56 % (Table S9). Comparison of the observed adjusted $R^2$ values in the observational study’s multi-variable regression models to the predicted $R^2$ values of the validation study’s multi-variable regression models resulted in underestimation of the observed of aminoglycosides, beta-lactams, lincosamides, sulfonamides and tetracyclines by 88 %, 61 %, 24 %, 111 % and 66 %, respectively. In contrast, macrolides and MLSb were overestimated by 3 % and 11 %, respectively (Table S9).

The predictive modelling performed with the coefficient obtained from the multi-variable regression models of the validation study provided results within the 95 % CI of the observational study for all three scenarios (Table 2).

4. Discussion

A reliable model for predicting precisely the most efficient intervention targeting AMU in reducing AMR gene abundance would be highly valuable for guiding political decision making. The study determined the relationships between AMU and AMR at pig-farm level in finisher batches close to slaughter and used this to establish a predictive model for how different changes in AMU at class level would influence AMR gene abundance at national level. The validity of generalizing the results from the observational study against the predictive modelling was strengthened by a retrospective evaluation. This showed that the lifetime AMUs in the finisher batches included in the observational study was dispersed throughout the range of lifetime AMUs in all finisher batches used in the modelling. In other words, the batches included in the observational study can be considered representative for the investigation of the general association between lifetime AMU and AMR gene abundance in conventional finishers in Danish pig production. The observed associations can thereby be used to predict the effect of different lifetime AMU scenarios across conventional pig production.

The majority of predictive studies of occurrence of AMR have different structures in terms of complexity and inherent assumptions. Conversely, these models share the assumption that the treatment effect is constant (Spicknall et al., 2013; Arepyeva et al., 2015, 2017; Blanquart et al., 2017). By applying the actual information regarding the AMU at unit level on each farm, the movement of pigs between farms and the number of pigs delivered for slaughter from each farm, the predicted results in our study were anchored in the actual conditions in Danish pig production (Birkegård et al., 2017a).

As expected in the light of previous studies, the observational study found a significant association between lifetime AMU and AMR gene abundance. The association could be described using a simple linear association. AMU is low in Denmark, therefore, extrapolation of the observed linear relationship between lifetime AMU and AMR gene abundance to higher levels of AMU should be conducted with caution. It is important to note that in the regression models, the intercepts are not zero, which is of major importance for the modelling. The associations between AMU and AMR also differed between the various AM-classes, suggesting that similar reductions in AMU for different classes will have different effects. Interestingly, the study found a higher effect on class-specific AMR for parenteral AMU, and a lower but broader effect for peroral AMU. The broader effect of peroral AMUs may be due to their widespread but intermittent usage during the weaner and finisher rearing periods, which is supported by findings in other studies (Wiuff et al., 2003; Zhang et al., 2013). Conversely, the lack of effect of parenteral extended-spectrum penicillins, macrolides and sulfonamides could result from their usage primarily in the piglet-rearing period.

Although significant associations were found between lifetime AMU and AMR gene abundance of six AM-classes, the usage of AMs could only explain between 9 %–52 % of the variation in AMR. While some of the unexplained variations are due to measurement error. Overall, a substantial part of the variation still requires explanation.

The significantly lower AMU and AMR gene abundances of most AM-classes in the organic production compared to conventional...
The predicted 95% CI of change of resistance (observational study) and the predicted % change of resistance (validation study) using three different hypothetical reduction scenarios of lifetime AMU in Danish finisher batches. The outcome of all models was AMR abundance of selected AM-classes in the gut microbiome close to slaughter at national level.

<table>
<thead>
<tr>
<th>Change in lifetime AMU (%)</th>
<th>Predicted change in AMR gene abundance (%)</th>
<th>Predictive modelling</th>
<th>AMU classes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Penicillins (ext.)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>1. Ceased parenteral and peroral tetracycline usage</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2. Reduction by the top 10% users of peroral tetracycline usage and replacement with peroral macrolide usage</td>
<td>–</td>
<td>–</td>
<td>24</td>
</tr>
<tr>
<td>3. Ceased peroral and parenteral extended-spectrum penicillin usage and replacement with parenteral lincosamide usage</td>
<td>−100</td>
<td>194</td>
<td>–</td>
</tr>
<tr>
<td>Validation</td>
<td>–</td>
<td>–</td>
<td>24</td>
</tr>
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</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>Predictive modelling scenario</th>
<th>Penicillins (ext.)</th>
<th>Lincosamides</th>
<th>Macrolides</th>
<th>MLSb</th>
<th>Tetracyclines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Observed parenteral and general tetracycline usage</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2. Reduction by the top 10% users of general tetracycline usage</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3. Ceased peroral and parenteral extended-spectrum penicillin usage</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

The study showed, that these measurement errors also biased the effect estimates towards zero, and the predicted effects of different lifetime AMUs are therefore biased towards zero (Andersen et al., 2018). As the predictions have not been adjusted for this bias, the predicted relative effects of different AMU in pig production were conservative predictions.

The validation study revealed limited value at individual farm level, but very high predictive value when considering all farms. Thus, though it might be very difficult to predict exactly what will happen at individual farms, it is nonetheless possible to predict changes reliable for groups of farms or at national level using the study’s multi-variable regression models.

The study has a number of limitations related to farm selection, calculation of AMU and measurement of AMR, which should be considered when drawing broader conclusions. The restrictive farm selection and stratification yielded a source population representative of the vast majority of farms delivering pigs for slaughter in Denmark. The study had an over-representation of larger farms, but with a size and production system in alignment with the structural development in pig farms expected by the pig industry in the near future (Christiansen, 2014).

Lifetime AMUs were calculated as total amounts throughout the rearing period at dispensing-type and AM-class level. Consequently, differences in AMU within the three rearing periods could not be distinguished. For that reason, finisher batches with similar AMUs might relate to usage in different units. Thus, the AMR gene abundances in the gut microbiomes of these pigs may be different as changes in AMU can happen over short periods (Dawson et al., 1984; Cavaco et al., 2008; Munk et al., 2017). Subsequently, the lifetime AMUs do not take into account the influence of usage in the rearing period of piglets compared to usage closer to slaughter. Nothing indicated that the updated design variables; annual number of slaughtered pigs or the annual number of pig suppliers interacted with the effect of lifetime AMUs on the AMR gene abundances. However, these variables take into account only the finisher units and not the entire rearing pathways, and may not apply as traditional confounders for a finisher batch (Figure S1).
Shotgun metagenomic sequencing was used to measure the relative AMR gene abundances in the microbial communities of feces from finishers. This method does not distinguish between intrinsic and acquired (transferable) resistance genes in a bacterial population (Martinez et al., 2015). Moreover, as the ResFinder database contains mainly AMR genes detected in clinically relevant bacteria, a considerable number of intrinsic AMR genes may have been missed (Marshall and Levy, 2011; Munk et al., 2017). Therefore, although not all detected AMR genes necessarily pose a risk to human health, their presence in feces from pigs represents an available gene pool from which zoonotic bacteria and human pathogenic bacteria may obtain resistance genes (SCENHR, 2009; Cavaco et al., 2011; Collineau et al., 2017).

Further observational and longitudinal studies are required to determine the importance of these limitations as well as the value of the predictive model.

5. Conclusions

This study shows that the association between AMU and AMR under real-life conditions can be described by linear models. It also reveals that these models can be applied in predictive modelling for a huge population, in our case the majority of pigs delivered for slaughter in Denmark, where potential scenarios can be tested. In itself, this provides a significant tool for the Danish authorities and other stakeholders, but it also demonstrate what is possible and which data will be needed in order to provide guidance for major political and targeted interventions in production animals and humans globally. Thus, this study provides a sound framework for further development, which could eventually assist in reducing AMR and safeguard AMs for the future.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.prevetmed.2019.104853.

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