Antimicrobial Resistance Modeling

Bangsgaard, Elisabeth Ottesen

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

Antimicrobial Resistance Modeling

Elisabeth Ottesen Bangsgaard

Ph.D. Thesis
Kongens Lyngby, August 2022

Technical University of Denmark
Department of Applied Mathematics and Computer Science
Richard Petersens Plads, building 324,
2800 Kongens Lyngby, Denmark
Phone +45 4525 3031
compute@compute.dtu.dk
www.compute.dtu.dk
ISSN: 0909-3192

Summary (English)

Antimicrobial resistance is a growing concern in connection with treatment of infections and the World Health Organization (WHO) now recognizes it as being amongst the ten biggest threats to the global health. Antimicrobial resistance bacteria can cause treatment extension or failure due to resistance against antimicrobial drugs. The spread of antimicrobial resistance in livestock constitutes a risk for animal welfare and increases the risk of transferring antimicrobial resistant bacteria from animals to humans.

The overall aim of the Ph.D. thesis is to gain more knowledge on the influence of antimicrobial use and management factors on antimicrobial resistance in Danish slaughter pigs. This is achieved by analyzing and modeling the spread of antimicrobial resistance genes in fecal samples from Danish slaughter pigs in relation to antimicrobial exposure and management factors. The data on antimicrobial resistance originates from a study, where faeces from Danish pigs were sampled at the time of slaughter. The collected fecal samples were analyzed by qPCR quantifying the amount of antimicrobial resistance genes.

An algorithm was develop for estimating the antimicrobial exposure of the sampled pigs. In Denmark, pigs are categorized into three phases based on their weight: piglets, weaners and finishers. The algorithm estimates the average antimicrobial exposure for a Danish pig in each rearing period. This is done by tracing the location(s) based on movements of the pigs registered in the Pig Movement Database and subsequently estimating the antimicrobial exposure derived from the national VetStat-register, which contains information on purchased veterinary drugs.

Mixed e□ect models were applied to data to examine the relationship between an-

timicrobial exposure, management factors and antimicrobial resistance in the pig production. Tetracyclines are one of the most used antimicrobial classes in the pig production. The total resistance level against tetracycline, calculated as a sum of the genes coding for resistance against tetracycline and the level of individual resistance genes coding for resistance against di□erent antimicrobial classes were modeled. The measured individual resistance genes, which were observed in at least 50% of the samples were modeled and the results were compared to metagenomic data of an antimicrobial resistance context study.

The key findings are that management factors in the form of movement patterns and ownership of the farms are crucial for the resistance levels of Danish slaughter pigs. In addition, some observed complex antimicrobial exposure and resistance patterns described by the models, might be explained by co-occurrence of antimicrobial resistance genes i.e. antimicrobial resistance genes which occur together in a genomic context. The modeling suggested that the occurrence of the resistance gene tet(X) is a□ected only by the antimicrobial exposure of macrolide and lincosamide classes, which is in contrast to the commonly accepted hypothesis that it provides resistance against tetracyclines. This result might be a consequence of observed co-occurrence with erm(F), that provides resistance against several antimicrobial classes including macrolides and lincosamides.

The results could contribute to qualified discussions on treatment strategies and targeted interventions in Danish pig production. Reductions in usage of certain antimicrobial classes do not necessarily yield a lower abundance of resistance genes for these classes and the genomic context should be considered in assessments. Furthermore, there should be a focus on more systematic data collection in future studies and the surveillance of the development in antimicrobial resistant bacteria should continue with high quality.

Summary (Danish)

Antibiotikaresistens skaber voksende bekymring i forbindelse med behandling af infektioner og Verdenssundhedsorganisationen (WHO) anser nu antibiotikaresistens for at være blandt de ti største trusler mod den globale sundhed. Antibiotikaresistente bakterier kan forårsage forlænget eller mislykket behandling grundet resistens mod antibiotiske lægemidler. Udbredelsen af antibiotikaresistens i husdyrproduktionen udgør en risiko for dyrevelfærden og øger risikoen for at antibiotikaresistente bakterier overføres fra dyr til mennesker.

Det overordnede mål med denne Ph.D.-afhandling er at opnå større viden om antibiotikaforbrug og mangementfaktorers indflydelse på antibiotikaresistens i danske slagtesvin. Dette er opnået ved at analysere og modellere udbredelsen af antibiotikaresistensgener i fækale prøver fra danske slagtesvin, i relation til antibiotikaeksponering og managementfaktorer. Antibiotikaresistensdata stammer fra et studie, hvor danske slagtesvin fik taget fækale prøver ved slagtning. De indsamlede prøver blev analyseret ved qPCR for at kvantificere mængden af antibiotikaresistesgener.

En algoritme blev udviklet til estimering af antibiotikaeksponeringen for de prøvetagne grise. I Danmark, kategoriseres grise i tre faser baseret på deres vægt: smågrise, fravænningsgrise og slagtesvin. Algoritmen estimerer middel antibiotikaeksponeringen for en dansk gris i hver periode. Dette bliver gjort ved at opspore lokation(er) på baggrund af flytninger af grisene i svineflytteregisteret og derefter estimere antibiotikaeksponering ved hjælp af det nationale VetStat-register, som indeholder information om indkøbte veterinære lægemidler.

Mixed e□ect modeller blev anvendt til at undersøge sammenhængen mellem antibiotikaeksponering, managementfaktorer og antibiotikaresistens i svineproduktionen.

Tetracykliner er en af de mest udbredte antibiotikaklasser i svineproduktionen. Det totale resistensniveau mod tetracyklin, udregnet som en sum af gener der koder for resistens mod tetracyklin, og niveauet af individuelle resistensgener mod forskellige antibiotikaklasser blev modelleret. De målte individuelle resistensgener, som blev observeret i mindst 50% af prøverne blev modelleret og resultaterne blev sammenlignet med metagenomisk data fra et antibiotikaresistens-kontektsstudie.

De væsentligste resultater er, at managementfaktorer i form af yttemønstre og gårdenes ejerforhold er afgørende for resistensniveau hos danske slagtesvin. Derudover kan observerede komplekse antibiotika-eksponering og resistens mønstre beskrevet af modellerne delvist forklares ved co-occurrence af antibiotikaresistensgener, dvs. de optræder sammen i genetisk sammenhæng. Modelleringen viser, at forekomsten af resistensgenet tet(X) kun er påvirket af antibiotikaeksponering af makrolid- og lincosamid-klasser, hvilket står i kontrast til den udbredte hypotese, at tet(X) giver resistens mod tetracykliner. Dette resultat formentlig en konsekvens af observeret co-occurrence med erm(F), som giver resistens mod ere antibiotikaklasser inklusiv macrolider og lincosamider.

Resultaterne kan bidrage til kvali cerede diskussioner om behandlingsstrategier og målrettede interventioner i dansk svineproduktion. Reduktioner i forbrug af visse antibiotikaklasser medfører ikke nødvendigvis en lavere forekomst af resistensgener for disse klasser og genomiske kontekster af resistensgenerne bør medtages i vurderinger. Derudover bør mere systematisk dataindsamling være i fokus i fremtidige studier og overvågning af udviklingen i antibiotikaresistente bakterier bør fortsætte med høj kvalitet.

Preface

This Ph.D. thesis was composed at the department of Applied Mathematics and Computer Science at the Technical University of Denmark in fulfillment of the requirements for acquiring an PhD degree. The research conducted in this PhD project was carried out in the Section for Dynamical Systems in the period November 1st 2018 to August 4th 2022 interrupted by a maternity leave.

The Ph.D. project was performed under the supervision of Senior Researcher Lasse E. Christiansen and Senior Researcher Kaare Græsbøll. This work was supported by The Danish Ministry of Food, Agriculture and Fisheries as part of the Veterinær-forlig III project.

In this thesis, research related to antimicrobial resistance in Danish pig production is presented. The intention of this Ph.D. thesis is to provide a brief overview of the background and motivation for the three articles written during the PhD study as well as a summarized presentation of the published work, additional results together with discussions and perspectives.

Lyngby, 04-August-2022

Elisabeth Ottesen Bangsgaard

Scientific Contribution

The research conducted during the Ph.D. study resulted in three publications, one accepted, one submitted and one in preparation for submission.

Research articles covered in this thesis

(I) Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen and Lasse E. Christiansen "The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way." In: Preventive Veterinary Medicine submitted, 2022

(II) Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen, Julie Clasen, Dziuginta Jasinskyt, Julie E. Hansen, Anders Folkesson and Lasse E. Christiansen "Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs" In: Preventive Veterinary Medicine, 2021

(III) Elisabeth O. Bangsgaard, Patrick Munk, Saria Otani, Kaare Græsbøll and Lasse E. Christiansen, "Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context", Journal of Antimicrobial Agents final draft ready for submission, 2022

Acknowledgements

I would like to thank my supervisors Lasse E. Christiansen and Kaare Græsbøll for their advice with this Ph.D. I would also like to thank the project group of "Veterinærforlig III", especially Vibe D. Andersen for her great support with the VetStat data and Patrick Munk for his knowledge sharing on metagenomics. Finally, I would like to say a special thanks to my friends and family supporting me throughout this period.

Abbreviations

ADD	Animal De ned Daily Dose
AIC	Akaike Information Criterion
ALEX	Average Lifetime Exposure Algorithm
AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
AMU	Antimicrobial Use
CHR	Central Husbandry Register
DANMAP	Danish Integrated Antimicrobial Resistance Monitoring and Research Programme
HGT	Horizontal Gene Transfer
MGE	Mobile Genetic Element
PMD	Pig Movement Database
qPCR	Quantitative Real-time Polymerase Chain Reaction
VetStat	Veterinary Medicine Statistic Program database

Contents

Chapter 1

Introduction

Antimicrobial resistance (AMR) is a major global threat for humans as well as for animals. In 2019, the World Health Organization (WHO) recognized AMR as being amongst the ten biggest threats to the global health [1]. AMR bacteria can lead to ine ective treatments of severe diseases resulting in prolonged illness, extended treatment, and even untreatable infections [2]. Gaining more insight into the key drivers af AMR would yield useful information about the spread of AMR and how to reduce it.

It is an increasing concern that AMR in livestock, including pig rearing, constitutes a notable risk of transferring AMR bacteria to humans [3, 4, 5]. Accumulating evidence suggests that overuse and misuse of antimicrobials are leading to ine ective treatment of diseases by the selection-pressure of antimicrobial resistance genes (ARGs) [6, 7]. The antimicrobial usage (AMU) in livestock and other potential in uencing factors have therefore obtained an increased attention lately.

AMR is a broad research eld, however, in this thesis, only ARGs in bacteria found in fecal samples of Danish slaughter pigs were considered.

1.1 Aim of the Thesis

The aim of this thesis is to investigate the in uence of antimicrobial exposure and management factors on the occurrence of antimicrobial resistance genes in fecal samples of Danish pigs at the time of slaughter by exploiting available data.

1.2 Antimicrobial Resistance

AMR is the microorganisms' defense against antimicrobial drugs which are developed to eliminate them. Even though this mechanism occurs naturally in some microorganisms as a self-defense, it can also rise from an alternation in the genome [8, 9]. In bacteria the genome consists of the chromosal DNA and small, circular DNA molecules (plasmids), both where the antimicrobial resistance genes (ARGs) causing the resistance can be located. ARGs in chromosomes can be inherited by the daughter cell while ARGs in the plasmids can be transferred between bacteria.

Co-occurrence. If more than one ARG is located on the same plasmid or other genetic elements in the same bacteria, the ARGs are said to be co-occurring. This means, that the bacteria have the ability of being multidrug-resistant, which is a big issue when trying to defeat them.

Co-selection. Bacteria can carry several ARGs providing resistance against more than one antimicrobialclass. Co-selection means that one ARG can select for another ARG, for example if they are co-occurring. A resistance gene against a particular antimicrobial class might therefore be selected even in the absent of a drug from that antimicrobial class. This also implies that using one antimicrobial drug class can preserve the AMR against all the drug classes for the co-occurring ARGs i.e. co-resistance [10].

Horizontal gene transfer. It is possible for bacteria to pass on resistance by transferring plasmids, this is called horizontal gene transfer (HGT) [11]. On a smaller scale, mobility of ARGs within a single cell can be facilitated by mobile genetic elements (MGEs) located close to the ARGs [12].

1.3 Measuring Antimicrobial Resistance

AMR can be de ned and measured in various ways. The detection of AMR was traditionally culture-based and evaluating the phenotypic resistance. However, DNA-

based methods quantifying the generic resistance are more commonly applied today. The advances of DNA-based methods includes lower cost, many samples can be processed fast, and the opportunity of detecting ARGs in all (known) bacteria in samples and not only in the subset that can be cultivated [13, 14]. However, it is not possible to determine if the ARGs are expressed i.e. if the gene results in phenotypic AMR. In this Ph.D. project, data from two DNA-based methods, qPCR and Metagenomics, formed the base of the statistical analyses.

The primary focus is on the qPCR data, while the metagenomic data is used to con rm ndings from the qPCR data. The qPCR data was collected, analyzed and prepared by DTU Bioengineering, while the collection of the metagenomic data and the related bioinformatic part were performed by DTU FOOD.

qPCR. The quantitative Polymerase Chain Reaction method is capable of quantifying an ARG based on a designed DNA template called a primer. The basic idea behind the method is that the part of the sample DNA in interest is ampli ed in each cycle [15]. In each cycle, the sample DNA is rst separated into single strings and then replicated by help from the selected primer. This results in a copy i.e. a doubling of the DNA segment which is measured by ourescent dye. The rst cycle where the number of copies is su cient for the ourescence to be detected is denoted C_q (quanti cation cycle). In this way, the C_q-value can be used to quantify the amount of DNA: A low C_q-value indicates a high initial amount of the gene of interest, while a high C_q-value suggests an low initial amount. In this project, the C_q-values were normalized by the reference gene 16S, this is explained in detail in section 3.2. For further explanation of the qPCR procedure used for the data in this project, see [16].

Metagenomics. The principle of the metagenomic method is to extract DNA from a sample, perform sequencing and then classify it according to an established reference database. There exist a number of di erent approaches and various computer tools for performing metagenomic calculations [17]. A concise overview of the method, necessary to follow the work in this project is as follows: after sequencing the DNA, the small DNA pieces are assembled into contiguous sequences called contigs. These contigs consist of the overlapping reads and represent larger parts of the DNA. The contigs are then matched with known DNA sequences from a database in order to predict and classify the genes. It is also possible to get information about the origin of the gene as well as the genomic context by binning them into individual metagenome-assembled genomes. For more detail on the exact method applied to the data considered in this project see [18].

1.4 Antimicrobial Use in Danish Pig Production

The pig sector in Denmark is well established and constitutes a population of 13 million animals in total, which is more than double the size of the human population of Denmark in 2022 [19].

Rearing periods. In general, the rearing periods can be divided into three groups based on the weight of the pigs: the *piglet* period (birth until approximately 7 kg), the *weaner* period (approximately 7 to 30 kg) and the *finisher* period (30 kg until slaughter). A herd is the pig population on a farm whereas a group of pigs reared together in the same period is referred to as a batch.

There exist many different types of farms in Denmark, such as fully integrated (including all three rearing groups), partly integrated (including piglets and weaners or weaners and finishers) or farms rearing only one of the three. This result in many different production networks transferring pigs between farms. The pigs are usually moved, when they enter a new rearing period, if they are transferred between different farms. There exist multiple complex trading patterns in the Danish pig industry, which covers several locations and owners [20].

AMU survillance. DANMAP is a systematic surveillance program for antimicrobial usage and resistance established in Denmark [21]. The main purpose of DANMAP is to collect veterinary data on AMU and AMR as a basis for research and counseling within the field. The abundance of AMR in Denmark is lower than for other European countries [22], most likely due to the initiatives that has been implemented during the years, to control and limit AMR bacteria in the Danish pig sector [23].

However, the pig production in Denmark still constitutes the main part of veterinary antimicrobial consumption, even though it only accounts for 47% of estimated live biomass. In 2020, it was reported that the Danish pig industry accounted for 76% of all antimicrobial drugs prescribed, this corresponds to 75.9 tonnes active compound [21]. The AMU data reported by DANMAP is build on the national VetStat registry, described in more detail in section 2.1.

Antimicrobial drugs. Antimicrobials are medicines used to prevent and treat infections caused by bacteria, viruses, fungi or parasites. There are several antimicrobial drugs on the market, however, in this thesis AMU is estimated for each of the twelve major antimicrobial classes: Aminoglycosides, Amphenicols, Lincosamides, Macrolides, Simple penicillins, Extended penicillins, Sulfonamides (incl. trimethoprim), Tetracyclines, Pleuromutilins, Cephalosporins, Fluoroquinolones, and Other according to the definitions in VetStat.

Animal defined daily dose. As an attempt to standardize the use, measurement

and reporting of antimicrobial consumption, the animal de ned daily dose (ADD) was introduced in VetStat [24]. The ADD de nes the average maintenance dose of the drug for the main indication within an age group of a species. Based on this, the antimicrobial exposure in this project is calculated as the average dose of the speci ed antimicrobial used for treatment of one kilogram pig for each rearing period of the pig [25]. The calculations are explained in section 3.1. Estimating antimicrobial exposure in this way allows for a comparison between the AMU in the di erent rearing periods, as well as a summation over the rearing periods to describe the lifetime exposure.

Yellow card initiative. The implementation of the yellow card initiative in 2010 has caused a reduction in antimicrobial use in the Danish pig production. The main idea is that farms using too much antimicrobials, get a yellow card by the authorities followed by a 9 months period to reduce it and a follow up action plan, in case of no improvement [23]. The antimicrobial thresholds are determined by the authorities and are di erentiated between the drug classes, depending on how critical they are for human treatments.

1.5 Factors In uencing AMR

Besides the direct e ect of AMU on AMR occurrence in pig production, it has been indicated that several other factors contribute to the occurrence. Management factors associated with the pig production have gained an increasing importance when studying AMR levels at pig farms. These include size of the farms, movement patterns, quality of feed and, in general, the daily routines such as number of people working on the farm, treatment strategies and applications [26, 27, 28, 29].

However, some of these factors can be di cult to quantify or collect information about. In this thesis, only management factors that are possible to retrieve through register data are considered. These factors are: the total number of farms in the production network (network size), the movement patterns including the ownership (production type) and number of di erent antimicrobial drug classes.

1.6 Project Structure and Role

This Ph.D. project was conducted as part of the Veterinærforlig III project, nanced by the Danish Ministry of Food, Agriculture and Fisheries in collaboration with DTU Bioengineering and DTU FOOD. The main objective was to develop a model as a

supporting tool for prediction of AMR in the Danish pig production as an input to the overall project goal of reducing AMU and AMR in veterinary context. In this setting, the main contribution of this Ph.D. project was the developed algorithm for antimicrobial exposure as well as obtained knowledge of AMU-AMR associations based on analysis and modeling of the collected data in Danish pigs.

The metagenomic data sampling and the accompanying bioinformatic analyses were carried out by DTU FOOD. The study in which pigs were sampled at the slaughter line and the related qPCR analyses were performed by DTU Bioengineering.

Large parts of the work conducted during the project period concerned understanding and quality checking the register data and the qPCR data. A major challenge was to merge and prepare the data from the di erent registries in order to get useful results for the modeling part. Data collected in the real world, especially self reported, can be quite messy, and combining all the collected information into useful exposure estimates and including this in the analysis of the qPCR data, compromised a signi cant proportion of the work. In this way, the project has been a combination of understanding and analyzing data of various sources and structure, programming, visualization, and interpretation, as well as an interdisciplinary project uniting antimicrobial resistance, Danish pig production, statistical modeling and plenty of programming.

Chapter 2

Data Sources

This chapter brie y introduces the underlying data sources that forms the basis of the work in this Ph.D. project.

The data for the analyses performed in this project is derived from fecal samples collected in Danish slaughter pigs, in combination with available register data. In Denmark, it is mandatory to report information about pig herds in the Central Husbandry Register (CHR), information about all movements of pigs in the Pig Movement Database (PMD) and information about antimicrobial purchases in Vet-Stat. The registers are maintained by the Danish Ministry of Food, Agriculture and Fisheries. They are of high quality, however, they do still contain some errors, such as wrongly entered or missing data and inconsistencies across the registries. Examples of this are: failed upload, late registration, and manual changes of errors combined with di erent data extraction times [30]. In this project, all needed data covering the period 2014-2019 was extracted and prepared for analysis only once.

2.1 Danish Data Registries

CHR
All livestock in Denmark are registered in the CHR, by a unique CHR number linked

to the geographical location. The CHR number can, for example, be used to identify transfers of pigs and antimicrobial purchases in the two other registers. In the CHR, demographics such as animal species on the farm, number of animals, farm type and ownership are registered. For pigs, the farm type distinguishes between, for example, slaughter houses, production farms, breeding farms, and exporting facilities. The number of pigs is reported within the three groups: sows, weaners and nishers. The ownership of the farms is registered by a unique CVR number. This means that more than one farm can be associated with a CVR number (i.e. having the same owner), but only one farm can be associated with a CHR number. It is also possible to have two CVR numbers linked to one CHR number i.e. two owners sharing a farm location. [31]

Pig Movement Database
The PMD is part of the CHR and holds all information about pigs moved between farms in Denmark. Usually pigs are transferred between farms when they are entering a new rearing period or sent for slaughter. The movements are reported on farm and rearing group level i.e. it is not possible to trace a unique pig through the database. Information about the sending and receiving farm including CHR and CVR number, number of moved pigs and the date of transfer is to be reported within 7 days. [32]

VetStat
The antimicrobial veterinary use in Denmark is monitored based on the VetStat registry. VetStat is a national registry for all prescription drugs for veterinary use. The register is used for regulation of AMU as well as for research purposes. VetStat contains detailed information on the purchased drugs including active compound, strength, amount, administration route, and intended use. In addition, the standard ADD for drugs is also found here, indicating the average dose for the main indication in the speci c animal type and age group. [33]

2.2 Fecal Samples From Danish Slaughter Pigs

As part of the Veterinærforlig II and III initiated by the Danish Ministry of Food, Agriculture and Fisheries, fecal samples from Danish slaughter pigs were collected at the slaughter line in 2015, 2017 and 2019. Five individual pigs from the same farm were sampled and these samples were mixed. The mixing resulted in a pooled sample describing the resistance level at in the herd, rather than in the individual pig [34, 35]. The pooled samples were analyzed with the qPCR method by DTU Bioengineering, for further detail see [16]. However, unforeseen budget cut on the overall project a ected the number of collected samples (planned sampling of sows) and the number of samples analyzed with qPCR. It was therefore not possible to include all collected samples in the Ph.D. project due to the non-analyzed samples

together with missing access to the list linking the sample IDs to the CHR numbers.

In total, 1153 samples from 749 farms were eligible for analysis in this project. Herds from 205 farms were sampled multiple times, see Table 2.1 for details.

Number of farms	Number of samples
122	2
34	3
18	4
15	5
15	6, 7, 8
1	14

Table 2.1: Number of re-sampled slaughter pig farms in the Veterinærforlig II and III project.

Chapter 3

Summary of Methods in the Articles

This chapter presents the main objectives of the research articles I-III and gives a summary of the used methods.

3.1 Research Article I

The objective of research article I ("The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way") was to present and discuss, the developed algorithm for estimating antimicrobial exposure and production type in Danish pig production. The motivation behind the article was to share findings and solutions for others in the area to use.

The purpose of developing the Average Lifetime Antimicrobial Exposure algorithm (ALEX) was to provide a faster, automated and more robust procedure for calculating the average exposure of antimicrobials in Danish pig production based on a previous method (the LEA algorithm [20]). The algorithm is designed to work purely on register data.

Back-tracing. All plausible routes of pigs from piglet to slaughter are identi ed by searching the PMD. In the rst step, farms moving pigs to the slaughter farm in a speci ed time window are searched. In the second step, movements to these farms are searched. This identi es the production network by movements of piglet to weaner and weaner to nisher farms. Internal movements are estimated in the case of missing movements, based on what is registered in the CHR on speci c rearing groups for the particular farm. Internal movements are also estimated in case where two, or more, rearing groups are registered on one farm according to the CHR. The time windows of the moving period for back tracing the movements in the PMD are based on ndings in [20]. Figure 3.1 depicts the steps and time windows in the ALEX algorithm.

Figure 3.1: Illustration of the steps in the ALEX algorithm [36]. In the rst step (1) movements of weaners to nisher farms are traced and based on this, movements of piglets to the identi ed weaner farms are traced (2). The last step of the algorithm is to calculate and weight the average AMU on these farms in speci c time windows (3).

Estimating. The antimicrobials purchased for all the identi ed farms in the production network in the relevant periods are extracted from VetStat. The AMU is smoothed over the relevant rearing period extended by 180 days prior. The exposure is calculated by,

$$AMU_x = \frac{\text{purchased antimicrobials}}{\text{number of pigs} \quad ADD_x \quad \text{purchase time window}} \text{ time in rearing period}$$

wherex is the antimicrobial drug class. The purchased antimicrobials are measured by the total amount of compound in mg. DTU FOOD converted the strength, amount and number of packages into the total amount (mg) and adjusted the standard ADD values for combination preparations and converted the unit into

mg/kg. The number of pigs is based on the registration of the herd size for the rearing group in CHR.

As mentioned, the AMU measure is smoothed by including all prescriptions 180 day before the rearing period, together with the prescriptions during the rearing period. These are averaged over the prescription period and multiplied to re ect the average exposure of the speci c antimicrobial drug class in the particular rearing period. The unit of AMU_x is [kg pig day] and re ects the total average exposure in the respective rearing period. In this way, a lifetime exposure can easily be calculated by adding the three estimates from each rearing period, which is possible, because the unit is not depended on the weight of the pigs in the di erent rearing periods.

Weighting. The AMU estimates are weighted according to registered number of pigs moved between the farms to get an average exposure for each rearing group. The weighting is only performed in the case of multiple suppliers including internal movements. The AMU estimates are adjusted and weighted for production network, involving multiple piglet or weaner farms, by calculating the proportion of pigs transferred to the speci c rearing group out of the total pigs moved to the rearing group on that farm. The sum of the average exposure in each rearing period constitutes the average lifetime exposure for a slaughter pig from the given farm.

Management factors. The number of farms identi ed in the PMD and the ownership information retrieved from the CHR for the pig networks are documented in parallel to the exposure estimation. The number of piglet farms and weaner farms together with the number of registered CVR numbers in the production network is based on the results of the back-tracing.

Successful traces. Several checks are implemented for quality insurance and validation. Traces where no movements from weaner to nisher or piglet to weaner farm are identi ed, are of course, if no internal movement could be estimated, regarded as failed traces. In addition, the traces should be su cient which was de ned as:

(1) the proportion between estimated number of moved piglets/weaners and estimated number of weaners/ nishers is higher than 50%,

(2) the fraction of the movement chain that is complete is higher than 80%,

(3) the number of farms detected in a network is less than 8.

Criteria (1) and (2) are implemented to allow for discrepancies between the registries and the real world. Criterion (3) is based on investigations carried out in connection with research article II [16].

3.2 Research Article II

The main objective of research article II (Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs [15]) was to investigate the influence of management factors on tetracycline resistance, in addition to tetracycline exposure, in Danish pig production. The focus on tetracycline arose from the fact, that it is one of the most commonly used antimicrobials in pig production [21, 26].

Resistance measure. In this work, the qPCR data, described in section 2.1, was used for modeling. As mentioned, the samples were collected on the slaughter line at Danish slaughterhouses in 2015, 2017 and 2019.

In this PhD project, all the qPCR results are normalized by 16S rRNA by calculating: $C_q = C_{q;16s} - C_{q;ARG}$. This normalization allows for a comparison between samples by considering the amount of bacterial DNA. In other words, 2^{C_q} represents the proportion of the sample, containing the ARG relative to the 16S gene, since the C_q-values are naturally on a $\log_2$-scale. The interpretation of $C_q = 1$ is therefore, that it is detected half as often as the reference 16S gene. In the case of three available technical replicates, the median was calculated and used.

To summarize the total tetracycline level it was chosen to make a variable summing up the individual qPCR results of the measured tetracycline resistance genes,

$$R(tet) = \sum_k 2^{-C_{(q;k)}}$$

where k covers the 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). Summing over the 2^{-C_q} gives an additive measure of the total tetracycline resistance level rather than just summing over the C_q, which is just the difference between running cycles of the ARG and the reference gene. The additive nature of the variable means, that the value can exceed 1 as it represents the sum of the relative proportions of the individual ARGs, since multiple ARGs can be present in the same bacteria.

Influential factors. The exposure to tetracycline and other antimicrobials were estimated using the algorithm ALEX. Likewise, the investigated management factors, were estimated by the ALEX algorithm. The management factors included network size, production type and number of antimicrobial classes used. The network size was constructed by counting the number of farms detected in the production network, the production type was based on ownership details retrieved from the CHR registry and the number of antimicrobial classes was counted for each rearing group, as an indicator of the general management hygiene level.

The production type was categorized into the following four groups:

1) CHR integrated
   Pigs stay on the same farm during all of the production,

2) CVR integrated
   Pigs are moved between at least two farms with the same owner,

3) other - 2:
   Pigs are only moved between two farms, however, with di erent owners,

4) others - 3+:
   The production network consists of three or more farms and multiple owners.

Mixed e ect models. Four non-nested linear mixed e ect models were formulated, reduced and compared. The response variable was $\log_2$-transformed due to the natural origin of the data. All formulated models included exposure variables divided into the di erent antimicrobial classes and rearing groups as xed e ects and the farms as random e ect. The rst model only included the exposure variables, the second model included a variable describing the size of the production network, the third model included a production type variable and the fourth model included a counting variable for the number of antimicrobial classes used. The models were reduced by backwards elimination and the non-nested models were then compared by the Akaike Information Criterion (AIC) and log-likelihood to evaluate the models t to data. To counteract for the multiple comparisons in the backwards elimination process the Bonferroni correction method was used with an overall alpha level of 0.05.

The Bonferroni method was implemented by dividing the signi cance level by the number of tests i.e. number of variables in the initial models tested in the backwards elimination process, $_{Bon:} = \frac{0:05}{\text{Number of variables tested in model}}$: The backwards elimination was carried out by using the step function from the LmerTest package in R. The full models, including all potential variables, were estimated and then provided to the step function which tested potential reductions of the models. The reduced, non-nested models were then compared by AIC which provides a relative measure of the quality of the model ts to the dataset and constitutes a good aid in model selection. The model with the lowest AIC was chosen, because it ts data best in the simplest way i.e. with the minimum amount of variables amongst the tested models. Model checking plots for the nal model were examined and gave no concern.

3.3 Research Article III

The objective of research article III (Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context) was to explain observed

AMU-AMR patterns in a broad pig population study by ndings in a more narrow metagenomic study.

The work presented in research article III, can be divided into two major parts, depending on the analysis of data collected in: the antimicrobial usage (AMU) study and the resistance gene context study. The AMU study refers to the previously described qPCR study sampling slaughter pigs in 2015, 2017 and 2019, while the context study refers to a metagenomic study performed on one farm over 11 weeks. The data collected and qPCR analyses in the AMU study were carried out by DTU Bioengineering, while the data collection and bioinformatic analyses in the context study were performed by DTU FOOD, as mentioned in the introduction. In this article, individual antimicrobial resistance genes measured in the AMU study, were analyzed by mixed e ect modeling and the results were compared to the ndings of the context study.

Mixed e ect models. Based on the results of research article II, mixed e ect models were formulated. The main di erence between the two articles is the response variable. With the previous work as a starting point, mixed e ect models were developed and reduced for each of the measured resistance genes for di erent antimicrobial classes (with su cient number of observations), rather than for the sum of tetracycline resistance genes. The individual models were backwards reduced using Bonferroni correction, similar to the procedure in research article II. Model checking plots were examined for all reduced models and none of them caused a signi cant concern.

Box-Cox transformation. The response variable of the individual models was the C_q-values for each resistance gene and no $\log_2$ transformation was therefore not applied, in contrast to the models in research article II. However, an investigation of Box-Cox transformation of the exposure variables for various suggested, that a square root transformation was appropriate. This was achieved by optimizing the value of in the range [0.1, 1], based on the log-likelihood of the models, implemented as a for-loop and utilizing the build-in function optimize in R. The exposure variables included variables, describing the antimicrobial exposure for each of the 12 antimicrobial drug classes, in each of the three rearing periods i.e. 36 variables.

Co-occurrence matrix. A matrix describing and illustrating the co-occurrences of the ARGs measured in the context study was constructed, based on the metagenomic data from the context study. The ARGs were determined to be co-occurring, when they were detected close to each other on the same contig, according to the de nitions by DTU FOOD [18].

Chapter 4

Summary of Results in the Articles

This chapter presents the most important findings of the research articles I-III. It includes a condensed version of the main results and a highlight of the key conclusions.

4.1 Research Article I

To obtain fast and reliable estimates of antimicrobial exposure, for investigating the AMU-AMR relationship in this project, an algorithm (ALEX) was implemented. ALEX is capable of estimating AMU, within the different rearing periods of the pigs, and mapping the production networks in a transparent way.

ALEX was compared to the published algorithm DREA [37], which showed, that ALEX is faster and more robust. Sensitivity analyses of the parameters related to the window sizes of movements and antimicrobial purchases were carried out underlining the robustness. The effectiveness of the ALEX algorithm was studied, this included a trace of all batches, from meat producing farms, sending at least one batch for slaughter during 2019 in each quarter. In 2019, on average 3346 different farms moved slaughter batches of minimum 20 pigs to a slaughter house each quarter,

according to the PMD. The successful trace rates were between 73-82% for each of the periods. Figure 4.1 depicts the data selection based on all slaughters in the PMD and the structures of the failed ALEX traces for the rst quarter of 2019. The

Figure 4.1: Flow chart of the failed ALEX traces of slaughter batches in rst quarter of 2019. Out of the 3497 identi ed farms sending to slaughter in rst quarter, 2559 were successfully traced resulting in a success rate of 73%.

structure of the failed traces in the rst quarter exempli es, that a signi cant share of the failures are due to missing registrations of pig movements in the PMD, within the de ned time windows. for unknown reasons some of these lacks of movements were over a 2 months period, and would therefore not have been traceable by extending the windows slightly. Extending the windows too much, would probably result in nding unintended movements for the particular trace.

Typing errors in both the CHR, PMD and VetStat introduces challenges when estimating the antimicrobial exposure. Inconsistencies across the registries were encountered multiple times in this project. A few examples of these inconsistencies are: a registration of a piglet to weaner movement in the PMD, where the receiver farm does not have weaners registered in the CHR. A movement in the PMD of

more pigs than what is registered on either the sender or receiver farm in the CHR. In general, many strange registrations were discovered when investigating special cases in connection with validation of the results and understanding of the failures of the algorithm. In addition, inconsistencies were found within registries, e.g. in the CHR, where a farm for some months changed the production type by reporting zero pigs in the weaner period and then suddenly changing it back again. This was partly dealt with by using a running median of 3 months for each rearing group registered in the CHR. Furthermore, updates of CHR is supposed to be registered on an ongoing basis, however, some updates are performed in connection with the yearly update.

Figure 4.2 shows the AMU of tetracycline, estimated by ALEX, in the four quarters of 2019. The AMU is grouped by the type of production. The results show no evidence of major seasonal variation in the lifetime tetracycline usage and only a slightly increasing tendency in the median of the production type, however, there is no signi cant di erence.

Figure 4.2: Tetracycline lifetime exposure for all slaughter pigs, traced by ALEX, in the four quarters of 2019. The tetracycline AMU is grouped by the production type

Two direct usages of the ALEX results were presented in the article. A visualization tool (see Figure 4.3) and a comparison of tetracycline and macrolide use in 2019 in relation to 2015. The comparison includes all farms, having at least one traceable slaughter batch in 2015 and 2019, with the ALEX algorithm.

Figure 4.3: Screen shot of the shiny app developed for visualizing the identi ed production networks, movements and AMU estimates provided by the ALEX algorithm.

Figure 4.4 presents the changes in tetracycline and macrolide exposure from 2015 to 2019, separated into groups depending on the exposure level in 2015. Overall this shows, that the pigs from production networks, having a high exposure in 2015, are the ones, where the biggest reduction is observed. The median of the change in macrolide exposure is above zero for the group, with low macrolide exposure in 2015. This was further investigated by plotting the distributions of changes in macrolide exposure divided into groups, based on the magnitude of the change in tetracycline exposure (see [36]). This showed, that the farms reducing tetracycline use the most, also were the farms increasing the macrolide use for the weaner and nisher rearing groups.

(a) (b)

Figure 4.4: Plots showing the changes from 2015 to 2019, in tetracycline use (a) and macrolide use (b), separated into groups based on the magnitude of the exposure in 2015.

4.2 Research Article II

It was found, that a mixed effect model including tetracycline exposure in the weaner and finisher period, pleuromutilins exposure in the finisher period, and a variable classifying the production type, was the most suitable, amongst the tested models, for describing the total tetracycline resistance level in Danish slaughter pigs.

The final model was selected based on AIC and the correlation between the observed and fitted total tetracycline resistance level ($R(tet)$) was 0.89, see Figure 4.5.

Figure 4.5: Correlation between fitted $R(tet)$ and observed $R(tet)$. The final model was a linear mixed effect model, with tetracycline exposure in the weaner and finisher period, pleuromutilins in the finisher period and production type as fixed effects and farm as random effect.

The fit of the final model seems to capture the overall trend of the data, even though the prediction variance is increasing for higher $R(tet)$ values, which is expected, due to the fitting on $\log_2$-scale.

The estimates of the production type suggest, that CHR integrated farms have a lower reference level of tetracycline resistance. Furthermore, CVR integrated farms seem to have a lower background level, compared to non-CHR and non-CVR integrated farms. According to these results, it appears that complicated moving patterns and production types, with several farms and owners have an increasing effect, on the level of the total tetracycline resistance.

The $R(tet)$ sum is primarily dominated by the tet(O) genes, tet(W), tet(Q), and tet(32). tet(W) was the most abundant gene and contributed by 45% (8%) on average, in each sample, to the total tetracycline resistance sum in this study.

4.3 Research Article III

The ndings showed that combing results from two AMR studies could explain some complex observed AMU-AMR relationships. The two studies were performed in the broad national pig population, quantifying the relationship between AMU and AMR by mixed e ect modeling, and in a single herd, investigating the genomic context, respectively.

Figure 4.6 presents the results of the reduced mixed e ect models for the individual ARGs. The estimates for each of the models are shown, together with an indication of the signi cance. This suggest that di erent ARGs are associated with di erent AMU, in di erent rearing periods. The most frequently occurring variables across the models are tetracycline and macrolide AMU in the weaner and nisher rearing periods. Two things to have in mind is, that the pigs are sampled close to nisher rearing period (at slaughter) and the extensive use of these drugs in these rearing periods. The variable 'Type' covering the production type is signi cant in seven out of the 14 models.

Figure 4.6: Results of the mixed e ect models of the individual ARGs measured by qPCR in the AMU study. The C_q-values for the measured ARGs are modeled as function of antimicrobial exposure for each antimicrobial class in each rearing period of the pig, and the type of production. Each column contains the estimates from the reduced mixed e ect model for the given ARG. Note, that the AMU variables in the models are square root transformed. The stars represent the level of signi cance of the estimates: 0 '***' 0.001 '**' 0.01 '*' 0.05. For the models, where the variable 'Type' is included, the intercept value is covering intercept for CHR integrated farms. The estimates and signi cance for 'CVR int.', 'Other 2' and 'Other 3' all have the CHR integrated intercept value as reference.

Figure 4.7 provides the context table showing co-occurring ARGs. The numbers indicate how many times the particular ARGs were detected together i.e. on the same contig. The numbers in the diagonal, can therefore be interpreted as total number of detections of the given ARG in the context study.

Figure 4.7: Context table indicating how many times ARGs were detected together on a contig. The yellow background color on the ARG labels indicates ARGs, that were also measured in the AMU study. The diagonal illustrates, how many times the ARG was identi ed in the context study, and the o -diagonal shows number of co-occurrences with other ARGs on a contig.

The most interesting ndings of this article included tet(X) and aph(3')-III.

In the AMU study, it was observed that tet(X) only was associated with macrolide and lincosamide AMU, despite the expected in uence of tetracycline exposure (see Figure 4.6). This could be explained by the genomic context study, where tet(X) was found co-occurring with erm(F) in four out of the ve detections (see Figure 4.7). erm(F) is commonly known to provide resistance against macrolide, lincosamide and

streptogramin B. This result might therefore be an example of co-selection.

The aminoglycoside ARG aph(3')-III was also found to be a ected by multiple antimicrobials in the AMU study. This matches the observation in the context study, where it was co-occurring with multiple ARGs. This again highlights the complexity of antimicrobial exposure and resistance patterns.

Chapter 5

Additional Results

This chapter introduces further results, not included in research articles I-III, yet relevant and important for the general discussion of the previous results. First distribution plots of tetracycline resistance levels are presented, next, an investigation of repeated measurements in the collected data is carried out and lastly, a modest comparison of qPCR and metagenomic data is presented, together with examination of antimicrobial exposure patterns.

5.1 Tetracycline Resistance Distribution

The distributions of tetracycline resistance levels over the three collection years (2015, 2017 and 2019) are investigated to get a better understanding of the trends. The data in this section represents all sampled farms in the qPCR study, irregardless of the traceability of the batches using the ALEX algorithm. In 2015, pigs from 443 unique farms were sampled, while pigs from 249 and 202 unique slaughter farms were sampled in 2017 and 2019, respectively. Only the first sample of each farm each year is included in the following.

Yearly distributions. Figure 5.1 illustrates the distribution of the total tetracycline resistance levels on the sampled farms by year. The total tetracycline resistance

level shows a slightly decreasing trend over time, even though the level actually seemed to increase vaguely from 2015 to 2017. Figure 5.2 shows the individual

Figure 5.1: Distribution plot of the total resistance level of tetracycline ($R_{(tet)}$) in Danish slaughter pigs samples, collected in 2015, 2017 and 2019. The vertical lines represent the mean $R_{(tet)}$ value each year. This shows an overall decreasing trend in the mean value of the total tetracycline resistance levels.

distributions of tet(W) and tet(X) ARGs reported in 2^{C_q}. Here the differences, in the yearly trends of the two individual tetracycline ARGs, are exposed. tet(W) is one of the most prevalent ARGs, showing a minor decreasing trend, while tet(X) exhibits an increasing trend, despite the observed decrease in tetracycline AMU. This exemplifies how the different tetracycline ARGs exhibits different distribution patterns over the years.

The total tetracycline resistance level sum ($R_{(tet)}$) is, however, heavily dominated by tet(W). Table 5.1 reports the fraction of the total resistance level accounted for by each of the ARGs each year. From this it is clear, that the total tetracycline level is greatly influenced by tet(W). In 2019, the relative tet(W) proportion composed 48% on average in the samples, while tet(O)-2 and tet(Q) accounted for 15% and 18%, respectively. This highlights the prevalence of tet(W) in the collected samples. In contrast, tet(X) only contributed by 1% on average in 2019, but it seems like it is getting an increasing (but still low) importance in the total tetracycline level.

(a)

(b)

Figure 5.2: The yearly distributions of (a) tet(W) and (b) tet(X) (2^{-C_q}). The vertical lines represent the mean 2^{-C_q} value by year. The tet(W) distribution shows an overall decrease in the mean value, tet(X) shows an overall increase in the mean value, from 2015 to 2019.

	Percentage of $R(tet)$ (mean and standard deviations)		
Gene	2015	2017	2019
tet(A)	0.06% (0.21%)	0.08% (0.16%)	0.07% (0.15%)
tet(B)	0.01% (0.19%)	0.01% (0.12%)	0.01% (0.13%)
tet(C)	0.12% (0.20%)	0.08% (0.09%)	0.09% (0.11%)
tet(L)	3.44% (3.25%)	3.90% (3.22%)	4.37% (4.03%)
tet(M)	0.25% (0.26%)	0.27% (0.23%)	0.46% (0.92%)
tet(O)-1	7.16% (2.98%)	6.59% (2.64%)	6.02% (2.60%)
tet(O)-2	17.79% (6.05%)	16.89% (6.06%)	14.95% (6.58%)
tet(PA)	0.41% (0.35%)	0.51% (0.40%)	1.06% (0.79%)
tet(Q)	17.93% (8.42%)	18.59% (7.30%)	17.58% (7.67%)
tet(W)	44.03% (7.38%)	44.99% (8.02%)	48.04% (9.05%)
tet(X)	0.50% (0.58%)	0.56% (0.55%)	1.04% (1.37%)
tet(32)	8.72% (4.79%)	8.18% (5.14%)	7.53% (5.51%)

Table 5.1: The mean and standard deviation, across farms, of the proportion (in percentage) of the total tetracycline resistance levels (calculated by $R(tet) = \sum_k 2^{\,c(q;k)}$), for each of the tetracycline ARGs constituting the sum. Based on the first observation for each farm each year.

5.2 Repeated Samples

The number of repeated samples in the project was unfortunately not as high as expected and hoped, due to unforeseen nancial circumstances and project reorganization. In total, only 17 farms were sampled, analyzed and successfully traced with ALEX, across all three collection periods in 2015, 2017 and 2019. By expanding this criterion to include farms, sampled at least three times, in minimum two di erent collection years, 48 farms were identi ed. 24 of these farms were sampled exactly three times, while the rest of the farms were sampled between four and 14 times.

Information level. This subset of data is investigated in order to understand the information level over time and the possibility of building a dynamic model based on this. The main interest is, to study the magnitude of change in resistance levels for the individual farms over time. The ARG tet(W) was chosen as target, due to the high prevalence in the samples.

Production type. There is an overrepresentation of farms with complicated trading patterns, in this subset of data i.e. productions with multiple owners and several farms in the network. This might be an expression of larger farms, which are sending pigs for slaughter more regularly, and therefore got sampled more often in this study. Seven of the production networks were estimated to have two di erent production types within one year, which might be a re ection of networks, that include additional farms when needed, to ll up all pens (i.e. changing production type from other - 2 to other - 3+).

Change in tetracycline exposure. A classi er (tet-classi er) was calculated to indicate, whether slaughter pigs have been exposed to relatively high or low tetracycline treatment. The classi er speci es, if the slaughter pigs have been exposed to more (over) or less (under), than the median lifetime tetracycline use, which was calculated based on this subset of data (33.5 AMU). The farms are then split into two groups: *no change in treatment strategy* (farms where the tet-classi er did not change) and *change in treatment strategy* (farms where the tet-classi er at some point changed).

The tet(W) measurements are averaged by year for each farm, assuming that the change in the lifetime AMU and production type is greater between the collection year, than within a collection year (2 years time span compared to 2 months time span).

Figure 5.3 shows the average tet(W) resistance level (C_q) as a function of time. To the left, farms that did not change the treatment strategy are shown (i.e. did not change the treatment strategy enough to cross the median use). It shows a weak

Figure 5.3: Average tet(W) resistance levels as function of time divided into groups of farms that did not change treatment strategy and farms that did. Change in treatment strategy is, in this section, de ned as changing the tetracycline exposure enough to cross the median tetracycline exposure in the data.

tendency for the farms, that had a relatively higher tetracycline exposure of the pigs (red dots), to also be the ones, that seem to have the highest tet(W) resistance levels. To the right, farms that did change their treatment strategy, enough to cross the median, are shown. From 2017 to 2019, it seem like, a number of the farms reduced the overall tetracycline treatment (red dots changed to blue dots), resulting in a minor reduction in tet(W) resistance.

Changes in C_q. Table 5.2 presents the summary statistics of the changes in C_q for tet(W). The minimum (-0.97) and maximum (1.13) values are corresponding to a reduction by half and a doubling, approximately. However, most of the changes in C_q are far from these values and might re ect, that most of the resampled farms did not have a big change in tet(W) resistance levels. The median (-0.18) and mean (-0.11) values of all farms suggest an average reduction of 12-15% tet(W) copies in the samples, compared to the previous taken samples. When looking at the farms, that did not change the treatment strategy considerably, the median of the changes in C_q (-0.01) re ects a reduction of 0.7% of relative tet(W), compared to previous measurement.

The results show, that there are few farms sampled during all three collection periods. In addition, the resistance levels for tet(W) and AMU, for the resampled farms, did change over time, but not to the extend of revealing signi cant associations. In the sampling, there was no systematic focus on resampling farms or sampling farms extensively changing their AMU patterns. The samples were taken at the slaughter line, from the pigs that were randomly send for slaughter on the sampling day. A

No change in treatment strategy (26 farms)					
Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
-0.84	-0.30	-0.01	-0.04	0.16	1.10

Change in treatment strategy (22 farms)					
Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
-0.97	-0.47	-0.23	-0.18	-0.04	1.13

All farms (48 farms)					
Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
-0.97	-0.39	-0.18	-0.11	0.11	1.13

Table 5.2: Summary statistics of the changes in C_q for tet(W) grouped by treatment changes and all farms. Change in treatment strategy is, in this section, defined as changing the tetracycline exposure enough to cross the median tetracycline exposure in the data.

more controlled sampling setting could be achieved, by sampling the pigs at the farms instead, however, this would be more costly. The data collected in this study, make a great basis for investigating the the resistance levels in the broader Danish pig population and more static AMU, however, it is not suitable for examine more complex dynamics e.g. by dynamical models, bacause the information level is not high enough, to support parameter estimation.

5.3 qPCR and Metagenomic Data Comparison

A small subset of the data collected in Veterinærforlig II was analyzed both by qPCR and metagenom (83 samples from 79 farms). The consistency between the results of the two methods is investigated by ranking profiles of each sample and each gene, for the ARGs detected by both methods. The ranking comparison was chosen, to find a common way of measuring the two methods' ARG quantification. The qPCR method offers relative gene proportion, while the metagenomic method provides gene counts.

Ranking of samples. The ARGs are ranked within each sample according to the measured level by each of the methods. The lowest ranking, i.e. 1, refers to the most widespread ARG in the given sample, measured with the specific method. In the cases where more ARGs were not detected in a sample, these were given equal (high) ranks. Figure 5.4 compares the ranking of the two methods' ARG quantification. The correspondence between the two rankings within the samples are in relatively

Figure 5.4: Ranking plot comparing qPCR and metagenome results, in order to investigate the consistency. The labels indicate the percentages of samples, where the speci c ARG was detected with the qPCR method.

good agreement, except for some speci c ARGs, especially, blaTEM, sul2, lnu(C), and vat(E). The results suggest that the methods to some extend observe similar resistance pro les. In addition, the comparison of rankings for lnu(C) reveals, that there might have been an issue with the primer.

Ranking of samples within ARGs. Figure 5.5 shows the rankings of the samples within the ARGs. The numbers in each plot indicate the average C_q-value for the particular ARG. In the case, where an ARG was not detected in any sample with the qPCR method, no number is shown on the sub gure. In this way, the ranking of the samples reveals, which of the samples the two methods found to have the highest abundance. The vertical lines in the plots, can be interpreted as non-detections by the qPCR method (i.e. they get the same ranking), but detections by the metagenomic method. For some of the ARGs, the ranking of samples are in a good agreement, while for other ARGs the rankings are deviating.

The results are compared based on the ARG classes de ned in the qPCR data. In the metagenomic data, the ARG results are categorized into the same classes. This could be an explanation of the vertical lines observed in Figure 5.5. These lines show, how the ranking of the metagenomic data manages to di erentiate between the samples, while the ranking of the qPCR does not (the samples get the same ranking, because of no detection). The qPCR method only detects the gene determined by the speci c primer, whereas the metagenomic method is capable of detecting di erent gene sequences for the same ARG class based on what is de ned

Figure 5.5: Comparison of the ranking of samples with qPCR and metagenome for each ARG. The numbers in the individual plots indicate the average C_q-value for all samples and the standard deviation. In case of no detection with the qPCR method of a speci c gene, the ranking value is the same for all samples.

in the data base.

The deviations in the rankings of the ARGs by each gene could partly be attributed the variation in abundance in the samples. The ranking of samples with a low standard deviation will naturally, be more prone to get a non-unique ranking.

5.4 AMU Exposure Patterns

Figure 5.6 shows the AMU exposure patterns of the sampled pigs in the qPCR study. The exposure is displayed as the total number of AMUs, separated between the three rearing periods and based on the results of the ALEX algorithm. To avoid too much bias in the plot, only the rst observation per farm per year is included.

It is noticed, that cephalosporins and uoroquinolones are not really used in any of the rearing periods. These drug classes are classi ed as critically important by the Danish Health Authority [38], and are therefore primary prescribed for pets and not production animals [21].

Figure 5.6: Total number of AMUs for the sampled farms in the qPCR study estimated by ALEX. The total number of AMUs is divided into the antimicrobial drug classes and the pig rearing periods.

Pleuromutilins, tetracyclines and macrolides are mostly used for weaners and n-ishers, while sulfonamides (incl. trimethoprim) and penicillins are more used in the piglet rearing period. Aminoglycosides and amphenicols are mainly used in the piglet and weaner rearing period. The observed usage patterns in the study population are similar to, what is reported for the national pig population by DANMAP [21].

Figure 5.7 presents boxplots for the 12 measured tetracycline ARGs grouped by the relative tetracycline exposure. This show the qPCR results in relation to the tetracycline exposure patterns. 16 batches were estimated by ALEX to have no tetracycline lifetime exposure, 356 to have a low exposure and 373 to have a high exposure. A C_q-value of -25 in the gure means, that the gene was not detected in the sample. The low and high exposure groups are created by comparing the estimated exposure to the median tetracycline exposure. It is observed, that the resistance levels seem to increase with the exposure, but with no signi cant di erence between the groups. The resistance levels for the no exposure group indicate, that several tetracycline ARGs are present, despite the estimated absent of exposure. The estimated exposure covers half a year before each of the rearing groups, this suggests that not using tetracycline (for at least half a year) lowers the resistance levels, but does not necessarily mean, that the resistance genes does not occur. Only the tetracycline exposure is included here, which means, that the relation to usage of other antimicrobials in these batches are not examined, in relation to pos-

Figure 5.7: Resistance levels of the 12 tetracycline ARGs, measured in the qPCR study, divided into tetracycline exposure groups. 'No exposure' means, that the pigs did not get any tetracycline exposure during their lifetime (estimated by ALEX). 'Low exposure' and 'High exposure' mean, that the pigs got less or more than the median tetracycline exposure. 16 batches were estimated by ALEX to have no tetracycline exposure, 356 to have low and 373 to have high. A C_q value of -25 means, that the gene was not detected in the sample.

sible co-selection. In addition, the exposure patterns of the farms with no exposure, have not been investigated i.e. if tetracycline was used just before the smoothing period (the purchase window in ALEX), or if tetracycline has not been used in a longer period on these farms. The life time exposure is considered for simpli cation, however, this does not reveal patterns in the exposure within the rearing periods.

Chapter 6

Discussion

This chapter contains extended discussions of the results and methods presented in research article I-III along with additional discussion points, limitations and perspectives.

The main goal of this thesis was to study and model antimicrobial resistance levels in Danish slaughter pigs. A method of estimating antimicrobial exposure per pig, in a fast and e cient way, was needed, in order to investigate the AMU-AMR associations for the large number of sampled slaughter pigs. In that context the ALEX algorithm presented in research article I was developed.

ALEX provides a fast, reliable and robust estimate purely based on register data, which is a big advantage when doing large scale studies due to lower time consumption and costs. Unfortunately, primary AMU data collected in Danish pig production networks documenting the exposures throughout the pigs rearing periods, were not available in this project. It was therefore not possible to do a direct validation of the traces and calculations in the algorithm. However, the ALEX estimates were validated against an already accepted lifetime exposure algorithm [36].

Another strength of the ALEX algorithm is, that it can provide a simple and fast way of comparing lifetime exposure as well as treatment strategies of Danish slaughter pigs over time. This was used to compare the changes on individual farms in tetracycline and macrolide lifetime exposure in 2015 and 2019, rather than overall

changes on a national level. Comparing the changes on farm level can give some valuable understandings on for example how legislation a ects the farmers treatment behavior. In the tetracycline-macrolide case investigated in research article I, tetracycline exposure was observed to decline from 2015 to 2019, most likely as a response to the yellow card implementation [21, 23]. Furthermore, it was observed, that the production networks with the biggest tetracycline reduction had an increased macrolide use [36], which could be di cult to discover without the ALEX approach.

There were a number of challenges and uncertainties associated with the register based algorithm ALEX, besides typing errors and inconsistencies in data [30]. Deviations between what is recorded in VetStat and what is actually used on the farms for the particular rearing groups also contributes to uncertainties in the estimates [25]. Another aspect is, that only the purchase date and the total purchased amount of antimicrobial drug are registered in VetStat. This means that the exact batch receiving the treatment, how many treated pigs in the batch, which dose or the length of the treatment period are not registered and have to be estimated. Furthermore, the pigs are not individually traceable through the production chain, which makes it di cult to obtain unique moving patterns for the traced pigs. The internal movements on farms are not registered in the PMD and were estimated based on information in the CHR. The number of piglets is not registered in the CHR and was calculated based on the average number of piglets per sow registered in the CHR. In VetStat there is no separation between the antimicrobial drugs purchased for sow and piglets, however, it was assumed that piglets and sows, to some extend, had a similar antimicrobial exposure [39].

One way to improve the register based AMU estimates is to increase the information level. This could include registration of antimicrobial exposure on batch, pen or even pig level, and individual movement registrations, as already implemented for cattle in Denmark [31]. However, this would increase administrative reporting tasks and not necessarily reduce the reported typing errors and inconsistencies across the registries.

There exists a discrepancy between the number of ownership registered in CHR and in Statistics Denmark Statistics Denmark has the opportunity of collecting more CVR numbers associated with one owner [40] and thereby revealing di erent production structures, than the CHR based production types estimated by ALEX. This minor deviation does not change the fact that the production type including management factors, appear to have a noticeable in uence on AMR levels. However, there seems to be an association between the AMU on farms and the biosecurity standards [41]. Both the production type and high AMU appear to be associated with low biosecurity [42, 43]. There seems to be a small tendency for the complex production types ('other - 3+'), to use more antimicrobial than the other production types, at least when considering tetracycline in the sampled farms in the qPCR study.

Having this in mind, the in uence of production type and the related biosecurity should be investigated further as the number of pig farms in Denmark is decreasing and developing into fewer, larger and more specialized farms [40].

In research article II, mixed e ect modeling of the total tetracycline resistance level was used to quantifying relationships between AMU, management factors and AMR in Danish slaughter pigs. Historically, tetracycline is one of the most used antimicrobial drugs for pigs, especially, for weaners and nishers, even though there has been an overall reduction on a nation scale [21]. However, the fecal samples collected in the qPCR study only show a minor reduction in the total tetracycline resistance level during the period of 2015 to 2019. Therefore the individual distributions of each tetracycline ARG was examined revealing that the level of some ARGs were decreasing while others were increasing. This highlights one of the issues of measuring, reporting and ghting AMR, namely, whether the ARGs should be considered individually or as an integrated part of an overall resistance pro le. The ARGs exhibit di erent patterns, but in the end, the goal is to reduce the total resistance level. It is also important to consider the individual ARGs in the light of co-selection and even more important, to take into account, in which bacteria the ARGs are present and how likely they are to be expressed i.e. providing phenotypical resistance.

In research article III, associations between AMU and AMR were investigated by individually mixed e ect modeling of the most prevalent and abundant ARGs measured with qPCR. Similar patterns of dependence on production type were observed both when modeling the total tetracycline resistance level and in half of the individual ARG models. This implies that ownership and number of farms (number of transferred pigs and mixing of pigs), gives rise to higher resistance levels. However, it remains unclear if it is the actual mixing of pigs, the biosecurity standards on the farms (as discussed above), higher treatment frequencies, the feeding procedures or something else that is causing this. It should be further explored exactly which management factors behind the production type that are more in uential in order to implement strategies in the productions based on this.

The ndings of the individual modeling of the ARGs also show that they are associated with AMU in the di erent rearing periods. This demonstrates that AMU, during the full life time of the pig, should be considered when modeling. This could also act as an inspiration for decision makers, when discussing and planning treatment strategies, however, the results should be interpreted with some caution, because certain antimicrobials are mostly used in speci c rearing periods. This is exempli ed by the antimicrobial drug class aminoglycosides, which seems to only have an in uence on AMR levels in the weaner rearing period based on the modeling. However, comparing this with the AMU exposure patterns show that aminoglycosides are most commonly used in this rearing group. Therefore it should not be concluded, based on these results, that it is better to expose pigs in the piglet and nisher period to aminoglycosides. Furthermore, the variables that are most

often identi ed as statistically signi cant from the models are the tetracycline and macrolide exposure in the weaner and nisher rearing periods. Part of this could, however, be due to the sampling of the pigs just after slaughter i.e. closest to the nisher rearing period, and the extensive use of these two classes in these rearing periods.

It is important to understand AMR, both from an epidemiological perspective, but also on a more detailed genomic level. The epidemiological perspective provides information on how the AMR is developing in the pig population, while the genomic data can help explaining the observed patterns. Furthermore, there is a trade-o when conducting studies. Well-controlled and detailed data collection will often lead to small-scale studies, due to cost and time consumption. In this thesis, a combination of information from di erent studies was utilized to investigate some unexpected AMU-AMR patterns of tet(X). The distribution of tet(X) shows an increasing trend of the mean value during the years, despite a reduction in tetracycline AMU nation wide. Modeling tet(X) individually shows that it is in uenced by macrolide and lincosamide exposure, rather than tetracycline. This might be a result of co-selection. The modeling results might be explained by combining it with observations from the smaller context study, where tet(X) is often co-occurring with erm(F), which is coding for macrolide and lincosamide AMR. The data originated from two di erent studies with di erent study populations as well as data collection and analysis methods. The context study only included a single farm, but the indications of the combination of the results clearly reveals, that this might be an interesting issue for future research to investigate.

With the growing accessibly of gathered AMU data and detailed analyses of AMR samples, it is important to keep an eye on the quality and the di erences introduced over time [44]. The ADD values provided in VetStat has been updated and changed over time by the Ministry of Food, Agriculture and Fisheries, so a recalculation of exposure must be performed, when considering longer time periods for comparisons [45, 46]. Changing the ADD values means that the average dose for the given antimicrobials has been changed, and since many AMU measures includes the ADD as part of the calculations, this might a ect the exposure estimates. In this project, the register data covering the full study period was retrieved only once and the same ADD values were used for all data. There are also some precautions that must be made, when evaluating AMR measurements over time. It has been documented that both the qPCR equipment and the procedures followed by the lab technician performing the analysis have an impact on the results [47]. In this project, a consistent bias in the reruns of 2015 samples and inconsistency in the reruns of 2017 samples were observed in connection with the qPCR analyses of the 2019 data. , Based on these observations, it was decided to rerun all collected samples to obtain a more reliable comparison of AMR over the years. This emphasizes the value of quality checking the data and demonstrates that improved procedures and methods are needed.

Both types of methods for detecting and quantifying AMR in this project were DNA-based. The methods are very sensitive and excellent in detecting even very small amounts of ARGs in samples [48]. However, they are not capable of determine whether the detected ARGs are expressed or not i.e. resulting in phenotypic resistance. Nevertheless, the intrinsically existence of ARGs might constitute a hazard due to potential expression and/or horizontal gene transfer [49].

A relating issue to the qPCR method is imperfect matching of primers and targets, which was observed in the small substudy comparing qPCR and metagenomic resistance ranking pro les. This was clear for lnu(C). Moreover, the primers nd the ARGs based on the exact design of the primer, whereas the metagenomic method can match against all versions (mutations) of the gene stored in the database. As an example, this appeared to be the case for blaOXA in the ranking comparison, where rankings for the qPCR results showed equally ranking of all samples due to no detections, while the rankings for the metagenomic results distinguished between the samples. In general, the qPCR method o ers highly sensitive results by being able to detect ARGs even in the presence of only a few gene copies, but the metagenomic method seems to provide a better coverage of the ARGs, by being able of detecting diverse versions of the genes [50, 51].

As mentioned, the qPCR method only detects exactly what is searched for, determined by the selected primers. The metagenomic method detects what can be matched according to a database. This means that both methods are only enabled of nding previously described ARGs [52], which might be an challenge when studying new AMR mechanisms and ARGs. However, the focus in this thesis was on already known antimicrobials and ARGs due to the relative restrictive legislation in Denmark.

The collection period of the key study (qPCR) in this project was spanning over four years in total, including unsystematic collections at slaughter houses in 2015, 2017 and 2019. However, further data collection is required to determine more accurately how AMU, and other management factors, exactly a ect AMR in the pig production. The collected data demonstrates how AMR steadily continues on farms with low AMU within a certain drug class. This is most likely interlinked with the co-selection caused by co-occurrence. Consequently, the ultimate impact of co-selection is that even banning a particular antimicrobial drug is not enough to eliminate the resistance against that drug class, even though a reduction might introduce a reduction in the long term [53, 54].

Several recent research studies focus on association between metal and antimicrobial resistance in the light of co-selection [55, 56, 57]. As a reaction, the European Commission has banned medical zinc, which is mainly used for prophylactic treatment of diarrhea, in pig production from 2022 [21]. Based on this, medical zinc has been phased out in the previous years in Danish pig production. Diarrhea is often

observed in the weaner rearing period when changing feed. In Denmark, actions on management and feed concepts to replace medical zink, are already being implemented. This highlights the complexity of AMR and the importance of including other factors than simply AMU in studies. It also show that alternatives to AMU should be implemented with caution and monitored. It appears that no easy solution exists on solving the AMR issue, because banning certain antimicrobial classes or introducing alternative treatments, most likely does not solve the problem with co-selection, at least not in the short term.

Although the data analyzed in this thesis was collected in the Danish pig sector, the relevance for humans is high. The human risk is a ected directly by the potential spread from pig to humans through farmers [58] or the food chain [59, 60]. Moreover, pigs can be used as human models and based on this, the ndings of this project highlight the need for further research of co-occurrence and the consequences [61, 62].

Chapter 7

Concluding Remarks

The work presented in this thesis demonstrate that besides the association between AMU and AMR abundance, there also exists an association between AMR and production typein Danish pig production.

Mixed e ect modeling of the total tetracycline resistance level as well as individual ARG levels revealed complex AMU-AMR patterns in the Danish pig population, depending on both type of production and exposure of certain antimicrobial classes in speci c rearing periods. To some extend, parts of these complex patterns could be explained by observed co-occurrence in a small scale genomic study.

The average antimicrobial exposure of pigs, during all three rearing periods, was estimated by the ALEX algorithm, which provides a fast and robust estimate based solely on register data. More quality checking in the data registers as well as improved collection of exposure and movement details can potentially result in more accurate AMU estimates.

The results of this research should be interpreted and implemented with some caution. The ndings can, however, contribute as inspiration for discussions on treatment strategies or as a basis for future studies. Future work should include more systematic data collection over an extended period, ensuring multiple samples per farm. Monthly resampling of farms that are changing the treatment strategies drastically would be to prefer in order to obtain more knowledge on the AMU-AMR dynamics.

Continuing the monitoring of antimicrobial resistance levels is necessary, since lowering antimicrobial exposure does not automatically lower ARGs as a consequence of co-selection. Alternative treatments should also be implemented with care and the implications should be tracked to avoid situations like the one with medical zink, causing co-selection between metal and antimicrobial resistance genes. Investigating the genomic context of the ARGs in the sense of co-occurrence, bacterial host species and horizontal gene transfer is important for assessing the human risk and could be an important part of future studies.

Bibliography

[1] The World Health Organization (WHO), Ten threats to global health 2019. https://www.who.int/news-room/spotlight/ten-threats-to-global-health-in-2019, 2022. Accessed: 2022-26-07.

[2] C. J. L. Murray, K. S. Ikuta, F. Sharara, and et al., Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis, Lancet, vol. 399, pp. 629 655, 2022.

[3] F. M. Aarestrup, The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward, Trans. R. Soc. B, vol. 370, 2015.

[4] A. E. van den Bogaard and E. E. Stobberingh, Epidemiology of resistance to antibiotics: Links between animals and humans, Int. J. Antimicrob. Agents, vol. 14, no. 4, pp. 327 335, 2000.

[5] M. Woolhouse, M. Ward, B. van Bunnik, and J. Farrar, Antimicrobial resistance in humans, livestock and the wider environment, Philos. Trans. R Soc. Lond. B Biol. Sci., vol. 370, no. 1670, 2015.

[6] A. H. Holmes, L. S. P. Moore, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P. J. Guerin, and L. J. V. Piddock, Understanding the mechanisms and drivers of antimicrobial resistance, Lancet, vol. 387, no. 10014, pp. 176 187, 2016.

[7] A. MacGowan and E. Macnaughton, Antibiotic resistance, Medicine, vol. 45, no. 10, pp. 622 628, 2017.

[8] B. Catry, H. Laevens, L. A. Devriese, G. Opsomer, and A. De Kruif, Antimicrobial resistance in livestock, J. Vet. Pharmacol. Ther., vol. 26, pp. 81 93, 2003.

[9] B. Wall, L. Marshall, A. Mateus, and D. P. et al., Drivers, dynamics and epidemiology of antimicrobial resistance in animal production. Rome: FAO, 2016.

[10] R. Cantón and P. Ruiz-Garbajosa, Co-resistance: an opportunity for the bacteria and resistance genes, Curr. Opin. Pharmacol., vol. 11, no. 5, pp. 477 485, 2011.

[11] H. Nikaido, Multidrug resistance in bacteria, Annu. Rev. Biochem., vol. 78, pp. 119 146, 2009.

[12] S. R. Partridge, S. M. Kwong, N. Firth, and S. O. Jensen, Mobile Genetic Elements Associated with Antimicrobial Resistance, Clin. Microbiol. Rev., vol. 31, no. 4, pp. e00088 17, 2018.

[13] M. F. Anjum, E. Zankari, H. Hasman, F. M. Aarestrup, S. Schwarz, J. Shen, and L. Cavaco, Molecular methods for detection of antimicrobial resistance, Microbiol. Spectr., vol. 5, no. 6, p. 5.6.02, 2017.

[14] P. Munk, V. D. Andersen, L. de Knegt, M. S. Jensen, O. Knudsen, B. E. and Lukjancenko, H. Mordhorst, J. Clasen, Y. Agersø, A. Folkesson, S. J. Pamp, H. Vigre, and F. M. Aarestrup, A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds, J. Antimicrob. Chemother., vol. 72, pp. 385 392, 2017.

[15] P. Kralik and M. Ricchi, A Basic Guide to Real Time PCR in Microbial Diagnostics: De nitions, Parameters, and Everything, Front. Microbiol., vol. 8, 2017.

[16] E. O. Bangsgaard, K. Græsbøll, V. D. Andersen, J. Clasen, D. Jasinskyte, J. E. Hansen, A. Folkesson, and L. E. Christiansen, Mixed e ect modeling of tetracycline resistance levels in Danish slaughter pigs, Prev. Vet. Med., vol. 191, p. 105362, 2021.

[17] N. Segata, D. Boernigen, T. L. Tickle, X. C. Morgan, W. S. Garrett, and C. Huttenhower, Computational meta'omics for microbial community studies, Mol. Syst. Biol., vol. 9, no. 666, 2013.

[18] P. Munk et al, Temporal evolution of the gut microbiomes and resistomes in fattening pigs, 2022. [In preparation].

[19] Danmarks Statistik, Svinebestanden 1. januar 2022. https://www.dst.dk/da/Statistik/nyheder-analyser-publ/nyt/NytHtml?cid=33200 [In Danish], 2022. Accessed: 2022-13-07.

[20] A. C. Birkegård, V. D. Andersen, T. Halasa, V. F. Jensen, N. Toft, and H. Vigre, Computional algorithm for lifetime exposure to antimicrobials in pigs using register data - the LEA algorithm, Prev. Vet. Med, vol. 146, pp. 173 180, 2017.

[21] DANMAP, DANMAP 2020 - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark, tech. rep., Statens Serum Institut and National Food Institute, DTU, 2021.

[22] P. Munk, B. E. Knudsen, O. Lukjancenko, A. Duarte, L. Van Gompel, R. Luiken, L. Smit, H. Schmitt, R. B. Garcia, A. D.and Hansen, T. N. Petersen, A. Bossers, E. Ruppé, E. Group;, O. Lund, T. Hald, S. J. Pamp, H. Vigre, D. Heederik, J. A. Wagenaar, M. D., and F. M. Aarestrup, Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries, Nat. Microbiol., vol. 3, no. 8, pp. 898 908, 2018.

[23] A. C. L. Antunes and V. F. Jensen, Close to a Decade of Decrease in Antimicrobial Usage in Danish Pig Production Evaluating the E ect of the Yellow Card Scheme, Front. Vet. Sci, vol. 7, 2020.

[24] V. F. Jensen, E. Jacobsen, and F. Bager, Veterinary antimicrobial-usage statistics based on standardized measures of dosage, Prev. Vet. Med, vol. 64, no. 2-4, pp. 201 215, 2004.

[25] V. D. Andersen, P. Munk, L. V. de Knegt, M. S. Jensen, F. M. Aarestrup, and H. Vigre, Validation of the register-based lifetime antimicrobial usage measurement for nisher batches based on comparison with recorded antimicrobial usage at farm level, Epidemiol. Infect., vol. 146, no. 4, p. 515 523, 2018.

[26] A. Lekagul, V. Tangcharoensathien, and S. Yeung, Patterns of antibiotic use in global pig production: A systematic review, Vet. Anim. Sci, vol. 7, p. 100058, 2019.

[27] A. I. V. Sørensen, V. F. Jensen, A. Boklund, T. Halasa, H. Christensen, and N. Toft, Risk factors for the occurrence of livestock-associated methicillin-resistant Staphylococcus aureus (LA-MRSA) in Danish pig herds, Prev. Vet. Med., vol. 159, 2018.

[28] C. Varga, A. Rajic, M. E. McFall, R. J. Reid-Smith, A. E. Deckert, S. L. Checkley, and S. A. McEwena, Associations between reported on-farm antimicrobial use practices and observed antimicrobial resistance in generic fecal Escherichia coli isolated from Alberta nishing swine farms, Prev. Vet. Med, vol. 88, pp. 185 192, 2009.

[29] A. Vieira, H. Houe, H. Wegener, D. Wong, and H.-D. Emborg, Association Between Tetracycline Consumption and Tetracycline Resistance in Escherichia

coli from Healthy Danish Slaughter Pigs," Foodborne Pathog. Dis., vol. 6, no. 1, pp. 99 109, 2009.

[30] N. Dupont, M. Fertner, A. Birkegaard, V. Andersen, G. Nielsen, A. Kruse, and L. de Knegt, "Improving institutional memory on challenges and methods for estimation of pig herd antimicrobial exposure based on data from the Danish Veterinary Medicines Statistics Program (VetStat)," ArXiv, 2017.

[31] Fødevarestyrelsen, "Det Centrale Husdyrsbrugsregister (CHR)." https://chr.fvst.dk [In Danish], 2022. Accessed: 2022-17-06.

[32] Fødevarestyrelsen, "Svine yttedatabasen (PDM)." https://svineflyt.fvst.dk [In Danish], 2022. Accessed: 2022-17-06.

[33] H. Stege, F. Bager, E. Jacobsen, and A. Thougaard, "VETSTAT - the Danish system for surveillance of the veterinary use of drugs for production animals," Prev. Vet. Med., vol. 57, no. 3, pp. 105 115, 2003.

[34] J. Clasen, A. Mellerup, J. Olsen, A. Ø., A. Folkesson, T. Halasa, N. Toft, and A. Birkegård, "Determining the optimal number of individual samples to pool for quanti cation of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR," Vet. Microbiol., vol. 189, pp. 46 51, 2016.

[35] G. V. Schmidt, A. Mellerup, L. E. Christiansen, M. Ståhl, J. E. Olsen, and Ø. Angen, "Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches," PLoS ONE, vol. 10, pp. 1 22, 06 2015.

[36] E. O. Bangsgaard, K. Græsbøll, V. D. Andersen, and L. E. Christiansen, "The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way." [Submitted 01-JUN-2022], 2022.

[37] A. Birkegård, T. H. B. H., K. Græsbøll, J. Clasen, A. Folkesson, and N. Toft, "Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms," Sci. Rep., vol. 7, no. 1, 2017.

[38] Danish Ministry of Health", "NATIONAL ACTION PLAN ON ANTIBIOTICS IN HUMAN HEALTHCARE," tech. rep., 2017.

[39] B. Callens, C. Faes, D. Maes, and et al, "Presence of antimicrobial resistance and antimicrobial use in sows are risk factors for antimicrobial resistance in their o spring," Microb. Drug. Resist., vol. 21, no. 1, pp. 50 58, 2015.

[40] M. G. Christiansen, "Strukturudvikling i dansk griseproduktion 2021," March 2021. SEGES: Notat 2201.

[41] S. Raasch, M. Postma, J. Dewulf, K. D. C. Stärk, and E. grosse Beilage, Association between antimicrobial usage, biosecurity measures as well as farm performance in German farrow-to- nish farms, Porc. Health Manag., vol. 4, no. 30, 2018.

[42] M. Postma, A. Backhans, L. Collineau, and et al, Evaluation of the relationship between the biosecurity status, production parameters, herd characteristics and antimicrobial usage in farrow-to- nish pig production in four EU countries, Porc. Health. Manag., vol. 2, no. 9, 2016.

[43] M. Laanen, D. Persoons, S. Ribbens, E. de Jong, B. Callens, M. Strubbe, D. Maes, and J. Dewulf, Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds, Vet J., vol. 198, no. 2, pp. 508 512, 2013.

[44] L. Collineau, C. Belloc, K. D. Stärk, and et al., Guidance on the Selection of Appropriate Indicators for Quanti cation of Antimicrobial Usage in Humans and Animals, Zoonoses Public Health, vol. 64, no. 3, pp. 165 184, 2017.

[45] N. Dupont, M. Fertner, K. Pedersen, N. Toft, and H. Stege, Rapportering af danske svins antibiotikaforbrug - hvor stor en betydning har beregningsmetoden?, Dansk Veterinærtidsskrift, pp. 33 38, 2014.

[46] N. Dupont, M. Fertner, and C. e. a. Kristensen, Reporting the national antimicrobial consumption in danish pigs: in uence of assigned daily dosage values and population measurement, Acta. Vet. Scand., vol. 58, no. 27, 2015.

[47] K. R. Gri ths, D. G. Burke, and K. R. Emslie, Quantitative polymerase chain reaction: a framework for improving the quality of results and estimating uncertainty of measurement, Anal. Methods, vol. 3, pp. 2201 2211, 2011.

[48] U. Ki, T. Suzuki, S. Nakazawa, and et al., Evaluation method for asymmetric uncertainty of quantitative polymerase chain reaction measurements of deoxyribonucleic acids with low copy number, Sci. Rep., vol. 11, no. 11550, 2021.

[49] H. Harbottle, S. Thakur, S. Zhao, and D. G. White, Genetics of antimicrobial resistance, Anim. Biotechnol., vol. 17, no. 2, pp. 111 124, 2006.

[50] E. Crossette, J. Gumm, K. Langenfeld, L. Raskin, M. Duhaime, and K. Wigginton, Metagenomic Quanti cation of Genes with Internal Standards, mBio., vol. 12, no. 1, pp. e03173 20, 2021.

[51] C. Ferreira, S. Otani, F. M. Aarestrup, and C. M. Manaia, Quantitative PCR versus metagenomics for monitoring antibiotic resistance genes: balancing high sensitivity and broad coverage, FEMS Microbes, 2022. [submitted].

[52] M. F. Anjum, Screening methods for the detection of antimicrobial resistance genes present in bacterial isolates and the microbiota, Future Microbiol., vol. 10, no. 3, pp. 317 320, 2015.

[53] N. A. Duggett, L. P. Randall, R. A. Horton, F. Lemma, M. Kirchner, J. Nunez-Garcia, C. Brena, S. M. Williamson, C. Teale, and M. F. Anjum, Molecular epidemiology of isolates with multiple mcr plasmids from a pig farm in Great Britain: the e ects of colistin withdrawal in the short and long term, J. Antimicrob. Chemother., vol. 73, no. 11, pp. 3025 3033, 2018.

[54] A. De Lucia, R. Card, N. Duggett, R. Smith, R. Davies, S. Cawthraw, M. Anjum, M. Rambaldi, F. Ostanello, and F. Martelli, Reduction in antimicrobial resistance prevalence in Escherichia coli from a pig farm following withdrawal of group antimicrobial treatment, Vet. Microbiol., vol. 258, pp. 109 125, 2021.

[55] C. Pal, K. Asiani, S. Arya, C. Rensing, D. J. Stekel, D. J. Larsson, and J. L. Hobman, Chapter seven - metal resistance and its association with antibiotic resistance, in Microbiology of Metal Ions (R. K. Poole, ed.), vol. 70 of Adv. in Microb. Physiol., pp. 261 313, 2017.

[56] M. Imran, K. R. Das, and M. M. Naik, Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat, Chemosphere, vol. 215, pp. 846 857, 2018.

[57] A. G. Wiggins, S. P. LaVoie, J. Wireman, and A. O. Summers, Thinking outside the (pill) box: Does toxic metal exposure thwart antibiotic stewardship best practices?, Plasmid, vol. 99, pp. 68 71, 2018.

[58] R. Köck, B. Loth, M. Köksal, J. Schulte-Wülwer, J. Harlizius, and A. Friedrich, Persistence of nasal colonization with livestock-associated methicillin-resistant Staphylococcus aureus in pig farmers after holidays from pig exposure, Appl. Environ. Microbiol., vol. 78, pp. 4046 4047, 2012.

[59] M. Barza, Potential Mechanisms of Increased Disease in Humans from Antimicrobial Resistance in Food Animals, Clin. Infect. Dis., vol. 34, pp. S123 S125, 2002.

[60] A. M. Hammerum and O. E. Heuer, Human Health Hazards from Antimicrobial-Resistant Escherichia coli of Animal Origin, Clin. Infect. Dis., vol. 48, pp. 916 921, 2009.

[61] M. M. Swindle, A. Makin, A. J. Herron, J. Clubb, F. J., and K. S. Frazier, Swine as models in biomedical research and toxicology testing, Vet. Pathol., vol. 49, no. 2, pp. 344 356, 2012.

[62] J. K. Lunney, A. V. Goor, K. E. Walker, T. Hailstock, J. Franklin, and C. Dai, Importance of the pig as a human biomedical model, Sci. Transl. Med., vol. 13, no. 621, p. eabd5758, 2021.

Research Article I

Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen and Lasse E. Christiansen "The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way" *Preventive Veterinary Medicine* submitted, 2022

Supplementary material

Supplementary material for "The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way" by Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen and Lasse E. Christiansen submitted to Preventive Veterinary Medicine, 2022

Research Article II

Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen, Julie Clasen, Dziuginta Jasinskyt, Julie E. Hansen, Anders Folkesson and Lasse E. Christiansen "Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs" Preventive Veterinary Medicine, 2021

Supplementary material

Supplementary material for "Mixed effect modeling of tetracycline resistance levels in Danish slaughter pigs" by Elisabeth O. Bangsgaard, Kaare Græsbøll, Vibe D. Andersen, Julie Clasen, Dziuginta Jasinskyt, Julie E. Hansen, Anders Folkesson and Lasse E. Christiansen published in Preventive Veterinary Medicine, 2021

Research Article III

Elisabeth O. Bangsgaard, Patrick Munk, Saria Otani, Kaare Græsbøll and Lasse E. Christiansen, "Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context", Prepared for Journal of Antimicrobial Agents nal draft ready for submission, 2022

4.2. The context study

As previously described, 66 samples were taken from the six pigs over 11 weeks and used to generate within-pig co-assemblies. The polished Nanopore assemblies from the six pigs were ltered for 1Kb+ sca olds and were 3627 Mb long, with an N50 of 42 Kb. 4228 contigs were at least 100Kb long.

The distributions of detected ARGs within each pig can be found in a table in the supplementary material. The ARGs are found in all six pigs in the range of 69-112 ARGs per pig. No clear di erence between the pigs was observed, and especially the ARGs observed at least eight times in the study appear to be well distributed and detected in all six pigs.

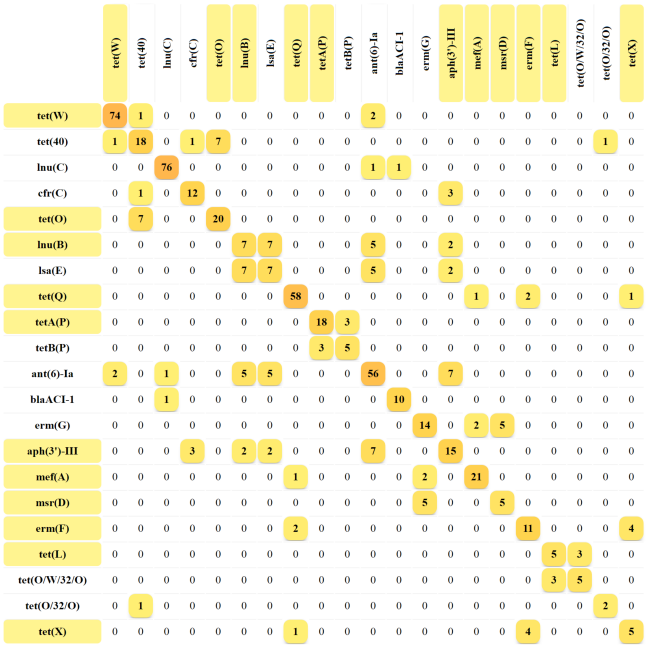
Figure 3 presents the number of co-occurrences of ARGs on the same contig. The number in the diagonal represents how many times the ARG was found in the context study and the o -diagonal shows how many times the ARG was found on a contig with another ARG.

Some contigs included three or four ARGs, however, this cannot be observed in this gure. In total, 441 contigs included only one ARG, 53 contigs included two ARGs, four contigs included three ARGs and two contigs included four ARGs i.e. in total 569 ARGs were identi ed in the metagenomic assemblies.

tet(W), lnu(C), tet(Q), and ant(6)-Ia were the most frequently detected ARGs in the context study. Even though tet(W) was found on 74 contigs, it was only found once on a contig with tet(40) and twice with ant(6)-Ia. The same applies for lnu(C) and tet(Q): tet(40) was found together with tet(O) in 7 out of the 18 contigs.

lnu(B) and lsa(E) were found co-occurring in seven contigs, in ve of these cases they were found in a contig also including ant(6)-Ia and in two of these ve cases they were found with the additional ARG aph(3')-III. This means that this group of ARGs constitutes three out of the four contigs including three ARGs and two contigs with four ARGs. The last contig including three ARGs contained tet(X), tet(Q) and erm(F).

tetB(P) was found together with tetA(P) in three out of ve contigs. aph(3')-III co-occurred with three other ARGs, but most frequently with ant(6)-Ia. msr(D) was only found co-occurring on contigs including erm(G). erm(F) was found in four out of the ve contigs where tet(X) was found.



	tet(W)	tet(40)	lnu(C)	cfr(C)	tet(O)	lnu(B)	lsa(E)	tet(Q)	tetA(P)	tetB(P)	ant(6)-Ia	blaACI-1	erm(G)	aph(3')-III	mef(A)	msr(D)	erm(F)	tet(L)	tet(O/W/32/O)	tet(O/32/O)	tet(X)
tet(W)	74	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0
tet(40)	1	18	0	1	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
lnu(C)	0	0	76	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
cfr(C)	0	1	0	12	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0
tet(O)	0	7	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(B)	0	0	0	0	0	7	7	0	0	0	5	0	0	2	0	0	0	0	0	0	0
lsa(E)	0	0	0	0	0	7	7	0	0	0	5	0	0	2	0	0	0	0	0	0	0
tet(Q)	0	0	0	0	0	0	0	58	0	0	0	0	0	0	1	0	2	0	0	0	1
tetA(P)	0	0	0	0	0	0	0	0	18	3	0	0	0	0	0	0	0	0	0	0	0
tetB(P)	0	0	0	0	0	0	0	0	3	5	0	0	0	0	0	0	0	0	0	0	0
ant(6)-Ia	2	0	1	0	0	5	5	0	0	0	56	0	0	7	0	0	0	0	0	0	0
blaACI-1	0	0	1	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0
erm(G)	0	0	0	0	0	0	0	0	0	0	0	0	14	0	2	5	0	0	0	0	0
aph(3')-III	0	0	0	3	0	2	2	0	0	0	7	0	0	15	0	0	0	0	0	0	0
mef(A)	0	0	0	0	0	0	0	1	0	0	0	0	2	0	21	0	0	0	0	0	0
msr(D)	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	5	0	0	0	0	0
erm(F)	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	11	0	0	0	4
tet(L)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3	0	0
tet(O/W/32/O)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	5	0	0
tet(O/32/O)	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
tet(X)	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	4	0	0	0	5

Figure 3: Table representing how many times ARGs were detected together in a contig. The yellow background color on the ARG labels indicates ARGs that were measured in the AMU study. The diagonal illustrates how many times the ARG was identi ed in the context study and the -diagonal shows the number of co-occurrences with other ARGs within a contig.

ARG	Number of detections	Number of contigs including the ARG and a MGE	Number of detections together with unique MGEs
tet(W)	74	8	6
tet(Q)	58	12	5
mef(A)	21	8	6
cfxA5	14	1	1
erm(F)	11	3	2
cfxA6	7	4	1
tet(L)	5	1	1
tet(X)	5	2	2
aph(3")-Ib	1	2	2

Table 2: Overview of ARGs found together with MGEs on the same contig in the context study.

Table 2 reports the number of ARG occurrences, the number of co-occurrences with MGEs, and the number of unique combinations of ARGs and MGEs in the context study. The full table with all ARGs in the context study found together with an MGE on a contig is included in the supplementary material.

tet(W) was detected in 74 of the contigs and in 8 (10.1%) of the cases it was found together with an MGE. tet(Q) was detected together with an MGE in 12 out of 58 (20.6%). mef(A) was found together with an MGE in 8 out of the 21 cases (38.1%). The cfxA5 variant was only found co-occurring with an MGE in 1 out of the 14 contigs, while the cfxA6 variant co-occurred with an MGE in 4 out of 7 contigs.

5. Discussion and Conclusion

The AMU study was a larger study performed on many slaughter pigs from different farms. Its data can be used to investigate the average resistance level nationwide. On the other hand, the context study was a small study conducted on fewer slaughter pigs all from the same herd. This study provided more detailed data which can be used to study the contexts and co-occurrence of the ARGs and MGEs in pigs. Combining the findings of the two different studies improves the understanding of the AMR dynamics in Danish slaughter pigs. It is important to emphasize that the context study expresses the findings on one specific Danish slaughter farm, but still the findings can be used to support the results of the wider AMU study.

In both studies, tet(W) and tet(Q) are amongst the most widespread ARGs. In the AMU study, they were found in 100% of the samples and had the highest C_{tq}-values, and in the context study they appeared on 74 and 58 contigs, respectively. Similar results are reported by Yang et al. [16], where the highest abundance of ARG in pigs was tet(W) in the study across different countries. lnu(C) was detected on most contigs in the context study. However, it was not measured in the AMU study and therefore it was not possible to do a comparison.

In the AMU study, the model for tet(X), the macrolide exposure and the lincosamide exposure in the piglet group were significant variables, while all tetracycline variables were eliminated in the final model. The AMU variables in the mixed effect models were all smoothed over a period of six months to capture the continuous exposure on the farm rather than an instantaneous estimate. This might also reduce the probability of wrongly associating specific AMU with an ARG due to the long decay in a herd [17, 18]. This result could be explained by the context study results, where tet(X) was found on a contig co-occurring with erm(F) in four out of the

ve detections. erm(F) is commonly known as an ARG giving rise to macrolide, lincosamide and streptogramin B resistance. Likewise, tet(Q) was found to be a ected by pleurimutilins and macrolides, where the latter might be a consequence of the observed co-occurrence with erm(F) in the context study. Similar results were reported by Chung et al. [19]. Munk et al. [20] and Van Gompel et al. [21] also reported similar associations between AMU exposure and different drug classes and ARGs.

aph(3')-III provides aminoglycoside resistance. In the AMU study, the level of aph(3')-III was found to be increased by exposure to not only aminoglycoside in the weaner period, but also lincomasides in the weaner period and macrolides in the nisher period. This could be a consequence of co-selection [4]. The in uence of lincosamides could arise from the co-occurrence of aph(3')-III and lnu(B), which was found in the context study. aph(3')-III was found co-occurring on two contigs with three other ARGs. This highlights the complexity of antimicrobial resistance in the sense of co-selection, cross-resistance, and multi-resistance.

The variant cfxA5 was detected on 14 contigs, while the variant cfxA6 was detected on seven contigs. However, the cfxA6 variant co-occurred with an MGE in approximately half of the cases. So even though cfxA6 was less widespread than cfxA5 in the context study, there might be a greater risk of mobilization to zoonotic bacteria.

The most frequently signi cant variables in the mixed-effect models appeared to be exposure to tetracyclines and macrolides in the nisher age group. Two things could explain parts of this, one is that the pigs are sampled at slaughter i.e. closest to the nisher period, the other could be the extended use of tetracyclines and macrolides in this age group. This also applies to the aminoglycosides that are mostly used to treat weaners, which is also re ected in the mixed-effect models.

Studying antimicrobial resistance by inspecting single genes can give some valuable insight into some of the dynamics. However, the results of this combination of two studies highlight the importance of analyzing the genomic context of the ARGs and the co-occurrence with MGEs to get a deeper understanding. ARGs co-occuring on the same DNA element can result in co-selection and ARGs captured by MGEs can potentially be transferred to a zoonotic bacteria, thereby posing a greater human risk. It is therefore of great importance to consider both the genomic context as well as the phenotypic resistance of the ARGs when planning and implementing treatment strategies. A treatment strategy in reducing resistance against tetracycline might be introduced by reducing exposure to tetracycline. However, since the level of tet(X) is driven by macrolides and lincosamides and not tetracycline, this might maintain the tetracycline resistance due to co-selection by erm(F). Such knowledge is also crucial when developing simulation models in order to get the most reliable results based on the mechanisms behind the resistance.

Both studies presented are based on DNA-based methods for detecting antimicrobial resistance. However, these methods are not capable of distinguishing between expressed and non-expressed ARGs, i.e. detecting ARGs by these methods does not necessarily result in phenotypic resistance. Having that said, the presence and prevalence of the ARGs will always constitute a risk of expressing resistance or gene transfer [6]. A challenge of working with the different methods is that, by the qPCR-method only pre-speci ed ARGs are detected by the pre-designed primers, while all ARGs in a chosen database are detectable with the metagenomic method. The qPCR method is very sensitive in detecting the ARGs and has a low quanti cation limit. This emphasizes that the qPCR method provides more sensitive results, but the metagenomic method provides better coverage of the different ARGs [22, 23]. This also means that we only nd exactly what we are looking for with the qPCR method and known ARGs matched in the database

with the metagenomic method but not any novel ARGs.

The AMU and context studies were performed on Danish slaughter pigs. Besides exploring the direct impact on human health by the potential risk of spreading multi-drug resistant bacteria [9], the studies also œr knowledge that can be used in a more indirect way. Using pigs as human models is very common, mostly due to the size and anatonomy of pigs [24, 25]. The achieved insights into potential drivers of speci c ARG genes are therefore of great value in future research.

6. Funding

This work was supported by the Danish Ministry of Food, Agriculture and Fisheries as part of the Veterinærforlig III project.

7. Acknowledgments

References

[1] P. Munk, B. E. Knudsen, O. Lukjancenko, A. Duarte, L. Van Gompel, R. Luiken, L. Smit, H. Schmitt, R. B. Garcia, A. D.and Hansen, T. N. Petersen, A. Bossers, E. RuppGroup;, O. Lund, T. Hald, S. J. Pamp, H. Vigre, D. Heederik, J. A. Wagenaar, M. D., F. M. Aarestrup, Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries, Nat. Microbiol. 3 (2018) 898–908. doi:https://doi.org/10.1038/s41564-018-0192-9.

[2] L. Frost, R. Leplae, A. Summers, A. Toussaint, Mobile genetic elements: the agents of open source evolution, Nat. Rev. Microbiol. 3 (2005) 722–732. doi:https://doi.org/10.1038/nrmicro1235.

[3] M. H. K. Johansson, V. Bortolaia, S. Tansirichaiya, F. M. Aarestrup, A. P. Roberts, T. N. Petersen, Detection of mobile genetic elements associated with antibiotic resistance in Salmonella enterica using a newly developed web tool: MobileElementFinder, J. Antimicrob. Chemother. 76 (2020) 101–109. doi:10.1093/jac/dkaa390.

[4] A. H. Holmes, L. S. P. Moore, A. Sundsfjord, M. Steinbakk, S. Regmi, A. Karkey, P. J. Guerin, L. J. V. Piddock, Understanding the mechanisms and drivers of antimicrobial resistance, Lancet 387 (2016) 176–187. doi:https://doi.org/10.1016/S0140-6736(15)00473-0.

[5] S. R. Partridge, S. M. Kwong, N. Firth, S. O. Jensen, Mobile Genetic Elements Associated with Antimicrobial Resistance, Clin. Microbiol. Rev. 31 (2018) e00088–17. doi:10.1128/CMR.00088-17.

[6] A. E. van den Bogaard, E. E. Stobberingh, Epidemiology of resistance to antibiotics: Links between animals and humans, Int. J. Antimicrob. Agents 14 (2000) 327–335. doi:https://doi.org/10.1016/S0924-8579(00)00145-X.

[7] S. Chen, S. Zhao, D. White, C. Schroeder, R. Lu, Y. H, P. McDermott, S. Ayers, J. Meng, Characterization of multiple-antimicrobial-resistant salmonella serovars isolated from retail meats, Appl. Environ. Microbiol. 70 (2004) 722–732. doi:10.1128/AEM.70.1.1-7.2004.

[8] S. M. Jajere, A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host speci city and antimicrobial resistance including multidrug resistance, Vet. World 12 (2019) 504–521. doi:https://doi.org/10.14202/vetworld.2019.504-521.

[9] M. Woolhouse, M. Ward, B. van Bunnik, J. Farrar, Antimicrobial resistance in humans, livestock and the wider environment, Phil.Trans. R. Soc. B 370 (2015). doi:10.1098/rstb.2014.0083.

[10] E. O. Bangsgaard, K. Græsbøll, V. D. Andersen, J. Clasen, D. Jasinskyte, E. Hansen, A. Folkesson, L. E. Christiansen, Mixed ect modeling of tetracycline resistance levels in Danish slaughter pigs, Prev. Vet. Med. 191 (2021) 105362. doi:https://doi.org/10.1016/j.prevetmed.2021.105362.

[11] P. Munk et al, Temporal evolution of the gut microbiomes and resistomes in fattening pigs (2022). [In preparation].

[12] Fødevarestyrelsen, Det Centrale Husdyrsbrugsregister (CHR), https://chr.fvst.dk; 2022, 2022. URL: https://chr.fvst.dk , [accessed 17 June 2022 (In Danish)].

[13] H. Stege, F. Bager, E. Jacobsen, A. Thougaard, VETSTAT - the Danish system for surveillance of the veterinary use of drugs for production animals, Prev. Vet. Med. 57 (2003) 105–115. doi:10.1016/S0167-5877(02)00233-7.

[14] Fødevarestyrelsen, Svine yttedatabasen (PDM), https://svine yt.fvst.dk; 2022, 2022. URL: https://svineflyt.fvst.dk , [accessed 17 June 2022 (in Danish)].

[15] E. O. Bangsgaard, K. Græsbøll, V. D. Andersen, L. E. Christiansen, The ALEX Algorithm - Estimating Average Lifetime Antimicrobial Exposure of Danish Slaughter Pigs in a Fast, Automated and Robust Way, Prev. Vet. Med. (2022). [submitted 01-JUN-2022].

[16] D. Yang, D. Heederik, D. Mevius, P. Scherpenisse, R. Luiken, L. Van Gompel, M. Zyskaa, K. Wadepohl, C. Chauvin, E. Van Heijnsbergen, I. Wouters, G. Greve, B. Jongerius-Gortemaker, M. Tersteeg-Zijderveld, M. Zajac, D. Wasyl, K. Juraschek, J. Fischer, J. Wagenaar, L. Smit, H. E. c. Schmitt, Risk factors for the abundance of antimicrobial resistance genes aph(3')-III, erm(B), sul2 and tet(W) in pig and broiler faeces in nine European countries, J. Antimicrob. Chemother. 77 (2022) 969–978. doi:10.1093/jac/dkac002.
[17] A. De Lucia, R. Card, N. Duggett, R. Smith, R. Davies, S. Cawthraw, M. Anjum, M. Rambaldi, F. Ostanello, F. Martelli, Reduction in antimicrobial resistance prevalence in escherichia coli from a pig farm following withdrawal of group antimicrobial treatment, Vet. Microbiol. 258 (2021) 109125. doi:https://doi.org/10.1016/j.vetmic.2021.109125.
[18] N. A. Duggett, L. P. Randall, R. A. Horton, F. Lemma, M. Kirchner, J. Nunez-Garcia, C. Brena, S. M. Williamson, C. Teale, M. F. Anjum, Molecular epidemiology of isolates with multiple mcr plasmids from a pig farm in Great Britain: the e ects of colistin withdrawal in the short and long term, J. Antimicrob. Chemother. 73 (2018) 3025–3033. doi:10.1093/jac/dky292.
[19] W. O. Chung, J. Gabany, G. R. Persson, M. C. Roberts, Distribution of erm(F) and tet(Q) genes in 4 oral bacterial species and genotypic variation between resistant and susceptible isolates, J. Clin. Periodontol. 29 (2002) 152–158. doi:https://doi.org/10.1034/j.1600-051x.2002.290210.x.
[20] P. Munk, V. D. Andersen, L. de Knegt, M. S. Jensen, B. E. Knudsen, O. Lukjancenko, H. Mordhorst, J. Clasen, Y. Agersø, A. Folkesson, S. J. Pamp, H. Vigre, F. M. Aarestrup, A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds, Journal of Antimicrobial Chemotherapy 72 (2016) 385–392. doi:10.1093/jac/dkw415.
[21] L. Van Gompel, R. E. C. Luiken, S. Sarrazin, P. Munk, B. E. Knudsen, R. B. Hansen, A. Bossers, F. M. Aarestrup, J. Dewulf, J. A. Wagenaar, D. J. Mevius, H. Schmitt, D. J. J. Heederik, A. Dorado-García, C. A. M. Smit, The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries, Journal of Antimicrobial Chemotherapy 74 (2019) 865–876. doi:10.1093/jac/dky518.
[22] E. Crossette, J. Gumm, K. Langenfeld, L. Raskin, M. Duhaime, K. Wigginton, Metagenomic Quanti cation of Genes with Internal Standards, mBio. 12 (2021) e03173–20. doi:https://doi.org/10.1128/mBio.03173-20.
[23] C. Ferreira, S. Otani, F. M. Aarestrup, C. M. Manaia, Quantitative PCR versus metagenomics for monitoring antibiotic resistance genes: balancing high sensitivity and broad coverage, FEMS Microbes (2022). [submitted].
[24] J. K. Lunney, A. V. Goor, K. E. Walker, T. Hailstock, J. Franklin, C. Dai, Importance of the pig as a human biomedical model, Sci. Transl. Med. 13 (2021) eabd5758. doi:10.1126/scitranslmed.abd5758.
[25] M. M. Swindle, A. Makin, A. J. Herron, J. Clubb, F. J., K. S. Frazier, Swine as models in biomedical research and toxicology testing, Vet. Pathol. 49 (2012) 344–356. doi:doi.org/10.1177/0300985811402846.

Supplementary material

Supplementary material for "Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context" by Elisabeth O. Bangsgaard, Patrick Munk, Saria Otani, Kaare Græsbøll and Lasse E. Christiansen prepared for *Journal of Antimicrobial Agents*, 2022

Supplementary material for Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context

Elisabeth Ottesen Bangsgaard[a], Patrick Munk[b], Saria Otani[b], Kaare Græsbøll[a], Lasse Engbo Christiansen[a,c]

[a] *Department of Applied Mathematics and Computer Science, Technical University of Denmark, Kongens Lyngby, Denmark*
[b] *Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark*
[c] *Department of Epidemiology Research, Statens Serum Institut, København, Denmark*

Supplementary material for Antimicrobial exposure and observed resistance patterns in slaughter pigs explained by genomic context by Elisabeth O. Bangsgaard et al. is provided in the following. The supplementary material covers selected results from the context study that are not presented in the article. The selected results include all genes found in the context study.

1. ARGs in the context study

Table 1 lists all the antimicrobial resistance genes (ARGs) detected in the context study. The genes in bold are genes also measured in the AMU study.

Aminoglycoside resistance
aac(6')-Im **aadA1** ant(6)-Ia ant(6)-Ib aph(2")-Ib aph(3")-Ib (alternative name: **strA**)
aph(3')-III aph(6)-Id (alternative name: **strB**)
Beta-lactam resistance
blaA blaACI-1 blaOXA-193 **cfxA5 cfxA6**
Glycopeptide / *Vancomycin resistance*
VanG2XY VanGXY
Lincosamide resistance
lnu(B) lnu(C) lnu(P)
Lincosamide / *Streptogramin A* / *Pleuromutilin resistance*
lsa(E)
Macrolide resistance
mef(A) mph(N)
Macrolide / *Lincosamide* / *Streptogramin B resistance*
erm(A) erm(B) **erm(F)** erm(G) erm(Q) **erm(T)**
Macrolide / *Streptogramin B resistance*
msr(D)
Nitroimidazole resistance
nimH nimJ
Oxazolidinone / *Amphenicol* / *Lincosamide* / *Streptogramin A* / *Pleuromutilin resistance*
cfr(C)
Streptogramin A resistance
vat(F)
Tetracycline resistance
tet(L) tet(O) tet(O/32/O) tet(O/W)-1 tet(O/W/32/O) **tetA(P) tetB(P) tet(Q)**
tet(W) tet(X) tet(32) tet(40) tet(44)

Table 1: Table of ARGs found in the context study. The ARGs in bold were also measured in the AMU study.

1.1. ARG distribution within the pigs

Table 2 shows the distribution of the detected ARGs within each pig.

	Pig A	Pig B	Pig C	Pig D	Pig E	Pig F	**Sum**
lnu(C)	14	14	7	21	14	6	**76**
tet(W)	17	9	12	17	11	8	**74**
tet(Q)	16	7	8	9	9	9	**58**
ant(6)-Ia	8	15	3	13	12	5	**56**
erm(B)	5	6	4	3	8	4	**30**
mef(A)	3	3	1	3	6	5	**21**
tet(O)	2	7	3	2	4	2	**20**
tet(40)	5	5	1	1	5	1	**18**
tetA(P)	3	1	2	2	7	3	**18**
aph(3')-III	3	3	1	2	4	2	**15**
cfxA5	4	2	2	3	1	2	**14**
erm(G)	3	3	2	3	1	2	**14**
cfr(C)	4	2	1	2	2	1	**12**
lnu(P)	3	1	1	2	3	1	**11**
erm(F)	1	0	2	3	2	3	**11**
blaACI-1	2	1	1	3	1	2	**10**
tet(44)	1	2	1	3	1	2	**10**
ant(6)-Ib	1	1	1	3	1	2	**9**
erm(Q)	1	2	2	1	1	1	**8**
lnu(B)	1	1	0	2	2	1	**7**
lsa(E)	1	1	0	2	2	1	**7**
cfxA6	1	0	0	1	1	4	**7**
tetB(P)	1	1	1	0	1	1	**5**
VanG2XY	1	0	1	1	1	1	**5**
msr(D)	1	1	1	1	0	1	**5**
nimH	1	1	0	0	2	1	**5**
tet(L)	1	1	1	1	1	0	**5**
tet(O/W/32/O)	1	1	1	0	1	1	**5**
tet(X)	0	0	2	1	1	1	**5**
mph(N)	0	1	0	1	1	1	**4**
erm(A)	1	0	1	1	0	0	**3**
tet(O/W)-1	1	1	1	0	0	0	**3**
aadA1	0	0	1	1	1	0	**3**
tet(O/32/O)	1	0	0	0	1	0	**2**
nimJ	1	0	0	0	0	0	**1**
erm(T)	1	0	0	0	0	0	**1**
VanGXY	0	1	0	0	0	0	**1**
aph(3")-Ib	0	0	1	0	0	0	**1**
aph(6)-Id	0	0	1	0	0	0	**1**
aac(6')-Im	0	0	1	0	0	0	**1**
aph(2")-Ib	0	0	1	0	0	0	**1**
vat(F)	0	0	0	1	0	0	**1**
blaA	0	0	0	1	0	0	**1**
blaOXA-193	0	0	0	1	0	0	**1**
tet(32)	0	0	0	1	0	0	**1**
Sum	**110**	**94**	**69**	**112**	**108**	**74**	**567**

Table 2: Number of detections of ARGs in each pig is presented in this table.

1.2. Co-occuring ARGs

Figure 1 illustrates all the ARGs co-occuring on a contig in the context study.

Figure 1: Figure representing how many times ARGs were found together. The yellow background color on the ARG labels indicates ARGs that were measured in the AMU study. The diagonal illustrates how many times the ARG was identified in the context study, while the o□-diagonal shows number of co-occurrences with other ARGs within a contig.