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Birkegård, Anna Camilla

Publication date:
2017

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

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Citation (APA):
Birkegård, A. C. (2017). *Antimicrobial resistance in Danish pigs: A cross sectional study of the association between antimicrobial resistance and geography, exposure to antimicrobials, and trade*. Technical University of Denmark.

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Antimicrobial resistance in Danish pigs: A cross sectional study of the association between antimicrobial resistance and geography, exposure to antimicrobials, and trade

PhD Thesis
Anna Camilla Birkegård
September, 2017

Antimicrobial resistance in Danish pigs: a cross sectional study of the association between antimicrobial resistance and geography, exposure to antimicrobials, and trade

PhD Thesis

Anna Camilla Birkegård

Lyngby, September 2017

Division for Diagnostics & Scientific Advice – Epidemiology, National Veterinary Institute – Technical University of Denmark, Lyngby, Denmark

Supervisors

Professor Nils Toft (Main supervisor)
Division for Diagnostics & Scientific Advice – Epidemiology
National Veterinary Institute
Technical University of Denmark
Denmark

Senior Researcher Tariq Halasa (Co-supervisor)
Division for Diagnostics & Scientific Advice – Epidemiology
National Veterinary Institute
Technical University of Denmark
Denmark

Assessment Committee

Senior Advisor Anette Boklund (Chairperson)
Division for Diagnostics & Scientific Advice – Epidemiology
National Veterinary Institute
Technical University of Denmark
Denmark

Professor Søren Saxmose Nielsen
Department of Veterinary and Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen
Denmark

Professor Jeroen Dewulf
Department of Obstetrics, reproduction and herd health
Faculty of Veterinary Medicine
Ghent University
Belgium

The cover art work was created by Anna Camilla Birkegård

PREFACE AND ACKNOWLEGEMENT

My PhD study has been an amazing journey with a very steep learning curve filled with wonderful experiences. I have enjoyed working with antimicrobial resistance that is one of the hot topics of the decade. It has been interesting and inspiring.

This thesis was funded by the Danish Veterinary and Food Administration in a project concerning antimicrobial resistance in Danish pig farms. I was enrolled at National Veterinary Institute, Technical University of Denmark from December 2013 to January 2017 with a short leave of absence. My enrolment included a three month stay at University of Glasgow, United Kingdom that was possible thanks to Louise Mathews, thank you for that opportunity.

I would like to thank Danish Crown and Tican for helping me setting up the cross-sectional study that was the foundation for my analyses. Especially, I would like to thank Vagner Bøge, Marie G. B. Hansen (Danish Crown) and Henrik Bækstrøm (Tican) for your cooperation and kind help. I would also like to thank the abattoir technicians and Margarida Arede for helping with the sampling process and likewise the owners of the sow farms, who were willing to send me samples. I would also like to acknowledge the data providers (the Danish Classification Inspection and the Danish Veterinary and Food Administration), especially Jesper Larsen and Mette Ingvordsen who repeatedly helped me understand the data. Without data from the national registers this thesis would not have been the same.

I am very grateful for the help provided by my two supervisors, Nils Toft and Tariq Halasa, who always had the time to assist and support me throughout the past three years. I have enjoyed working with you and it has been a pleasure to have you as my supervisors. I have learned a lot from both of you. Thank you for inspiring me, sharing your knowledge, and your patience with my endless emails with questions and request for feedback. I am very grateful that you gave me the opportunity to be part of this interesting research project. You have given me the perfect combination of project management and opportunity to grow as an independent researcher.

I would also like to acknowledge the “Vetforlig II” project group. From DTU-food, I would like to especially thank Håkan Vigre and Vibe Dalhoff Andersen for good and fruitful discussions. Furthermore, thanks to Annette Ersbøll for introducing me to spatial analyses and for helping me through some of the first analyses of my PhD project.

Thanks to Julie Clasen for our many work and non-work related discussions. It has been a pleasure to have you for moral support. You were missed during your maternity leave and it was good to get you back.

Thanks to Anders Folkesson for extremely helpful feedback and for being my go-to-source for feedback on the microbiologically parts of my PhD.

Thanks to the VetStat study group (Amanda Brinch Kruse, Gitte Blach Nielsen, Leonardo de Knegt, Mette Fertner, Nana Dupont, and Vibe Dalhoff Andersen) for the feedback, R-codes for calculating antimicrobial consumptions, and sharing for your knowledge regarding pig production and the challenges working with VetStat.

To my former and present colleagues at the former Epi-group at DTU Vet thank you for creating a supporting, inspiring, fun, and unique work environment. I have enjoyed being part of the section during these past years. Vibeke Frøkjær Jensen, thank you for constructive feedback on the thesis.

Last but not least thanks to my loving family and friends. My husband (Steen) and kids (Lea and Alex) for being my all and everything. Thanks to my mom and dad for helping me with the kids and for always being there when I need you. Thanks to my mother and father in law for always taking care of my kids when needed so I could concentrate on my work.

SUMMARY

Antimicrobial resistance is a worldwide problem of paramount importance for both humans and animals. To combat the emergence of antimicrobial resistance, the problem must be targeted in all major reservoirs as it is assumed that a high level of AMR genes in environmental reservoirs can increase the risk of human pathogens becoming resistant. Pigs might constitute an important reservoir. Therefore, it is important to manage antimicrobial resistance in pigs. Before effective actions can be initiated, it is crucial to know which factors are associated with the levels of antimicrobial resistance in pigs.

The Danish pig farm is an ideal place to study AMR, as the pigs are all kept together in a confined space and managed in a similar manner. Furthermore, movements of pigs between farms and the purchase of antimicrobials for use at the farm are registered in national databases, thus facilitating the collection of information on relevant factors.

The aim of this PhD project was to study the relationship between the levels of antimicrobial resistance genes and three factors in Danish pig farms: the geographical location of the farm, the exposure to antimicrobials, and the trade patterns. Data collection was necessary in order to fulfil the aim of the project, and early in the project it became evident that a thorough consideration of how the data should be collected was needed. This resulted in three different projects, each contributing to the planning process.

Throughout the entire PhD project, the focus was on seven antimicrobial resistance genes - *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O), and *tet*(W). The levels of these genes were determined using a quantitative real-time polymerase chain reaction (qPCR). The *erm* genes confer resistance against macrolides, the *sul* genes confer resistance against sulfonamides, and the *tet* genes confer resistance against tetracyclines.

It was necessary to determine the minimum number of individual faecal samples that should be pooled together to represent the average level of antimicrobial resistance genes in the farm. It was estimated that a sample pooled from individual samples from five pigs was optimal. The pooling

method was optimised, and was found to have strong agreement with previously used pooling methods.

Samples were collected from 687 Danish pig farms in February and March 2015. However, 6 farms did not fulfil the criteria for the target population and were excluded. The samples were collected at five abattoirs located on Zealand and in Jutland. The sample material was faeces, and it was collected by squeezing a small amount out of the rectum at the slaughter line. After collection the samples were sent to the laboratory. The samples were pooled into one aliquot per farm and DNA was extracted from the faecal material. The antimicrobial resistance gene levels were then measured using quantitative real-time PCR.

As the samples were collected at the abattoir it was not possible to know in advance which farms were available for sampling. Consequently, it was not possible to estimate whether the sampled farms were representative of the target and study populations prior to sampling. However, an evaluation of the representativeness of sampled farms (in terms of farm size and geographical location) was carried out post-sampling. It was found that the sampled farms were larger than non-sampled farms. Furthermore, there was an undersampled area in the western part of Jutland and an oversampled area in the northern part of Jutland. A simulation study showed that the sampling procedure would inevitably result in a bias towards larger farms and with some non-randomness in the spatial distribution.

One of the aims of this PhD project was to estimate the quantitative relationship between the antimicrobial resistance gene levels and antimicrobial exposure. Previous studies have indicated that antimicrobial exposure in early periods of a pig's life can influence the antimicrobial resistance genes levels found later in life. In order to quantify the relationship, an estimate of the antimicrobial exposure throughout the entire lifetime of a slaughter pig was required. An algorithm to estimate the LEA was therefore developed.

Previous studies have shown that livestock farms in close proximity share common pathogens. Furthermore, there have been indications that phenotypic antimicrobial resistance can be spatially clustered. Therefore, the spatial pattern of the antimicrobial resistance gene levels was assessed, and both areas with high levels and areas with low levels of some of the genes were found. However, it was concluded that the geographical location of the farm had only a minor effect on the antimicrobial resistance gene levels.

Antimicrobial exposure is the most important risk factor for the development of antimicrobial resistance. However, previous studies of the relationship between antimicrobial resistance and antimicrobial exposure have focused on phenotypic antimicrobial resistance in a small number of farms. In this PhD project, the focus was on genotypic antimicrobial resistance. The quantitative relationship between the levels of seven antimicrobial resistance genes and the lifetime exposure of 11 different antimicrobial classes was estimated. It was found that some antimicrobial classes had a positive correlation with the levels of some antimicrobial resistance genes, but a negative correlation with other antimicrobial resistance genes. In conclusion, it was found that even though exposure to antimicrobials was associated with the antimicrobial resistance gene levels, it could only explain 10% - 42% of the variation in the gene levels.

The microflora of a pig is established soon after birth. The source of the microflora is bacteria in the environment and from the sow. It has been shown that antimicrobial exposure of the sow will influence the antimicrobial resistance levels in the piglet. Therefore, the correlation between the levels of antimicrobial resistance genes in finisher farms and that of the sow farms with which they had trade connections was assessed. A significant correlation was found for most of the antimicrobial resistance genes with correlation coefficients ranging from 0.06 to 0.47.

Of the three factors that were studied in this PhD project, only the antimicrobial exposure and the level of antimicrobial resistance in the sow farm with a trade connection were found to have a considerable effect on the antimicrobial resistance genes. However, it became apparent during the project that many other factors could affect the levels of antimicrobial resistance genes. This could lay the foundation for future studies.

SAMMENDRAG (DANISH SUMMARY)

Antibiotikaresistens er et globalt problem, der påvirker både mennesker og dyr. Mange projekter er derfor iværksat for at løse problemet. Antibiotikaresistens i grise udgør et reservoir for antibiotikaresistens i mennesker. Det er derfor vigtigt at reducere niveauerne af antibiotikaresistens i grise. For at dette skal kunne lade sig gøre må man vide hvilke faktorer, der påvirker niveauerne af antibiotikaresistensen i grise.

De danske svinebesætninger udgør en ideel forskningsenhed for studier i antibiotikaresistens og antibiotikaforbrug. Dette skyldes, at grisene går sammen i sektioner og bliver håndteret ens. Derudover bliver det registeret, når grisene flyttes eller der indkøbes antibiotika til brug i besætningen.

Formålet med dette ph.d.-projekt var at belyse, hvilke faktorer, der associeret med niveauerne af antibiotikaresistens i de danske svinebesætninger. Tre faktorers sammenhæng med antibiotikaresistens blev undersøgt. De tre faktorer var den geografiske placering af besætningen, livstidseksponeringen for antibiotika samt handelsmønstre. Det var nødvendigt at indsamle data for at undersøge de ønskede faktorer. Indsamlingen af data var kompliceret, da der var flere ukendte faktorer forud for indsamlingen herunder, hvor mange grise, der skulle indsamles prøver fra, hvorvidt stikprøven ville være repræsentativ for målpopulationen og hvordan antibiotikaeksponeringen skulle måles. Derfor blev der i forbindelse med dataindsamlingen lavet tre undersøgelser af, hvordan data bedst kunne indsamles.

I hele projektet blev der taget udgangspunkt i syv antibiotikaresistensgener – *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O) og *tet*(W). Niveauerne af disse gener blev målt med qPCR (quantitative polymerase chain reaction). De to *erm*-gener koder for resistens mod makrolider, de to *sul*-gener koder for resistens mod sulfonamide, og de tre *tet*-gener koder for resistens mod tetracyklin.

Det blev undersøgt, hvor mange grise, der skulle tages prøver fra for at få et mål for niveauet af antibiotikarestens, der var repræsentativt for besætningsniveauet. Konklusionen var, at prøver fra 5 grise var tilstrækkeligt. Derudover blev det undersøgt, om der var overensstemmelse mellem

gentagne målinger af flere trin i prøveanalysen. De trin, der blev undersøgt, var: 2 opløsninger af materiale fra samme prøve, 2 poolinger af samme opløste prøve og 2 kørsler af samme prøve på qPCR'en. Der var god overensstemmelse på alle tre trin. Endeligt blev der også fundet en poolingsmetode, der var hurtigere end den, der tidligere har været brugt, og med sammenlignelige resultater.

Der blev indsamlet prøver fra 687 svinebesætninger i et tværsnitsstudie i februar og marts 2015. Der måtte udgår 6 besætninger, da de ikke levede op til de krav, der blev stillet til stikprøven. Prøverne blev indsamlet på fem slagterier på Sjælland og i Jylland. Prøvematerialet var fæces, som blev indsamlet ved at klemme en lille mænge fra endetarmsåbningen ud i et tomt prøveglas. Efter endt prøveindsamling blev DNA'et ekstraheret fra prøverne, og niveauerne af de syv antibiotikaresistensgener blev målt med qPCR.

Eftersom prøverne var indsamlet på slagterierne, var det ikke muligt at vide på forhånd, hvilke besætninger, der kunne tages prøver fra. Det indebærer, at det ikke er muligt at undersøge forud for prøveudtagningen, hvorvidt besætningerne, der indgik i stikprøven, var repræsentative for målpopulationen og studiepopulationen. Derfor blev det testet, om besætningerne var repræsentative mht. geografisk placering og størrelse. Besætningerne, der indgik i stikprøven, var større end de besætninger, der ikke indgik i stikprøven. Derudover blev det fundet, at der var et område i Vestjylland, hvor besætningerne i stikprøven var underrepræsenterede, og at der var et område i Nordjylland, hvor besætningerne i stikprøven var overrepræsenterede. Et simuleringsstudie viste, at det ikke er muligt at opnå en stikprøve, der vil være fuldstændig tilfældigt fordelt i landet, samt at det var uundgåeligt, at besætningerne i stikprøven ville være større end de, der ikke indgik i stikprøven.

Da det var et af formålene med dette ph.d.-studie at undersøge sammenhængen mellem antibiotikaeksponering og antibiotikaresistens, var der brug for et godt estimat for antibiotikaforbruget. Dette estimat skulle afspejle antibiotikaeksponeringen gennem hele grisens liv, da tidligere studier har indikereret, at dette er vigtigt. Derfor blev der konstrueret en algoritme, der estimerer livstidseksponeringen for antibiotika.

Det har tidligere været vist, at besætninger med husdyr, der ligger geografisk tæt på hinanden, har sammenlignelige niveauer af bakterier. Derudover har det også været vist, at visse former for fænotypisk antibiotikaresistens er blevet fundet i højere niveauer i visse geografiske områder.

Derfor blev der i dette ph.d.-projekt undersøgt, hvorvidt der var områder i Danmark, hvor niveauerne af de specifikke antibiotikaresistensgener var signifikant højere eller lavere end i resten af landet. Der blev fundet enkelte områder med højere niveauer af antibiotikaresistens, ligesom der blev fundet enkelt områder med lavere niveauer. På trods af dette blev det konkluderet, at den geografiske placering af en svinebesætning ikke er af afgørende betydning for niveauet af antibiotikaresistens i besætningen.

Antibiotikaeksponering er kendt for at være den vigtigste risikofaktor for forekomsten af antibiotikaresistens. Tidligere studier har primært fokuseret på at undersøge sammenhængen mellem antibiotikaeksponering og fænotypisk antibiotikaresistens i en lille stikprøve. Der blev i dette ph.d.-projekt undersøgt den kvantitative sammenhæng mellem eksponering af 11 forskellige antibiotikaklasser og niveauerne af de syv antibiotikaresistensgener. Nogle antibiotikaklasser var positiv korreleret med niveauerne af generne, mens andre var negativt korreleret. Selvom antibiotikaeksponering havde en effekt på niveauerne af antibiotikaresistensgenerne, forklarede det kun 10-42 % af variationen i niveauerne.

Mikrofloraren i mave-tarmsystemet dannes kort tid efter, at en gris bliver født. De bakterier, der er med til at danne denne mikroflora, kommer fra grisens omgivelser inklusiv soen. Der har været studier, der har vist, at niveauet af antibiotikaresistens i en pattegris er påvirket af den mængde antibiotika, som soen har indtaget. Derfor var det interessant at undersøge, hvorvidt niveauet af antibiotikaresistensgenerne i slagtesvin var korreleret med niveauet af antibiotikaresistensgener, der kunne findes i den sobesætning, hvor slagtesvinene var født. Der blev fundet signifikante korrelationer mellem niveauerne af visse antibiotikaresistensgener i slagtesvinebesætninger og i de sobesætninger, hvor slagtesvinene var født. Korrelationskoefficienterne for sammenhængen var mellem 0,06 og 0,47.

Af de tre faktorer, der blev undersøgt i dette ph.d.-studie, var det kun soens niveau af antibiotikaresistensgener og antibiotikaeksponeringen, der havde betydning for niveauet af antibiotikaresistens hos slagtesvinene. En stor del af variationen i resistensgenerne kan ikke forklares med de faktorer, der blev undersøgt i dette studie. Der er derfor meget at tage fat på i fremtidige studier.

LIST OF ABBREVIATIONS

AMR: Antimicrobial resistance

CHR: Central Husbandry Register

C_q: Cycle of quantification

E. coli: *Escherichia coli*

LEA: Lifetime Exposure to Antimicrobials

PBS: Phosphate buffer solution

PMD: Database for pig movements

qPCR: Quantitative real-time Polymerase Chain Reactions

RQ: Relative quantification

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APPENDIX 159

1. INTRODUCTION

Treatment of bacterial diseases with antimicrobial agents started out as a miracle cure. However, it soon became evident that there was a limit to the miracle as antimicrobial resistance (AMR) emerged. The efficacy of antimicrobials is essential for treating bacterial diseases, and relies on the susceptibility of the pathogen. However, AMR is an inevitable consequence of antimicrobial use, and occurs when bacteria become resistant to an antimicrobial agent due to AMR genes harboured in the bacteria[1]. It is assumed that a high level of AMR genes in environmental reservoirs can increase the risk of human pathogens becoming resistant. Therefore, pigs might constitute an important reservoir[2].

Choosing an efficient antimicrobial therapy protocol has become more challenging for a wide range of bacterial infections in recent times, as available options become more and more scarce. There is a societal interest in solving this problem, and various action plans have been implemented. For example, the White House National Action Plan for Combating Antibiotic-resistant Bacteria set five goals, including: slowing the emergence of AMR, surveillance of AMR from a One-Health perspective, and developing new antimicrobial agents[3]. In order to reduce the levels of AMR, it is essential to identify the factors that are associated with these levels. The main aim of this PhD thesis was to provide new knowledge about factors that may be associated with the occurrence of AMR in the Danish pig production. The studies described in this thesis were carried out from a One-Health perspective, focusing on selected AMR genes in Danish pig production that are also found in humans. AMR has the potential to be transferred from pigs to humans, as seen with livestock-associated methicillin resistant staphylococcus aureus (LA-MRSA)[4]. Furthermore, it is generally accepted that it is possible to transfer AMR genes from pigs to humans through faecal contamination of the carcass[5]. In addition, pigs can act as a model for humans. It is often easier to study AMR in pigs than in humans as pigs live in controlled environments.

Only selected AMR genes in faeces from slaughtered pigs are considered in this thesis. Faeces are considered to be a large reservoir for both commensals and pathogens and hence also for AMR

genes[6,7]. So it seems logical to consider AMR genes in porcine faeces at the time of slaughter when investigating the factors affecting AMR from a One-Health perspective.

A cross-sectional study design with sampling at the abattoir was used to collect the samples that were used in this study. This resulted in a much larger number of sampled farms than what would have been feasible through farm visits.

The goal of this thesis was to acquire knowledge about the association between AMR genes and the following three factors:

1) Geographical location of pig farms
2) Exposure to antimicrobials
3) Trade patterns

However, to meet the goal there was a need for a thorough consideration of how the data should be collected (Objective 1). This resulted in three sub-projects (Manuscripts 1-3, Fig. 1.1). To achieve the goals of the thesis, four main objectives were defined and described in the following sections.

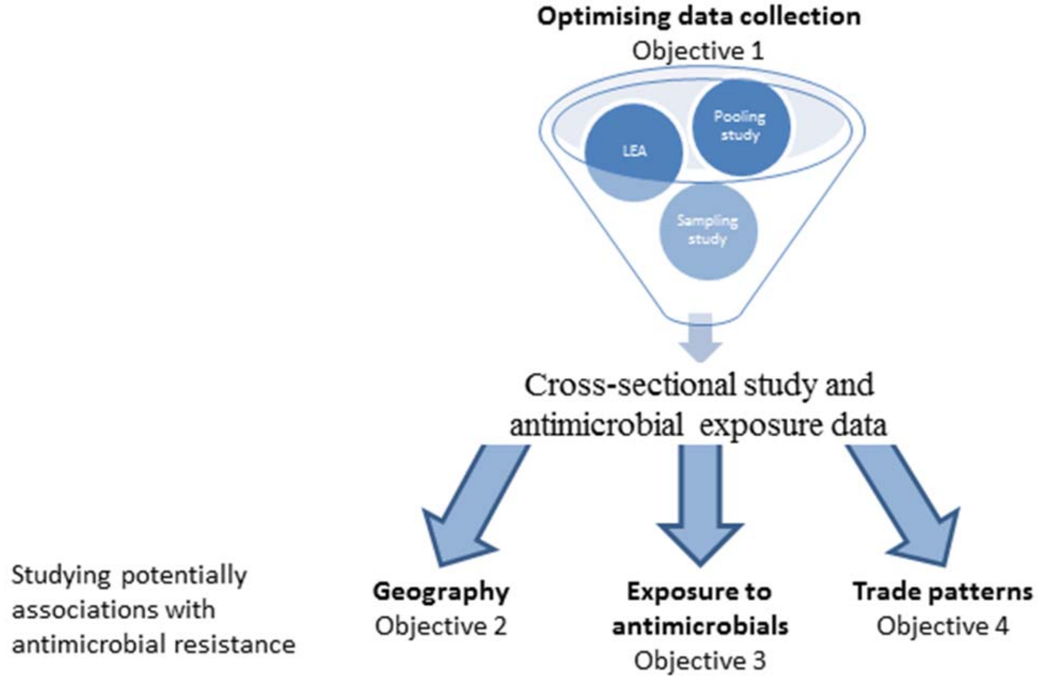

Figure 1.1 The objectives of the PhD study. LEA: lifetime exposure to antimicrobials.

1.1 Objective 1 – optimizing the data collection

The first objective was to estimate how data for studying the factors affecting AMR in pigs should be collected, given the restrictions in terms of time and budget. To achieve this objective, three questions had to be answered:

1.1.1 How many pigs should be sampled to obtain an estimate of the farm level of antimicrobial resistance genes?

The minimum number of individual faecal samples to pool together to obtain a representative sample for the farm-level quantification of AMR genes in a Danish pig farm was assessed (Manuscript 1[8], the "pooling study", Fig.1.1). Further goals of the pooling study were to test the agreement among several runs of the qPCR assay used in Manuscripts 4-6[9–11], and to assess whether a less time-consuming pooling method could be adopted.

1.1.2 Were the sampled farms representative of the target population?

Sampling at the abattoir was the chosen sampling procedure, because the strict biosecurity measures in pig farms in Denmark[12] make it difficult to sample a large number of farms in a short time span. Collecting faecal samples at the abattoir is cheaper than farm visits, and allows sampling from

slaughtered pigs that are consequently not harmed. The cross-sectional study design is one of the preferred methods of studying the status of a population, as it is relatively inexpensive, it can provide a snapshot of a large sample size, and it makes it possible to evaluate associations[13].

The sampling procedure was evaluated to assess whether the sampled farms were representative of the target population and whether selection bias could have been introduced (Manuscript 2[14], the "sample study", Fig.1.1). To do so a method for planning, conducting, and evaluating a cross-sectional study, where simple random sampling is impossible, was described. Hereunder, it was evaluated if the study criteria have been fulfilled for the obtained sample. This approach was exemplified using the cross-sectional study of pig farms sampled at the abattoir described in Manuscript 4-6[9–11].

1.1.3 How can exposure to antimicrobials be estimated?

An algorithm to estimate the lifetime exposure to antimicrobials (LEA) was developed (Manuscript 3[15], "LEA" in Fig.1.1). Reliable information on the consumption of antimicrobials is essential to understanding their association with the levels of AMR genes. Based on the relatively short lifetime of slaughter pigs (5-6 months in Denmark), it is assumed that the exposure to antimicrobials during the entire lifetime of a pig could influence the levels of AMR genes found at the date of slaughter. Several studies have shown that AMR at one time point is affected by antimicrobial exposure in earlier periods[16–18]. Andersen et al.[19] showed that the association between antimicrobial use and AMR is most efficiently estimated when the LEA of a batch of finishers is taken into account.

An estimate for the LEA must reflect the actual use during the lifetime of the pigs. This reflection can be difficult to obtain in Danish pig production where at least 50 % of slaughter pigs will be moved from one farm to another at some point in time and purchases of antimicrobial to use at the farm is reported at farm level. Furthermore, antimicrobial treatment strategies vary across rearing periods. In the majority of farms, the highest amounts of antimicrobials are used during the weaning period[20].

The objective of the Manuscript 3[15] was to develop and optimise an algorithm designed to implement the majority of slaughter pigs in Denmark in the LEA approach presented by Andersen et al[19]. The purpose of this implementation was to propose a method that translates data on purchase

of antimicrobials (register data) into antimicrobial exposure data. The word exposure is deliberately used here to take into account not only the direct consumption by the individual pigs but also the indirect exposure by excretion of antimicrobial residues and AMR genes from other pigs in the farm. This enabled the estimation of the antimicrobial exposure without the need to obtain the actual antimicrobial usage records at the farms.

1.2 Objective 2 – The association with the geographical location of pig farms

A single veterinarian practitioner or veterinary group will often visit pig farms located in spatial proximity to one another, and may use the same treatment protocols in all of their affiliated pig farms. Therefore, antimicrobial exposure might be similar for these farms. Furthermore, the farms may share common pathogens[21–25] and may be exposed to the same environmental risk factors, such as residual antimicrobials and AMR genes from the soil. Consequently, pig farms in close spatial proximity could potentially have similar AMR gene levels. Therefore, the second objective of the PhD study was to test if the selected AMR genes were spatially randomly distributed in Danish pig farms. This was done by describing the spatial patterns of the AMR genes and to test whether there was a random spatial distribution of the AMR genes or if spatial clustering could be detected (Manuscript 4[9]).

1.3 Objective 3 – The association with exposure to antimicrobials

Antimicrobial consumption is the most important risk factor for the development of AMR[7]. Previous studies have primarily focused on AMR in specific bacterial species, in particular *Escherichia coli* (*E. coli*) and *Enterococcus spp.*[16–18,26–29], yet this might underestimate the total level of AMR. Ever since DNA-based methods for assessing AMR were developed, it has been possible to consider AMR genes present in total bacterial community DNA, and this is the approach employed in this thesis.

The third objective of the PhD study was to quantify the relationship between the lifetime exposure to 11 different classes of antimicrobials and the level of the seven AMR genes in the faecal microflora (Manuscript 5[10]).

1.4 Objective 4 – The association with trade patterns

In this thesis, trade patterns are defined as the patterns of how finishers are moved throughout their life. The finishers could be moved internally, i.e. within the same farm to a new section, or externally, i.e. to a new farm. The environment would consequently change for the finishers regardless the move. The trade patterns might influence the AMR gene levels found in finishers at the time of slaughter. Many pigs in Demark are moved from their place of birth, (i.e. traded), and this often occurs early in the pig's life. The porcine intestine is bacteria-free prior to birth, and the normal flora is established during the delivery process and shortly after birth. The source is bacteria found in the surrounding environment, e.g. the normal flora of the sow and the surroundings where the piglet was housed at birth[30–32]. Furthermore, antimicrobials consumed by the sow have been shown to affect AMR levels in their offspring[33,34]. Therefore, AMR can be transferred from sows to piglets that go on to become slaughter pigs.

The fourth objective of the PhD study was to describe the relationship between AMR gene levels found in finishers at slaughter, and those found in the sow farms where the finishers were found (Manuscript 6[11]).

2. ANTIMICROBIAL RESISTANCE AND CONSUMPTION

The term AMR refers to resistance in microorganisms to anti-bacterial, anti-viral, and other medical agents used to treat diseases caused by pathogenic microorganisms. However, most of the public focus has been on AMR in bacteria[35], which is the only type of AMR considered in this thesis. Furthermore, the term resistant bacteria are here used to describe bacteria resistant to antimicrobial agents.

AMR is a naturally occurring phenomenon and has even been found in environments never exposed to anthropogenic use of antimicrobials[36]. When antimicrobial exposure is present to a bacterial population there is a risk that it will select for AMR in both pathogenic and commensal bacteria[1]. AMR can be either a natural property of the bacteria, i.e. the wild type harbour the AMR genes, or a secondarily acquired mechanism, i.e. mutations or transferral of AMR genes from the external (e.g. the surroundings) or internal (e.g. commensals inside the host) environment[37–41]. Furthermore, certain strains of bacteria produce antimicrobials that can exerts elective pressure on neighbouring organisms[42,43], thus contributing to the selection of AMR.

There are numerous definitions of AMR based on different criteria that do not necessarily perfectly overlap, such as genetic, biochemical, microbiological, and clinical criteria[37,44–46]. The two most commonly used definitions are based on microbiological and clinical criteria[44]. However, in this thesis, AMR is based on genetic criteria, i.e. the AMR gene levels.

The transfer of AMR genes between different bacterial species is known to occur in the natural environment, and it has also been shown that exchange can occur between cells of different species and between bacteria from diverse ecological niches[47]. All AMR genes therefore represent a potential hazard. The intestines constitute a huge reservoir of normal flora bacteria[48] and AMR genes. Faecal bacteria are therefore good indicators of the AMR levels in a host[6,7]. For this reason, AMR genes in faecal samples have been analysed in this PhD project.

2.1 The relevance to humans of antimicrobial resistance in pigs

The Swann Report was published in 1969[49] by a committee appointed by the UK government, warning that use of antimicrobials in livestock could impair their efficacy in humans through the development of AMR. This was supported by several authors of peer-reviewed articles in the late 1960s, who also raised concerns that AMR could be transferred from livestock to humans via the food chain[50,51]. It is now generally accepted that AMR bacteria can be transferred from animals to humans through meat consumption[5,52–54]. In addition, AMR can also be transferred to humans through direct contact with pigs[54–56], and the use of pig manure as fertiliser [54,57,58]. The relative importance of transmission through meat compared to other transmission routes is not known[59]. However, the direction of the link between the prevalence of AMR in animals and the prevalence of AMR in humans has been questioned[60].

2.2 Surveillance of AMR and antimicrobial consumption

National AMR surveillance is mandatory for members of the European Union[61]. In Denmark, the surveillance program is called The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP)[20]. It is a close collaboration between veterinary, food, and human health institutes and was established in 1995. The annual DANMAP report targets antimicrobial consumption in humans and animals, as well as the occurrence of AMR in livestock, food products of animal origin, and humans[20].

There has been several initiatives to reduce the antimicrobial consumption in Danish pig farms, including seizing the use of antimicrobial growth promoters in 1995-1999 and the Danish action plan for reducing the use of antimicrobials in food animals in 2005[20]. An ongoing initiative is the yellow card initiative that was implemented in July 2010 by the Danish Veterinary and Food Administration. The initiative is based on threshold levels for antimicrobial consumption calculated as a running nine-month average. There are separate threshold values for weaners, finishers, and sows (including piglets, gilts and boars). If one of the threshold values is exceeded, the farmer receives a yellow card and can expect increased supervision by the veterinary authorities together with a potential fine if the antimicrobial consumption is not lowered within nine months[62].

2.3 Detecting antimicrobial resistance

Traditionally, studies focusing on the quantification of AMR have used culture-based methods and assessed phenotypic AMR in indicator bacteria such as *E. coli* or *Enterococcus spp*.[20]. This is because they are part of the normal intestinal flora enabling continuous monitoring and comparison between populations. Furthermore, they are commonly present on raw meat, making them a good indicator for the risk of AMR transfer from pigs to humans[20,53]. Culture-based methods involve isolating the bacteria on general or selective media, followed by assessment of their growth in presence of antimicrobials. However, most culture-based testing approaches are both labour intensive and economically expensive[46]. Furthermore, the development of DNA-based methods has shown that the complexity of microbial communities is much greater than previously thought, and that many currently uncultivable bacteria are present in the intestinal microflora[42,48].

There are more than 400 different bacterial species in the mammalian gut, and *E. coli* constitutes less than 1% of these[63]. Furthermore, there is a large variation in the abundance of *E. coli* among faecal samples from pigs[64]. The overall AMR level in faecal samples might therefore be underestimated when only specific indicator bacteria are used to assess AMR. DNA-based methods make it possible to quantify the AMR gene levels in total community DNA. Genotypic determination of AMR has the advantage that the level of AMR of practically all bacteria can be assessed, given that the genotype is known. Furthermore, extracted DNA can be stored for later use[48,65]. However, it is not possible to determine whether or not the AMR genes identified using DNA-based methods are expressed. The silent (non-expressed) genes do not contribute to phenotypic AMR, and are therefore without clinical importance in the silent state. Furthermore, currently unknown AMR genes are not detected, while the resulting phenotypic AMR would be.

Although various DNA-based methods for quantification of AMR exist, the method chosen for this study was qPCR.

2.3.1 Quantitative Real-Time Polymerase Chain Reaction

Since the commercial introduction of qPCR, the technique has become popular due to its quantitative precision, low contamination risk, high sensitivity, broad dynamic range, and relatively low cost[66,67]. High-throughput qPCR systems make it possible to detect and quantify AMR genes within the total community DNA of complex samples such as faeces[66,68], even when the sample size

is large. It has also been shown that the quantitative level of AMR obtained by qPCR is representative of the general AMR level[68].

In brief, qPCR is used to measure the expression of a set of target genes in a given sample through repeated cycles of sequence-specific DNA amplification, followed by expression measurements. Between cycles, the amount of each target transcript approximately doubles during the exponential phase of amplification. The cycle at which the observed expression first exceeds a fixed threshold is commonly called the quantification cycle (C_q). The C_q values represent a quantitative assessment of gene expression. The resulting relative quantitative (RQ) values of the gene levels must be assessed in relation to the total amount of bacterial DNA present in the sample, due to a large variation in the abundance of the bacteria present in faeces[30]. This RQ is based on the expression levels of a target gene versus a reference gene measuring the total amount of bacterial DNA. This allows the gene values to be compared across multiple samples. There are several possible methods to determine the RQ value[69]. In this thesis, the RQ value was calculated using a modified Livak-method[70], taking into account the effectiveness of the genes and inter-plate correctors with the use of 16S as reference gene.

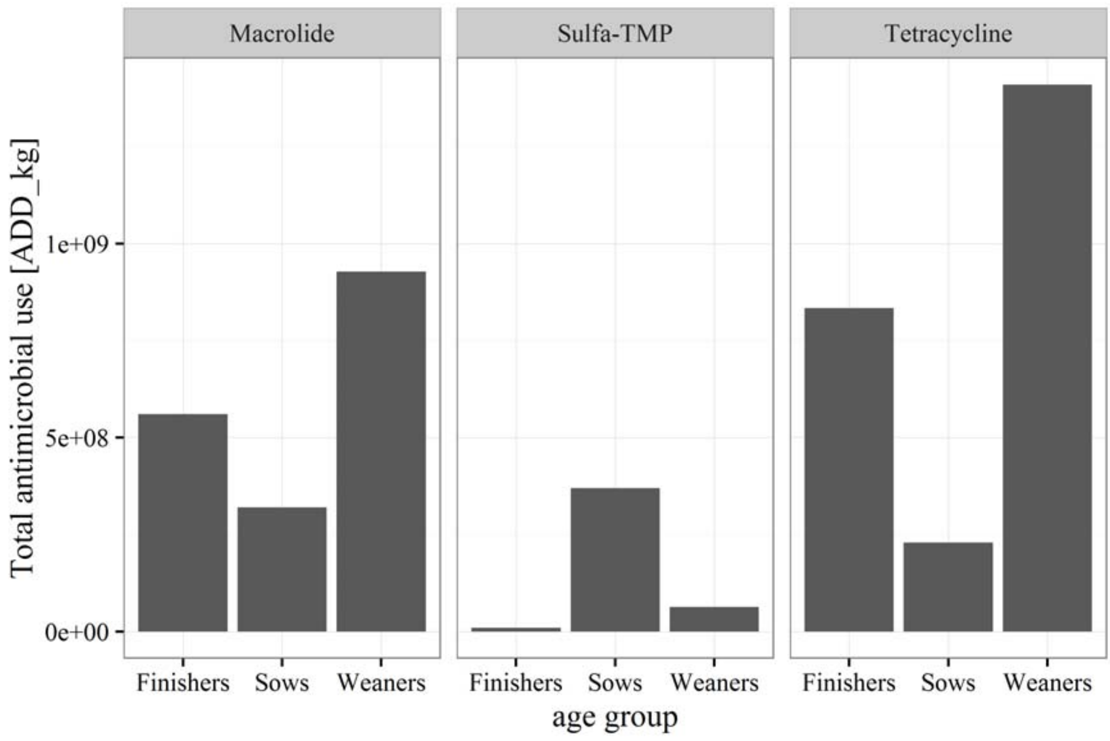

Figure 2.1: The total macrolide, sulfonamide, tetracycline consumption by all pig farms in Denmark for sows (including piglets), weaners, and finishers respectively. The figure is based on VetStat data on purchases of antimicrobials at the pharmacy in the period from 1st of May 2014 to 1st of July 2015.

2.3.2 Antimicrobial resistance genes included

This thesis focuses on seven AMR genes: *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O), and *tet*(W). These genes encode AMR against macrolides (*erm* genes), sulfonamides (*sul* genes), and tetracyclines (*tet* genes). The three sets of AMR genes bestow resistance against the three most commonly used antimicrobial agents in Danish pig production in different rearing periods (Fig 2.1). The seven AMR genes were chosen because an assay had been validated for these specific genes[68]. The inclusion criteria for the genes in the validation were: the genes confer resistance towards the antimicrobial classes used in the Danish pig production, the genes are widespread in total community DNA from porcine faeces, and the possibility of designing a qPCR assay for the chosen genes utilizing the same temperature profile[68].

The first macrolide antimicrobial intended for use in livestock was introduced in the early 1960s. In the Danish pig production, macrolides are most used to treat weaners. They are the second most used antimicrobial class in weaners and the third or fourth in finishers and sows[20]. The abbreviation

erm stands for erythromycin ribosome methylation which relates to their function. Ribosome methylation modify the ribosome thus hindering the binding of the macrolide peptides[59] The *erm* genes have been isolated from a variety of gram-negative bacterial species, gram-positive bacterial species, and even from spirochetes. The *erm* genes are found both as chromosomal genes and on plasmids[71]. Around twenty different *erm* genes have been identified and there exists even more genes encoding for macrolide resistance[71].

Sulfonamides were the first antimicrobial to be use systemically that act very selectively on bacteria[72]. However, history shows that sulfonamide resistance is quick to emerge – for example, when sulphonamides were first introduced in 1937, they were highly effective against gonorrhoea, yet some years later they became almost completely ineffective[73]. In Danish pig production, sulfonamides are primarily used to treat sows in combination with trimethoprim (sulfa-TMP)[20]. Sulfa-TMP is the second most used antimicrobial class in sows in Denmark, but it is seldom used in weaners and almost never used in finishers[20]. The *sulI* and *sulII* genes are two plasmid-encoded AMR genes[74]. Sulfonamides inhibit the folic acid synthesis which is a vital compound in the bacterial synthesis. Sulfonamide resistance alter the binding site for sulfonamide. Thereby, the sulfonamide cannot act on the bacteria and the folic synthesis continues[74]. To my knowledge only three sul genes has been identified (*sulI*, *sulII* and *sulIII*)[75].

Tetracyclines were discovered in the late 1940s[76], and are now used extensively in pig production. In Denmark, they are by far the most antimicrobial class used in pig production[20]. Tetracycline is known to easily select for resistance[77]. The *tet* genes are generally found in either gram-negative or gram-positive bacteria. Most of the *tet* genes have been connected to transferable elements, and more than 40 different *tet* genes have been identified[77]. There is a high background level of tetracycline resistance caused by its extensive use in pig production since the 1950s[78] and tetracycline was rarely found before the that timepoint[79].

All seven genes pose a potential zoonotic hazard as they have all been reported in bacteria known to cause food poisoning. Hereunder, the *sulI* and *sulII* genes have frequently been isolated from *Salmonella* ssp[80,81], and the *ermB*, *ermF*, *tet*(M), *tet*(O) *tet*(W), and *ermB* genes have been isolated from *Campylobacter* spp. originating from pigs[71,82,83]. Resistant *Salmonella* and *Campylobacter* spp. have been associated with more severe infections compared to infection with susceptible strains[5]. Furthermore, all genes have been reported in *E. coli* or *Enterococcus spp*. which are, as

previously mentioned, commonly present on raw meat and represent a hazard of transfer of AMR from pigs to humans[20,53]. From these bacteria there is a possibility for transfer of AMR genes to pathogenic bacteria as all seven AMR genes have been found on mobile elements. The *sulI* and *sulII* genes have been found in *E. coli* from samples obtained from porcine faeces, pork, and humans [84–86]. The *ermB*, *tet*(M), *tet*(O), and *tet*(W) genes have been found in *Enterococcus* spp. on porcine carcasses and/or in pork[87–89]. Furthermore, a study has shown that *ermF* genes can be transferred to bacteria of different species including *Enterococcus faecalis*[90].

2.4 Factors influencing antimicrobial resistance in pig farms

Antimicrobial consumption is the most important risk factor for the development of AMR[7]. The association between tetracycline consumption and the occurrence of phenotypic tetracycline resistance in pigs in specific bacteria (*Salmonella* spp.[91], *E. coli*[17,26–28] and lacto-positive enteric coliforms[92]) is supported by numerous studies. However, high levels of phenotypic tetracycline resistance have also been found in farms that do not use antimicrobials[18,26,93], and equal levels of phenotypic tetracycline resistance have been found in farms that used tetracycline and farms that did not[27]. Furthermore, there seems to be no clear association between the reported dosage and AMR towards tetracycline[94]. At gene level, *tet*(M)[28,95,96], *tet*(O)[95,96], and *tet*(W)[96] have frequently been detected in pigs that are not directly exposed to antimicrobials. It is likely that this is due to a high background level of tetracycline resistance[78], as previously stated. It has been found that the macrolide resistance genes *ermB*[78,95,97] and *ermF*[97] have frequently been detected in pigs that are not directly exposed to antimicrobials. Other studies have indicated a high incidence of erythromycin resistant *Enterococcus* spp. in pig farms that used macrolides as a growth promoter[98,99]. Macrolide exposure has furthermore been shown to increase the incidence of *ermF*[95] detection, and has been associated with an increased risk of detecting phenotypic sulfonamide resistance[17]. To my knowledge, the effect of sulfonamide usage on sulfonamide resistance has not been extensively studied in pigs. However, a significant positive association between sulfonamide consumption and phenotypic sulfonamide resistance has been found in veal calves[100].

Factors other than antimicrobial exposure have also been known to affect AMR levels. Non-antimicrobial risk factors include: temperature in the pen[101–103], the number of pigs housed on the premises[101,104], feeding strategies[104], pen hygiene[92], production system[104], movement of the pigs[92], age[93,102] and season[105]. Furthermore, metals can co- or cross-select for AMR[38,106].

3. PIG PRODUCTION IN DENMARK

In 2015, almost 19 million pigs were slaughtered in Denmark. In addition, 12 million pigs were exported, 95% of which were exported at approximately 30 kg[107–109]. Denmark is one of the largest producers and exporters of live pigs in the world, and pig production has a considerable impact on the national economy[110]. The success of the Danish pig industry is partly due to a world-class breeding strategy, meat quality, food safety, and traceability[109,111,112]. Another explanation can be found in the coordination and cooperation between partners throughout the production chain[109,112], with most pigs in Denmark being slaughtered through farmer-owned cooperatives[109].

There are many different production systems for raising slaughter pigs in Denmark. These belong to three major categories: organic, free range, and non-organic indoor pig production. The latter is commonly referred to as "conventional pig production" and constitutes 99.3% of the total production of slaughter pigs in Denmark based on data from July 2014 to March 2015. Farm management, including space requirements, feeding practices, weaning age, and legislative regulations, differs considerably among the three major production categories[113]. Within the conventional farms there are different specialised production systems, both in relation to specific farm management and the type of pork produced.

In addition to farms with slaughter pig production, there are also different types of breeding farms. The Danish pig industry is organised in a strict pyramidal structure, with purebred farms at the top, multiplying farms in the middle, and production farms at the bottom.

Only conventional farms were considered in this thesis. The publicly available registers do not include information on any specialised farm management practices (e.g. multisite operations and wean-to-finish stables) or meat production (e.g. heavy pigs for the German market and specialized pig produced to the British market) at the individual farms, and their effect was therefore not considered.

Movements of pigs between farms producing slaughter pigs often occurs within a short distance[114]. Approximately 80% of pig-producing farms purchase weaners from only one sow farm[12], and most

trade takes place with fixed delivery contracts[115]. In Denmark, all pigs must be labelled with an approved ear tag when they are moved. However, the following exceptions to this rule exist: pigs that are tattooed on the ham and transported directly to an abattoir; pigs that are transported directly for destruction; batches of pigs that are transported to farms with the same owner (e.g. in the multi-site operations), and batches of weaners that are transported on the basis of a fixed delivery contracts[116]. Consequently, younger pigs, up to slaughter weight, are rarely ear tagged.

Pigs raised for pork production in conventional farms are often housed in large groups with high animal densities and with a minimum requirement of space ranging from 0.4 m^2 to 1 m^2 per pig weighing from 30 kg to 110 kg[117]. In Denmark, finisher pigs are often housed in sections of around 10-20 pens with 15-20 pigs per pen. Advances in the Danish breeding stock have led to a rapidly growing slaughter pig that is fed a specialised diet to enhance growth. These practices result in slaughter weight being reached long before physical maturity. There is a high chance of disease occurrence due to the young age of the pigs, stress from frequent movements, and mixing pigs from different litters. Rapidly initialised antimicrobial treatment of clinical symptoms, and metaphylactic treatment of pigs are common practice in Danish pig production[118], with the result that most pigs are treated at least once during their lifetime. However, the organised structure of Danish pig production facilitates the monitoring of antimicrobial usage[111].

In this thesis, a slaughter pig is defined as a pig produced for slaughter, weighing approximately 100 kg. Weights during other production periods are: 1) piglet: birth - 7 kg, 2) weaner: 7 kg - 30 kg, and 3) finisher: 30 kg - slaughter.

3.1 Register data

Pig production in Denmark is tightly regulated by ministerial orders, including environmental legislation dictating how many pigs may be kept at the farm given the size of the land owned by the farmer[119]. Furthermore, all farms with pigs must be registered in the Central Husbandry Register (CHR), all movement of pigs must be registered in the database for pig movements (PMD), and all purchases of medical products, vaccines, and some minerals to use in veterinary medicine must be registered in VetStat[116,120,121]. Since July 2010, it has been mandatory for farmers with more than 300 sows, gilts and boars, 3,000 finisher pigs, and/or 6,000 weaners to have a veterinary advisory service contract. However, most Danish commercial pig farms have a contract regardless of their

size. This arrangement includes mandatory visits by the veterinarian at intervals dictated by the agreement and by the farm type[122].

In this thesis, data from three nationwide databases owned and maintained by the Danish Veterinary and Food Administrations are used. The three registers are: the CHR on farm demographics, the PMD on movements of pigs, and the VetStat on antimicrobial purchases. It is mandatory to register the relevant information in each of the databases.

3.1.1 Central Husbandry Register (CHR)

All livestock farms in Denmark are registered in the CHR with a unique farm number (the CHR number) for identification. The CHR number is the key ID used in all national livestock databases.

The CHR contains, among others, information on the number of pigs in three age groups: sows (all adult breeding pigs), weaners (7 kg - 30 kg), and finishers (> 30 kg, but not adult breeding animals) on a normal production day, as well as the production type. All holdings that deal with pigs (e.g. abattoirs, rendering plants, cooling facilities for dead pigs, production farms, boar stations, breeding farms, and export facilities) are registered in the CHR. The "production type" can be used to distinguish between the different types of holdings. The CHR also contains information about the owner and manager of the farm.

Once a year, all farmers are asked to confirm or correct the registered information in the CHR database. However, owners of larger farms (more than 300 sows, gilts and boars, 3,000 finisher pigs, and/or 6,000 weaners) must update the CHR twice a year. In addition, a newly established pig farm, or the addition of a new age group must be registered within 7 days, and farm closures must be registered no later than 6 months after the last pig is moved from the premises. It is the responsibility of the farm owner to register the farm and keep the records up-to-date. Farmers who do not follow the regulations may face legal action[120].

3.1.2 Database for pig movements (PMD)

Since 2002, movements of pigs have been recorded in the PMD. The PMD is structurally a subset of the CHR.

The PMD contains information about movements of pigs within Denmark, as well as export to foreign countries. Information includes the CHR-number of both the sending and receiving

holdings, the date of movement, and the number of pigs moved. The register also contains information on the number of dead finishers and dead sows moved together with the number of containers of dead weaners or piglets moved registered by the rendering plant.

The recipient of the pigs is required to register the movement within 7 days. However, when pigs are exported, it is mandatory for the sender to register the movement, still within 7 days[116].

3.1.3 VetStat

Since 2000, information about all purchased drugs prescribed by a veterinarian to be administered to pigs has been collected in the central database, VetStat. The data originate from: (i) pharmacies, which are obliged to report all sales of drugs for veterinary use, (ii) veterinarians, who are obliged to report their own administration of drugs to livestock, and (iii) feed mills, which are obliged to report all sales of medicated feed[123]. In Denmark, antimicrobials for use in animals can only be purchased with a valid prescription from a veterinarian[124]. Therefore, records of all purchases of antimicrobials in Denmark for use in pig farms should be in the VetStat data.

The information available in VetStat includes among others: the purchase date, the CHR number of recipient farm, the animal species code, the age group code, the product ID, the amount of product, and other information about the product such as the Anatomical Therapeutic Chemical classification code[125] for veterinary drugs. Only antimicrobial usage registered by pharmacies is included in this thesis, as this accounts for 99.9% of all antimicrobial purchases for use in pig farms[126].

4. MANUSCRIPTS 1-6

1. Julie Clasen, Anders Mellerup, John Elmerdahl Olsen, Øystein Angen, Anders Folkesson, Tariq Halasa, Nils Toft and **Anna Camilla Birkegård**.
   Determining the optimal number of individual samples to pool for quantification of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR
   Veterinary Microbiology. 2016;189:46–51, doi: 10.1016/j.vetmic.2016.04.017.

2. **Anna Camilla Birkegård**, Tariq Halasa, and Nils Toft.
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4. **Anna Camilla Birkegård,** Annette Kjær Ersbøll, Tariq Halasa, Julie Clasen, Anders Folkesson, Håkan Vigre, snd Nils Toft.
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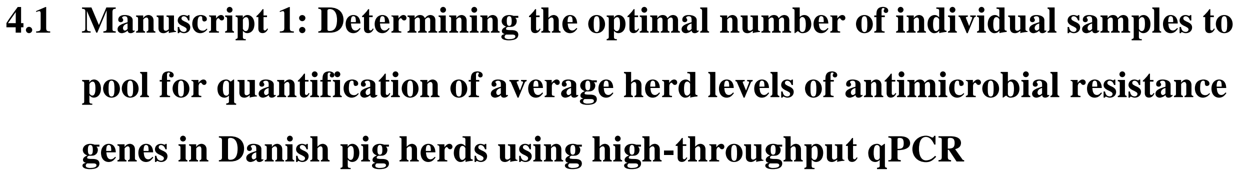

4.1 Manuscript 1: Determining the optimal number of individual samples to pool for quantification of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR

Julie Clasen, Anders Mellerup, John Elmerdahl Olsen, Øystein Angen, Anders Folkesson, Tariq Halasa, Nils Toft and **Anna Camilla Birkegård**

Veterinary Microbiology. 2016;189:46–51, doi: 10.1016/j.vetmic.2016.04.01

Veterinary Microbiology 189 (2016) 46–51

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Short communication

Determining the optimal number of individual samples to pool for quantification of average herd levels of antimicrobial resistance genes in Danish pig herds using high-throughput qPCR

Julie Clasen[a], Anders Mellerup[a], John Elmerdahl Olsen[b], Øystein Angen[c], Anders Folkesson[a], Tariq Halasa[a], Nils Toft[a], Anna Camilla Birkegård[a,*]

[a] National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark
[b] Department of Veterinary Disease Biology, University of Copenhagen, Frederiksberg, Denmark
[c] Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 16 February 2016
Received in revised form 19 April 2016
Accepted 21 April 2016

Keywords:
Pooling
Antimicrobial resistance gene
Pig
High-throughput qPCR
Sample size
Herd level

ABSTRACT

The primary objective of this study was to determine the minimum number of individual fecal samples to pool together in order to obtain a representative sample for herd level quantification of antimicrobial resistance (AMR) genes in a Danish pig herd, using a novel high-throughput qPCR assay. The secondary objective was to assess the agreement between different methods of sample pooling. Quantification of AMR was achieved using a high-throughput qPCR method to quantify the levels of seven AMR genes (*ermB*,*ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O) and *tet*(W)). A large variation in the levels of AMR genes was found between individual samples. As the number of samples in a pool increased, a decrease in sample variation was observed. It was concluded that the optimal pooling size is five samples, as an almost steady state in the variation was observed when pooling this number of samples. Good agreement between different pooling methods was found and the least time-consuming method of pooling, by transferring feces from each individual sample to a tube using a 10 μl inoculation loop and adding 3.5 ml of PBS, approximating a 10% solution, can therefore be used in future studies.

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1. Introduction

There is a considerable societal interest in the careful monitoring of AMR levels in humans and animals, and both national surveillance programs such as DANMAP in Denmark (DANMAP, 2014) and European surveillance programs (EFSA, 2015) exist. Surveillance programs most often monitor AMR in one of the three major categories of organisms: animal bacterial pathogens; zoonotic bacteria or commensal bacteria (Franklin et al., 2001). In Denmark, AMR is monitored in *Escherichia coli* and *Enterococcus* spp. (DANMAP, 2014). These are considered good indicator bacteria as they are part of the normal gut flora and constitute a reservoir of resistance genes (Franklin et al., 2001). With more than 400 different bacterial species in the gut, *E. coli* constitutes less than 1% of these (Berg, 1996) and a large variation in the abundance of *E. coli* (Dunlop et al., 1999), the AMR levels might be underestimated using indicator bacteria. DNA-based methods make it possible to investigate total community DNA and quantify the AMR gene levels in complex samples such as porcine feces (Schmidt et al., 2015). Sample pooling has proven beneficial, including screening for presence or absence for a range of diseases (Arnold and Cook, 2009; Rovira et al., 2008; Tavornpanich et al., 2004; Weinstock et al., 2001). Few studies have assessed the value of pooling to quantify the herd level for a disease (Davies et al., 2003; Pedersen et al., 2014; Schmidt et al., 2015). However, they do not establish how many individual samples (IS) would be optimal to pool, which is necessary in order to represent the true herd AMR status. Minimizing the number of individual samples is important, because the sampling process is time-consuming and therefore expensive.

The primary objective of this study was to determine the minimum number of individual fecal samples to pool together in order to obtain a representative sample for herd level

* Corresponding author at: National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg, Denmark.
E-mail addresses: jucl@vet.dtu.dk (J. Clasen), amellerup84@gmail.com (A. Mellerup), jeo@sund.ku.dk (J.E. Olsen), ysan@ssi.dk (Ø. Angen), afol@vet.dtu.dk (A. Folkesson), tahbh@vet.dtu.dk (T. Halasa), ntoft@vet.dtu.dk (N. Toft), acbir@vet.dtu.dk (A.C. Birkegård).

http://dx.doi.org/10.1016/j.vetmic.2016.04.017
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quantification of AMR genes in a Danish pig herd, using a novel high-throughput qPCR assay. The secondary objective was to assess the agreement between different methods of sample pooling.

2. Materials and methods

2.1. Study design

Two studies were conducted. In study one, the minimum number of IS to pool in order to obtain a representative sample for herd level quantification of AMR genes in a Danish pig herd was estimated. In study two, three different pooling methods were compared.

2.2. Sample collection

In study one, 20 IS were collected in November 2014 from a pig herd on Funen, housing 1700 finisher pigs and 1150 weaner pigs. Fecal samples were collected from one section with finisher pigs close to slaughter. The section had eight pens housing 3–18 pigs. Between one and five IS were taken from each pen, depending on the number of pigs in the pen. The samples were collected from the rectum of the pig using disposable plastic gloves, which were changed between samplings. As the sampling did not involve invasive handling of the animals, permission for sampling was not required by Danish law. The samples were stored in plastic containers with tight lids and immediately placed in a Styrofoam box with cooling elements, then transported and stored overnight at 5 °C before pooling.

In study two, five IS from five different pig herds (i.e. 25 IS), were collected in January 2015 at an abattoir in Jutland in the lairage, just prior to slaughter. The samples were collected and transported as done in study one but pooled the same day as the sampling.

2.3. Pooling of samples

In study one, a 10% Phosphate Buffered Saline (PBS) solution was made twice for each of the 20 IS by transferring 1 g of feces to 9 ml of PBS. Pooled samples (PS) were then made from the first dilution of the 20 IS by mixing 0.5 ml of the diluted samples. A total of 48 pools were made: 11 pools of two IS, 11 pools of three IS, 11 pools of four IS, six pools of five IS, four pools of ten IS, four pools of 15 IS and one pool of 20 IS. The pools with the same number of IS were made of different IS, but for the pools of two, three and four IS, two duplicates of pools were made resulting in six pairs of identical pools. This was done to validate the consistency of the pooling method. All samples (IS and PS) were stored at −20 °C until DNA extraction.

In study two, three pooling methods were used. Pooling method 1: the same method as for study one. Pooling method 2: feces from each IS were transferred to a tube using a 10 μl inoculation loop. The PS was weighed and the amount of PBS required for a 10% solution was calculated and added. Pooling method 3: using a 10 μl inoculation loop, feces from each IS were transferred to a tube and a fixed amount of PBS was added, approximating a 10% solution. The amount of PBS used in pooling method 3 was calculated as the mean of the amount of PBS used for the samples in pooling method 2. The PS were stored at −20 °C before DNA extraction. The time spent making ten samples was measured for each method.

2.4. Quantification of AMR genes using real-time qPCR

Total DNA was extracted from the 20 IS and PS, using the Maxwell® 16 LEV Blood DNA Kit (Promega), details can be obtained by the corresponding author. Samples were diluted to 40 ng/μl in nuclease-free water (Qiagen, Hilden, Germany) and stored at −20 °C until further processing. Seven AMR genes, *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O) and *tet*(W), were included in the array. Primers and probes have been validated by Schmidt et al. (2015). 16S rDNA primers and probes were included in the assay as a reference gene; here were used as forward primer: TGGAGCATGTGGTTTAATTCGA, as reverse primer: TGCGGGACTTAACCCAACA and as probe: CCTTTGACAACTCTAGAGATAGAGCCTTCCC, all synthesized by DNA Technology A/S (Aarhus Denmark). qPCR amplifications for the quantification of the included genes were performed with the Fluidigm HD Biomark system. The PCR protocol was as follows: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 60 s at 59 °C for extension and annealing, where the fluorescence was measured after each cycle. Standard curves for the qPCR was generated from 10-fold and 2-fold serial dilutions of a fecal samples containing target DNA for each primer set for determination of efficiency, i.e. limit of detection (LOD) and limit of quantification (LOQ).

2.5. Data analysis

Raw quantification cycle (C_q) values generated by the qPCR were exported from the Fluidigm Real-Time PCR Analysis Software, version 4.2.1 (Fluidigm, 2014) to R (R Core Team, 2015). Samples with technical replicates with a discrepancy exceeding 0.5, C_q values exceeding primer-specific LODs or with non-detects of one or both of the technical replicates were excluded. The mean of C_q values for technical replicates were calculated, then corrected with the IPCs included in all runs, along with an efficiency calibration (Ståhlberg et al., 2013) based on standard curves generated. Relative quantification (RQ) was then determined for each of the samples using a modified Livak-method (Eq. (1))(Livak and Schmittgen, 2001):

$$RQ = 2^{-(C_{q,\text{AMRgene}} - C_{q,\text{reference gene}})} \quad (1)$$

Table 1
Primer efficiency, R^2, dynamic range, LOD and LOQ for qPCR assays.

Gene	Efficiency	R^2	Dynamic Range	LOD (C_q value)	LOQ (C_q value)
ermB	98.0%	0.9896	9-fold	23	23
ermF	94.5%	0.9739	7-fold	24	24
sulI	100.0%	0.9510	7-fold	26	26
sulII	102.7%	0.9698	7-fold	23	23
tet(M)	108.2%	0.9547	4-fold	25	25
tet(O)	94.9%	0.9900	10-fold	23	23
tet(W)	90.9%	0.9929	12-fold	24	24
16S rDNA	94.2%	0.9953	9-fold	24	18

LOD: limit of detection, LOQ: limit of quantification. R^2: determinant coefficient. C_q: Cycle of quantification.

2.6. Statistical analyses

All statistical analyses and data management were performed using R version 3.2.1 (R Core Team, 2015) with a significance level of 5%.

In study one, boxplots with jittered data were created from the RQ values for each AMR gene as a function of the number of IS in the pool. The plots were visually evaluated to assess the number of IS in the pool needed to stabilize the variation in the RQ value and this was chosen as the optimal sample size. The "wilcox.test"-function in R (R Core Team, 2015) was used to compare the RQ value for pools containing the optimal sample size and those with more IS. Likewise, pools with fewer IS than the optimal sample size were compared to those with more than the optimal sample size. Bland–Altman plots were created to assess if there was agreement between; runs of the same DNA extraction; DNA extractions of the same IS and pools of the same IS. In a Bland-Altman plot, the agreement between two measures is evaluated by plotting the mean observation from the two methods against the difference

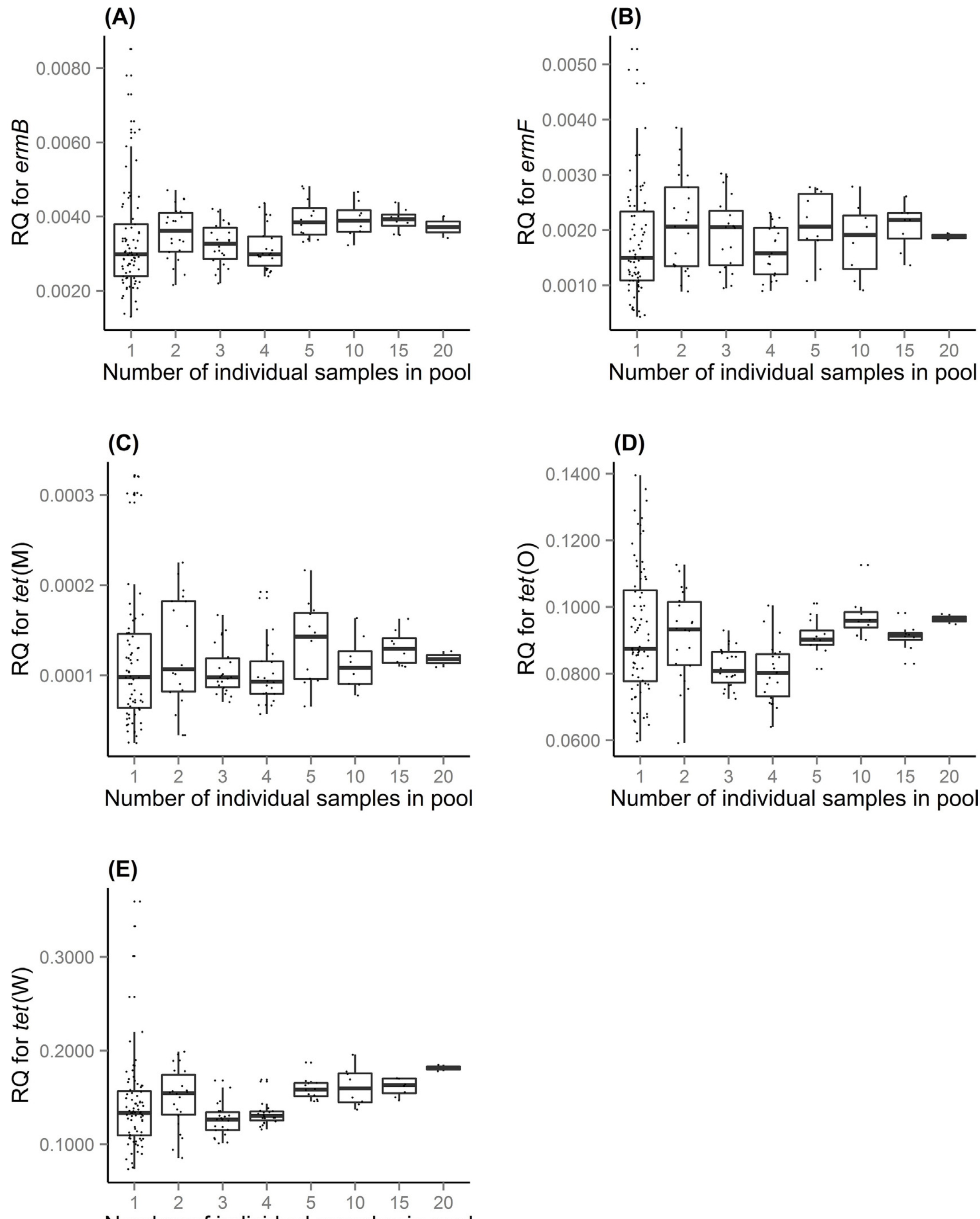


Fig. 1. Boxplot with jittered data showing relative quantification (RQ-value) of AMR genes compared with 16S rDNA (study 1). Number of individual samples in a pool plotted against the RQ-value of each of the genes: (A) *ermB*; (B) *ermF*; (C) *tet*(M); (D) *tet*(O); (E) *tet*(W). It should be noted that the y-axis of the boxplots are on different scales due to a large variation in RQ-values between the genes.

between the observations from the two methods (Altman and Bland, 1983).

In study two, Bland–Altman plots were created to assess if there was agreement between the three pooling methods using pairwise comparison.

3. Results

3.1. Accuracy of the qPCR assays

The efficiency of the qPCR assays, R^2, dynamic range, LOD and LOQ are shown in Table 1. No contamination from the DNA extraction was observed.

Statistical analyses could not be made for *sulI* and *sulII* due to a large number of non-detects.

3.2. Study 1

RQ values of the AMR genes for the IS showed a large variation (Fig. 1). Increasing the number of IS in the pools resulted in a decreased variation of AMR genes levels. Using five IS in a pool stabilized the variation of the AMR gene levels for all genes included. There were no significant differences between the RQ values of any of the AMR genes for the pools with five IS and the pools with more than five IS. In contrast, a significant difference was found for all AMR genes (with the exception of *ermF*), between pools with less than five IS and those with five or more IS (*p*-values: *ermB* and *tet*(W): <0.0001, *tet*(M): 0.014 and *tet*(O): 0.006).

The Bland-Altman plots showed a moderate to good agreement for all genes (Fig. 2, only shown for *ermB*).

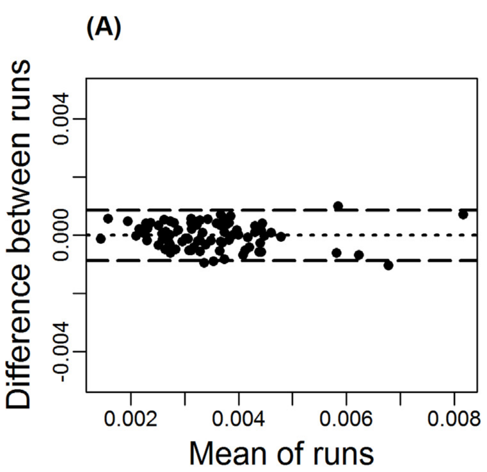
3.3. Study 2

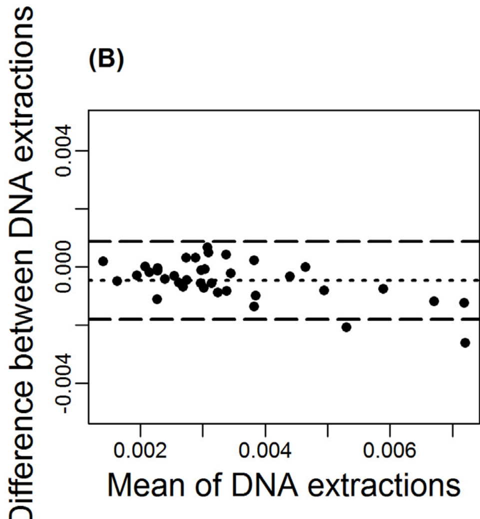
Bland-Altman plots for all genes showed good agreement between all three methods (Fig. 3, only shown for *ermB*).

For pooling method 3, the amount of PBS added to the pooled feces was 3.5 ml. The time taken to make ten PS was measured to: 60 min; 40 min; and 20 min for pooling method 1, 2 and 3, respectively.

4. Discussion

In this study, a novel high-throughput qPCR assay was used to determine the optimal pooling strategy for quantification of the average level of AMR genes in Danish pig herds. It was found that pooling five samples was optimal with respect to reducing variation.





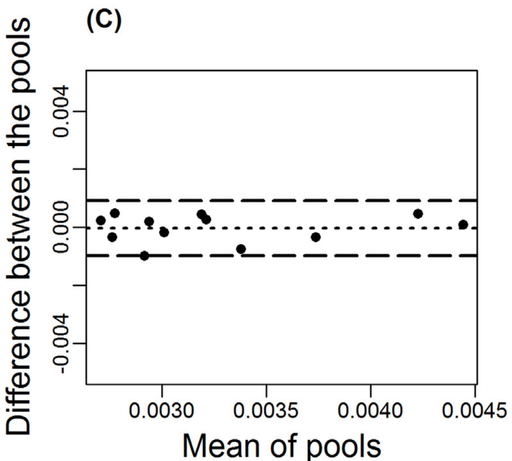


Fig. 2. Bland-Altman plots for ermB from study 1. Bland-Altman plot of agreement between: (A) different qPCR runs of the same sample; (B) two DNA extractions of the same individual sample; (C) pools of the same sample. —: Observed average agreement. —: 95% limits of agreement.

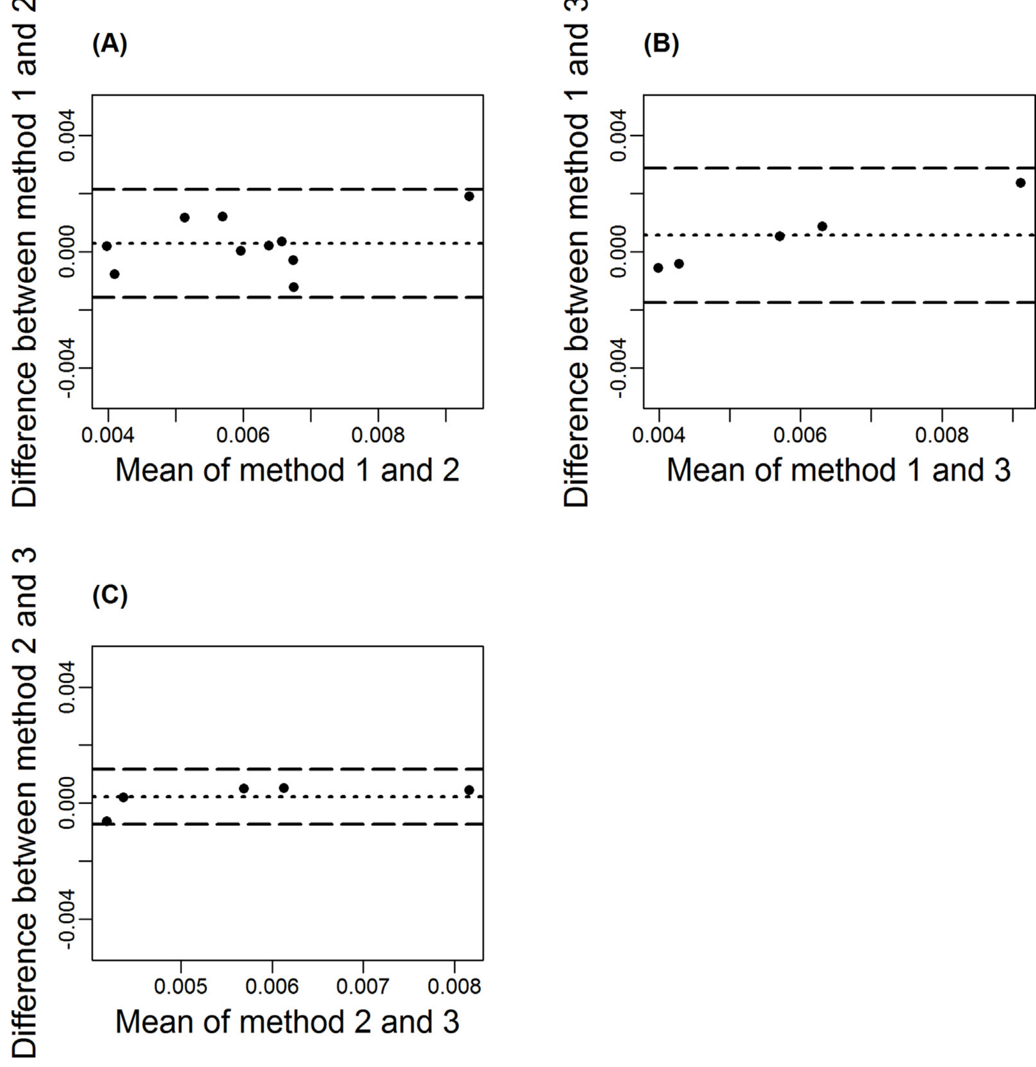


Fig. 3. Bland-Altman plots for ermB from study 2. Bland-Altman plot of pairwise agreement between different pooling methods by: (A) methods 1 and 2; (B) methods 1 and 3; (C) methods 2 and 3. –: Observed average agreement. –: 95% limits of agreement.

Analysis of samples is time-consuming and expensive, and it may therefore be beneficial to use pooling of samples. It is therefore essential to determine the smallest number of samples required to represent the herd in order to reduce time for sampling, diminish disruption of normal procedures in the pig herd, and subsequently reduce laboratory work. Large variation was observed in the levels of AMR genes between individual samples, emphasizing the importance of collecting more than one sample, in order to adequately describe the average levels of AMR in a particular herd. Increasing the number of individual samples in a pool reduced sample variation around a visually estimated herd average for the AMR gene. An almost stable variation of AMR gene levels was found when five individual samples were pooled. This was in accordance with the results found by Dunlop et al. (1999) when evaluating the tetracycline resistance in *E. coli*. The latter study used bootstrapping and the results were not confirmed using biological samples. Cortey et al. (2011) and Pedersen et al. (2014) have both compared theoretical pools with biological pools and found good agreement between the two. It is therefore presumed that pooling of samples in this study as well will provide an average of the individual samples.

A limitation of this study is that it was carried out in only one farm. Thus the results may not be representative for all finisher herds in Denmark, as variation between herds in the levels of the different genes is expected. However, the samples did display differences in levels of the examined genes, and the results for all suggested pooling of five samples as optimal. Thus, the results found in this farm, should be generally valid, as levels of genes are expected to be comparable, to the levels of (potentially other) genes in finisher herds. Therefore, it would be reasonable to suggest that the results of this study can be used as a guideline to determine the number of samples in a pool when evaluating herd levels of AMR. Further research may be needed to investigate the required number of IS in a pool from sow herds, where animals may have different levels of genes.

In the presented work, we tested for repeatability on three different levels: running the same DNA extraction twice on the qPCR; DNA extraction of the same individual sample twice; and creating the same pool twice. Good agreement was found for all three levels. This indicates that the novel qPCR method used in this study is reliable for the quantification of AMR genes *ermB, ermF, tet*(M), *tet*(O) and *tet*(W). It was not possible to evaluate results for *sulI* and *sulII* due to the high number of non-detects.

Pooling method 3 was the least time-consuming, it is therefore suggested that it is used instead of pooling method 1, which has

been used in previous studies (Pedersen et al., 2014; Schmidt et al., 2015).

In conclusion we suggest that the optimal sample size in pools from finisher herds is five individual samples, and we present an easier and quicker pooling method than previously used.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

We would like to thank Rasmus Kenneth Boysen for helping with the collection of samples; Karin Tarp and Sophia Rasmussen for their technical assistance; and Kerstin Skovgaard for guidance and help with data analysis.

The study was funded by the Danish Veterinary and Food Administration. The funders had no role in study design, data collection and analyses, decision to publish, or preparation of the manuscript.

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4.2 Manuscript 2: Sampling pig farms at the abattoir in a cross-sectional study − Evaluation of a sampling method.

Anna Camilla Birkegård, Tariq Halasa, and Nils Toft.

Preventive Veterinary Medicine. 2017;145:83–90, doi: 10.1016/j.prevetmed.2017.07.002

Preventive Veterinary Medicine 145 (2017) 83–90

Contents lists available at ScienceDirect

Preventive Veterinary Medicine

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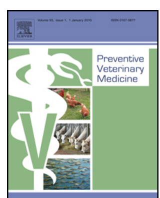

Sampling pig farms at the abattoir in a cross-sectional study – Evaluation of a sampling method

Anna Camilla Birkegård*, Tariq Halasa, Nils Toft

Department for Diagnostics and Scientific Advice, National Veterinary Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs Lyngby, Denmark

ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form 14 June 2017
Accepted 2 July 2017

Keywords:
Sampling procedure
Pig farm
Cross-Sectional study
Slaughterhouse
Abattoir

ABSTRACT

A cross-sectional study design is relatively inexpensive, fast and easy to conduct when compared to other study designs. Careful planning is essential to obtaining a representative sample of the population, and the recommended approach is to use simple random sampling from an exhaustive list of units in the target population. This approach is rarely feasible in practice, and other sampling procedures must often be adopted. For example, when slaughter pigs are the target population, sampling the pigs on the slaughter line may be an alternative to on-site sampling at a list of farms. However, it is difficult to sample a large number of farms from an exact predefined list, due to the logistics and workflow of an abattoir. Therefore, it is necessary to have a systematic sampling procedure and to evaluate the obtained sample with respect to the study objective.

We propose a method for 1) planning, 2) conducting, and 3) evaluating the representativeness and reproducibility of a cross-sectional study when simple random sampling is not possible. We used an example of a cross-sectional study with the aim of quantifying the association of antimicrobial resistance and antimicrobial consumption in Danish slaughter pigs. It was not possible to visit farms within the designated timeframe. Therefore, it was decided to use convenience sampling at the abattoir. Our approach was carried out in three steps: 1) planning: using data from meat inspection to plan at which abattoirs and how many farms to sample; 2) conducting: sampling was carried out at five abattoirs; 3) evaluation: representativeness was evaluated by comparing sampled and non-sampled farms, and the reproducibility of the study was assessed through simulated sampling based on meat inspection data from the period where the actual data collection was carried out.

In the cross-sectional study samples were taken from 681 Danish pig farms, during five weeks from February to March 2015. The evaluation showed that the sampling procedure was reproducible with results comparable to the collected sample. However, the sampling procedure favoured sampling of large farms. Furthermore, both under-sampled and over-sampled areas were found using scan statistics.

In conclusion, sampling conducted at abattoirs can provide a spatially representative sample. Hence it is a possible cost-effective alternative to simple random sampling. However, it is important to assess the properties of the resulting sample so that any potential selection bias can be addressed when reporting the findings.

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1. Introduction

The cross-sectional study is a commonly used design in veterinary epidemiology. Compared to other study designs, it is relatively inexpensive, fast, and easy to conduct. An ideal sample would be obtained through simple random sampling, where samples are obtained by randomly selecting from a list of all units in the target population. In simple random sampling every subject has the same chance to be sampled (Dohoo et al., 2010). Other random sampling procedures such as stratified or systematic random sampling also exist (Dohoo et al., 2010). When sampling from live individuals, the random component of the sampling is often impeded by logistic, financial, or biological constraints. Many that have designed and carried out a study involving sampling of live animals, for example at farms, would agree on that the resulting sample is often not obtained completely at random or the characteristics of the farms have changed after they were chosen for sampling. Therefore, in reality many resort to non-probability sampling when it is not pos-

* Corresponding author.
E-mail address: acbir@vet.dtu.dk (A.C. Birkegård).

http://dx.doi.org/10.1016/j.prevetmed.2017.07.002
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sible to use an exact method for determining a subjects probability for sampling (Dohoo et al., 2010).

Regardless of the sampling procedure, the sample must be representative of the target population or at least the part of the target population that the researcher wishes to draw conclusions about. While careful planning is essential to obtaining a sample that meets the criteria of the study (Houe et al., 2004), an evaluation of the representativeness of the sample after collection could provide valuable information. In the 1960s, John Tukey (1962) introduced the idea that it is important to assess if your assumptions are correct before initiating statistical analyses. When reporting a cross-sectional study, the STROBE statement (von Elm et al., 2007) suggests that the eligibility criteria, as well as the sources and methods of sample selection are defined, together with a descriptive summary of the sample. However, an evaluation of the representativeness of sample and the extent to which it meets the eligibility criteria is not required. Therefore, it could be valuable to evaluate the level of sample bias that might be introduced as a result of limited access to the entire target population or due to the sampling procedure, particularly in studies where practical constraints prohibit simple random sampling. For example, the most practical way to obtain a random sample of pig farms would be to sample at the farms. However, due to the strict biosecurity measures at pig farms (Boklund et al., 2004), it is difficult to sample a large number of farms in a short period of time. Depending on the purpose of the study, a convenience sampling procedure (such as sampling pigs at the abattoir) could be a valuable substitute. Convenience sampling is a non-probability approach where the samples are collected because they are easy to obtain rather than sampling at random (Dohoo et al., 2010). Although convenience sampling at the abattoir has previously been explored (DANMAP, 2016; van den Bogaard et al., 2000; Wegener et al., 2003), this study describes how the idea can be used on a larger scale in order to obtain samples from a bigger number of farms. Convenience sampling at the abattoir is inexpensive compared to farm visits and has no negative impact on animal welfare, as sampling from slaughtered pigs would not harm the pigs. However, the consequence of sampling over a short time period at the abattoir is that it is not possible to plan in advance which farms to sample. Records of the farms sending pigs to slaughter are only available close to the date of slaughter. The representativeness of the sample must therefore be evaluated after sampling.

The objective of the study was to present a method for planning and conducting a cross-sectional study when simple random sampling is not possible and to evaluate if the study criteria have been fulfilled for the obtained sample. Our approach is exemplified using a cross-sectional study of pig farms sampled at the abattoir with the aim of quantifying the association of antimicrobial resistance and antimicrobial consumption in Danish pigs.

2. Materials and methods

2.1. Study set-up

To demonstrate our approach, a cross-sectional study of the association between antimicrobial resistance genes at farm level in pigs, and factors such as geographical location, farm size, and antimicrobial consumption was used. To meet the objectives of such a study, it was necessary to obtain a random sample of farms that were representative of the Danish pig population in terms of geographical location and farm size.

We conducted our study in three consecutive steps:

1) Planning the cross-sectional study
2) Conducting the cross-sectional study
3) Evaluating the sampling procedure
   a) Evaluation of the collected sample from the cross-sectional study in terms of farm size and spatial randomness
   b) Evaluation of the reproducibility of the sampling procedure by simulating it using meat inspection data. The sampling procedure was assessed to be reproducible if repeating the sampling would result in a sample that was comparable with the collected sample in terms of farm size and spatial distribution

Thereby, two set of samples were considered: a) the sample obtained in the cross-sectional study, where actual faecal samples were collected, hereafter referred to as the collected sample; b) simulated samples, used to examine the reproducibility of the sampling procedure, where information about the farms were obtained from the meat inspection data.

2.2. Register data

Data from three registers (the Central Husbandry Register (CHR), meat inspection data, and the database for pig movements) were used.

All pig farms in Denmark are registered in the national CHR database with a unique identification number (CHR-number). The CHR is owned and maintained by the Danish Veterinary and Food Administration. Information in the CHR includes Cartesian coordinates (given in “UTM EUREF89 zone 32” format) and the number of pigs for three age groups: breeding animals, weaners (7–30 kg) and finishers (pigs above 30 kg excluding breeding animals) (Anon, 2016a). The CHR also contains information on abattoirs, rendering plants, cooling facilities for dead animals, animal fairs and export stables.

The meat inspection data are owned and maintained by the Danish Classification inspection. The data include delivery number, CHR-number, date of slaughter, and the ID of the abattoir (Anon, 2016b). The delivery number is tattooed on the ham of the pig and can be read on the carcass on the slaughter line. The delivery number was used during sampling to identify the farm from which the pigs originated.

The database for pig movements contains information about of movement of dead and live pigs. The database includes CHR-numbers of receiving and sending farms or holdings, the number of pigs moved and the date of the movement (Anon, 2016c).

2.3. Step one: planning the cross-sectional study

The cross-sectional study serving as an example for our approach was designed to meet the following criteria:

1 Representativeness based on farm size, which was estimated using two different variables – the productivity and the number of finisher pigs registered in the CHR. Productivity was calculated as the cumulative delivery over 26 weeks of pigs for slaughter, traced back from the first day of sampling. This approximately corresponds to two batches of finishers, in order to reduce the influence of seasonal variation (Moodley et al., 2011). Both number of finishers and productivity were used in order to account for the inaccuracy in the CHR and the fact that not all pigs were slaughtered in Denmark, which would influence the estimated productivity of some farms.
2 Spatial randomness of locations of sampled farms compared to non-sampled farms in the target population.

The target population is the population that we want to draw conclusions on (Houe et al., 2004). For the cross-sectional study the target population was Danish farms with a conventional production

of finishers. The target population was defined as farms included in a data extraction from the CHR database on 19th January 2015. Farms that did not have finishers registered or that were registered as organic, free-range, trade or hobby farms were excluded from the target population because these farm types have a farm management system that differ from the conventional production farms. Furthermore, all farms located on Bornholm (124 farms) and farms without coordinates in the CHR data extraction (3 farms) were excluded. The information for the target population was obtained from the CHR which optimally only contain information on active farms. However, it happens that the farmer have not inactivated the farm in the register while the farm is empty. Therefore, we used the database for pig movements to estimate if the farm was active or inactive. The farms were considered to be active if at least one movement (either into or out of the farm) was registered in the movement data during the period between September 2014 and March 2015. Only active farms were included in the target population, resulting in the exclusion of 358 inactive farms. The final target population consisted of 5654 farms.

The sampling frame did not cover all farms in the target population, as not all farms sent pigs to the sampling sites. The 3274 farms included in the sampling frame were defined as the study population, i.e. the population that the samples are taken from (Houe et al., 2004). The study population was used to evaluate the choice of sampling site. The remaining farms did not send any pigs to slaughter during the period, sent pigs to slaughter outside of Denmark, or sent pigs to an abattoir that was not in the sampling frame.

It was decided that only five of the seven major Danish-owned abattoirs for finisher pigs would be used as sampling sites. Statistics Denmark estimated that a total of 1,531,600 finishers were slaughtered in Denmark in February 2015 (Statistics Denmark, 2015), and 1,365,963 (90%) of these were slaughtered at the seven abattoirs (calculated based on meat inspection data). The reasons for excluding two of the abattoirs were that one primarily slaughtered pigs from free-range and organic farms, and the other was located on the remote island of Bornholm. By mapping the location of the farms that sent pigs to the sampling sites in 2014, it was apparent that each of the five chosen sampling sites covered a certain region of Denmark (Fig. 1).

The next step in planning the sampling was to determine the number of farms that should be sampled per abattoir. There was a chance of sampling the same farms more than once and a chance of sampling farms that did not match the target population. Given the resources available to the project, we therefore planned to have 800 farms with finishers in the sample. The number of farms per sampling site was calculated based on the number of farms that sent pigs to the abattoir in weeks 6–10 and weeks 46–50 of 2014 using the meat inspection data. These two periods were chosen in order to take into account seasonal variation and a re-organisation of the abattoir structure in the autumn of 2014.

2.4. Step two: conducting the cross-sectional study

Sampling took place during weeks 6–10 of 2015. Reaching the sample size required a total of 4–7 h per day over 16 active sampling days with sampling from one or two abattoirs per day. A previous study (Clasen et al., 2016) showed that a pooled sample of faeces from five individual pigs was sufficient to represent the antimicrobial resistance level of the farm. Our sampling approach therefore required that at least five pigs from the same farm were slaughtered on the same day.

2.5. Step three: evaluation of the sampling procedure

Evaluation of the sampling procedure was conducted in two parts. First, the representativeness of the farms in the collected

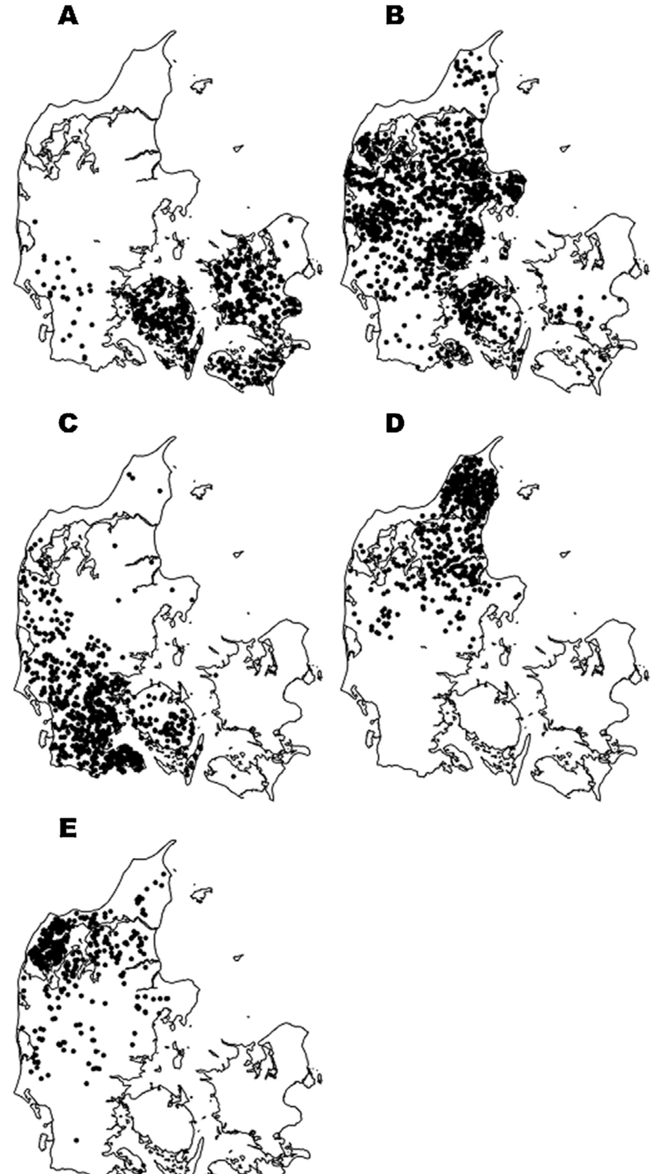

Fig. 1. Location of farms sending pigs to the five abattoirs (A–E) included as sampling sites. The maps are showing the location of the farms that have sent pigs to slaughter at the abattoirs included in the study. Black dot indicates the location of a pig farm.

sample was assessed by comparing farm size and spatial distribution with that of the non-sampled farms in the target population and with the non-sampled farms in the study population. Reproducibility was assessed by simulating samples using information from the meat inspection data about farms sending pigs to slaughter on the days when of data collection occurred.

2.5.1. Evaluation of representativeness of the collected sample

In order to evaluate the representativeness of the farms in the collected sample with regard to farm size, the registered number of finishers and estimated productivity (estimated as the number of pigs delivered during 26 weeks for slaughter) of the farms included in the collected sample were compared to the farms in the target and study populations that were not included in the collected sample. A Wilcoxon test was used to compare the median productivity of the farms in the collected sample with both the non-sampled farms in the target population, and the study population as well as to compare the number of finishers at the farms in the col-

lected sample with the non-sampled farms in the study and target population.

To evaluate whether or not the farms in the collected sample were randomly spatially distributed among the non-sampled farms, a purely spatial cluster analysis was performed using the "rsatscan" package (Kleinman, 2015), which allows the spatial cluster analysis to be run in SatScan (Kulldorff and Information Management Services Inc, 2015) from R (R Core Team, 2017). The Scan statistics were based on a Bernoulli model (Kulldorff, 1997). The sampled farms were defined as cases and the non-sampled farms in the target and study populations were defined as controls. In the Bernoulli model, the null hypothesis is that there is an equal distribution of cases and controls both inside and outside of the search window (Kulldorff, 1997). The spatial scanning window was set to be circular, include a maximum level of 25% of the population, and centre on the coordinates of each farm in turn. Only secondary spatial clusters with no geographical overlap were allowed. The analysis was run as two-sided tests, scanning for areas with sampling rates that were higher or lower than expected under the null hypothesis. The relative risk (RR) to be sampled for a specific site within an identified cluster in the cross-sectional study was calculated as the ratio of the observed to the expected number of sampled farms within that cluster. The spatial scan statistics used 999 Monte Carlo replications to estimate the significance levels of these clusters. The spatial cluster was considered to be significant if the p-value was below 0.05. Spatial clusters with RR >1 were defined as over-sampled areas, and clusters with RR <1 were defined as under-sampled areas. The SatScan analysis gives the point location of the centre and the radius of the clusters (here defined as circles). The results of the scan statistics were plotted onto a map of Denmark (excluding Bornholm) using the "sp" package (Pebesma and Bivand, 2005) in R (R Core Team, 2017).

2.5.2. Evaluating the reproducibility of the sampling procedure through simulation

In order to evaluate the reproducibility of the proposed sampling procedure, a simulation study was conducted by sampling from meat inspection data. To simulate the sampling, the following three assumptions were made:

1) Sampling would take place in the same period as the cross-sectional study
2) For the total number of farms to sample, three scenarios were chosen:
   - i Using the same number of farms to sample per abattoir as in the cross-sectional study.
   - ii Doubling the number of farms to sample compared to the cross-sectional study.
   - iii Recalculating the number of farms to sample per abattoir using meat inspection data from the sampling period instead of using previous data. This resulted in a target of 120, 290, 180, 120 and 90 farms to sample for each of the abattoirs A-E, respectively.
3) Two cut-off values were chosen in order to determine the smallest number of pigs a farm could send to the abattoir on the day of sampling before being included in the sampling:
   - a.) A cut-off value of 20 pigs sent for slaughter per day. This number was chosen as five was sampled per farm to be sampled and it was estimated that it would be possible to sample every second pig arriving from the same farm, and that the abattoirs would divide each batch into two slaughter lines.
   - b.) An abattoir-specific cut-off value was defined as the minimum number of pigs sent to slaughter on the day of sampling in the cross-sectional study for the sampled farms. The cut-off values for abattoirs A to E were 21, 10, 15, 8 and 18, respectively.

A subset of the meat inspection data (containing only farms delivering more than the chosen cut-off value during the sampling period) was constructed for each simulation scenario. From this subset, the required number of farms were sampled at random, using the "sample" function in R (R Core Team, 2017) without replacement. This was repeated 1000 times for each simulation scenario. This resulted in 5000 samples (5 simulation scenarios with 1000 repetitions of the sampling).

The farms that did not match the target population were excluded. For each simulated sample the productivity and number of finishers was calculated and summarized.

To further evaluate the reproducibility of the sampling procedure, we evaluated whether the sampled farms in each of the 5000 samples were randomly distributed in space. Spatial cluster analyses were conducted for the farms in the simulated samples using the same approach as described for the collected sample, where the sampled farms were compared with their respective set of non-sampled farms.

2.6. Descriptive analyses

For each of the 5000 simulated samples summary statistics were calculated for the number of finishers and the productivity for the sampled farms. Hereafter, boxplots were made for the distribution of the first quantile, median, and third quantile within the five simulation scenarios. The summary statistic for the number of finishers and the productivity for the farms in the collected sample, farms in the target population, and farms in the study population were also calculated and the information was added to the graphs.

The results of the spatial cluster analyses for the farms in the simulated samples were mapped together with the results of the spatial cluster analyses for the farms in the collected sample.

3. Results

3.1. Step one: planning the cross-sectional study

A sample size of 800 farms was initially decided as a target. Calculating the number of farms to sample from abattoirs A-E resulted in the following distribution: A: 140, B: 300, C: 160, D: 120 and E: 80 farms.

3.2. Step two: conducting the cross-sectional study

In the cross-sectional study 800 faecal samples were collected. However, 94 farms were sampled two to four times. In four observations, it was not possible to match the delivery number with a CHR-number, and these were excluded. A further six farms were excluded – four because they did not have any finishers registered in the CHR, and two because they did not match the target population. This resulted in a collected sample of 681 farms.

3.3. Step three: evaluating the sampling procedure

3.3.1. Evaluation of representativeness of the collected sample

For the cross-sectional study, the median number of finishers was 1500 pigs for the farms in the collected sample and 800 pigs for the non-sampled farms in the target population, and 1000 pigs for the non-sampled farms in the study population. The median productivity was 2439 pigs for the farms in the collected sample and 939 pigs for the non-sampled farms in the target population, and 1554 pigs for the non-sampled farms in the study population.

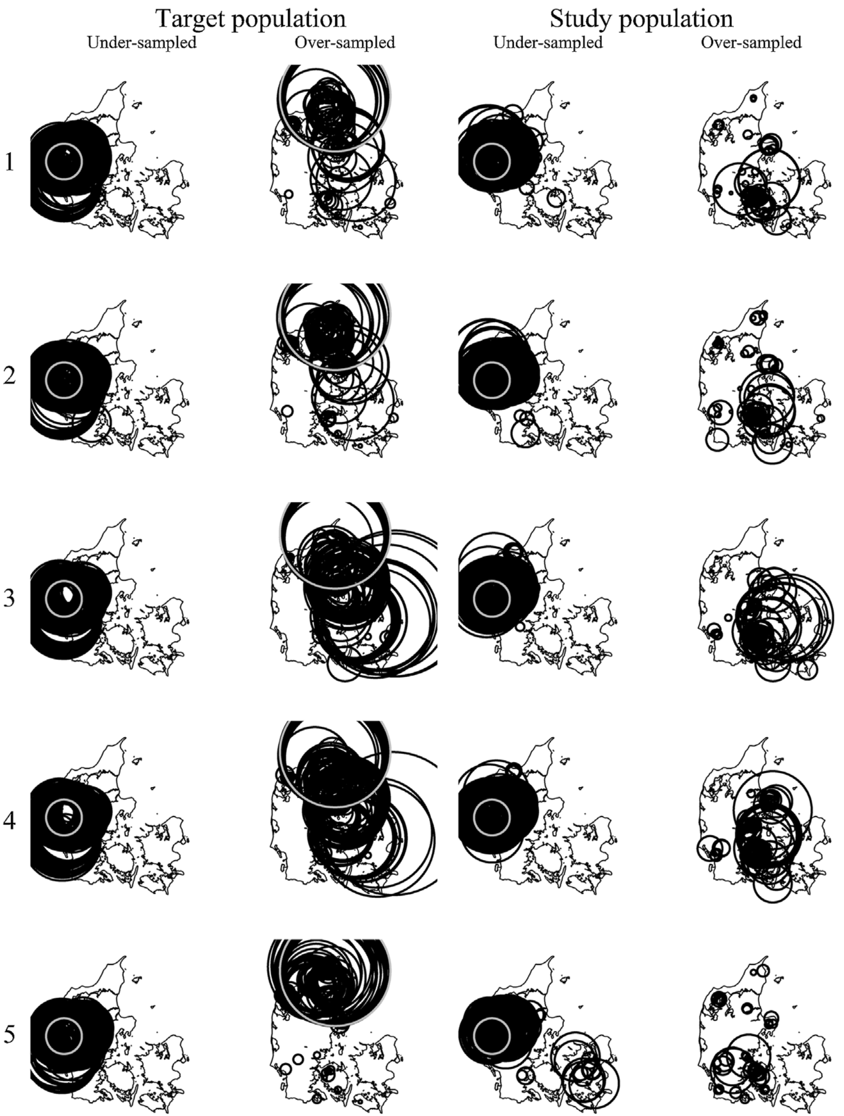

Fig. 2. Results of the spatial cluster analyses for the cross-sectional study and the simulated samples when compared to either the target population or the study population. The sampling procedure was simulated using five different sampling scenarios. These were different combinations of how many farms to sample per sampling site (i: using the same number of farms to sample per abattoir as in the cross-sectional study, ii: doubling the number of farms to sample compared to the cross-sectional study, iii: recalculating the number of farms to sample per abattoir by using meat inspection data from the sampling period) and how many pigs a farm should send to slaughter, that the farm would be sampled (a: 20 pigs sent for slaughter per day or b: abattoir-specific cut-off value). The five simulated samples were, 1: i + a, 2: i + b, 3: ii + a, 4: ii + b, 5; iii + a. The numbers on the left side of the figure refer to the simulation scenarios. Each simulated scenario was repeated for1000 iterations and for each iteration; the spatial cluster analysis was performed. The grey circles show the over- and under-sampled areas found from the spatial cluster analysis of the collected sample. The black circles show the locations of significant clusters (over- and under-sampled areas) found for the farms from the simulated samples. Each black circle represents the result from one iteration out of the 1000 iterations per simulated scenario.

The differences were significant for all variables and for both populations, with $p < 0.001$.

One under-sampled area in the western part of Jutland and one over-sampled area in the northern part of Jutland were found for the farms in the collected sample (Fig. 2).

3.3.2. Evaluation of random spatial distribution

Repeating the sampling procedure through simulation resulted in samples that were consistent in terms of farm size measured as the number of finishers and the productivity (Fig. 3, boxplots) and comparable with the collected sample (Fig. 3, black lines), but the sampled farms were larger than those in the target population (Fig. 3, grey solid lines) and study population (Fig. 3, grey dashed lines).

For the simulation scenarios, different numbers of over- and under-sampled areas were found, and the under-sampled areas were found in a higher percentage of iterations than the over-sampled areas (Table 1). The spatial clusters were often found in the same areas as for the collected sample, and sampling in Zealand seemed to lead to a randomly spatially distributed sample, except when the number of farms to sample per abattoir was recalculated (Fig. 2 simulation scenario 5).

4. Discussion

This study presents a method for planning, conducting and evaluating a non-probability sampling procedure exemplified through a cross-sectional study of pig farms at the abattoir. The sampling procedure was efficient, simple and cost-effective, as samples were obtained from 681 farms in just 36 man-days, whereas sampling directly at farms would require 681 man-days, representing an 18-fold increase in costs.

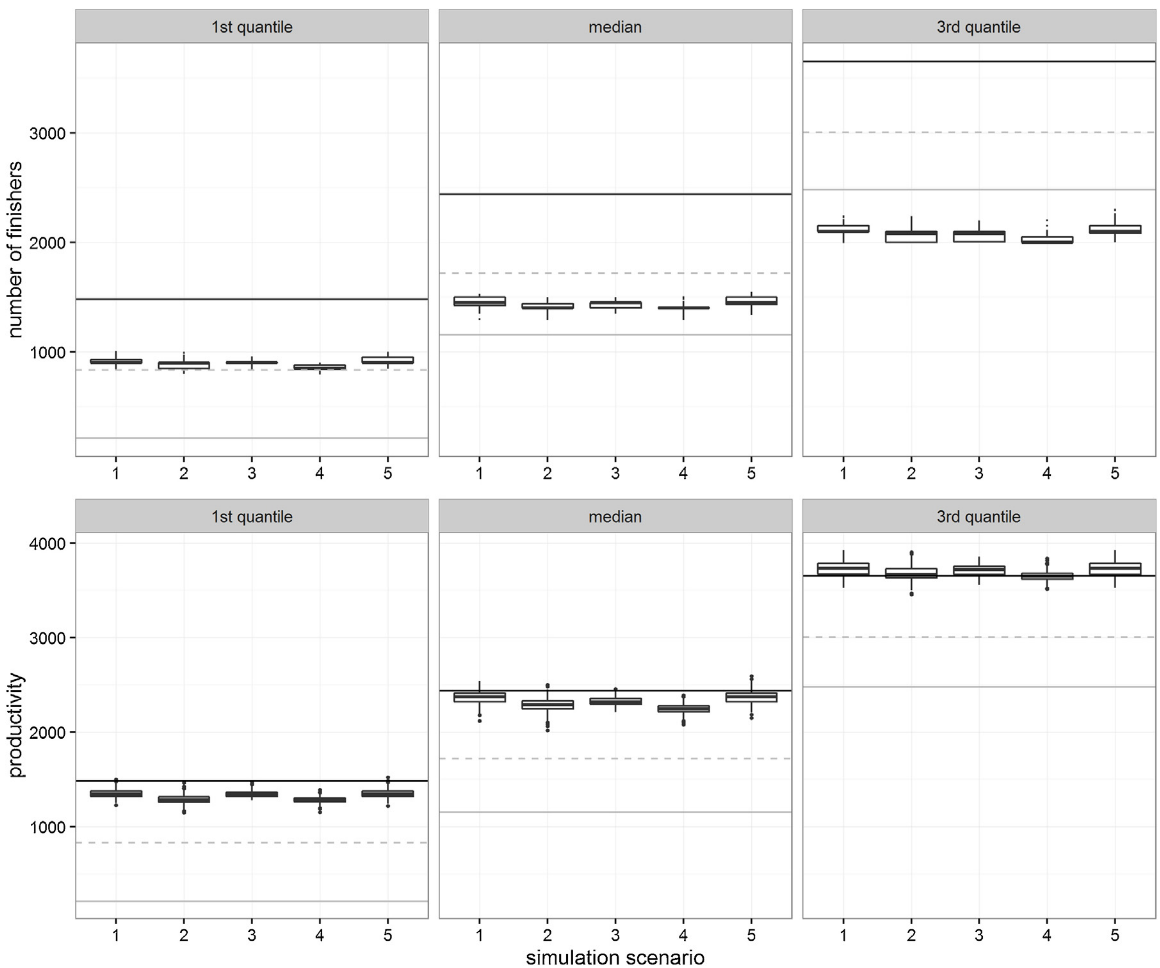

Fig. 3. Comparison of farm sizes between the collected sample and the simulated samples. The sampling procedure was simulated using five different sampling scenarios. These were different combinations of how many farms to sample per sampling site (i: using the same number of farms to sample per abattoir as in the cross-sectional study, ii: doubling the number of farms to sample compared to the cross-sectional study, iii: recalculating the number of farms to sample per abattoir by using meat inspection data from the sampling period) and how many pigs a farm should send to slaughter, that the farm would be sampled (a: 20 pigs sent for slaughter per day or b: abattoir-specific cut-off value). The five simulated samples were, 1: i + a, 2: i + b, 3: ii + a, 4: ii + b, 5; iii + a. Each simulated scenario was repeated in 1000 iterations and for each iteration summary statistics was calculated for the number of finishers and the productivity of the farms in the resulting sample. Per simulated sample, the distribution (boxplots) in the first quantile, median and third quantile for the number of finishers registered in CHR (top) and estimated productivity (delivery of pigs to slaughter over 26 weeks) (bottom) is shown. The black lines refer to the farms in the collected sample, the grey solid lines to the farms in the target population, and the grey dashed lines to the farms in the study population.

Simple random sampling is the most correct way of obtaining a cross-sectional sample. However, it can be difficult to retain a completely random sample due to time, logistic, economic or legislative restrictions. Therefore, a non-probability sampling procedure is often adopted – either on purpose or as an ad hoc solution to sampling difficulties. Dohoo et al. (2010) discuss that using a non-probability sampling procedure limits the external validity of the study. We suggest making a post-sampling evaluation to assess the potential problem with external validity. Our study highlights the importance of evaluating the characteristics for the farms in the resulting sample. We show how this can be achieved using two evaluation criteria: farm size and a spatial component. However, other sampling criteria can be evaluated in a similar manner, in order to assess if appropriate conclusions can be drawn based on the sampled data.

When evaluating the representativeness of the farms in the sample it is important to choose the appropriate evaluation parameters, so they match the objectives of the study. In this study, the representativeness of the farms in the samples was based on farm size. This is an important confounder for several farm-related parameters, such as the level of antimicrobial consumption per pig (Fertner et al., 2015; van der Fels-Klerx et al., 2011), the prevalence of diseases such as PRRSV (Evans et al., 2010), movement patterns (Nöremark et al., 2011) and biosecurity measures (Boklund et al., 2004; Laanen et al., 2013). We showed that the sampling procedure introduced sampling bias, since larger farms were over-represented in the collected sample from the cross-sectional study. Doubling the simulated sample size did not remove this selection bias (Fig. 3 –simulation scenario 3 and 4). It is not surprising that larger farms were over-represented in the sample, as they are expected to deliver pigs for slaughter more often and in larger batches, leading to a higher chance of being sampled at the abattoir. In Denmark, the number of small pig farms is decreasing (Christiansen, 2015). In addition, large farms have a higher impact on human health due to the high number of slaughtered pigs that originate from these farms. Therefore, large farms represent the most relevant segment of the Danish pig population. However, this is still a sampling bias, and results from non-probability sampling at abattoirs should be evaluated with care and the results interpreted accordingly. Our example study aims to explain possible associations between farm size, geographical location, antimicrobial consumption, and the level of antimicrobial resistance at a

Table 1
Results from the spatial cluster analyses

Simulation scenario	Target population				Study population			
	Over-sampled area, %[a]	Under-sampled area, %[a]	Mean number of farms in cluster(+/−sd)	Mean radius (+/−sd), km	Over-sampled area, %[a]	Under-sampled area,%[a]	Mean number of farms in cluster(+/−sd)	Mean radius (+/−sd), km
1	23.7 (1.6)	94.2 (0.8)	863 (+/−475)	47 (+/−21)	7.2 (0)	32.9 (0.2)	496 (+/−288)	42 (+/−20)
2	24.8 (2.4)	95.5 (1.8)	840 (+/−464)	46 (+/−20)	9.4 (0.2)	35.6 (0.9)	373 (+/−293)	40 (+/−21)
3	46.2 (3.8)	99.8 (2.0)	881 (+/−446)	60 (+/−27)	11.0 (0.1)	41.2 (1.6)	354 (+/−272)	42 (+/−24)
4	52.8 (6.2)	100 (3.1)	803 (+/−466)	50 (+/−27)	13.6 (0.5)	51.0 (1.0)	381 (+/−262)	44 (+/−22)
5	17.9 (0.7)	89.5 (0.1)	812 (+/−490)	44 (+/−23)	7.4 (0.4)	31.9 (0.8)	331 (+/−275)	37 (+/−20)

The sampling procedure was simulated using five different sampling scenarios. These were different combinations of how many farms to sample per sampling site (i: using the same number of farms to sample per abattoir as in the cross-sectional study, ii: doubling the number of farms to sample compared to the cross-sectional study, iii: recalculating the number of farms to sample per abattoir by using meat inspection data from the sampling period) and how many pigs a farm should send to slaughter, that the farm would be sampled (a: 20 pigs sent for slaughter per day or b: abattoir-specific cut-off value). The five simulated samples were, 1: i + a, 2: i + b, 3: ii + a, 4: ii + b, 5: iii + a. The numbers on the left refer to the simulation scenarios. Each simulated scenario was repeated for 1000 iterations and for each iteration, the spatial cluster analysis was performed.

[a] Over- and under-sampled areas: percentage of iterations where a significant spatial cluster was found out of 1000 iterations. Numbers in parentheses indicate the percentage of iterations where more than one spatial cluster was found.

farm, thus selecting predominantly larger farms will restrict the validity of the conclusions to larger farms.

Although the sampling method aimed to cover the entire country, it appears that an uneven spatial distribution of the sampled farms was unavoidable. Both under- and over-sampled areas were found for the cross-sectional study, and the trend remained in the simulated samples (Fig. 2). The locations of the under- and over-sampled areas were not consistent, and it appears that there is a smaller chance of finding an over-sampled area than an under-sampled area (Table 1). For the farms in the collected sample, an under-sampled area in the western part of Jutland was found both when comparing the locations of the sampled farms of all Danish pig farms with finishers (target population) and with the only farms that send pigs for slaughter at the sampling sites (study population) (Fig. 2). It is therefore unlikely that the choice of sampling sites caused the uneven spatial distribution of the sampled farms. However, we actually do not have an explanation for this phenomenon.

An over-sampled area was found when the sampled farms were compared to the target population, but not when they were compared to the study population. This might indicate that the farms in the northern part of Jutland primarily sent pigs to the two abattoirs located in that area that was also included in the study. One possible explanation for the occurrence of the under- and over-sampled areas is that the number of farms to sample per abattoir was calculated using historical meat inspection data. The first calculation might have influenced the results and been misleading, as the distribution of farms between the abattoirs might have changed in the meantime. Recalculating the number of farms to sample per abattoir did not remove the uneven spatial distribution of sampled farms in relation to non-sampled farms. However, the location of the clusters was changed and there was a reduction in the percentage of samples in which a significant spatial cluster was found (Table 1 and Fig. 3 –simulation scenario 5).

The sampling procedure was shown to be reproducible, as simulation of the sampling procedure resulted in simulated samples that were comparable (in terms of farm size) with the collected sample. In addition, there was little variation in farm size among the farms in the simulated samples (Fig. 3).

Using the non-probability sampling strategy inevitably meant sampling some farms more than once. In our collected sample, 119 observations were not used for further analysis. This is equivalent to a loss of 17% of the observations, meaning that if a predetermined number must be met, roughly extra 20% should be added to the sample size.

In conclusion, sampling at abattoirs in order to obtain a cross-sectional sample of finisher farms (with no prior knowledge of the farms sending pigs for slaughter) is a fast, inexpensive and logistically less demanding option than on-farm sampling. However, the sampling procedure will target larger farms, additional samples may be needed in order to reach the minimum required number of farms, and a potential bias may occur due to the farm-size effect. We have described a method for post-sample evaluation, thus making it possible to assess and adjust for potential selection bias. In our example, we used farm size and spatial randomness as criteria, but it is possible to test any property of the sample using our methodology.

Acknowledgements

This study was funded by the Danish Veterinary and Food Administration. The authors would like to thank the employees of Danish Crown and Tican for their cooperation and assistance with sampling and in setting up the study, in particular Marie B. Hansen, Vagner Bøge and Henrik Bækstrøm. The authors would also like to thank Jesper Larsen from the Danish Classification Inspection, for support with the meat inspection data and Margarida Arede for help with sampling.

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4.3 Manuscript 3: Computional algorithm for lifetime exposure to antimicrobials in pigs using register data—The LEA algorithm.

Anna Camilla Birkegård, Vibe Dalhoff Andersen, Tariq Halasa, Vibeke Frøkjær Jensen, Nils Toft, and Håkan Vigre.

Preventive Veterinary Medicine, 2017, 146: 173-180, 10.1016/j.prevetmed.2017.08.008

Preventive Veterinary Medicine 146 (2017) 173–180

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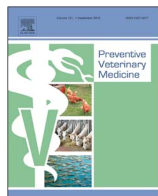

Computional algorithm for lifetime exposure to antimicrobials in pigs using register data—The LEA algorithm

Anna Camilla Birkegård[a,*], Vibe Dalhoff Andersen[b], Tariq Halasa[a], Vibeke Frøkjær Jensen[a], Nils Toft[a], Håkan Vigre[b]

[a] National Veterinary Institute, Technical University of Denmark, Kemitorvet, 2800 Kgs., Lyngby, Denmark
[b] National Food Institute, Technical University of Denmark, Kemitorvet, 2800 Kgs., Lyngby, Denmark

A R T I C L E I N F O

Keywords:
Antimicrobial data
Pig population
Algorithm
Movement pattern

A B S T R A C T

Accurate and detailed data on antimicrobial exposure in pig production are essential when studying the association between antimicrobial exposure and antimicrobial resistance. Due to difficulties in obtaining primary data on antimicrobial exposure in a large number of farms, there is a need for a robust and valid method to estimate the exposure using register data.

An approach that estimates the antimicrobial exposure in every rearing period during the lifetime of a pig using register data was developed into a computational algorithm. In this approach data from national registers on antimicrobial purchases, movements of pigs and farm demographics registered at farm level are used. The algorithm traces batches of pigs retrospectively from slaughter to the farm(s) that housed the pigs during their finisher, weaner, and piglet period. Subsequently, the algorithm estimates the antimicrobial exposure as the number of Animal Defined Daily Doses for treatment of one kg pig in each of the rearing periods. Thus, the antimicrobial purchase data at farm level are translated into antimicrobial exposure estimates at batch level. A batch of pigs is defined here as pigs sent to slaughter at the same day from the same farm.

In this study we present, validate, and optimise a computational algorithm that calculate the lifetime exposure of antimicrobials for slaughter pigs. The algorithm was evaluated by comparing the computed estimates to data on antimicrobial usage from farm records in 15 farm units. We found a good positive correlation between the two estimates.

The algorithm was run for Danish slaughter pigs sent to slaughter in January to March 2015 from farms with more than 200 finishers to estimate the proportion of farms that it was applicable for. In the final process, the algorithm was successfully run for batches of pigs originating from 3026 farms with finisher units (77% of the initial population). This number can be increased if more accurate register data can be obtained.

The algorithm provides a systematic and repeatable approach to estimating the antimicrobial exposure throughout the rearing period, independent of rearing site for finisher batches, as a lifetime exposure measurement.

1. Introduction

In recent decades, authorities have enforced regulatory initiatives to regulate the usage of antimicrobials in an attempt to reduce antimicrobial resistance (AMR) in livestock animals. However, there is a general need for a quantitative assessment of the relationship between AMR and antimicrobial exposure in livestock animals. In studies of this relationship, reliable estimates of antimicrobial exposure in batches of livestock animals during different rearing periods are needed. Here, we define a batch of animals as animals sent to slaughter on the same day from the same farm. In modern conventional pig production, the time from birth to slaughter is approximately six months. Therefore, we assume that the antimicrobial exposure during the entire lifetime of pigs influences the levels of AMR found at slaughter. Previous studies performed in pig farms have shown that AMR at one time point was associated with antimicrobial exposure in previous rearing periods (Dunlop et al., 1998; Rosengren et al., 2007; Varga et al., 2009). Furthermore, in population studies estimates of antimicrobial exposure

Abbreviations: LEA, lifetime exposure to antimicrobial; CHR, Central Husbandry Register; PMD, Database of Pig Movements; ADD, $_{kg}$Animal Defined Daily Dose for treatment of one kg pig

* Corresponding author.
E-mail address: acbir@vet.dtu.dk (A.C. Birkegård).

http://dx.doi.org/10.1016/j.prevetmed.2017.08.008
Received 21 September 2016; Received in revised form 7 August 2017; Accepted 10 August 2017
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must reflect the actual use in batches during the lifetime of the pigs. This can be difficult to obtain when using farm level register data because pigs are often moved from the farm, where they were born.

In this study, we present a computational algorithm that use data from registers to translate antimicrobial purchase data on farm level to antimicrobial exposure on batch level. The antimicrobial exposure is calculated for three rearing periods (piglet, weaner, and finisher period) and as a total estimate of the amount of antimicrobial exposure. We call this the LEA approach. The concept of the LEA approach was presented by Andersen et al. (2017), who showed that the LEA estimate explained more of the variation in the AMR levels in finishers than estimates of the antimicrobial usage in finisher units only or at the farms in general did. The principle of the LEA approach presented by Andersen et al. (2017) was a database estimation of the amount of antimicrobials that finishers at ten farms had been exposed to during their lifetime and independent of rearing site. Here, we further develop the approach to provide the LEA estimates for larger population using a systematic computational approach.

In Denmark, all purchases of antimicrobials for use in livestock are registered in the national veterinary medicine register (VetStat) (Stege et al., 2003). Antimicrobials are prescription-only drugs and only sold through veterinarians, pharmacies, or feed mills. The prescribing veterinarian has to reach a diagnosis prior to prescription. Thus, antimicrobials used for prophylactic treatments and as growth promoters are prohibited in Denmark (Anonymous, 2016a). All movements of pigs in Denmark are mandatory to register in the national database for pig movements (PMD). Coupling information on movements of pigs and antimicrobial purchases allows tracing pigs to the farms where they have been and calculate the amount of antimicrobials they were exposed to in the different rearing periods. This is the essence of the LEA approach, which generates data on antimicrobial exposure to batches of pigs with a high resolution both in time (exposure in different rearing periods) and space (usage at different farms). High temporal and spatial resolution is critical in the study of how antimicrobial exposure influences the levels of AMR in livestock animals (Collineau et al., 2017). Henceforth, the LEA estimates may be used for studying the association between antimicrobial exposure and AMR at batch level in population studies and risk assessments where collection of primary data about the antimicrobial usage at farm level is not feasible.

The objective of the presented work was to develop and optimise an algorithm designed to implement the LEA approach presented by Andersen et al. (2017) to the majority of slaughter pigs in Denmark. Furthermore, we validate the algorithm using farm data on actual antimicrobial usage. The algorithm was run on Danish farms with a conventional production of slaughter pigs to estimate the proportion of farms for which the algorithm is applicable.

2. Materials and methods

The LEA algorithm estimates the total amount of antimicrobial exposure during the lifetime of a batch of pigs. A batch was defined as a group of pigs sent from the same farm to the same slaughterhouse on the same day. Briefly, the algorithm was run for batches of finisher pigs starting at their date of slaughter. Based on the date of slaughter and identity of the farm with the finisher unit, the algorithm retrospectively estimates at which farm(s) they were reared as piglets and weaners. This tracing process is Step 1 (Estimating the age group of moved pigs) and Step 2 (Production chain trace back) of the algorithm. Subsequently, the antimicrobial exposure per pig per day was estimated for the batches for each of the rearing periods, as described in Step 3 (herd size calculation) and Step 4 (antimicrobial exposure smoothing) of the algorithm (Fig. 1). Finally, the lifetime exposure to antimicrobials was calculated as the sum of antimicrobial exposure in the three rearing periods.

2.1. Register data used by the LEA algorithm

The LEA algorithm used data from three national registers: VetStat, the PMD, and the CHR. The databases are owned by the Danish Veterinary and Food Administration. More information regarding these three databases can be found in Supplement material 1.

Data from the CHR were extracted twice – in September 2014 and in March 2015 – to account for changes in the farm demographics between the beginning and end of the study period. The number of pigs in each age group (weaners, finishers, and sows) was calculated as the mean of the two data extractions. The PMD data were extracted in April 2015, and data from VetStat were extracted twice – observations from 1st of September 2013 to 30th of April 2015 were extracted in June 2015 and observations for May 1st to July 1st were extracted in October 2015. The two subsets were subsequently merged.

In this paper, we define a farm as a premise housing pigs according to the CHR.

The algorithm only uses data from pharmacies, accounting for more than 99.9% of antimicrobials sold for use in pigs in the farms included in the present study (based on calculations from VetStat data).

2.2. LEA algorithm

The LEA algorithm operationally runs in four steps (Fig. 1): 1) Estimating the age group of moved pigs; 2) Production chain trace back; 3) Herd size calculation; 4) Antimicrobial exposure smoothing.

The algorithm deals with three rearing periods of a pig produced for slaughter at approximately 100 kg: the piglet period (birth-7 kg), the weaner period (7–30 kg weight) and the finisher period (30–100 kg, approximately).

In this study the LEA algorithm was applied to all Danish pig farms with more than 200 finishers (according to the CHR register data) who sent pigs to slaughter between January 1st and March 2015, 31st. For each farm, the batch of pigs that were sent to slaughter closest to the 15th of February 2015 was chosen.

The LEA algorithm was written and run in R version 3.2.2 (R Core Team, 2017). A brief description of each of the four steps follows, and further details are provided in Supplement 2.

2.2.1. Step 1: estimating the age group of moved pigs

The PMD contains no information about the age of the pigs moved. In order to calculate the antimicrobial exposure defined by age group, this information must be estimated in order to track the pigs correctly and calculate the number of pigs. The age group was estimated based on:

i) The assumption that a slaughter pig is moved up to three times during its lifetime. The pig is first moved from piglet unit to weaner unit at a bodyweight of approximately 7 kg and then from the weaner unit to a finisher unit at a bodyweight of approximately 30 kg. The movement from a unit to the subsequent unit can happen within the same farm or between farms. The third and final move is from the finisher unit to the slaughterhouse at a bodyweight of approximately 100 kg. The only certain movement is to the slaughterhouse.
ii) The number of pigs moved. There is a limit to the number of pigs of a specific age group a vehicle may transport at once. We assumed that, in the majority of cases, the number of pigs transported would be as close to this limit as possible and consequently that the number of pigs moved would reflect the age group of the moved animals. We do not have an exact estimate for number of pigs moved that would reflect a specific age group. Therefore, we tested different settings (see the section "test of the parameter settings"). To distinguish between movements of sows and movement of finishers, the cut-off$_{sow}$ was defined as either 40 or 60 pigs per movement. To distinguish between movements of finishers and

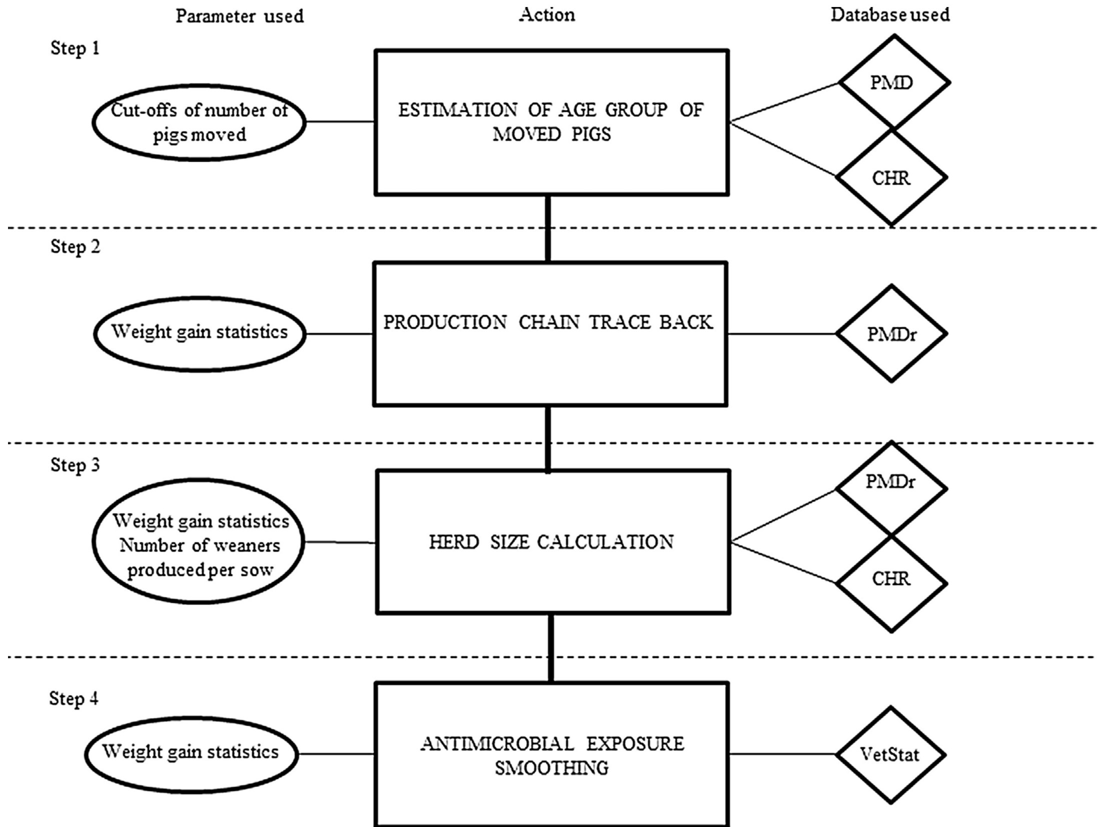

Fig. 1. Overview of the four steps in the LEA algorithm. The figure shows the register data and assumptions used in each step. PMD: Database of Pig Movements, CHR: Central Husbandry Register, PMDr: revised Database of Pig Movements (after estimating the age group of moved pigs). The weight gain statistics were obtained from Danish production statistics (Jessen, 2015).

movement of weaners, the cut-off$_{weaner}$ for the number of pigs moved was defined as either 250 or 350 pigs per movement. The values for the cut-offs were based on the median number of pigs moved to and from farms with only one age group. Only one set was used in the final algorithm

iii) The information about sending and recipient farms. In the algorithm, a farm could only send and receive pigs of an age group present on the farm according to the CHR. This criterion ensured that when pigs were moved into a farm only having finishers, the pigs would be defined as finishers, irrespective of the number of pigs moved. Furthermore, pigs sent to a slaughterhouse only for finishers, were defined as finishers. Likewise, pigs sent to a slaughterhouse that only slaughtered sows were defined as sows.

2.2.2. Step 2: production chain trace back

The algorithm works retrospectively for each batch of pigs by first identifying the farm(s) with the weaner unit and then the farm(s) with the piglet unit.

Transfer windows were defined in order to estimate the timeframe at which a pig would progress from the piglet unit to the weaner unit and from the weaner unit to the finisher unit (Fig. S1). The timeframe of the transfer windows were defined using the weight gain statistic for farms with the highest and lowest weight gain. The transfer window was defined as the difference in days between fast (median for 25% fastest growing pigs) and slow growing pigs (median for the 25% slowest growing pigs) (Jessen, 2015). The daily weight gain statistic, which describes how fast the pigs are growing in different rearing periods were used to estimate the duration (in number of days) of the rearing period within each rearing unit (piglet, weaner, and finisher unit). The transfer windows were extended to fit irregular movement patterns. Three sets of increases (either 50%, 100%, or 250% of the original transfer window) were assessed. Only one value was used in the final algorithm.

With the date of slaughter as onset, the algorithm retrospectively identified weaner and piglet units where the pigs were housed during their weaner and piglet period, respectively. If the pigs were moved to a new farm when changing rearing periods, the new farm was identified based on the transfer window and the PMD (see step 2 in the Supplementary material 2).

2.2.3. Step 3: herd size calculation

The algorithm estimates the LEA for a batch of pigs. However, pigs within the batch are housed with other pigs, and the purchase of antimicrobials is registered at farm level. Therefore, the exposure for the batch has to be calculated as an average for all pigs in the same age group present at the farm during the rearing period. In the CHR, the number of pigs are reported as the average number of pigs on a normal day of production (Anonymous, 2016b), which is most likely an imprecise estimate of the true number of pigs. Therefore, the LEA algorithm aims at obtaining a better estimate of the average number of pigs at the farm in each of the rearing periods. This is calculated based on information found in the CHR, corrected using records of the movements into and out of the farm (see Supplement 2).

2.2.4. Step 4: antimicrobial exposure smoothing

The LEA algorithm calculates the antimicrobial exposure per batch. This is done for each of the three rearing periods. The estimates for antimicrobial exposure are calculated as a sum of average daily ADD_{kg} per pig across the period when the batch was assumed to be present in the rearing unit. The antimicrobial exposure is calculated per class of antimicrobial for 11 different classes, and as a total of all antimicrobials used during each rearing period. These 11 classes of antimicrobials cover all antimicrobial usage in the Danish pig production. For the combination products, the amount of active product was calculated per

antimicrobial class and included in the sum of that class.

Antimicrobial exposure is measured in Animal Defined Daily Doses for treatment of one kg (ADD_{kg}). The ADD_{kg} is defined as the average approved dose for the main indication in the particular animal species for treatment of one kilogram pig. The ADD_{kg} can be used across age groups, as it is independent of animal bodyweight (Jensen et al., 2011). The antimicrobial exposure is calculated as the sum of ADD_{kg} used per day per pig in the batch. Thus, the estimated exposure does not take into account, when the pigs were treated, but merely estimates the amounts of antimicrobial used for the production of a given pig.

In VetStat, antimicrobial purchases for use in sows and piglets are registered in the same age group category. Previous studies have shown that antimicrobial exposure of the sow will affect the AMR pattern in the piglet (Callens et al., 2015; Mathew et al., 2005). Therefore, we use the antimicrobial purchase for use in sows and piglets as an estimate for the antimicrobial exposure of piglets, thus including both the direct and indirect selection pressure on the piglet microbiota.

No information about the duration of treatment, number of pigs treated, or exact dates of treatment are available in the VetStat data. In general, the veterinarian is allowed to prescribe antimicrobials that are expected to be needed until next planned visit. Therefore, it was assumed that antimicrobials would be used between the purchase date of a product and the following purchase date of a product with the same antimicrobial class and dispersing form (parenteral or oral). We call this our "smoothing method". Details about the smoothing and calculation of antimicrobial exposure can be found in Supplementary material 2.

2.3. Test of the parameter settings

Only batches of pigs where the LEA algorithm could identify at least one weaner unit and one piglet unit were included in the further analyses. The tracing of pigs back to the weaner and piglet units depended on the setting of the transfer windows and the identification of the age group of the pigs moved in the PMD. The LEA algorithm was run 12 times with different settings for the transfer window, cut-off$_{weaner}$ and cut-off$_{sow}$ to evaluate the effectiveness of the algorithm to trace the pigs back to weaner and piglet units. The parameter settings optimizing the number of batches where both weaner and piglet units could be identified were chosen for the final LEA algorithm.

2.4. Adjustments to the number of pigs calculated

The LEA algorithm does not accurately calculate the number of pigs present for all of the farms. The number of finishers and weaners registered in the CHR is used as an alternative to the LEA estimate of the number of pigs present in the following circumstances:

i) If the number of weaners and finishers is calculated to be zero or below zero.
ii) If the absolute difference between the calculated number of pigs and the number of pigs registered in the CHR for the age group, divided by the number of pigs registered in the CHR, is calculated to be above 0.9.
iii) If the absolute value for the movement balance ratio is calculated to be above 1. The movement balance ratio is calculated as the sum of pigs produced at the farm and the number of pigs sent out, subtracted by the number of pigs received, and subsequently divided by the total number of pigs registered at the farm.

All above of the above are results of missing information of movements of pigs into or out of the farm causing an underestimation or overestimation of the number of pigs present at the farm; or because the weight gain parameters did not fit the specific farm.

2.5. Validation of the smoothing method

The validity of our smoothing method was assessed by comparing the estimated value of exposure to actual farm registrations of antimicrobial usage.

We obtained data from 7 finisher units, 4 weaner units, and 4 piglet units, including one farm with all three units (an integrated farm). The finisher and weaner units received pigs from the weaner and piglet units, respectively. The following information was available in the data registered on the farms: the farm number (CHR-number), age group of treated animals, date of start of treatment, date of end of treatment, product name, and amount of product used in the duration of the treatment.

For the period, where farm registrations were available, up to 15 dates of slaughter was chosen at random using the "sample"-function in R (R Core Team, 2017). These dates were used to set the periods for which the antimicrobial exposure should be calculated. The periods were set to be 75 days (number of days in the finisher period, see Supplementary material 2) prior to the date of slaughter. This was done to be able to run the smoothing part of the LEA algorithm.

For the piglet and weaner units, we chose up to 30 slaughter dates at random for the finisher units that received pigs from the piglet and weaner units. These were sampled among slaughter dates ensuring that there was farm data available. Hereafter, with use of the sampled slaughter dates and number of days in each period (30 and 58 days, defined in Supplement material 2) the dates for the piglet and weaner periods were defined. This was done to be able to run the smoothing part of the LEA algorithm for the defined dates.

The smoothing step of the LEA algorithm (included in Step 4) was run for the defined periods for the finisher, weaner, and piglet units to obtain estimates of antimicrobial exposure. The farm records were used to calculate the amount of antimicrobials used in each period. Both antimicrobial estimates were calculated in ADD_{kg} without taking into account the number of pigs. This was done to be able to only validate the smoothing method. The two antimicrobial estimates (smoothed and farm data) were compared in scatterplots and the spearman correlation was calculated between the two estimates for the piglet, weaner, and finisher units, respectively.

2.6. Lifetime exposure to antimicrobials and movement patterns

The output of the LEA algorithm was used to describe the movement patterns and, the patterns of antimicrobial exposure in the Danish pig population.

The distribution of antimicrobial exposure was visualized using boxplots. To make the visual presentation of the output of the LEA algorithm comparable to official reports of the antimicrobial usage in Denmark (Danish Veterinary and Food Administration, 2017) the antimicrobial exposure was standardized to ADD/100 pigs/day. This was calculated as the ADD_{kg} divided by the standard weight at treatment (sows: 200 kg, weaners: 15 kg, finishers: 50 kg) and divided by the number of days in each period.

All analyses were done in R (R Core Team, 2017) using RStudio (RStudio team, 2016).

3. Results

3.1. Test of the parameter settings

The settings for the transfer window and the settings for the cut-offs affected the number of farms that were traceable. The optimal parameter settings were as follows: the transfer window was increased by 250% compared to the original transfer window (Fig. S1), the cut-off$_{weaner}$ was set to 250 pigs, and the cut-off$_{sow}$ was set to 60 pigs (Fig. 2). The percentage of the farms, where an associated weaner unit could be identified was slightly higher (94.33%) for the chosen setting

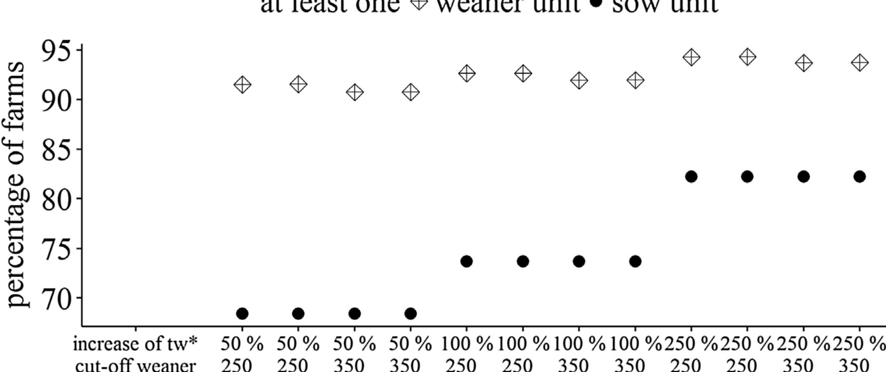

Fig. 2. Performance of the traceability of the LEA algorithm with different parameter settings. The percentage of the traceable batches of pigs is shown as a function of the parameter settings: Increase of the transfer window (tw): percentage of transfer window extension; cut-off$_{weaner}$: cut-off for the number of pigs moved used to distinguish between weaners and finishers; cut-off$_{sow}$: cut-off for the number of pigs moved used to distinguish between sows and finishers. *tw: transfer window.

of 250/250/60, than the setting of 250/250/40 (94.28%). The settings for the transfer window affected the number of instances where no weaner units could be identified. The settings for the cut-off$_{weaner}$ affected the number of batches of pigs that were traceable to the piglet unit.

3.2. Validation of smoothing estimation

Fig. 3 shows the comparison between the LEA smoothed purchase data and actual farm usage data used to validate the smoothing method. The spearman correlations were piglet period: $\rho = 0.9$, weaner period: $\rho = 0.6$, finisher period: $\rho = 0.6$, all p-values < 0.0001. The correlations were higher for the weaner and finisher periods, when pleuromutilins were not included in the calculations, $\rho = 0.8$ and p-values < 0.0001 for both. The correlations were based on 101 observations from the 7 finisher units, 51 observations from the 4 weaner units, and 99 observations for the 4 piglet units.

3.3. Lifetime exposure to antimicrobials and movement patterns

There were 3954 conventional farms, with more than 200 finisher pigs registered in CHR, sending pigs to slaughter between January 1st and March 31st 2015. Hereof, 928 (23%) were excluded because they were not traceable by the LEA-algorithm.

In total, we estimated the LEA for batches of pigs originating from finisher units at 3026 farms (77%), hereafter referred to as the traceable batches of pigs. The batches of pigs had spent their weaner period at 1991 different farms and their piglet period at 1524 different farms.

For 962 of the farms with finisher units for the traceable batches, the number of finishers registered in the CHR was used instead of the calculated number of finishers. The calculated number of weaners was exchanged for the number of weaners registered in the CHR for 412 of the 1991 farms with weaner units.

Fig. 4 illustrates the different lifetime movement patterns for 80% of

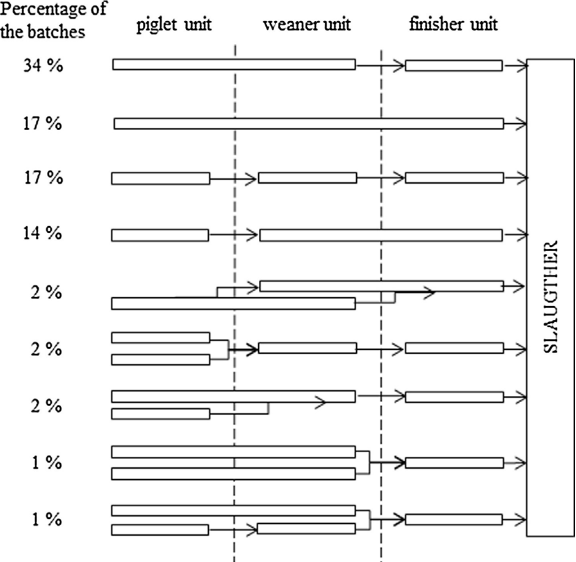

Fig. 4. Lifetime movement patterns for the traceable batches of pigs, and the percentage of the batches with the specific movement pattern. "Traceable" refer to that the LEA algorithm could assign at least one weaner and one piglet unit to the batch. The figure illustrates approximately 80% of the traceable batches of pigs. The remaining 20% had very diverse movement patterns and each of these patterns represented less than 1% of the farms. One horisontal bar is one physical farm. An arrow indicate a movement to a new farm or a movement to the slaughterhouse.

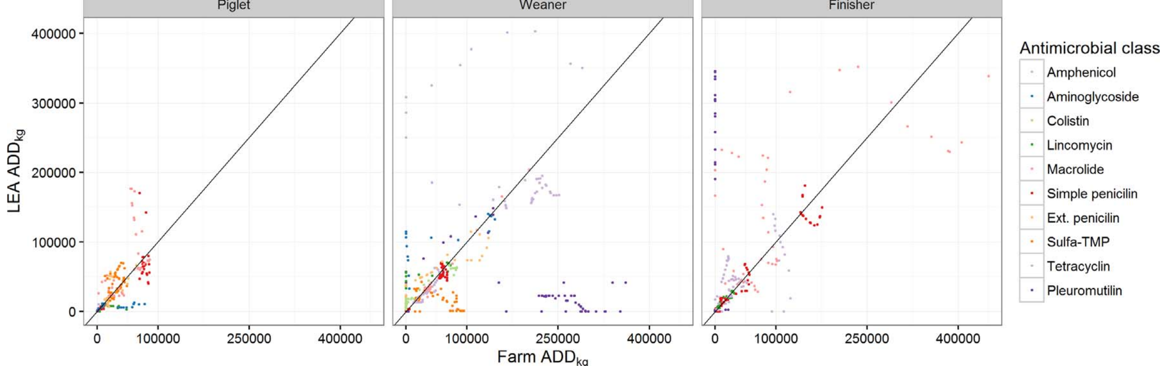

Fig. 3. Validation of the smoothing method. Scatterplot of the correlation between smoothed antimicrobial exposures estimated from the LEA-algorithm (LEA ADD$_{kg}$) and data on antimicrobial usage from farm records (farm ADD$_{kg}$). The line indicates the perfect linear association between the two measures. Ext.: extended.

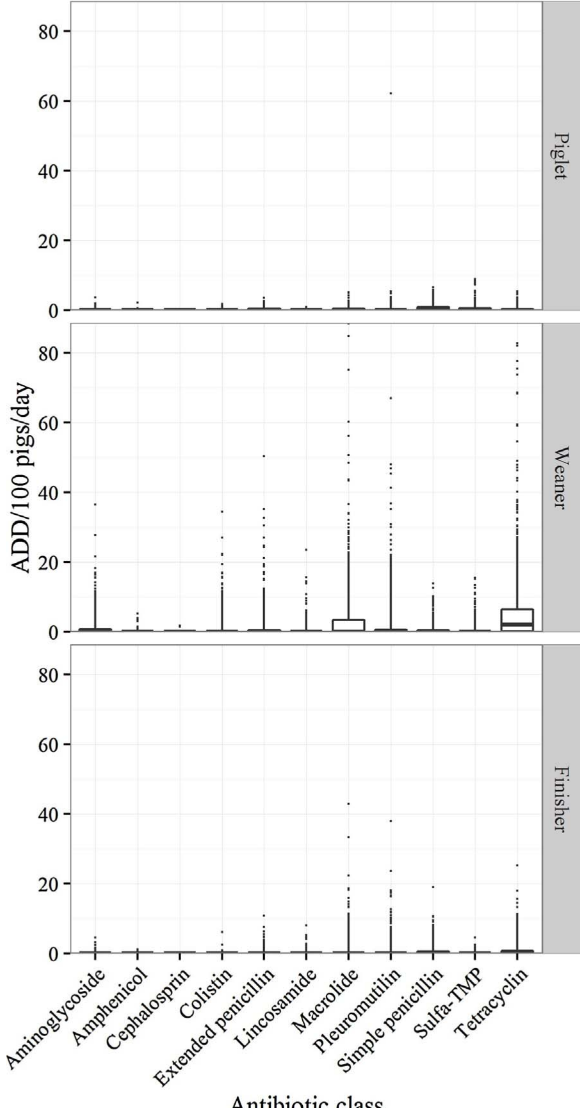

Fig. 5. Antimicrobial exposure in the three rearing periods, described for each antimicrobial type. The antimicrobial exposure was standardized to ADD/100 pigs/day. This was calculated as the ADD_{kg} divided by the standard weight at treatment (sows: 200 kg, weaners: 15 kg, finishers: 50 kg) and divided by the number of days in each rearing period. Two observations of antimicrobial exposure in the weaner period were removed due to high numbers (ADD/100 pigs/day of 1) that masked the overall distributions of the remaining observations. Lower and upper hinges describe the 25th and 75th percentiles and the middle hinge describes the median. The lower and upper whiskers extend from the lower and upper hinges to $+/-1.5$ times the distance between the 25th and 75th percentiles. Values below the lower whiskers or above the higher whiskers are considered outliers, and are plotted as dots (Wickham, 2009).

the traceable batches of pigs together with the percentage of batches showing a particular movement pattern. The remaining 20% of the batches had very different movement patterns, each describing < 1% of the batches. The movement of the majority slaughter pigs can be described by 9 different patterns (Fig. 4).

The distribution of exposure (measured as average amount of ADD per 100 pigs per day in the batch) to different antimicrobial classes in each rearing period can be seen in Fig. 5. The lifetime exposure to antimicrobials can be seen in Fig. 6.

4. Discussion

There were 3954 farms with conventional production of finishers in the register data used in this study. This paper presents an implementation of the LEA approach on the routinely registered data about antimicrobial purchase and movement of pigs between farms in a computational algorithm that enabled estimation of the antimicrobial exposure from birth to slaughter at batch level for 80% of Danish pig farms with finisher units. The LEA algorithm facilitates estimation of the antimicrobial exposure for a batch of pigs slaughtered on a given date and is based on statistics regarding production parameters and the use of register data. The LEA algorithm therefore enables retrospective estimation of the antimicrobial exposure during different rearing periods and in total in a large population. As shown in this study, 80% of finisher pigs, which could be traces back, are moved to a new farm at some point in time. Studies have shown that antimicrobials consumed in the early stages of a pig's life affect the level of antimicrobial resistance found in later stages (Dunlop et al., 1998; Rosengren et al., 2007; Varga et al., 2009). It is therefore important to consider all rearing periods when assessing the effect of antimicrobial exposure on AMR in slaughter pigs.

In the LEA algorithm, there are assumptions about age groups of moved pigs, number of days spent in each rearing unit, and the duration of treatment. These assumptions were necessary because we lack specific information at farm level regarding these parameters. Therefore, the estimated exposure to antimicrobials in the different batches is surrounded with some uncertainty. The assumption with highest influence on the traceability of the batches concerned the increase of the transfer windows (Fig. 2). The cut-off values used to define the age group in PMD could be rendered unnecessary by implementing the age group in the PMD.

In the data on antimicrobial purchases from VetStat, no information is available on the duration of treatment. Therefore, we assumed that the amount of antimicrobials of a given antimicrobial class and dispensing form (parenteral or oral) will be used until the next date when a product of same antimicrobial class and dispensing form was purchased. This assumption was validated using data from farm records on antimicrobial usage. We found generally a good correlation between the smoothed estimate and the farm data. This indicates that the smoothing method gives a sufficiently good estimation of the actual exposure level and duration of treatments. This is recommended by Collineau et al. (2017) to include in studies of the relationship between AMR and antimicrobial exposure, which is the intended use of the LEA algorithm. However, differences between the smoothed and the actual farm data were observed, most profound for pleuromutilins (Fig. 3). Whether this difference is caused by the algorithm or that pleuromutilins sometimes are purchased for use in one age group but used in another is unknown. The difference was particularly observed in the batches from the integrated farm (results not shown). The LEA algorithm cannot take into account whether antimicrobial products are purchased for use in one age group, but in reality used in another age group, because only purchasing information is available in the register data. Our results show that in some farms sometimes antimicrobials are used for another age group than the age group it was prescribed and purchased for. For instance, in Fig. 3, according to the LEA algorithm pleuromutilins are often used in finisher units. However, in several of these farms the real farm data showed a zero ADD value for pleuromutilins. In the weaner units, the pattern is opposite. Several of the weaner units using pleuromutilins according to the real farm data had no use according to the LEA algorithm. In 90% of the finisher units purchasing but not using pleuromutilins in finishers, the pleuromutilins were used in a weaner unit at the same farm. Other antimicrobial classes were also purchased for use in one age group but used in another. However, the pattern was most pronounced for pleuromutilins. Considering the estimation of lifetime exposure, the discrepancy between which age group the antimicrobials has been prescribed to and the actual use will be partly excluded from the estimate of the LEA algorithm, because lifetime exposure ignores the actual age when the pigs have been exposed to the antimicrobial.

Another assumption in the LEA algorithm was the number of days in

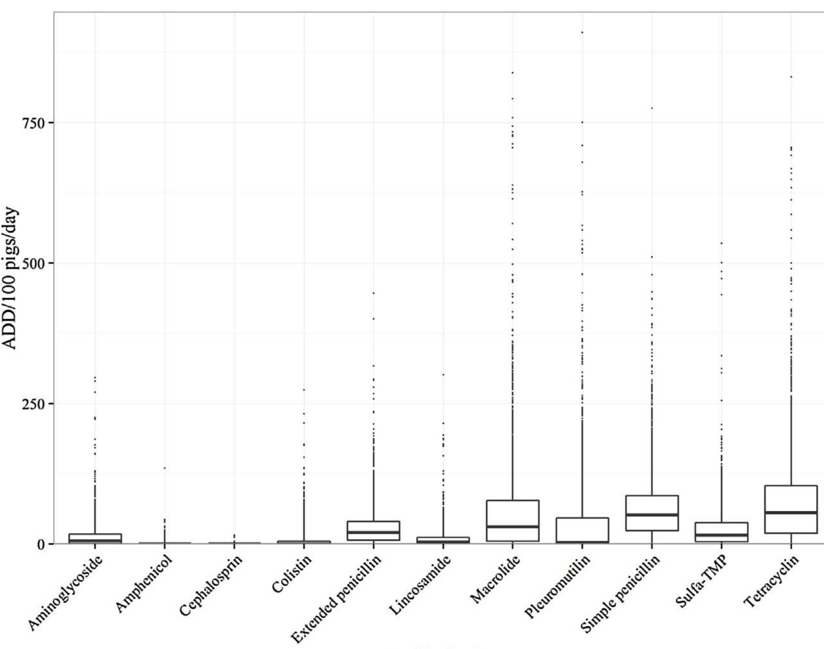

Fig. 6. Lifetime exposure to 11 different antimicrobial classes. Five observations of lifetime exposure were removed due to high numbers (ADD_{kg} above 1000) that masked the overall distributions of the remaining observations. Lower and upper hinges describe the 25th and 75th percentiles and the middle hinge describes the median. The lower and upper whiskers extend from the lower and upper hinges to $+/-1.5$ times the distance between the 25th and 75th percentiles. Values below the lower whiskers or above the higher whiskers are considered outliers, and are plotted as dots (Wickham, 2009).

each rearing period. This assumption was based on the median weight gain per rearing period of Danish pig farms (Jessen, 2015). These values were fixed, meaning that the same values were used for all farms. This was due to the lack of information of farm specific weight gains, which could be obtain through productivity data. Applying productivity data at farm level would increase the validity of the LEA algorithm. However, this estimate cannot be obtained from all farms and we therefore did not endeavour further to integrate it into the LEA algorithm at this stage. Furthermore, there is only a three-nine day difference in time between the median growing pigs and the fastest or slowest growing pig depending on the rearing period (Fig. S1). Therefore, we believe that using fixed values for number of days in each rearing period do not add a substantially error.

We found that the LEA algorithm could be used to trace back to the farm of origin for 77% of Danish pig farms with a conventional production of finishers and with more than 200 finishers registered in CHR. The excluded farms were found to be significantly smaller than the included ones. It is the nature of the algorithm that batches of pigs from integrated farms will not be excluded as they do not require data from the movement database to be assigned a piglet and weaner unit. It was estimated that 20% of the excluded farms were excluded due to lack of registrations in the PMD. The remaining cases were excluded due to production systems not covered by the algorithm. These production systems could have a shorter or longer nursing time as well as a lower or higher daily weight gain. Although registers on farm demographics and the movement of pigs in Denmark do exist, they are neither perfect nor complete, as reflected in this study.

Using the data on antimicrobial purchases available from VetStat poses further challenges. One challenge is that antimicrobials purchased for use in piglets will be registered for use in sows. Thus, the LEA algorithm estimates the antimicrobial use for production of slaughter pigs including the antimicrobial use for the sows in the piglet unit. However, the selection pressure on the environment of the piglet (including the sow) is also of relevance for the occurrence of AMR in the piglet (Callens et al., 2015; Mathew et al., 2005). Nevertheless, the predictive value would presumably be better if the antimicrobial use in the sow and the piglet could be separated.

The LEA algorithm was used to describe the pattern of antimicrobial exposure in Danish batches of slaughter pigs (Figs. 5 and 6). Raw values of the sum of average daily amount of antimicrobial exposure showed that the pigs are exposed to larger amounts of antimicrobials in the finisher period than in the weaner period (results not shown). However, standardizing the values to ADD/100 pigs/day showed that the weaners are exposed to a higher dose per day than the finishers (Fig. 5) due to lower bodyweight and a lower number of days in the weaner period. The algorithm estimates only pigs slaughtered in Denmark. Consequently, the antimicrobial consumption of weaners exported to be fattened abroad are not part the antimicrobial estimates in the weaner units.

5. Conclusions

We have developed an algorithm (LEA), which based purely on register data, production parameter statistics, and relatively few assumptions estimates the lifetime antimicrobial exposure for a batch of pigs, regardless of whether the pigs remained at the same farm or were moved to other farms during their lifetime. The LEA algorithm works for the majority of pig farms in Denmark. This gives us the opportunity to study the association between antimicrobial exposure and AMR at batch level in large population studies and to perform risk assessments at national level.

Acknowledgements

We would like to thank Mette Fertner and Nana Dupont for help calculating the ADD_{kg} from the VetStat data, and discussions about how to calculate the LEA. Furthermore, we would like to thank Amanda Kruse, Gitte Blach Nielsen and Leonardo de Knegt for a valuable discussion leading to the assumption used in the LEA algorithm. Finally, we would like to thank Mette Ingvordsen, Lisbeth Harm Nielsen and Jan Dahl for feedback on the initial LEA algorithm. This study was funded by the Danish Veterinary and Food Administration. The funding party had no influence on the design of the study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.prevetmed.2017.08.008.

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Supplement material 1 – details on register data used

All pig farms in Denmark are registered in the Central Husbandry Register (CHR) with a unique identification number, which is the key ID used in all national register databases for livestock animals. The CHR includes information on the number of pigs on a normal production day in three age groups: sows (all adult breeding animals), weaners (7-30 kg) and finishers (>30 kg, but not including sows), as well as the production type. The production type is the type of holding defined by the farmer, such as production pig farm, organic pig farm, free-range pig farm, slaughterhouse, rendering plant, exporting stable or animal fair. By law, all pig farmers are obliged to register data in the CHR and update the information one or two times per year depending on the farm size (Anonymous, 2016a). However, the number of pigs registered in the CHR might differ from the actual number of pigs present in a given rearing period.

The Database of Pig Movements (PMD) contains information about all movements of pigs within, and exports from, Denmark. Movements of pigs are mandatory to register in the PMD, including movements of pigs to slaughterhouses and rendering plants. Information includes the CHR-number of the sending and the recipient farms, the date of movement and the number of pigs moved. For movements to the slaughterhouse the movement date is equal to the date of slaughter.

VetStat contains data on all purchases of prescription-only drugs to use in treatment of livestock animals. In Denmark, antimicrobials for use in animals can only be purchased with a prescription from a veterinarian (Anonymous, 2016b). Antimicrobials for use in livestock are sold through pharmacies, veterinary practitioners and feed mills (Stege et al., 2003). The information available in VetStat includes (among other) purchase date, CHR number of the recipient farm, animal-species code, age group code, amount of product, and Anatomical Therapeutic Chemical Classification system code (ATC code). The age group code can be matched to the three age groups registered in the CHR. The ATC code can be used to identify products as antimicrobials.

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Supplementary Material 2 – detailed description of the LEA algorithm

The LEA algorithm encompasses the following 4 steps: 1) Estimating the age group of moved pigs; 2) Production chain trace back; 3) Herd size calculation; 4) Antimicrobial exposure smoothing.

The number of days that the batches of pigs spent in the piglet, weaner, and finisher units was specified in the LEA algorithm and calculated using estimates of the average daily weight gain from industry statistics. The pigs were expected to spend 30 days in the piglet unit, 53 days in the weaner unit (gaining 23 kg with a daily weight gain of 438 g) and 75 days in the finisher unit (gaining 70 kg with a daily weight gain of 931 g) (Jessen, 2015). Furthermore, it was assumed that 30.6 pigs were weaned per sow-year and that there was a mortality of 2.9% during the weaner period and 3.7% during the finisher period (Jessen, 2015). Finally, it was assumed that 4.4 weaners were produced on average per sow throughout the weaner period (30.6 weaners per sow per year/365 days per year * 53 days per weaner period = 4.4 weaner per sow per weaner period).

Step 1: Estimating the age group of moved pigs

The PMD contains no information on the age group of the moved pigs, and assumptions about the age group were therefore made in order to improve traceability and to calculate the number of pigs per age group. The age group of the moved pigs was determined based on information about the sending and recipient farms as well as the number of pigs moved (see main text).

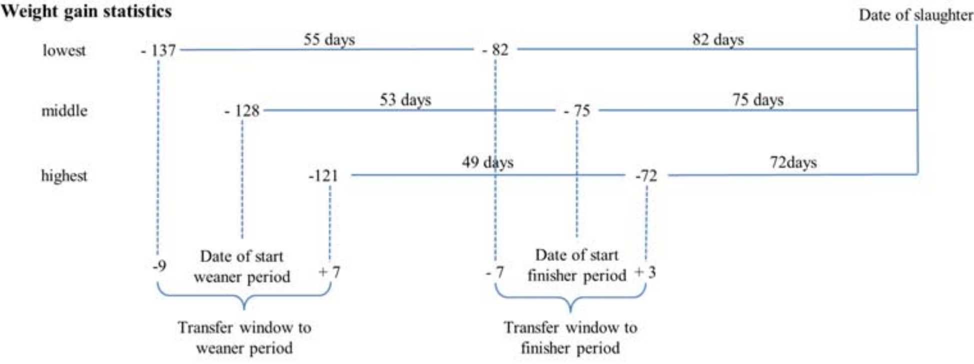

Figure S1 - Calculation of the transfer window before increase. The number used in the figure is based on how many days it takes a weaner to go from 7-30 kg, and a finisher to go from 30-100 kg, based on the average weight for the farm with the 25% lowest, 50% middle and 25% highest weight gain (Jessen, 2015). The figure shows how the transfer windows were defined. The onset is the date of slaughter and the transfer window from the weaner to finisher unit is between 82 days to 72 days before slaughter. The pigs in the batch will have been moved from the weaner units to the finisher units within this window.

Step 2: Production chain trace back

In order to know when a pig was moved from the weaner period to the finisher period, a transfer window was defined using the weight gain statistic for the farms with the highest and lowest weight gain (Jessen, 2015). The transfer window for the weaner period was defined in the same way (Fig. S1). Farms with weaner units were identified as farms moving weaners into finisher units during the transfer window to the finisher period. Farms with piglet units were identified in a similar manner. In some cases, pigs remained at the same farm throughout different periods of their life and additional pigs could also be added to the farm. The algorithm was designed to handle these different production systems.

The in-house production of pigs was estimated (Equations S1 and S2) to determine whether the farm should be identified as having the weaner or piglet unit on the same farm (i.e. the in-house production was greater than zero). If the LEA algorithm did not allocate a piglet unit in another farm to a weaner unit and the farm of this unit had a piglet unit, then the piglet unit of that farm was allocated to the weaner unit, irrespective of the in-house production. A likewise allocation was done for finisher units.

$$\text{in-house production}_{\text{weaner}} = \left(n_{\text{sow}} * \text{weaner/sow} - \sum_{i=1}^{N_w} \text{weaner}_{\text{out}}\right) * \left(1 - \text{mortality}_{\text{weaner}}\right) \quad \text{(Equation S1)}$$

$$\text{in-house production}_{\text{finisher}} = \left(\text{in-house production}_{\text{weaner}} - \sum_{i=1}^{N_f} \text{weaner}_{\text{out}}\right) * \left(1 - \text{mortality}_{\text{finisher}}\right) * \frac{N_f}{N_w}$$

(Equation S2)

where *weaner/sow* is the number of weaned pigs produced per sow in the weaner period; $mortality_{weaner}$ is the average mortality in the weaner period; N_w is the number of days in the weaner period; N_f is the number of days in the finisher period; $mortality_{finisher}$ is the average mortality in the finisher period; $weaner_{out}$ is the number of piglets moved out of the farms.

The sum of pigs moved to the finisher or weaner units from each weaner or piglet unit was calculated for the timeframe of the increased transfer window. For the farms identified as having their own weaner or piglet unit, the in-house production was used as the sum of movement. If the sum of movement was below zero for these farms, then the number of pen places for the respective age group was used instead. The sum of moved pigs was used to calculate the percentage of contribution (POC) (Equation S3 and S4). The POC was used to adjust the antimicrobial usage for the proportion of pigs in the finisher units that originated from each of the farms with weaner units (Step 4).

$$\mathrm{POC_y} = \frac{\mathrm{movement_{y\to x}}}{\mathrm{movement_{total,x}}} \qquad \text{(Equation S3)}$$

$$\mathrm{POC_z} = \frac{\mathrm{movement_{z\to y}}}{\mathrm{movement_{total,y}}} * \mathrm{POC_y} \qquad \text{(Equation S4)}$$

where $movement_{y \to x}$ is the sum of pigs moved from weaner unit to finisher unit; $movement_{total,x}$ is the total sum of pigs moved to finisher unit; $movement_{z \to y}$ is the sum of pigs moved from piglet unit to weaner unit; $movement_{total,y}$ is the total sum of pigs moved to weaner unit.

Step 3: Herd size calculation

The LEA algorithm calculates the number of pigs as the average of the daily number of pigs actually present during the rearing period. For each day of the finisher and weaner periods, the numbers of finishers or weaners, respectively, were calculated as the number of pigs moved out of the farm subtracted from the sum of the in-house production and the number of pigs moved into the farm. The mean number of pigs was then calculated for the period. Equation S5 and Figure S2 show how the mean number of finishers was calculated; the number of weaners was calculated in the same way.

$$\mathrm{n_{finisher}} = \frac{1}{\mathrm{N_f}}\left(\text{in-house production}_{\mathrm{finisher}} + \sum_{n=1}^{\mathrm{N_f}}\left[\mathrm{finisher_{in}} - \mathrm{finisher_{out}}\right]\right) \qquad \text{(Equation S5)}$$

where $finisher_{in}$ is the number of finishers moved into the finisher unit from another farm; $finisher_{out}$ is the number of finishers moved out of the finisher unit to another farm or slaughterhouse.

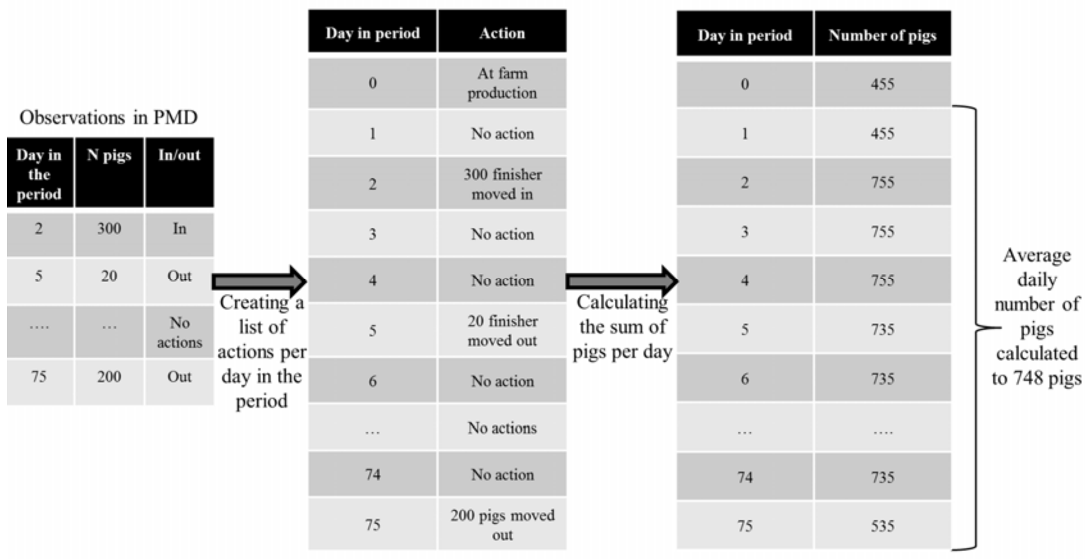

Figure S2 - Calculation of the average number of finishers per day during the finisher period.

….: dates in the period not shown in the table. PMD: Database of Pig Movements.

Step 4: Antimicrobial exposure smoothing

Antimicrobial exposure was calculated as an average for the number of pigs in the age group present at the farm and smoothed between two consecutive dates of purchase of a product of the same antimicrobial class and dispensing form. Therefore, the amount of antimicrobials purchased was smoothed out between the first and second date of prescription. The smoothing was done separately for 11 antimicrobial classes (amphenicol, aminoglycoside, cephalosporin, colistin, lincomycin, macrolide, extended-spectrum penicillin, simple penicillin, sulfa-TMP, pleuromutilin and tetracycline) and dispensing forms (parenteral and oral). Figure S3 shows an example of how antimicrobial exposure was calculated in the weaner period, and equation S6 shows how the daily smoothed ADD_{kg} was calculated.

$$\mathrm{ADD_{kg,smoothed}} = \frac{\mathrm{ADD_{kg,day,x}}}{\mathrm{n_{x,y}} * \mathrm{n_{pigs}}} * \mathrm{POC} \qquad \text{(Equation S6)}$$

where $ADD_{kg,day,x}$ is the amount in ADD_{kg} purchased at day x; $n_{x,y}$ is the number of days between day x and the next purchase at day y; n_{pigs} is the number of pigs in the age group.

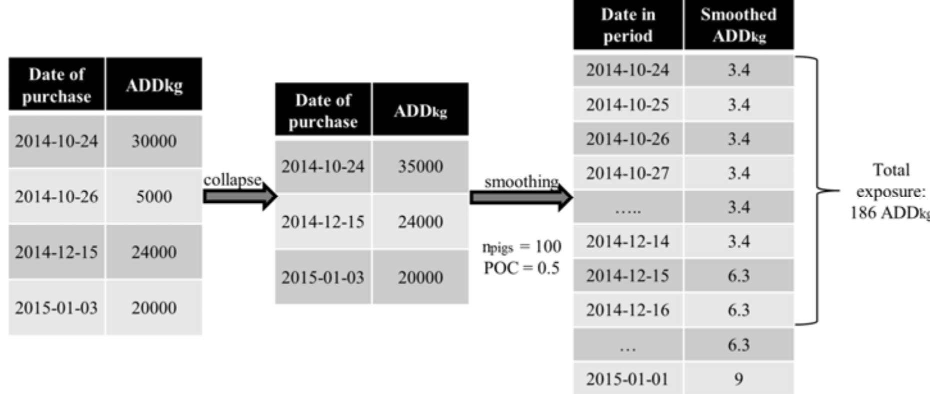

Figure S3 - Example of an antimicrobial exposure calculation during the weaner period.

….: dates in the period not shown in the table

The first step in calculating antimicrobial exposure was to combine antimicrobials purchased within 8 days, as we assumed these were used within the same treatment period. The average dose per day during the period within the two dates of prescription was calculated as the dose purchased divided by the number of days until next prescription. The dose was then divided by the number of pigs in the respective age group and multiplied by the POC (Equation S6). This step was completed for

weaner and piglet units because a batch could contain pigs originating from different farms. In the weaner and finisher units, the calculated numbers of pigs were used. In the piglet units, the number of sows registered in the CHR was used, as the actual number of sows at the farm was assumed to be approximately constant and in accordance with the number registered in CHR. It is illegal to store antimicrobials at a pig farm for more than 63 days for treatment of finishers, or 50 days for treatment of sows and weaners without a renewed prescription for the stored item (Anonymous, 2016). This renewal is not registered in VetStat. If the number of days between two purchases of the same type of antimicrobial exceeded the 50/63 days, these limits were used instead.

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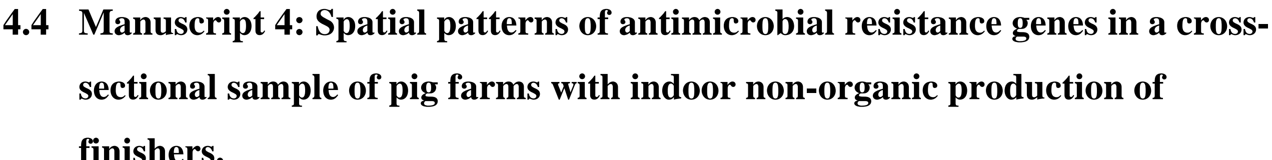

4.4 Manuscript 4: Spatial patterns of antimicrobial resistance genes in a cross-sectional sample of pig farms with indoor non-organic production of finishers.

Anna Camilla Birkegård, Annette Kjær Ersbøll, Tariq Halasa, Julie Clasen, Anders Folkesson, Håkan Vigre, snd Nils Toft

Epidemiology and infection. 2017;145(7):1418–1430, doi: 10.1017/S0950268817000206 .

Epidemiol. Infect., Page 1 of 13. © Cambridge University Press 2017
doi:10.1017/S0950268817000206

Spatial patterns of antimicrobial resistance genes in a cross-sectional sample of pig farms with indoor non-organic production of finishers

A. C. BIRKEGÅRD[1]*, A. K. ERSBØLL[2], T. HALASA[1], J. CLASEN[1], A. FOLKESSON[1], H. VIGRE[3] AND N. TOFT[1]

[1] *Technical University of Denmark, Frederiksberg, Denmark*
[2] *University of Southern Denmark, Copenhagen, Denmark*
[3] *Technical University of Denmark, Lyngby, Denmark*

Received 30 May 2016; Final revision 4 January 2017; Accepted 16 January 2017

SUMMARY

Antimicrobial resistance (AMR) in pig populations is a public health concern. There is a lack of information of spatial distributions of AMR genes in pig populations at large scales. The objective of the study was to describe the spatial pattern of AMR genes in faecal samples from pig farms and to test if the AMR genes were spatially randomly distributed with respect to the geographic distribution of the pig farm population at risk. Faecal samples from 687 Danish pig farms were collected in February and March 2015. DNA was extracted and the levels of seven AMR genes (*ermB, ermF, sulI, sulII, tet*(M), *tet*(O) and *tet*(W)) were quantified on a high-throughput real-time PCR array. Spatial differences for the levels of the AMR genes measured as relative quantities were evaluated by spatial cluster analysis and creating of risk maps using kriging analysis and kernel density estimation. Significant spatial clusters were identified for *ermB, ermF, sulII* and *tet*(W). The broad spatial trends in AMR resistance evident in the risk maps were in agreement with the results of the cluster analysis. However, they also showed that there were only small scale spatial differences in the gene levels. We conclude that the geographical location of a pig farm is not a major determinant of the presence or high levels of AMR genes assessed in this study.

Key words: Antimicrobial resistance genes, Denmark, pig farms, spatial patterns.

INTRODUCTION

After the Swann Report was published in 1969 [1], antimicrobial resistant (AMR) bacteria and the use of antibiotics in animals have been under scrutiny for their potentially negative effects on human health. Every year in Europe, more than 25 000 people die of diseases caused by AMR bacteria [2]. Management of this problem would benefit from an epidemiological approach to identify both direct and indirect causes of human infections arising from AMR bacteria.

Bacteria harbouring AMR genes are present in porcine faeces [3, 4], and it is generally accepted that AMR bacteria can be transferred from animals to humans through meat consumption [5] and via direct contact with pigs [6, 7]. Spreading slurry on farmland for the purposes of crop fertilisation might be a third way of transferring AMR genes from pigs to humans as fertilisation with porcine manure can increase the

* Author for correspondence: A. C. Birkegård, Section for Diagnostics and Scientific Advice, National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C, Denmark.
(Email: acbir@vet.dtu.dk)

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AMR levels in soil [8, 9]. The relative importance of transmission through meat compared with other transmission routes vary from gene to gene depending on the bacterial host of the gene.

Some bacteria are intrinsically resistance to AMR. Therefore, the bacterial composition of the porcine gut will affect the levels of the AMR genes. Previous studies have identified spatial patterns in the distribution of the different bacterial pathogens of livestock [10–13]. Thus, this study was planned with the hypothesis that AMR genes show a non-random spatial distribution. This hypothesis is supported by previous studies that have found spatial patterns of phenotypic AMR in enteric pathogens [14] and indicator bacteria [15]. In this study, we report the spatial patterns of the endemic levels of seven selected AMR genes in faecal total community DNA from pig farms. To the best of our knowledge, this is the first study of its kind. The pigs from which the samples were obtained had no clinical signs of disease. Therefore, the levels of AMR genes are assumed to reflect the background level of AMR in the Danish pig population, potentially acting as a reservoir for AMR in humans. The present study was designed to assess whether the spatial distribution of seven selected AMR genes was random with respect to the geographic distribution of the pig farm population at risk.

Seven genes, *ermB, ermF, sulI, sulII, tet*(M), *tet*(O) and *tet*(W) were included in this study because they have previously been identified as being present on Danish pig farms and a validated qPCR assay was available for testing for the presence of these genes [4]. The genes included in the assay comprise genes coding for two of the three most commonly used antimicrobial classes in Danish pig production, tetracyclines and macrolides [16]. The *ermF* and *ermB* genes code for resistance against macrolides whereas the *tet*(M), *tet*(O) and *tet*(W) genes encode resistance against tetracycline. These genes were included because they are expected to be found at high levels in some farms and could be used for detecting potential differences between farms. This might not be the case if the differences were below the sensitivity of the qPCR. Furthermore, the assay included two genes that are relatively rare in finisher pigs, i.e. the *sulI* and *sulII*. Sulphonamides, the antimicrobial class that these two genes encode resistance against, are rarely used in finisher in Danish pig production [16]. Evaluation of the levels of AMR genes using spatial statistical and geostatistical methods can be useful in generation of hypotheses regarding how the genes might spread through pig populations. Identification of spatial clustering of farms according to a specific AMR gene would provide a foundation for further analyses to explain the presence of these clusters, aiding our understanding of determinants of AMR genetics among Danish pig farms. This would help in the introduction of surveillance and monitoring systems, as well as preventive initiatives to limit the extent of AMR genes in pig farms. Furthermore, the findings of risk areas for specific AMR genes would indicate that the AMR genes are spread from farm to farm.

The objectives of the study were to describe the spatial patterns of AMR genes in faecal samples from pig farms and to test if the AMR genes were spatially randomly distributed.

METHODS

Study design and sampling

This was a cross-sectional study with sampling carried out from 2 February 2015 to 3 March 2015. The sampling period was restricted to these months to avoid seasonal changes in the level of AMR in pig farms [17].

Sampling took place at five of the seven largest Danish-owned slaughterhouses for finisher pigs in Denmark to ensure spatial randomness. Previous investigations showed that these slaughterhouses primarily received pigs from local farms [unpublished data]. The remaining two slaughterhouses were excluded because one primarily slaughtered pigs from free-range and organic farms, and the other was located on Bornholm. This remote island was excluded for all analysis, and therefore in this study 'Denmark' refers to 'Denmark excluding Bornholm'.

The number of farms to sample at each slaughterhouse was weighted according to the average number of farms sending pigs to slaughter during two 5-week periods starting from February and November 2014. The data used to plan the sampling were meat inspection data. These data were obtained from the Danish Classification Inspection and include details of individual pigs slaughtered at each of the study slaughterhouses. A total of 15 31 600 finishers were slaughtered in Denmark in February 2015 [18]. Of these, 13 65 963 (89%) were slaughtered at the seven major slaughterhouses.

In Clasen *et al.* [4], it was demonstrated that samples from five pigs were sufficient to obtain a representative sample of AMR genes at farm level. However, it

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was not known in advance which farms were sending animals for slaughter on a given day or how many pigs they would send. Hence, a purposive sampling strategy was adopted: when a number of pigs from the same farm were identified at the slaughter line, five of the pigs were sampled. That this approach resulted in a random sample that was later verified using meat inspection data from the sampling period [unpublished data]. Slaughterhouse technicians, who were introduced to the sampling methods by the first author on the first sampling day, collected the samples. The samples were taken at the slaughter line after the gut was removed from the carcass by squeezing a small amount of faeces out of the rectum into an empty 12·5 ml sample glass. The samples were kept at room temperature until all samples were collected for the day, and were then placed in a Styrofoam box with cooling elements and mailed overnight to the laboratory. Some deliveries were delayed by one day, but the cooling element was still frozen at arrival and the samples were deemed to be valid.

Quantification of AMR gene levels

The five samples per farm were pooled into a single aliquot and AMR levels were quantified as described by Clasen *et al.* [4]. Pooling was performed by taking an amount fitting the eye of a 10 µl inoculation loop from each of the five samples and dissolving it in 3·5 ml phosphate buffered saline (PBS). The pooled samples were vortexed individually and 2 ml of them was stored at −20°C until further processing. DNA was extracted with the Maxwell 16 Blood DNA Purification Kit (Promega) and DNA concentrations were diluted to 40 ng/µl. Seven AMR genes (*ermB, ermF, sulI, sulII, tet*(M), *tet*(O) and *tet*(W)) were included in the study as a high-throughput real-time PCR (qPCR) assay was optimised and ready to use [4]. The genes were quantified using the high-capacity qPCR chip ‘Gene Expression 192 × 24’ (Fluidigm) with two technical replicates. The amplification efficiency of the primers was determined by standard curves and obtained results were normalised with 16S ribosomal DNA, which was used as the reference gene.

Data analysis

Raw quantification cycle (C_q) values generated by the qPCR were taken from the Fluidigm Real-Time PCR Analysis Software version 4.1.3 [19] and exported to R version 3.2.2 [20]. The mean of the C_q values for technical replicates for each sample per gene was calculated. The C_q values were corrected with the inter-plate calibrators included in all runs, along with an efficiency calibration [21] calculated from standard curves generated for each of the primer sets [4]. The C_q value reflects the number of PCR cycles until a predefined threshold is reached. Therefore, high C_q values reflect a low-level presence of the gene. Values above gene-specific limits of detection [4] were coded as non-detects. Relative quantification (RQ) values indicate the quantity of genes in relation to the total amount of bacterial DNA found in the sample. The latter was measured by the reference gene 16S. The RQ values were calculated using the Livak method [22] as follows:

$$\mathrm{RQprimer}_{\mathrm{setX}} = 2^{-(\mathrm{Cq,gene\ of\ interest} \pm \mathrm{Cq,reference\ gene})}.$$

The RQ value was calculated for all genes except *sulI* and *sulII*. Samples with non-detects were excluded before calculating the RQ values. Due to a large number of non-detects among the samples for *sulI* and *sulII*, these genes were dichotomised as present or absent and analysed on a binary scale. The gene was deemed to be present if the qPCR assay resulted in a C_q value even though it was above limit of detection.

Genes with RQ values were also grouped according to the quantiles of the RQ values, as it is not known whether quantitative levels of AMR genes measured by the C_q values show a linear relation to the amount of the gene present in the sample.

Spatial analyses

To test the hypothesis that the distribution of the seven AMR genes were not randomly spatially distributed two sets of complimentary spatial analyses were conducted. First, spatial cluster analysis using scan statistic was performed to identify significant areas with significantly higher or lower risk (or higher or lower mean RQ values) of the seven AMR genes. Secondly, risk maps created using kriging and kernel density estimation were developed to allow us to visualise and describe the geographic distribution of AMR genes.

Cartesian coordinates given in UTM EUREF89 zone 32 format were obtained from the national Central Husbandry Register where all pig farms in Denmark are registered with a unique identification number [23].

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Spatial cluster analysis

The spatial scan statistic is a non-parametric test for the presence of clustering of events, accounting for the geographically irregular distribution of (in this example) the Danish pig farm population at risk. The spatial scan statistic is a cluster detection test able both to identify and to test the significance of specific clusters while it simultaneously provides the location of the clusters. Purely spatial cluster analyses were performed to identify spatial clusters of low and high levels of the AMR genes. Briefly, the test sequentially centres a circle or an ellipse in each farm in the study population and compares the RQ values of the AMR genes inside the circle with the RQ values of the farms outside. This circle or ellipse is called the search window. The search window will be increased until it reaches a predefined maximum. The predefined maximum can either be a specified size the search window (i.e. radius of the circle) or a maximum proportion of the population at risk inside a cluster. Often the maximum is set by using existing epidemiological knowledge of the disease in question. However, in this study no such information was available and different settings were used. The likelihood function was computed for each search window. The cluster with the highest likelihood constitutes the most likely cluster. Spatial scan analysis was carried out for the seven AMR genes separately. Depending on the type of the variable used for the analysis different models (i.e. statistical distributions) can be selected. Three different models were used:

(1) A normal model [24] for continuous RQ values for *ermB, ermF, tet*(M), *tet*(O) and *tet* (W). The model calculates the mean within and outside the search window and the level of significance is calculated for the difference between the two means. The normal model implemented in SaTScan can also handle non-normal data [24].
(2) A multinomial model [25] for ordinal RQ values in quantiles for *ermB, ermF, tet*(M), *tet*(O) and *tet*(W). The model calculates the expected and observed number of observations within each category for each search window and thus results in a relative risk for each of the four categories of the genes in relation to the other categories.
(3) A Bernoulli model [26] for binary values for *sulI* and *sulII*. Samples where the gene was present were defined as cases, and samples where the gene was not present were defined as controls.

For each model and gene, the cluster analysis was run with different parameter settings for the shape of the search window (elliptic or circular) and the maximum percentage of the population at risk was included in clusters (1, 5, 12·5, 25 or 50% of the population). For the Bernoulli and normal models, the search for high- and low-level clusters was carried out simultaneously. The test statistics are generated using a randomisation process based on Monte Carlo simulation. The number of iterations for all tests was set to 999. The most likely cluster and a number of secondary clusters will be identified. Only secondary clusters that did not overlap with the most likely cluster were requested. If a cluster is identified the test determines its significance and the cluster declared statistically significant if the *P*-value was less than the α level of 0·05.

Risk maps

Kriging and kernel density estimation were used to estimate values of a variable at an unmeasured location from observed values at surrounding locations. Kriging was used for continuous variables (RQ values of the AMR gene levels) and kernel density estimation was used for binary variables (presence of *sulI* and *sulII* genes). Kriging and kernel density estimation techniques were used to describe the first-order trends in the spatial distribution of AMR genes.

For both kriging and kernel density estimation analyses a regular grid comprised of individual cells 5 km length east to west and 5 km north to south was superimposed over the geographic boundaries of Denmark.

The ordinary kriging and the kernel density estimation analyses were done according to Bihrmann *et al.* [10] where details on mathematical equations can be found. The methods are explained briefly in the following sections.

Kriging

Kriging is considered an optimal method of spatial prediction of variables representing a spatially continuous surface. It refers to a family of least-square linear regression algorithms that attempt to predict values of a variable at locations where data are not observed, based on the spatial pattern of the observed data. Ordinary kriging is a common method to use and it relies on the observations of the target variable and its corresponding spatial positions. Kriging has

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the advantage that along with a smooth surface of predicted values, prediction variance is also estimated. Kriging is a weighted average of observed values, where the weight function is based on the spatial variation between measurements which is modelled by the semivariogram. Kriging can be used to estimate the spatial distribution of a disease measured at farm level (e.g. farm level incidence or prevalence of infected animals) [10, 27–29]. Although the disease variable is measured in particular farms it is assumed that the disease variable represents a spatially continuous surface of the disease level. This can be interpreted as the disease level we would expect at the location of a virtual (or new) farm. The method assumes a stationary rate, but it has also been effective on non-stationary rates [30].

Semivariograms were derived to obtain estimates of three parameters (range of influence, nugget and partial sill) that were then used to estimate the spatial variation and the weight function for kriging. Semivariograms measure the degree of dissimilarity between observations as a function of the distance. Typically, semi-variance, half the variance, increases as the distance between the locations grows until at some point the locations are considered independent of each other and the semi-variance no longer increases. If neighbouring data points resemble each other more closely than those further apart spatial dependence is assessed to be present. This would be indicated by a rising curve in the semivariogram, which plateaus as the similarities diminish with increasing distance. A semivariogram is characterised by three parameters the nugget effect, the sill and the range of influence. The nugget effect refers to the variability in the variable that cannot be explained by distance between the observations. Many factors influence the magnitude of the nugget effect including imprecision in sampling techniques, underlying variability of the attribute that is being measured, and the minimum spacing between observations. The latter is due to no observations sampled close to each other, it is impossible to estimate spatial dependence at small distances. The sill refers to the maximum observed variability in the data and corresponds to the variance of the data. The difference between the sill and the nugget effect (the partial sill) represents the amount of observed variation that can be explained by distance between observations. Finally, the range of influence is the point at which the semi-variance stops increasing and represents the distance at which two observations on average are not correlated. Often a model is fit to the semivariogram to estimate the parameters and in order to make use of the spatial dependence in other statistical techniques, including the kriging analysis.

In the present study kriging has been used to estimate the spread patterns of AMR gene levels. The spatial dependence would be a result of neighbouring farms having more similar AMR gene levels than those that are further apart. For each gene, two semivariograms were created, one as a primary analysis and the other as a sensitivity analysis. The semivariograms were created in two ways, the first was chosen where possible and the second as an alternative:

(1) Two models with different parameter settings were fitted to the same semivariogram.
(2) Two semivariograms were fitted using different lag widths, to which models with equal settings were fitted.

An exponential semivariogram model was used and the best fitted model was chosen. The model semivariogram parameter estimates the partial sill, the nugget effect, and the practical range of influence (three times the range of influence reported by the fitted model) were reported.

Directional semivariograms in four directions (north, north-east, east and south-east) were estimated to visually evaluate anisotropy. Anisotropy exists if there are substantial differences between the semivariograms in different directions.

For each semivariogram model, ordinary kriging was performed using the grid and repeated with different numbers of nearest neighbours in the kriging estimation. The number of neighbours ranged from 15 to 50 farms with intervals of five farms. A smoothed map showing the distribution of AMR gene levels measured in RQ values across Denmark was then produced. Furthermore, the prediction variances were plotted as an estimate of the uncertainties in the maps.

Kernel density estimation

The first-order spatial trend in the distribution of pig farms with *sulI* and *sulII* genes was described using kernel density estimation methods. Kernel density estimation gives weighted means for each location in the study region. Here a Gaussian, edge-corrected kernel smoothed map of gene-positive farms (showing the number of gene-positive farms per square kilometre) was computed as the numerator and a kernel

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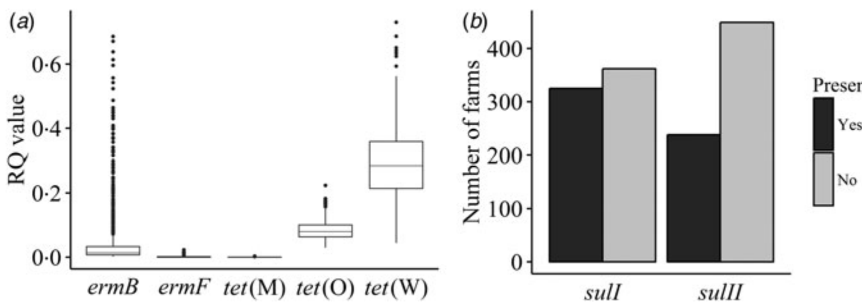

Fig. 1. Descriptive statistics of the genes. (*a*) The distribution of the RQ values for the *ermB, ermF, tet*(M), *tet*(O) and *tet* (W) genes. (*b*) The distribution of *sulI* and *sulII* genes; grey indicates the absence of the gene, while black indicates the presence of the gene.

smoothed map of all of the sampled pig farms computed as the denominator using the 'spatialkernel' package in R [20, 31]. A raster map showing the prevalence of *sulI*- and *sulII*-positive farms (expressed as the number of gene-positive farms per 100 farms per square kilometre) was produced by dividing the numerator raster map by the denominator. Bandwidths for each of the kernel smoothed maps were calculated using the normal optimal method and an average of the bandwidths for the positive and negative farms were used [32].

Software for spatial analyses

All data were handled in R version 3.2.2 [20]. Spatial cluster analysis was performed in SaTScan version 9.4.1 [33]. Maps were derived using the 'sp' package [20, 34]. Semivariograms, ordinary kriging and kernel density estimation were performed using the 'gstat' package in R version 3.2.2 [20, 35]. Bandwidths for the kernel density estimations were computed using 'sm' package in R version 3.2.2 [20, 32].

RESULTS

Study population

The cross-sectional study comprised a study population of 687 Danish indoor non-organic pig farms with finishers sent to slaughter in Denmark. Samples were collected from 129, 253, 125, 104 and 76 farms, respectively, from the five slaughterhouses. More information regarding the farms can be found elsewhere [unpublished data]. The sampling technique resulted in an almost random spatial distribution of the study population with respect to the Danish finisher pig farms at risk with relative under-sampling in the western part of Jutland. The spatial distribution of AMR genes in this area should be evaluated carefully [unpublished data].

Levels of the AMR genes

The distribution of the RQ values for each gene and the distributions of presence and absence of *sulI* and *sulII* can be seen in Figure 1. For *tet*(M), 43 samples were excluded from the analyses on the basis of non-detection, and for *ermF*, 19 samples were excluded from the analysis for the same reason. Of these samples, two were excluded from analysis for both *ermF* and *tet*(M). No samples were excluded for *ermB, tet* (O) and *tet*(W).

Spatial cluster analysis

Different parameter settings resulted in slightly different cluster locations and sizes. If two clusters were found in the same area the cluster including the highest number of farms was shown on the map (Fig. 2). The following significant spatial clusters were found: two high-risk clusters for *ermF*, one low-risk cluster for *ermF, ermB* and *tet*(W), and one high-risk cluster for *sulII*. For *ermB, ermF* and *tet*(W), the reported clusters were found with the multinomial model. No significant spatial clusters were found for *sulI, tet*(M) and *tet*(O).

Risk maps

Semivariograms for the *ermB, ermF, tet*(M), *tet*(O) and *tet*(W) genes are shown in Figure 3. Table 1 shows the parameters for the chosen exponential semivariogram model. The model estimates for *tet*(O) were very similar, whereas the model estimates for *tet*(W), *tet*(M), *ermB* and *ermF* differed between the two models (results not shown). The directional semivariograms showed no indication of anisotropy for any of the genes (results not shown).

Using the two models from the semivariogram and different numbers of nearest neighbours in the

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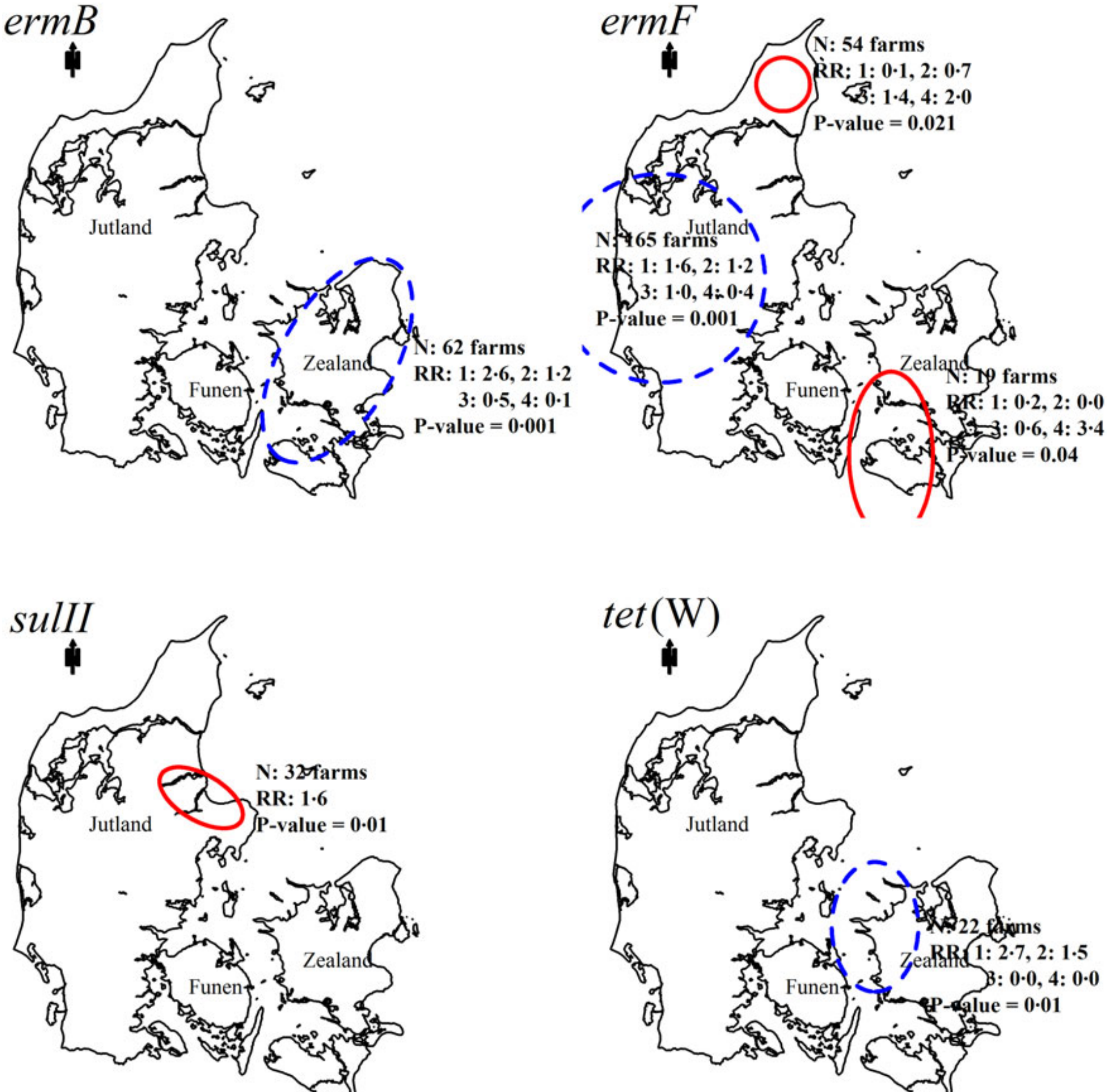

Fig. 2. Results of cluster analysis of AMR genes. Blue dashed lines indicate low-risk clusters, while red solid lines indicate high-risk clusters. Relative risk (RR) for multinomial models (i.e. *ermB, ermF* and *tet*(W)), the RR is indicated for each of the categories (1–4) in relation to the other models. For the Bernoulli model (i.e. *sulII*), the RR indicates the risk of being positive relative to the risk of being negative. *N*, number of farms in the cluster.

estimation of the RQ value only introduced minor changes to the estimated value. The visual patterns of high, medium and low levels for all genes did not change. A stable kriging map was produced with 40 nearest neighbours, so this number was chosen in the shown kriging maps (Fig. 4). Colours going form blue to increasing darker red on the maps indicate an increasing RQ value reflecting a higher level of the AMR gene.

Figure 5 shows the results of the kernel density estimation for *sulI* and *sulII*. Colours going from yellow to increasingly darker red indicate increasing population prevalence for the genes found in the area. The common bandwidth used for both genes was (22773 m and 32971 m, respectively).

DISCUSSION

This study showed that the some AMR genes found in faecal samples from pigs are not completely randomly spatially distributed. In the spatial cluster analysis, one low-risk cluster for *ermF, ermB* and *tet*(W) and two high-risk clusters for *ermF* were identified, together with one high-risk cluster for *sulII*. No clusters were found for *sulI, tet*(O) and *tet*(M). The size and location of the clusters varied among the genes.

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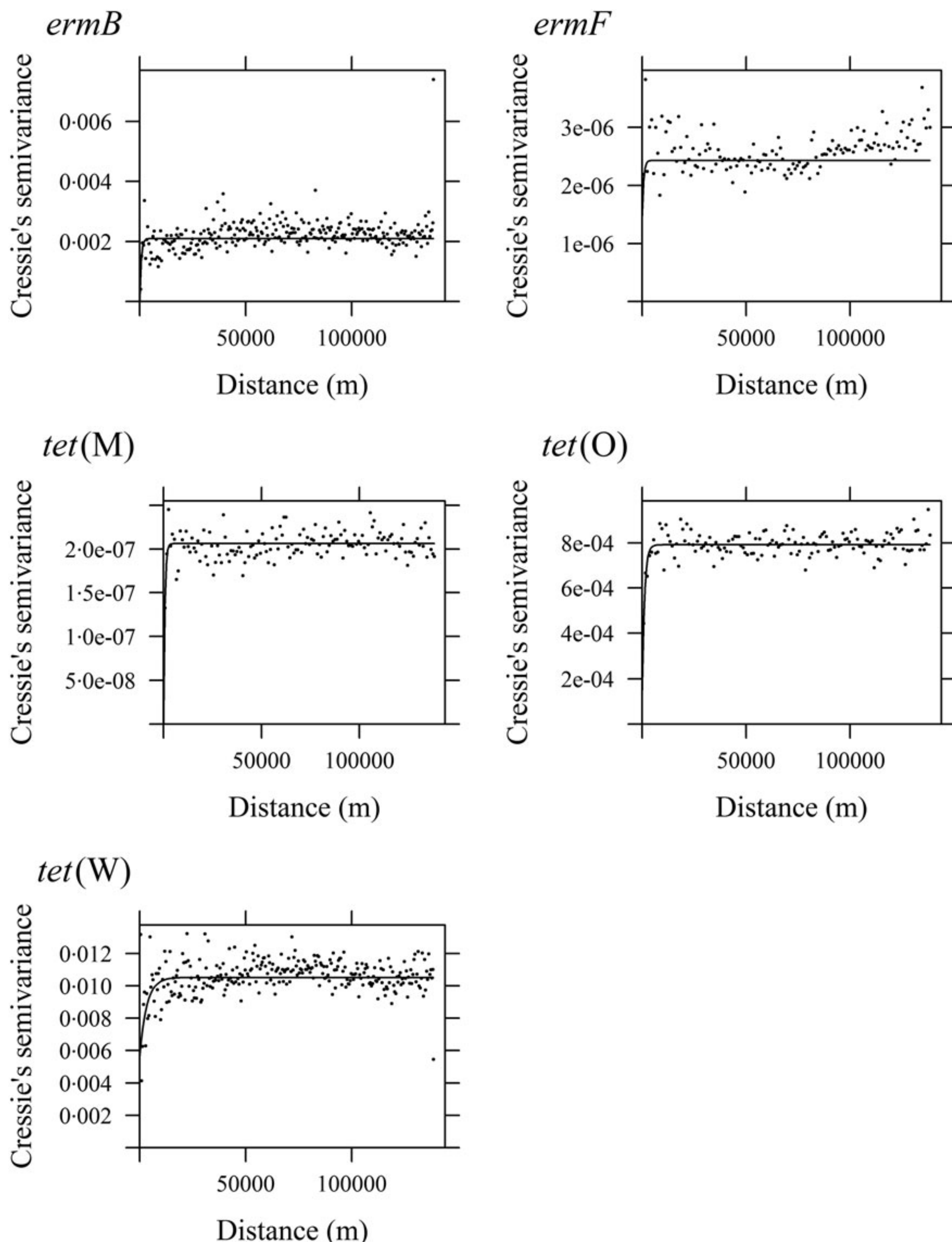

Fig. 3. Semivariograms. On each semivariogram, the fitted model is shown as black line. Each dot in the semivariogram cloud represents a point-pair of farms. Point-pairs comprised by farms within the distance of a specified lag width are plotted against the half of the variation (semi-variance) in the RQ values for the gene on the y-axis. When the cloud flattens out the relationship between the pairs of locations beyond this distance is no longer correlated. This distance is defined as the range of influence. However, when an exponential model is used the range of influence is multiplied by three to get the practical range of influence. The sill is defined as the semi-variance at the point where the semi-variance model flattens and the nugget effect is the intersection of the model and the y-axis. The partial sill is the sill minus the nugget.

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Table 1. *Semivariogram settings and parameter estimates*

Gene	Parameter estimates in fitted exponential semivariogram model			Model setting*	Semivariogram settings*	
	Nugget	Partial sill	Practical range of influence, km	Weighing	Lag width, m	Cut-off, m
ermB	0	0·0021	2·0	N_j/h_j^2	500	80 000
ermF	$1{\cdot}47e^{-6}$	$9{\cdot}5e^{-7}$	2·1	N_j/h_j^2	1000	None
tet(M)	0	$2{\cdot}06e^{-7}$	1·9	$N_j/\{\gamma(h_j)\}^2$	1000	None
tet(O)	0·00015	0·000640	3·4	N_j/h_j^2	1000	None
tet(W)	0·0057	0·0048	9·9	$N_j/\{\gamma(h_j)\}^2$	500	50 000

* Refers to settings in the programming in R. h_j represents the distance in metres; N_j represents the number of point-pairs; lag width represents the step size of distance intervals for creating the semivariogram; and cut-off represents the maximum distance at which pairs of data points will be considered for inclusion in the semivariogram.

The clusters on Zealand include fewer farms than clusters of a similar size in Jutland. This is due to an uneven distribution of farm locations in Denmark [36]. The clusters found by the multinomial model have a relative risk above one in either category one and two, or in category three and four, meaning that they are either high-risk clusters or low-risk clusters. No mixed clusters were found. It is possible that the current sample size is insufficient to show clustering for the three genes where no clusters were found, if they truly exist. The risk maps created with kriging analysis and the kernel density estimation were consistent with the results of the spatial cluster analysis. Both interpolation methods and the spatial cluster analysis showed consistent results with different parameter settings, indicating that the findings regarding the absence and presence of spatial differences for the genes could be considered reliable. The spatial scan statistic provides the location, size and significance of any clusters identified. Because its approach is circular or elliptic in nature, the assessment of clusters along natural or artificial borders may be biased to some extent. However, the spatial scan statistics can account for irregular dispersal of the farms over space which is the case for the distribution of pig farms in most countries including Denmark. On the other hand, this irregular dispersal of farms can lead to unreliable estimates of interpolation. The kriging analysis provide an error map and this show that for most parts of the country the predictive values are provided with the same error level. In the north-eastern part of Zealand very few pig farms are located why this area is associated with a higher prediction variance and thus a higher uncertainty is associated with the RQ values predicted in those areas.

To the best of our knowledge, this is the first study to report spatial patterns of AMR genes of total community DNA from porcine faeces. However, it is not the first to evaluate spatial patterns of AMR in Danish pig farms. A previous study in Denmark [15] evaluated spatial patterns in ampicillin resistance in *Escherichia coli*. However, *E. coli* only constitutes a small part of the porcine gut microbiota. The present study evaluates AMR genes in total community DNA, thereby taking into account all bacteria in porcine faeces and for several AMR genes. This means that there is no indication of which bacteria are found in the samples and in which bacteria the AMR genes are harboured. The AMR genes included in the study might be harboured in different bacterial species. The spatial distribution of the bacteria species would therefore affect the spatial distribution of the AMR genes. This could be the reason why spatial autocorrelation is found for some of the genes and not for other genes.

Many factors contribute to the occurrence of AMR in farms and in the environment. The different patterns in the semivariogram might suggest that the genes are spread by different mechanisms. Local differences in antimicrobial usage or in the presence of bacterial species are two factors that might explain the spatial patterns of AMR genes [37]. There is evidence for local variation in the prevalence of different bacteria in Danish pigs [38]. The local distribution of bacteria might be affected by introducing live pigs into the farm, as these movements of live pigs for meat production occur very locally in Denmark [39].

Antimicrobial usage has been shown to be spatially clustered in Denmark [36] and therefore AMR genes could be expected to cluster accordingly. It is

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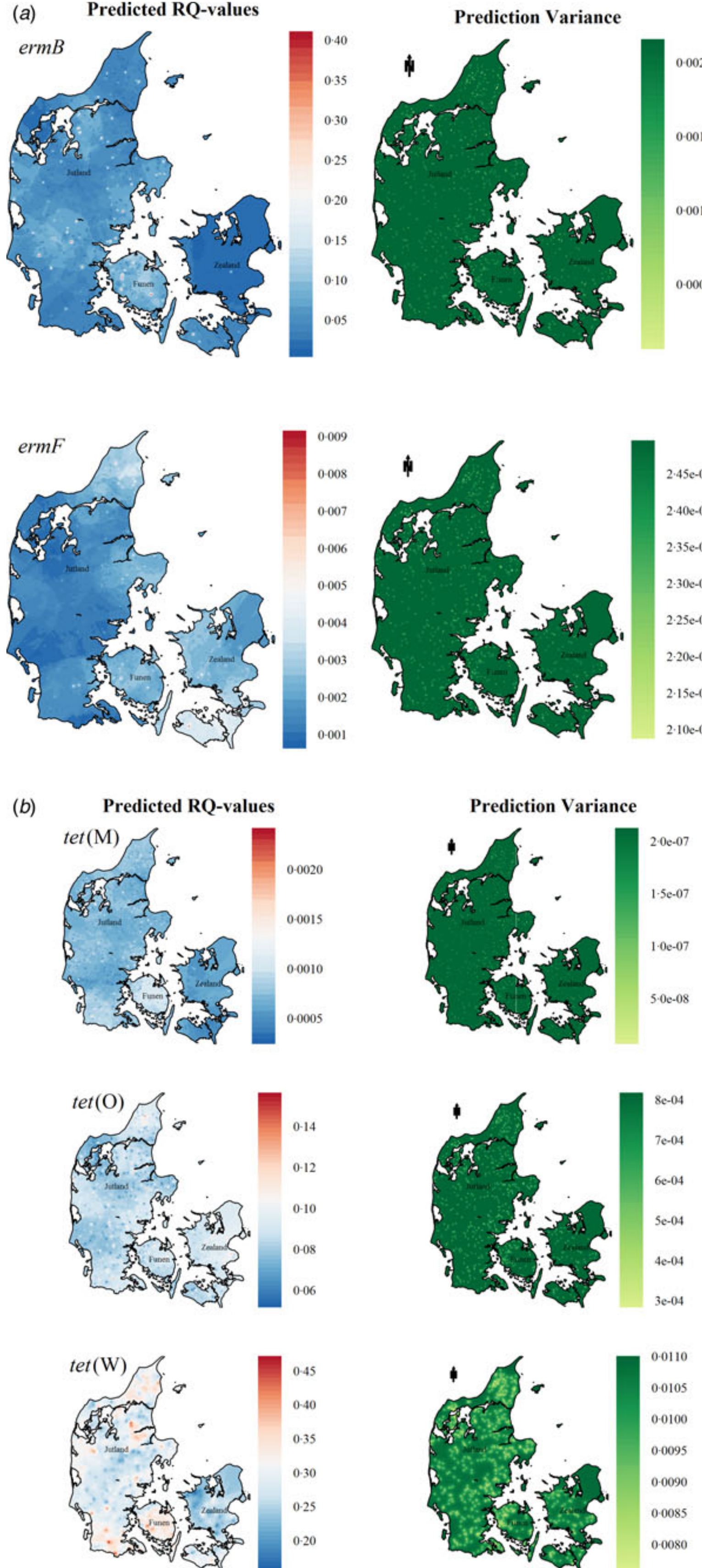

Fig. 4. (*a*) Risk maps for the levels of *ermB* and *ermF* genes produced by ordinary kriging. Each panel shows the distribution of predicted RQ values and the corresponding map for the prediction variance. The legends are unique for each gene due to the heterogeneous distributions of the genes even though same colour scale is used to produce the maps. (*b*) Risk maps for the levels of *tet*(M), *tet*(O) and *tet*(W) genes produced by ordinary kriging. Each panel shows the distribution of predicted RQ values and the corresponding map for the prediction variance. The legends are unique for each gene due to the heterogeneous distributions of the genes even though same colour scale is used to produce the maps.

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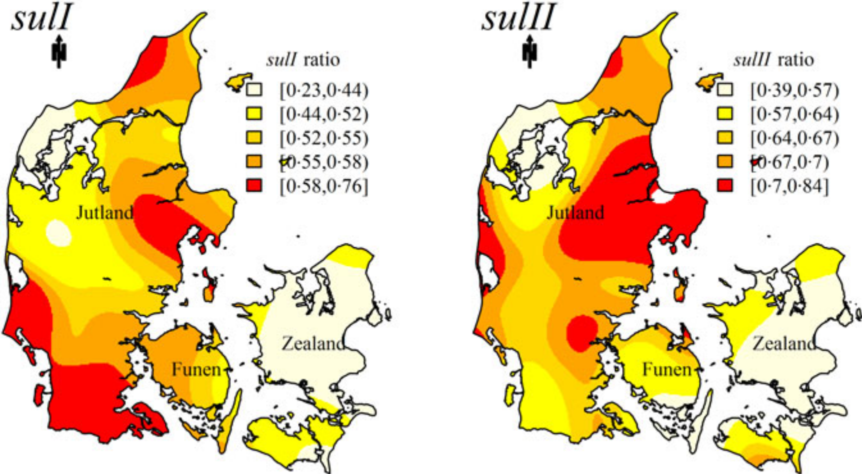

Fig. 5. Risk maps for the prevalence of the *sulI* and *sulII* genes. The maps are created using kernel density estimation.

interesting to note that for genes coding for resistance to the same antimicrobial class, significant spatial clusters were found for some of the genes, such as *sulII* and *tet*(W), but not for others such as *sulI* and *tet*(M) or *tet*(O), indicating that local patterns of antimicrobial usage cannot explain the spatial clusters alone. Another explanation for spatial patterns of AMR could be local differences in feeding strategies. It was not possible to assess this factor in the current study, as feeding practice is not recorded in any nationwide Danish register. Feeding strategies can alter the composition of the pig gut microbiome, leading to an increase in *Bifidobacterium* spp., which constitute a large part of the animal gut microbiota and promotes gut health [40]. The *tet*(W) or *tet*(M) genes are highly prevalent among *Bifidobacterium* spp. [41]. Furthermore, *tet*(M), *tet*(O) and *tet*(W) have been found in different types of swine feed [42] and might be present in probiotic microorganisms also used in some feeding schedules [43] which could significantly affect the distribution. The distribution of the *tet* genes in particular might be caused by differences in feeding practices or gut microbiota of the pigs. This study has shown that the *tet*(W) gene is present at the highest levels, and that there is a large variation in these levels among the sampled farms.

Within the practical range of influence, the farms are correlated, but due to the methods used in this study, it is not possible to estimate the size of the autocorrelations by for example a correlation coefficient. We deemed this to be beyond the scope of the paper as it is a purely descriptive study. Furthermore, there is no available method for assessing the adequacy of a fitted semivariogram model, and these results should therefore be treated with caution. In addition the accuracy of the semivariogram at small scale is weak because it is not possible to sample within a smaller distance than the distance between two pig farms. It is also important to note that only indoor, non-organic finisher farms were included in the study, and the spatial relationship might not be applicable for all Danish pig farms.

The risk maps showed results consistent of the cluster analysis, but also that the spatial difference were at small scale. Spatial clusters were found for specific AMR genes in Danish pig farms. However, the spatial distribution does not reveal major cold or hotspots in Denmark for the genes in question.

The conclusion was that the geographical location of a pig farm is not the major risk factor for presence or high levels of the AMR genes included in the study. Further analyses are needed to explain the clusters found in this study.

ACKNOWLEDGEMENTS

This research was carried out as part of a grant from Danish Veterinary and Food administration regarding science-based advice on AMR. The authors would like to thank the employees of Danish Crown and Tican, especially Marie B. Hansen, Vagner Bøge and Henrik Bækstrøm, for their cooperation, assistance with sampling, and help in setting up the study. They thank Jesper Larsen for support with the meat inspection data, Margarida Arede for help with sampling, Joanna Zeitman Amenuvor for help with sample pooling, and Karin Tarp and Sophia Rasmussen for their technical assistance. They would also like

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to thank Kerstin Skovgaard for advice on handling qPCR data.

DECLARATION OF INTEREST

None.

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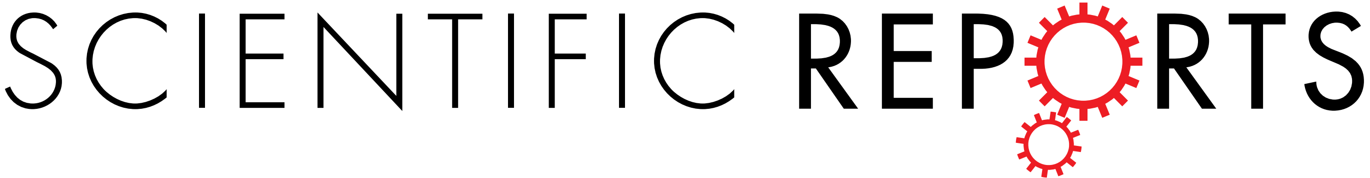
4.5 Manuscript 5: Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms.

Anna Camilla Birkegård, Tariq Halasa, Kaare Græsbøl, Julie Clasen, Anders Folkesson, and Nils Toft.

Scientific Reports. 2017; 7(1), 9683, doi: 10.1038/s41598-017-10092-9



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Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms

Received: 1 June 2017
Accepted: 3 August 2017
Published online: 29 August 2017

Anna Camilla Birkegård, Tariq Halasa, Kaare Græsbøll, Julie Clasen, Anders Folkesson & Nils Toft

Bacterial antimicrobial resistance (AMR) in pigs is an important public health concern due to its possible transfer to humans. We aimed at quantifying the relationship between the lifetime exposure of antimicrobials and seven antimicrobial resistance genes in Danish slaughter pig farms. AMR gene levels were quantified by qPCR of total-community DNA in faecal samples obtained from 681 batches of slaughter pigs. The lifetime exposure to antimicrobials was estimated at batch level for the piglet, weaner, and finisher periods individually for the sampled batches. We showed that the effect of antimicrobial exposure on the levels of AMR genes was complex and unique for each individual gene. Several antimicrobial classes had both negative and positive correlations with the AMR genes. From 10–42% of the variation in AMR gene levels could be explained in the final regression models, indicating that antimicrobial exposure is not the only important determinant of the AMR gene levels.

Antimicrobial consumption in pigs is a major contributor to the global antimicrobial consumption in livestock[1]. In Denmark, approximately 66% of the antimicrobials consumed are purchased for use in livestock of which 76% are used in pig production[2]. High levels of antimicrobial resistance (AMR) are therefore expected in Danish pig farms due to the selective pressure of the antimicrobials consumed. Pigs constitute a potential reservoir of AMR that can be transferred to pathogenic bacteria in humans through pork, direct contact with pigs, or release of porcine manure into the environment[3,4]. The rapid increase of AMR in recent decades has intensified the discussion about the prudent use of antimicrobials, especially in the pig production.

AMR is a natural consequence of the selective pressure of antimicrobials. However, the relationship between antimicrobial exposure and AMR is not easy to quantify[5]. Many AMR-associated genes have other functions not related to AMR when antimicrobial exposure is absent[6]. Epidemiological factors, including the size and age group of the population at risk and contact between farms, further complicate the quantification of the relationship between antimicrobial exposure and AMR. The population size is important as it directly relates to the antimicrobial exposure[5,7], and age is important as the composition of the intestinal microflora changes with the age of the pig[8]. On a pig farm, animals are normally housed in groups based on age, so information about the size and age of the population at risk is easily obtainable. Data on the purchase of antimicrobials and information on contacts between farms in Danish pig production are available, making the pig farm an ideal study unit for quantifying the relationship between antimicrobial exposure and AMR.

Previous studies estimating the relationship between AMR and antimicrobial exposure in pig populations have primarily focused on phenotypic resistance in one or few bacterial species[9–15]. This method underestimates the risk of AMR genes present in porcine faeces, as large parts of the gut microbiota cannot be cultured by traditional means[16]. Quantitative Real-Time Polymerase Chain Reaction (qPCR) is a DNA-based method extensively used to monitor gene levels due to its quantitative precision, low contamination risk, high sensitivity and broad dynamic range[17]. With qPCR, it is possible to quantify the levels of AMR genes from total-community DNA, even in complex samples such as porcine faeces[18,19].

Division for Diagnostics & Scientific Advice, National Veterinary Institute, Technical University of Denmark, Kemitorvet Building 204, 2800 Kgs. Lyngby, Denmark. Correspondence and requests for materials should be addressed to A.C.B. (email: acbir@vet.dtu.dk)

Period	Exposure levels				
	No	Very low	Low	High	Very high
Piglet	0	[0;87]	[87;136]	[136;186]	[186;522]
Weaner	0	[0;52]	[52;100]	[100;167]	[167;2777]
Finisher	0	[0;17]	[17;66]	[66;137]	[137;1367]
Lifetime	0	[0;239]	[239;348]	[348;474]	[474;2900]

Table 1. Average animal daily dose (ADD) per kg pig intervals used to categorise the antimicrobial exposure. The intervals are given by the summary statistics for the total antimicrobial exposure in each rearing period (piglet, weaner, finisher and lifetime) No exposure: a value of zero; Very low exposure > 0–25th percentile; Low exposure (>25th percentile - Median); High exposure (>Median - 75th percentile); Very High exposure (>75th percentile).

The factors driving AMR in pig production requires investigation in order to introduce efficient initiatives to reduce the levels. These factors are best studied in environments reflecting real-life practices. The cross-sectional study design is one of the preferred methods of studying the status of a population as it is relatively cheap and thus enables a large sample size.

The objective of this study was to quantify the association between the lifetime exposure of pigs to antimicrobials and the levels of seven AMR genes, *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O), and *tet*(W) in Danish slaughter pig farms.

Results

Population. Faecal samples were obtained from 681 batches of slaughter pig from Danish pig farms. Samples from one batch per farm were included in the study. The samples were collected at five abattoirs that only slaughtered pigs weighing approximately 100–120 kg (five to six months of age[20]). Thus, the influence of age on the AMR level[21] could be excluded as a bias.

The antimicrobial exposure for the batches was calculated as the average amount of antimicrobials to which pigs in the batch were exposed during their lifetime. The antimicrobial exposure estimates were calculated using information about antimicrobial purchases, farm demographics, and pig movements obtained from national registers (see methods section for details). However, it was not possible to calculate the antimicrobial exposure for 46 batches (7%), due to missing data in the registers. These were excluded from further analysis, resulting in a total of 635 batches of slaughter pigs included in the final analysis. Non-detects for *tet*(M) were found in samples from 35 batches (6%) and for *ermF* in samples from 15 batches (3%). These observations were excluded from the analyses where *ermF* and *tet*(M) were included.

Descriptive analyses. The antimicrobial exposure was estimated for each batch of pigs as the average animal daily dose for treatment of one kg pig (ADD_{kg}) for each of three rearing periods; piglet (birth-7 kg), weaner (7–30 kg), and finisher (30 kg-slaughter) period and as a lifetime total. Due to the heterogeneous pattern of antimicrobial consumption in Danish pig production, antimicrobial variables were categorised using the values given in Table 1. The resulting number of groups per antimicrobial exposure variable can be seen in Fig. 1. Groups were merged if one group represented less than five percent of the batches. Antimicrobial exposure variables were excluded from the analyses when all batches were in the same group, which among others was the case for cephalosporins (all usages), sulfa-TMP as group treatment, and colistin as individual treatment (Fig. 1).

Regression analyses. Regression analyses were made to assess the quantitative relationship between antimicrobial exposure and AMR gene levels. Logistic multivariable regression analyses were made for *ermB* (low/high levels) and for *sulI* and *sulII* (absence/presence). Linear multivariable regression analyses were made for *ermF*, *tet*(M), *tet*(O), and *tet*(W) with the levels presented as log(RQ-value). The effect of the variables in the final regression models can be seen in Supplementary Tables S1–S7 for the *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O), and *tet*(W) genes, respectively. We showed that batches exposed to high levels of macrolides in the finisher period had 66 times higher odds of having a high level of *ermB* than baseline batches that were not exposed to macrolides in the finisher period (Supplementary Table S1). Furthermore, the RQ value of *ermF* that was increased with 2.5 in batches that were exposed to high levels of macrolides compared to batches that had not been exposed to macrolides in the finisher period (Supplementary Table S2, 2.5 is a value of 0.92 on the log scale). The number of explanatory variables that were significant in the final models ranged from three (the *tet*(O) model, Supplementary Table S6) to eight (the *ermF* and *tet*(W) models, Supplementary Tables S2 and S7). The proportion of the gene variation explained by each model was as follows: *ermB* = 42%; *ermF* = 29%; *sulI* = 10%; *sulII* = 10%; *tet*(M) = 10%; *tet*(O) = 18%; *tet*(W) = 35%. Collinearity was not found between any of the continuous explanatory variables. Furthermore, exposure to amphenicol, colistin, and sulfa-TMP exposure did not correlate with any of the AMR genes.

The complexity of the association between antimicrobial exposure and the seven AMR genes is summarised in Fig. 2. We found 23 positive correlations (Fig. 2, red solid lines) and 8 negative correlations (Fig. 2, blue dotted lines). Exposure to tetracycline was negatively correlated with *ermB*, and *tet*(O), while being positively correlated with *sulII*, and *tet*(W). This mixed correlation with AMR genes was also the found for exposure to extended penicillins and tetracyclines. Exposure to macrolides, simple penicillins, lincomycins, and aminoglycosides was positively correlated with several AMR genes. Exposure to simple penicillins was negatively correlated with *tet*(W).

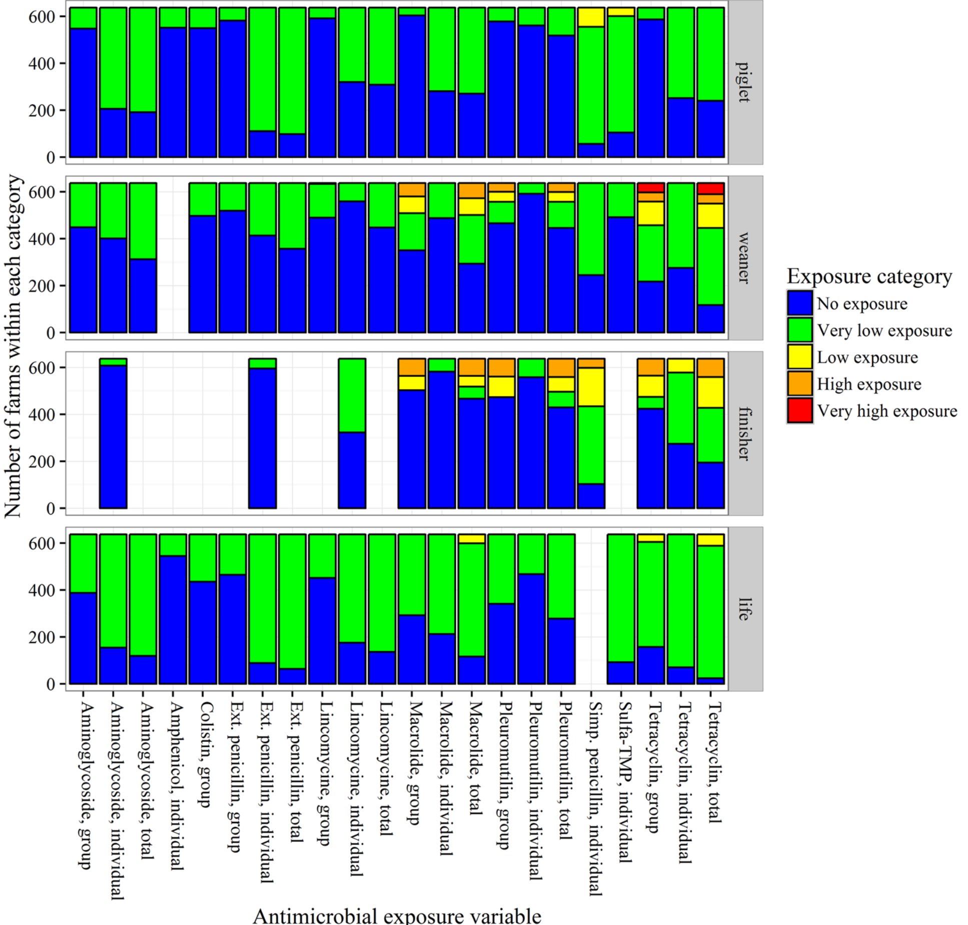

Figure 1. Categories for antimicrobial exposure variables. The number of categories within each antimicrobial exposure variable and the distribution of batches within the categories. Simp.: simple. Ext.: extended. Cephalosporins were given to very few batches and therefore not included.

Discussion

This study highlights the complexity of the relationship between antimicrobial exposure and AMR genes. The quantitative association of antimicrobial exposure and AMR genes depended on the specific gene as well as the antimicrobial class. While the exposure to tetracycline and extended penicillin was positively correlated with the level of certain AMR genes, it was negatively correlated with the level of others (Fig. 2). Even genes conferring resistance to the same antimicrobial class showed differently correlation patterns with exposure to antimicrobial classes in our study (Supplementary Tables S1–S7).

The association between antimicrobial class and AMR genes encoding resistance against that particular class was expected. We found that exposure to macrolides and lincomycin was positively correlated with *ermB* and *ermF*, and tetracycline exposure was positively correlated with the levels of *tet*(W). The association could be the result of selective pressure by the antimicrobial agent on the bacterial flora increasing the AMR gene levels, or the presence of the AMR gene necessitating a higher dose of the antimicrobial agent in order to treat the bacterial infection. However, tetracycline exposure was not correlated with *tet*(M), nor was sulfa-TMP correlated with the *sul* genes. Perhaps because sulfa-TMP is rarely given to younger pigs (7 kg–100 kg), but is often used to treat sows[2]. Furthermore, we found a negative correlation between exposure to tetracycline and *tet*(O). This could be due to tetracycline concentrations in the pig gut are above the minimal inhibitory concentration that this gene confers. Alternatively, antimicrobial treatment may change the composition of the gut microbiome so that microbes harbouring *tet*(O) decrease in number relatively to the total number of microorganisms in the gut even though the genes confers increased protection to tetracycline. Furthermore, this could be explained by the inclusion of the *tet*(W) gene in the final regression model.

Tetracycline exposure has repeatedly been shown to increase levels of phenotypic tetracycline resistance[10–12,14,22,23]. However, high levels of phenotypic[9,10,12,21] and genotypic[14,24,25] tetracycline resistance have also been found in pigs that have not been exposed to tetracycline. This inconsistent relationship between antimicrobial exposure and AMR has also been shown for macrolides[24,26,27]. This corresponds well to a recent systematic review, where no clear association was identified between antimicrobial exposure and phenotypic AMR[28], perhaps due to different specific AMR genes driving the phenotypic AMR.

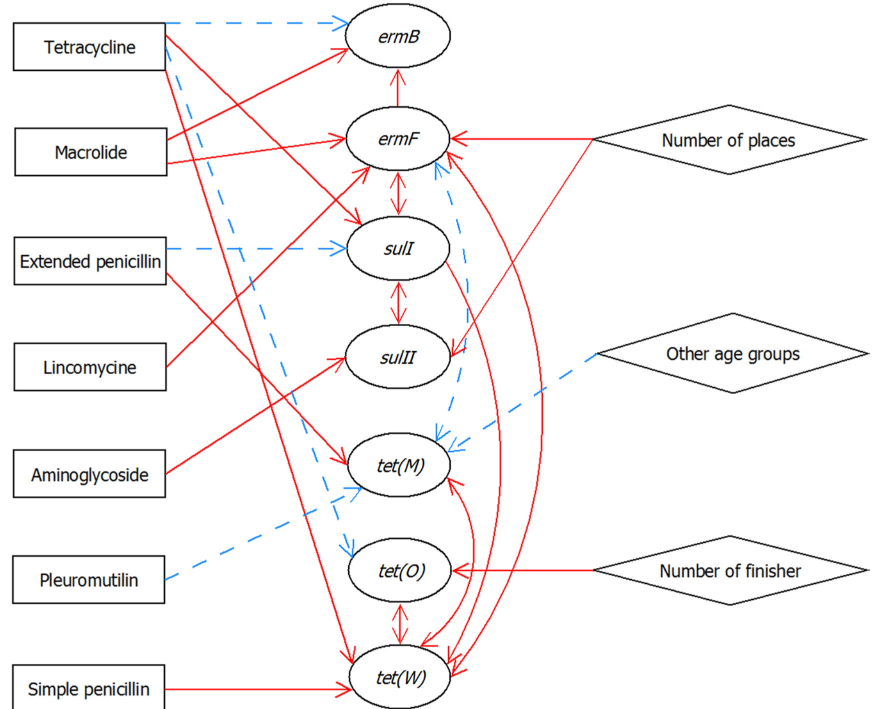

Figure 2. Association between antimicrobial exposure and antimicrobial resistance genes. Map of association showing the effect of antimicrobial exposure variables and other factors on the levels of antimicrobial resistance genes. The figure is a summary of the effect of antimicrobial class and other variables included in the final regression analyses given in Supplementary Tables S1–S7. A dotted blue line indicates a negative correlation and a solid red line indicates a positive correlation.

We found that some antimicrobials decrease the prevalence of some genes (e.g. the effect of tetracycline exposure on *ermB* and aminoglycoside exposure on *sulI*, Fig. 2). This might be because a decrease in the use of one antimicrobial class will normally be accompanied by an increase in another[29]. For example, farms using few tetracyclines might use more macrolides. Therefore, the decreasing effect of tetracycline on *ermB* could have been a hidden effect of macrolide use.

To our knowledge, this is the first study to quantify the association between antimicrobial use and AMR in pigs using a lifetime antimicrobial exposure estimate and AMR genes in total-community DNA. Although the relationship between AMR levels and antimicrobial exposure in pigs has been subject to several studies, the present study differs in some important aspects. Previous studies have either: monitored a relatively narrow period in the production cycle[27]; focused on one or few bacterial species[9, 22, 23, 30]; included only few antimicrobials[24]; omitted information on the antimicrobial exposure prior to the study period[26]; or lacked information about antimicrobial exposure[30, 31]. Furthermore, because detailed register data were available at farm level, it was possible to calculate antimicrobial exposure for almost all batches of pigs from which we had samples.

Our findings suggest that AMR genes in pigs at the time of slaughter potentially were affected by antimicrobial exposure during the entire rearing period. This was expected given the relatively short lifespan of a slaughter pig, which is five to six months in Denmark and consistent with what has been previously shown[9, 11, 13].

Determining the AMR gene levels in the total-community DNA is challenging, since neither the proportion of AMR genes nor the bacteria that harbour the genes are known. However, all seven AMR genes have been shown to occur on mobile elements, and can therefore be transferred from one bacterium to another[32–34].

We used lifetime antimicrobial exposure at batch level as a proxy for antimicrobial consumption for the pigs. The term 'exposure' is deliberately chosen as the exact consumption of the individual pig cannot be established based on register data. In a pig farm, the animals are exposed to antimicrobial residues excreted by treated pigs as well as antimicrobials administered orally or parenteral. Furthermore, pigs excrete AMR genes to the environment in the faecal droppings, which might be indigested by other pigs resulting in spread of AMR genes within a pig pen. Therefore, our hypothesis was that all antimicrobials used at a farm can contribute to the AMR gene levels in the pigs present at the farm.

We also showed that factors other than antimicrobial exposure were associated with the AMR gene levels. For example, *tet*(O) was positively correlated with the number of slaughter pigs present at the farm, which has also been shown for phenotypic tetracycline resistance[10]. In our study, we used the number of farms from which the pigs originated as a proxy for the degree of mixing and transportation. This number was correlated with the levels of *sulII* and *ermF* (Fig. 2, Supplementary Tables S2 and S4). We also found that the levels of AMR genes were correlated with those of other AMR genes, which could be the result of either co- or cross-resistance. This was

further supported by the correlations found between exposure to penicillins, simple and extended, was correlated with *sulI*, *tet*(O) and *tet*(W) (Fig. 2).

We were only able to explain 10–42% of the variation in AMR gene levels by factors included in the statistical analyses. A possible reason could be non-antimicrobial risk factors known to affect the AMR levels, but not included in this study due to information not being obtainable from available registers. These include transportation[35], housing temperature[35], farm management[23], and the consumption of metals[36]. Furthermore, the bacterial composition of the porcine gut and feeding strategies also affect the levels of the AMR genes because many bacteria are intrinsically carrying AMR genes[6].

In conclusion, using lifetime estimations of antimicrobial exposure and levels of AMR genes in total community DNA we quantified the associations between antimicrobial exposure and the level of AMR genes. These associations were found to be more complex than previously described and depended both on the specific AMR genes and antimicrobial classes in question. Furthermore, our results indicate that antimicrobial exposure is not the only important determinant of the AMR gene levels.

Materials and Methods

Study design. This paper follows the recommendations to optimise reporting of epidemiological studies on antimicrobial resistance (STROBE-ASM guidelines[37]).

The study design was cross-sectional with a target population of Danish pig farms with conventional production of slaughter pigs. The samples were obtained by a tested and validated method for sampling at abattoirs in order to ensure that the resulting samples were representative of the target population as explained by Birkegård *et al.*[38]. Information about the farms was obtained through national registers after sampling. The sampled pigs had no clinical signs of disease as they were assessed suitable for slaughter, and antimicrobial exposure was a result of treatments for diseases occurring in a normal Danish pig production. Almost all pigs were exposed to antimicrobials in one or more rearing periods. In Denmark, antimicrobial use for growth promoting or prophylaxis is not permitted[39] and therefor the reported antimicrobial usage are for treatment of diseases or use in metaphylaxis.

The study unit was a batch – defined as a group of pigs slaughtered on the same day and originating from the same farm.

Faecal samples from pigs slaughtered in Denmark were collected in February and March 2015 at five abattoirs. The number of farms to sample was determined by the available resources, and sampled farms were selected randomly, as previously described[38,40]. In brief, the faecal samples were collected at the slaughter line after removal of the gut from the carcass. A small amount of faecal material was squeezed out of the rectum of the removed gut and into an empty 12.5 mL sampling vial. Five pigs were sampled per farm as this was shown to be sufficient to account for the variation in AMR at farm level[18]. The five faecal samples were pooled resulting in one sample per farm.

The farmers were not informed of the sampling as their cooperative abattoir management gave permission. Therefore, selection bias in terms of willingness to participate in the study can be excluded. However, the sample scheme resulted in a sampling bias towards larger farms[38].

Level of antimicrobial resistance. In this study, the levels of seven AMR genes (*ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), tet(O), and *tet*(W)) were quantified. These genes were chosen because an assay had been validated for these specific genes in a previous study[19]. Inclusion criteria for the selected genes were: 1) the use of the antibiotic class in the Danish pig production, 2) the occurrence of the gene in a wide bacterial population and 3) the possibility of designing a qPCR assay for the chosen genes utilizing the same temperature profile[19].

Based on the pooled samples the AMR levels were quantified, as described by Clasen *et al.*[18]. DNA was extracted using the Maxwell 16 Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA) and DNA concentrations were diluted to 40 ng/μl. Levels of seven AMR genes were quantified using the high-capacity qPCR chip Gene Expression 192 × 24 (Fluidigm Corporation, South San Francisco, CA, USA) with two technical replicates using 16S as the reference gene, as previously described[38]. The average cycle of quantification (C_q) value for the two technical replicates was used in the further analyses. C_q values above the gene specific limit of quantification were regarded as non-detects. The gene specific limits of detection were 23 (*ermB*, *sulII*, *tet*(O)), 24 (*ermF*, *tet*(W), 16S), 25 (*tet*(M)) and 26 (*sulI*) respectively and efficiencies ranged from 90.9–108.2%[18]. After excluding samples with non-detects, obtained Cq values were corrected for variations in between runs, by the use interplate calibration followed by correction for efficiency of the genes. Relative quantification (RQ) values were calculated from cycle of corrected Cq values with the modified Livak method[41] (equation (1))

$$RQ_{\text{primer setX}} = 2^{-(\text{Cq,gene of interest} - \text{Cq,reference gene})} \tag{1}$$

Due to the large number of non-detects among the samples for *sulI* and *sulII*, these genes were dichotomised as present or absent[38]. The distribution of RQ values for the *ermB* genes was skewed[38], so the levels of RQ values were therefore classified as either low (below the 75th percentile) or high (above the 75th percentile).

Level of antimicrobial exposure. The lifetime exposure to antimicrobials was calculated as the estimated average amount of antimicrobials to which pigs within a batch were exposed during their lifetime using information about antimicrobial purchase, farm demographics, and pig movements from national registers.

Antimicrobial exposure was measured in animal defined doses per kilogram pig (ADD_{kg}).

The ADD_{kg} is defined as the average approved dose for the main indication in the particular animal species for treatment of one kilogram pig. The ADD_{kg} can be used across age groups, as it is independent of animal bodyweight[42]. The ADD is equivalent to defined daily doses used in human medicine[43]. VetStat (the register on antimicrobial purchases) contains information about the number of ADD_{kg} that a package or vial of product contain.

Combining this information with the number of packages purchased and the number of pigs makes it possible to calculate the ADD_{kg} per pig (equation (2)).

$$ADD_{kg} = \frac{\text{Amount of product sold mg/kg}}{ADD_{kg}/\text{mg} * \text{number of finshers in batch}} \quad (2)$$

Antimicrobial exposure was calculated per antimicrobial class and dispersing form. Antimicrobial agents were grouped following the classification structure provided by VetStat.

The use of antimicrobials in the Danish pig population is heterogeneous, with many farms using little or none of a specific antimicrobial class. Therefore, antimicrobial exposure (measured in ADD_{kg}) was categorised according to the quartiles for the total exposure of antimicrobials for each of the rearing periods: piglet (birth−7 kg), weaner (7 kg–30 kg), finisher (30 kg–slaughter), and aggregated lifetime exposure (the sum of exposure in each period). The following categories were used:

(1) No exposure (a value of zero)
(2) Very low exposure (>0–25th percentile)
(3) Low exposure (>25th percentile - Median)
(4) High exposure (>Median −75th percentile)
(5) Very High exposure (>75th percentile).

Other variables. The number of slaughter pigs present at the farm was calculated based on data in national registers. Farm size was added to the analyses using a calculated number of finishers present at the farm. The number of age groups (sows with piglets, weaners, and finishers) present at the farm was also included in the analyses.

The number of farms at which the pigs in the batch had been in was calculated by tracing the pigs back in time using pig movement data. The variable was categorised as one, two, or more than two.

Statistical analyses. All statistical analyses were carried out in R[44] using RStudio[45].

Multivariable logistic regression analyses were performed for *ermB* (low/high level), *sulI* (absence/presence), and *sulII* (absence/presence). In addition, multivariable linear regression analyses were performed for *ermF*, *tet*(M), *tet*(O), and *tet*(W), using log-transformed RQ levels to improve the homogeneity of variance and normality of residuals. Both the logistic and linear regression analyses were carried out in four steps. Backwards elimination was performed at each step, starting with the variables with the highest p-value and using a Bonferroni corrected significance level of 0.05 divided by the number of variables in the model for the β-estimates to eliminate the non-significant variables. The Bonferroni corrected p-value was used to correct for multiple comparisons. Furthermore, an ANOVA was used to test the overall effect of the final variables, and all non-significant variables were excluded, again using a Bonferroni correction. Antimicrobial classes already included in the model at the previous step were not included in the next step. The following variables were included in the models:

(1) Categorical explanatory variables: categorised antimicrobial exposure variables for the piglet, weaning and finishing periods for both group and individual treatment for the 11 classes of antimicrobials, the number of farms from which the pigs originated, and the categories for *ermB*, *sulI*, and *sulII*.
    a. Continuous explanatory variables: the number of slaughter pigs, the number of other age groups present at the farms, and the log transformed RQ values of *ermF*, *tet*(M), *tet*(O), and *tet*(W).
(2) The total amount of antimicrobials for the piglet, weaning, and finishing periods.
(3) The total amount of each antimicrobial class used over the lifetime of the pigs for both individual and group treatment.
(4) The total amount used per antimicrobial class over the lifetime of the pigs.

Some farms had non-detects for *ermF* and *tet*(M), which were included in the regression analyses as 'NA'.

To assess multicollinearity, we calculated Spearman's correlation coefficients among all continuous explanatory variables, using $\rho > 0.8$ as a cut-off.

The adjusted R^2 for the linear regression analyses of the final model was used to estimate the percentage of variation in the genes explained by the model. For the logistic regression analyses, McFadden's pseudo R^2 (calculated using the pR2 function in the pscl package[46]) was used as an equivalent.

Pairwise significant differences among individual levels of significant categorical variables were assessed using the LS-means package[47] for the variables in the final models.

Data availability. The data generated and analysed in the current study are not publicly available due to the agreement for obtaining data. Prior to collection of the data a written agreement was signed ensuring that no other than the project group (named persons) could obtain AMR data for individual farms, and subsequently follow the batches. However, summarised data are available from the corresponding author on reasonable request.

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Acknowledgements
The authors would like to thank the employees of Danish Crown (in particular Marie B. Hansen and Vagner Bøge) and Tican for their cooperation, assistance with sampling, and help in setting up the study. This research was carried out as part of a grant from the Danish Veterinary and Food Administration, relating to evidence-based advice on AMR.

Author Contributions
A.B. designed the study, collected data, performed data analysis, data interpretation and wrote the manuscript. T.H. and N.T. participated in the study design, data analysis and interpretation. K.G. participated in the data analysis and interpretation. A.F. and J.C. did the laboratory analyses and participated in the literature review and data interpretation. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-10092-9

Competing Interests: The authors declare that they have no competing interests.

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Title page

Title: Association between selected antimicrobial resistance genes and antimicrobial exposure in Danish pig farms

Authors and affiliations

Anna Camilla Birkegård[*], Tariq Halasa, Kaare Græsbøll, Julie Clasen, Anders Folkesson, Nils Toft

Division for Diagnostics & Scientific Advice, National Veterinary Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs. Lyngby

*corresponding author, acbir@vet.dtu.dk

Supplementary Tables showing the results of the final regression model

Table S1: Estimates of regression coefficient (β), Standard Error (SE), Odds Ratio (OR) together with the Confidence Interval (CI) and the p-value of the final multivariable logistic regression model of the association between antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors for having high levels of *ermB* compared to low levels

Category of explanatory variable	Explanatory variable	Level+	β-estimate	SE	OR	CI	p-value†
	Intercept	-	-0.015	0.82	-	-	-
AMR genes	*ermF*, log(RQ)*	-	0.35	0.11	1.42	[1.15;1.76]	0.001
Antimicrobial exposure, weaning period	Macrolides, group	No[b,d]	Ref	-	-	-	<0.0001
		Very low[a]	0.98	0.30	2.65	[1.48;4.80]	0.001
		Low	0.91	0.43	2.48	[1.05;5.68]	0.03
		High[a]	1.37	0.42	3.95	[1.73;8.88]	0.001
	Tetracycline, group	No	Ref	-	-	-	0.004
		Very low	-0.08	0.30	0.93	[0.51;1.68]	0.8
		Low[e]	0.52	0.37	1.69	[0.81;3.48]	0.2
		High	-0.48	0.61	0.62	[0.17;1.89]	0.4
		Very High[c]	-2.22	0.80	0.11	[0.02;0.47]	0.006
Antimicrobial exposure, finishing period	Macrolides, group	No[c,d]	Ref	-	-	-	<0.0001
		Low[a,d]	2.49	0.34	12.05	[6.26;23.93]	<0.0001
		High[a,c]	4.19	0.47	66.06	[28.11;181.55]	<0.0001
	Tetracycline, group	No[d]	Ref	-	-	-	0.002
		Very low[d]	0.44	0.42	1.55	[0.67;3.48]	0.3
		Low	-0.26	0.37	0.77	[0.36;1.55]	0.5
		High[a,b]	-1,88	0.63	0.15	[0.04;0.47]	0.003

SE: Standard error, OR: Odds ratio, CI: Confidence interval of odds ratio.
†The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.
+For categorical explanatory variables there are different levels. There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: a no exposure, b very low exposure, c low exposure, d high exposure, and e very high exposure.
*Continuous variable on log scale.
Ref: Reference level.
Group and individual refer to how the antimicrobial was administrated whereas the total refers to the total amount that the pigs have been exposed to.

Table S2: Estimates of regression coefficient (β), Standard Error (SE), statistic, and p-value of the final multivariable linear regression model of the association between of antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors on *ermF* levels measured as log(RQ-values)

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	Statistic	p-value[†]
	Intercept	-	-9.40	0.52	-	-
AMR genes	*sulI*	absent[i]	Ref	-	-	<0.0001
		present[h]	0.30	0.09	3.16	0.002
	tet(M), log(RQ)*	-	-0.24	0.07	-3.71	0.0002
	tet(W), log(RQ)*	-	0.69	0.13	5,37	<0.0001
Antimicrobial exposure, weaning period	Lincomycin, group	No[b]	Ref	-	-	<0.0001
		Very low[a]	0.67	0.11	6.06	<0.0001
		Low	1.03	0.79	1.29	0.20
	Macrolides, group	No[b]	Ref	-	-	<0.0001
		Very low[a]	0.38	0.12	3.29	0.001
		Low	0.36	0.15	2.33	0.02
		High	0.02	0.17	0.10	0.92
Antimicrobial exposure, finishing period	Lincomycin, individual	No[b]	Ref	-	-	<0.0001
		Very low[a]	0.35	0.09	3.72	0.0002
	Macrolides, group	No[c,d]	Ref	-	-	<0.0001
		Low[a]	0.67	0.16	4.25	<0.0001
		High[a]	0.92	0.15	6.19	<0.0001
Other variables	Number of farms	1[m,n]	Ref	-	-	0.0005
		2[l]	0.62	0.14	4.30	<0.0001
		>2[l]	0.66	0.16	4.23	<0.0001

SE: Standard error

[†]The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.

[+]For categorical explanatory variables there are different levels. The *ermB* gene is categorised as either high level or low level whereas the *sulI* gene is categorised as absent or present. The number of farms is referring to the number of farms that the pigs in the batch is originating from and is categorised into three groups: 1, 2 or more than 2 (2+). There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure, [b] very low exposure, [c] low exposure, [f] low level of ermB, [g] high level of *ermB*, [h] absence of *sulI*, [i] presence of *sulI*, [l] 1 farm, [m] 2 farms, and [n] more than two farms.

*Continuous variable on log scale.

Ref: Reference level.

Group and individual refer to how the antimicrobial was administrated.

Ext.: extended.

Sim: simple.

Table S3: Estimates of regression coefficient (β), Standard Error (SE), Odds Ratio (OR) together with the Confidence Interval (CI) and the p-value of the final multivariable logistic regression model of the association between antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors for presence of *sulI* compared to absence

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	OR	CI	p-value[†]
	Intercept	-	1.35	0.60	-	-	-
	*ermF** log(RQ)	-	0.24	0.07	1.28	[1.12;1.46]	<0.0001
	sulII	Absent[k]	Ref	-	-	-	<0.0001
		Present[j]	1.28	0.18	3.63	[2.54;5.24]	<0.0001
Antimicrobial exposure, piglet period	Ext. penicillin, total	No[b]	Ref	-	-	-	0.01
		Very low[a]	-0.79	0.26	0.46	[0.27;0.75]	0.004
	Tetracycline, total	No[b]	Ref	-	-	-	0.005
		Very low[a]	0.50	0.18	1.65	[1.16;2.35]	

SE: Standard error, OR: Odds ratio, CI: Confidence interval of odds ratio.
[†]The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.
[+]For categorical explanatory variables there are different levels. The *ermB* gene is categorised as either high level or low level whereas the *sulII* gene is categorised as absent or present. There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure, [b] very low exposure, [c] low exposure, [d] high exposure, [f] low level of ermB, [g] high level of *ermB*, [j] absence of *sulII*, and [k] presence of *sulII.*
*Continuous variable on log scale.
Ref: Reference level.
Individual refers to how the antimicrobial was administrated whereas the total refers to the total amount that the pigs have been exposed to.
Ext.: extended.

Table S4: Estimates of regression coefficient (β), Standard Error (SE), Odds Ratio (OR) together with the Confidence Interval (CI) and the p-value of the final multivariable logistic regression model of the association between antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors for presence of *sulII* compared to absence

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	OR	CI	p-value[†]
	Intercept	-	-0.75	0.24	-	-	-
AMR genes	*sulI*	Absent[i]	Ref	-	-	-	<0.0001
		Present[h]	1.31	0.18	3.72	[2.62;5.32]	<0.0001
Antimicrobial exposure, weaning period	Aminoglycoside, individual	No[b]	Ref	-	-	-	0.0002
		Very low[a]	0.63	0.20	1.87	[1.28;2.76]	0.001
Other variable	Number of farms	1[n]	Ref	-	-	-	0.03
		2	0.53	0.25	1.71	[01.04;2.79]	0.03
		2+[l]	0.86	0.28	2.35	[1.37;4.05]	0.002

SE: Standard error, OR: Odds ratio, CI: Confidence interval of odds ratio.
[†]The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.
[+] For categorical explanatory variables there are different levels. The *sulII* gene is categorised as absent or present. The number of farms is referring to the number of farms that the pigs in the batch is originating from and is categorised into three groups: 1, 2 or more than 2 (2+). There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure, [b] very low exposure, [h] absence of *sulI*, [i] presence of *sulI*, [l] 1 farm, and [n] more than two farms
*Continuous variable on log scale.
Ref: Reference level.
Group and individual refer to how the antimicrobial was administrated.

Table S5: Estimates of regression coefficient (β), Standard Error (SE), statistic, and p-value of the final multivariable linear regression model of the association between of antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors on *tet*(M) levels measured as log(RQ-values)

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	Statistic	p-value
	Intercept	-	-7.54	0.19	-	-
AMR genes	*ermF*, log(RQ)*	-	-0.09	0.02	-4.10	<0.0001
	tet(W) , log(RQ)*	-	0.41	0.08	5.25	<0.0001
Antimicrobial exposure, lifetime	Ext. penicillin, individual	No[b]	Ref	-	-	0.004
		Very low[a]	-0.24	0.06	-3.69	0.0002
	Pleuromutilin, individual1	No[b]	Ref	-	-	0.0002
		Very low[a]	0.24	0.06	3.69	0.0002
Other variables	Other age groups ‡		-0.15	0.04	-3.71	0.0002

SE: Standard error

†The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.

+For categorical explanatory variables there are different levels. There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure and [b] very low exposure.

*Continuous variable on log scale.

‡Continuous variable. Other age groups refer to the number of other age groups that are present at the farm.

Ref: Reference level.

Individual refers to how the antimicrobial was administrated whereas the total refers to the total amount that the pigs have been exposed to.

Ext.: extended.

Table S6: Estimates of regression coefficient (β), Standard Error (SE), statistic, and p-value of the final multivariable linear regression model of the association between of antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors on *tet*(O) levels measured as log(RQ-values)

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	Statistic	p-value[†]
	Intercept	-	-2.10	0.05	-	-
AMR genes	*tet*(W), log(RQ)*	-	0.34	0.05	10.45	<0.0001
Antimicrobial exposure, weaning period	Tetracycline, individual	No[b]	Ref	-	-	0.009
		Very low[a]	-0.07	0.03	-2.60	0.009
Other variable	Number of slaughter pigs per 1,000 pigs[‡]	-	0.04	0.009	4.28	<0.0001

SE: Standard error

[†]The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.

[+]For categorical explanatory variables there are different levels. There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure, [b] very low exposure, [c] low exposure, and [d] high exposure.

*Continuous variable on log scale.

‡Continuous variable.

Ref: Reference level.

Individual refers to how the antimicrobial was administrated whereas the total refers to the total amount that the pigs have been exposed to.

Ext.: extended.

Sim: simple.

Table S7: Estimates of regression coefficient (β), Standard Error (SE), statistic, and p-value of the final multivariable linear regression model of the association between of antimicrobial resistance (AMR) genes, antimicrobial exposure, and other factors on *tet*(W) levels measured as log(RQ-values)

Category of explanatory variable	Explanatory variable	Level[+]	β-estimate	SE	Statistic	p-value[†]
	Intercept	-	0.15	0.19	-	-
AMR genes	*ermF*, log(RQ)*	-	0.05	0.01	4.69	<0.0001
	sulI	Absent[i]	Ref	-	-	0.004
		Present[h]	0.09	0.03	3.36	0.0008
	tet(M), log(RQ)*	-	0.08	0.02	4.73	<0.0001
	tet(O), log(RQ)*	-	0.28	0.04	7.26	<0.0001
Antimicrobial exposure, weaning period	Sim. penicillin, individual	No[b]	Ref	-	-	0.0001
		Very low[a]	0.08	0.03	3.17	0.002
	Tetracycline, group	No[e]	Ref	-	-	<0.0001
		Very low	0.08	0.03	2.72	0.007
		Low	0.10	0.04	2.48	0.01
		High	0.13	0.06	2.38	0.02
		Very high[a]	0.23	0.05	4.26	<0.0001
Antimicrobial exposure, finishing period	Tetracycline, group	No[c,d]	Ref	-	-	<0.0001
		Very low[d]	0.13	0.05	2.68	0.008
		low[a,d]	0.20	0.04	5.47	<0.0001
		High[a,b,c]	0.32	0.04	7.78	<0.0001

SE: Standard error

[†]The p-value for the reference level is an overall test of significance with a Bonferroni correction to adjust for multiple comparisons.

[+]For categorical explanatory variables there are different levels. The *sulI* gene is categorised as absent or present. There are for the antimicrobial exposure levels up to five levels of exposure (see Fig. 1 for number of levels per antimicrobial exposure variable). Results from the LS-means analysis showed that the effect was significantly different from: [a] no exposure, [b] very low exposure, [c] low exposure, [d] high exposure, [e] very high exposure, [h] absence of *sulI*, and [i] presence of *sulI*.

*Continuous variable on log scale.

Ref: Reference level.

Group and individual refer to how the antimicrobial was administrated.

Ext.: extended.

Sim: simple.

4.6 Manuscript 6: Persistence of antimicrobial resistance genes from sows to finisher pigs.

Anna Camilla Birkegård, Tariq Halasa, Anders Folkesson, Julie Clasen, Kaare Græsbøl, and Nils Toft.

Submitted to Preventive Veterinary Medicine

1 **Title page**

2 Title: Persistence of antimicrobial resistance genes from sows to finisher pigs – short

3 communication

4 Authors: Anna Camilla Birkegård*, Tariq Halasa, Anders Folkesson, Julie Clasen, Kaare Græsbøll,

5 Nils Toft

6 Affiliation: National Veterinary Institute, Technical University of Denmark, Kemitorvet, Building

7 204, 2800 Kgs. Lyngby, Denmark

8 *Corresponding author: Anna Camilla Birkegård: acbir@vet.dtu.dk, +4535886286

9

Abstract

Antimicrobial resistance in pigs has been under scrutiny for many years. However, many questions remain unanswered, including whether the initial antimicrobial resistance level of a pig will influence the antimicrobial resistance found at slaughter. Faecal samples from finishers pigs from 681 farms and from sows from 82 farms were collected, and levels of seven antimicrobial resistance genes, *ermB*, *ermF*, *sulI*, *sulII*, *tet*(M), *tet*(O), and *tet*(W), were quantified by high-capacity qPCR. There were 40 pairs of observations where the finishers were born in the farms of the sows. We found a significant positive correlation between the levels of AMR genes in finishers and the sows in the farms where the pigs were born. Furthermore, there were differences between AMR gene levels for the sow and finisher populations for *ermB*, *ermF*, *sulI*, *sulII*, *tet*(O), and *tet*(W), though not for *tet*(M).

Keywords

Antimicrobial resistance genes, sows, offspring, slaughter pigs

Introduction

Antimicrobial resistance (AMR) is a growing global health concern. As a result, there is considerable interest in careful monitoring of AMR levels and understanding what factors influence them. The use of antimicrobial agents can lead to the development of AMR, both directly and indirectly in commensal and pathogenic bacteria. However, it is also clear that AMR genes are widespread in nature and that in absence of antimicrobials the natural function of these genes is not associated with AMR. Therefore, it is questionable whether reducing the use of antimicrobial agents is a sufficient measure to control AMR levels (Baquero et al., 2011). Factors influencing the abundance of AMR genes within a population include among others the rate of transfer of AMR genes from the environment, and the initial abundance of AMR genes (Zur Wiesch et al., 2011).

33 Furthermore, it has been shown that the use of antibiotics in sows can affect the level of AMR
34 found in offspring (Callens et al., 2015; Mathew et al., 2005). As is the case with all mammals,
35 piglets are born with a sterile gut that immediately after birth is colonised by a complex and diverse
36 microbial ecosystem originating from environment hereunder from the dam (Sekirov et al., 2010).

37 In this study, we consider seven AMR genes, *ermB, ermF, sulI, sulII, tet*(M), *tet*(O), and *tet*(W).
38 The three sets of AMR genes bestows resistance against three most commonly used antimicrobial
39 agents in Danish pig production but in different rearing periods. The two *erm* genes confer
40 resistance against macrolides; the most used antimicrobial class in weaners and the third and fourth
41 in finishers and sows. The *sulI and sulII* genes confer resistance towards sulfonamides; the second
42 most used antimicrobial class in sows in Denmark, seldom used in weaners and almost never used
43 in finishers. The three *tet* genes confer resistance towards tetracyclines; the most commonly used
44 antimicrobial class in both finishers and weaners (DANMAP, 2016).

45 The objective of this study was to evaluate whether i) the levels of seven AMR genes found in
46 finisher pigs at slaughter were associated with the levels in the farm where the finishers were born,
47 and ii) there were equal levels of the seven AMR genes in the sow and finisher pig populations.

48 Materials and methods

49 Sampling from finisher

50 Sampling from finisher farms took place in February and March 2015 at five different abattoirs.
51 Faecal samples were obtained from five pigs per farm and collected using a validated sampling
52 method as previously described (Birkegård et al., 2017a, 2017b). In brief: faecal samples were
53 collected at the slaughter line, after the removal of the gut, by squeezing out a small amount of
54 faeces into a 12.5 mL sampling vial. The pigs were sampled as they came along the slaughter line,

55 ensuring that five pigs per farm were sampled. The samples were placed in Styrofoam boxes with
56 cooling elements and sent by overnight mail to our laboratory.

57 **Sampling from sows**

58 Samples from the sows were obtained through two approaches. The first approach was sampling at
59 an abattoir only slaughtering sows. The yield of this approach was too small due to logistical
60 challenges. Hence, a new sampling approach was chosen with sampling at the farms.

61 *Sampling at the abattoir*

62 The sampling took place in the lairage over the same period as the sampling for finisher farms.
63 Farms sows to sample was chosen with a convenient approach, meaning that sows from farms
64 sending more than 10 sows in the batch was chosen for sampling when the abattoir technician had
65 time to sample and only approachable sows were sampled.

66 *Sampling at the farm*

67 A targeted sample approach was used to supplement the abattoir samples. A subsample of 180
68 farms with sampled finishers was chosen randomly. Farms with sows that according to the
69 movement database (Danish Veterinary and Food Administration, n.d.) sold pigs in the period of 1st
70 of September 2014 to 1st of January 2015 to the chosen farms with finishers together with the
71 chosen farms with finishers that also had sows were invited to participate in study. A sampling kit
72 (five 12.5 mL sampling vials, cooling elements and a response letter) was sent to each farm owner
73 who agreed to participate in the study. The owners were asked to collect samples from the oldest
74 sows and take samples during different production cycles (e.g. gestation, farrowing, mating, and
75 nursing sections). However, the age of sampled sows was not noted.

76 *Collecting the samples*

77 Both at the abattoir and at the farms five sows per farm were sampled and the faecal samples were
78 collected from the rectum of live sows by digital manipulation. After sampling, the samples were
79 placed in Styrofoam boxes with cooling elements and sent by overnight mail to our laboratory.

80 **Antimicrobial resistance gene levels**

81 The levels of seven AMR genes, *ermB, ermF, sulI, sulII, tet*(M), *tet*(O) and *tet*(W), were determined
82 as described by Clasen et al (2016). Briefly, the five samples collected per farm were pooled into
83 one aliquot. DNA was extracted from all samples and the levels of AMR genes were measured in
84 relative quantification values (RQ) describing the quantity of the genes relative to the amount of
85 bacterial DNA in the sample. The latter was measured by the reference gene 16S, ensuring that the
86 AMR genes were comparable between farms.

87 The *sulI* and *sulII* genes had many non-detects for the finisher farms and were dichotomised as
88 either present or absent, as in previous studies (Birkegård et al., 2017a).

89 **Birth farms**

90 Using the movement database (Danish Veterinary and Food Administration, n.d.) the farms where
91 the finishers were born were identified and overlap with farms with sampled farms was found. For
92 some of the farms the finishers had not been moved during their lifetime, i.e. both sows and the
93 finishers were sampled for the farm. Farms with finishers born at several different farms were
94 excluded from further analyses due to the complications of knowing the exact origin of the pigs.
95 The correlation between the levels of AMR genes in the pairs of farms with sampled finishers and
96 farms with sampled sows in the birth farm of the finishers were visualised using scatterplots for
97 *ermB*, *ermF*, *tet*(M), *tet*(O), and *tet*(W). Spearman correlation coefficients (ρ) and associated p-
98 values were calculated per gene. The correlations were estimated to be significant if the p-value was
99 below 0.05. A bootstrapping procedure was carried out to test if the correlation between the levels

100 of AMR genes in the pairs of finishers and sows from the birth farm happened by chance. Forty
101 pairs of unrelated sow and finisher farms were sampled 10,000 times from the farms with sampled
102 finishers or sows, respectively. For each sample the Spearman correlation coefficient (ρ) and
103 associated p-value were calculated per AMR gene.

104 **Comparing levels of genes in the sow and finisher populations**

105 The levels of the genes (RQ values) in the finisher and sow populations were visualised using
106 boxplots and compared using a two-sided Mann-Whitney-Wilcoxon Test for the *ermB*, *ermF*,
107 *tet*(M), *tet*(O), and *tet*(W) genes. The prevalence of the *sul* genes in the two populations was
108 compared using a Pearson's Chi-squared Test.

109 **Software**

110 All data management and statistical analyses were carried out in R version 3.2.2 (R Core Team,
111 2017) using RStudio (RStudio team, 2016).

112 **Results**

113 **Populations**

114 The samples from finishers were obtained through sampling at the abattoir from 681 pig farms with
115 indoor non-organic production of finishers (Birkegård et al., 2017a, 2017b).

116 The samples from sows were obtained from 82 farms, 65 farms at the abattoir and 17 at the farm.

117 The farms with sampled finishers were larger than farms that were not included in the
118 study (Birkegård et al., 2017b). Likewise, the farms with sampled sows were larger (median size:
119 700 sows) compared to the all farms with sows (median size: 500 sows). However, for both
120 populations the antimicrobial exposure in the farms with sampled finishers/sows was not
121 significantly different from that in the farm without sampled finishers/sows when tested with a
122 Wilcoxon signed-rank test.

123 **Birth farm**

124 The sampling resulted in 52 pairs of finishers and sows from their birth farm. In total, 12 pairs were
125 excluded because the finisher farms included a mixture of pigs born in more than one farm. In ten
126 of the remaining 40 pairs the, finisher and sow were sampled from the same farm, and in 30 of them
127 the finishers had been moved at least once, but could be traced back to only one birth farm.

128 Due to missing values for *ermF* in either the sows or the finishers, 13 pairs were excluded from the
129 *ermF* analyses. Likewise, 8 pairs were excluded from the *tet*(M) analyses.

130 Correlation coefficients ranging from 0.06 to 0.47 were found between the pairs of finishers and
131 sows from their birth farm for the different AMR genes (Fig. 1). Significant correlations between
132 the gene levels in the pairs of finishers and sows from their birth farm were found for
133 *ermB* (p-value = 0.002), *ermF* (p-value = 0.03), and *tet*(O) (p-value = 0.04), whereas the
134 correlations for *tet*(W) and *tet*(M) were found to be non-significant (Fig. 1).

135 Bootstrapping the pairs of unrelated finisher and sow, showed that the percentages of repetitions
136 where the correlations were found to be significant ranged from 4.6-5.3 % for the individual genes
137 for pairs of unrelated finishers and sows. Due to the high prevalence of *sulI* (98% positive farms)
138 and *sulII* (96% positive farms) in sow farms, it was not possible to assess whether the AMR levels
139 of the sows was associated with the levels in the finishers for these two genes.

140 **Antimicrobial resistance gene levels in finisher pigs and sows**

141 There were significantly higher levels of AMR genes in the finisher farms compared to the sow
142 farms for the *erm*- and the *tet*-genes (p-value <0.001), with the exception of *tet*(M) (p-value = 0.4;
143 Fig. 2). In contrast, there was a significantly higher prevalence of the *sul*-genes in the sow
144 population (*sulI*: 98.7% positive farms and *sulII*: 94.9% positive farms) compared to the finisher
145 population (*sulI*: 53.1% positive farms and *sulII*: 65.2% positive farms; p-value <0.001).

146 **Discussion**

147 We found a significant correlation between the levels of *ermB*, *ermF*, and *tet*(O) in the pairs of
148 finishers and sows from their birth farm (Fig. 1). This indicates that the starting level of AMR for a
149 pig, here approximated as the AMR level of the sows in the farm where the finishers were born,
150 influences the level of AMR found at slaughter. This conclusion was further supported by the
151 bootstrapping of pairs of unrelated finishers and sows to demonstrate that in at least 94.6 % of the
152 iterations there was no significant correlation between the unrelated finisher and sow farms. Similar
153 results have been observed in poultry farms (Dierikx et al., 2013).

154 We found a significant difference between sow and finisher farm populations in the level of most of
155 the AMR genes included in the study. For example, the *ermB*, *ermF*, *tet*(O), and *tet*(W) genes were
156 found in higher levels in finishers than in the sows (Fig. 2). The prevalence of *sul*-genes was
157 significantly higher (98.7% for *sulI* and 94.9% for *sulII*) in sows than in finishers (53.1% for *sulI*
158 and 65.2% for *sulII*). This corresponds with the pattern of antimicrobial consumption in Danish pig
159 farms. Tetracyclines and macrolides are mainly given to weaner and finisher pigs, whereas
160 sulfonamides are mainly given to sows (DANMAP, 2016).

161 Sows and finishers are fed differently, which might further contribute to the differing AMR gene
162 levels, as feeding strategies can alter the composition of the gut microbiota (Jensen et al., 2012), and
163 the AMR gene levels vary according to the distribution of bacterial species (Lanza et al., 2015).
164 Finishers are fed to grow fast, whereas sows are fed more restrictively, and have a more varied diet
165 as they are fed according to production cycle (Kyriazakis and Whittemore, 2006). Another potential
166 reason for the difference is the housing density, which is often highest for the finishers. The housing
167 density could influence the bacterial load and therefore the AMR levels. Furthermore, it is possible
168 that differences in AMR gene levels could be explained by the difference in age between finishers
169 and sows, as age is also known to affect the level of AMR (Dewulf et al., 2007).

170 In conclusion, we have found strong indications that the level of AMR genes in sows can influence
171 the AMR levels in their offspring at the time of slaughter. Furthermore, there is a difference
172 between AMR gene levels for the sow and finisher farm populations for *ermB*, *ermF*, *sulI*, *sulII*,
173 *tet*(O), and *tet*(W), though not for *tet*(M).

174 Acknowledgements

175 The authors would like to thank the employees of Danish Crown and Tican (in particular Marie B.
176 Hansen, Vagner Bøge, and Henrik Bækstrøm), Margarida Arede, and the owners of the sow farms
177 for their cooperation and assistance with sampling. The authors would also like to thank Joanna
178 Zeitman Amenuvor, Karin Tarp, and Sophia Rasmussen for their technical assistance together with
179 Kerstin Skovgaard for advice on handling qPCR data. This study was carried out as part of a grant
180 from the Danish Veterinary and Food Administration, relating to evidence-based advice on AMR.

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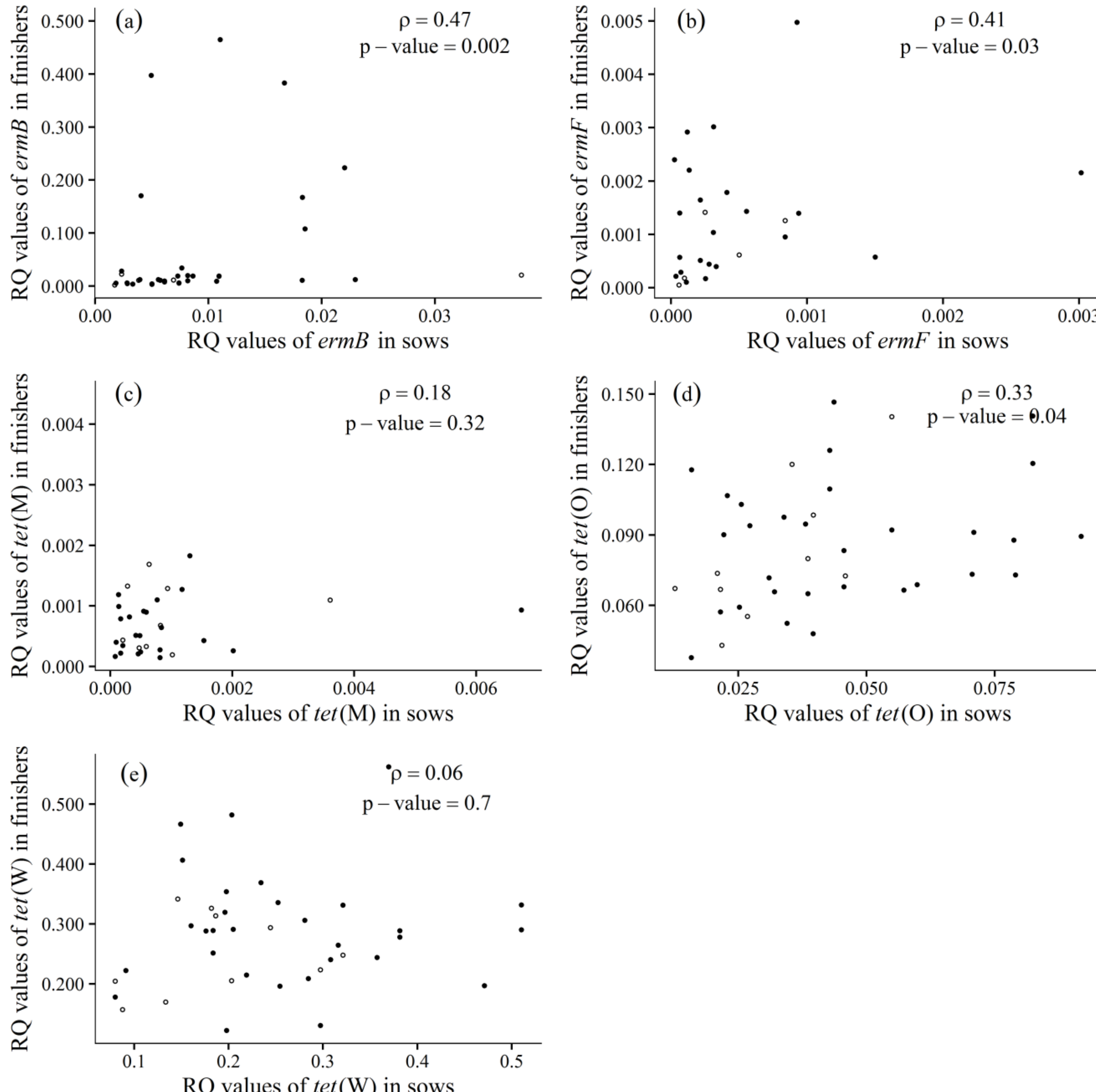
230

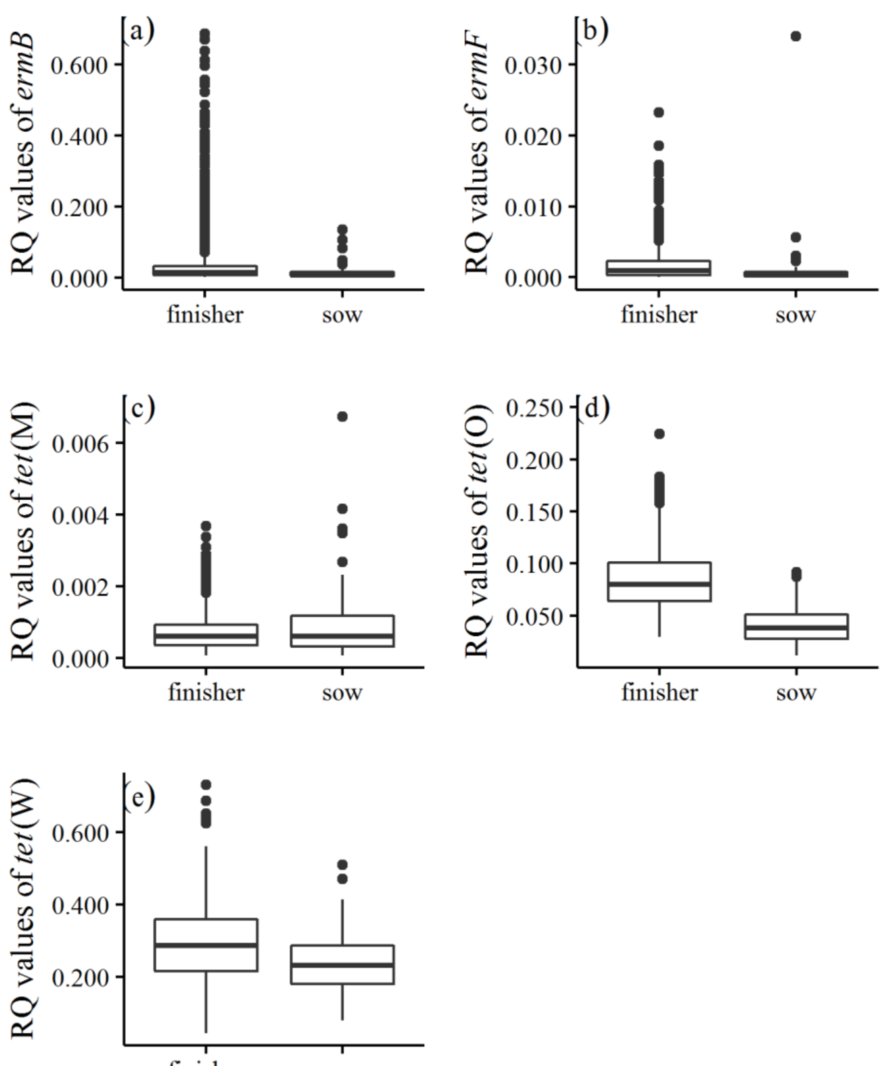
231 **Figures**



232

233 **Figure 1 Scatterplots of the association among AMR gene levels in finishers and sows from the birth farm of the finishers for**
234 **(a): *ermB*, (b): *ermF*, (c): *tet*(M), (d): *tet*(O), and (e): *tet*(W) genes.** For farms with both finishers and sows, the correlation is
235 indicated by an empty dot, whereas other pairs are indicated by a filled dot. The Spearman correlation coefficient (ρ) for the given
236 gene is indicated on the plot, together with the p-value. In the plot of correlations for *ermB* (a), three observations were removed due
237 to extreme levels (above 0.1) of *ermB* in the sow farms. Likewise, two observations were removed in the plot for *ermF* (b). The
238 excluded observations were included in the calculation and the Spearman correlations.

239



240

241 **Figure 2 AMR gene levels in sow and finisher farms for (a): ermB, (b): ermF, (c): tet(M), (d): tet(O), and (e): tet(W) genes.**

242

5. MATERIALS AND METHODS NOT INCLUDED IN THE MANUSCRIPTS

This chapter includes information about the materials and methods used in this PhD study. However, the chapter only contains information that is not included in the manuscripts, such as an elaboration of methods used in the manuscripts and additional analyses.

5.1 The pooling study

Manuscript 1[8] addressed the question of "how many pigs should be sampled to obtain a representative farm-level sample?". The study consisted of two parts with a total of three goals:

1) Part one:
   a. Answering the question: "how many pigs should be sampled to obtain a representative farm-level sample?"
   b. Testing the agreement among samples
2) Part two:
   c. Optimising the pooling method

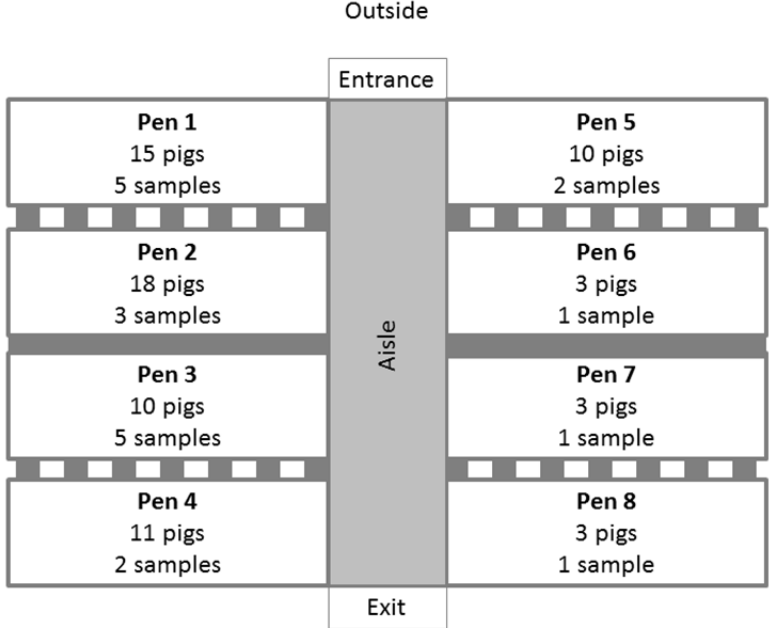


Figure 5.1 Sampling scheme. Schematic drawing showing the number of pens in the section of the pig farm where the samples were collected, the number of pigs in each pen, and the number of samples collected per pen. There was no contact between pigs in pens 2 and 3, or between pigs in pens 6 and 7, whereas bars between the other consecutive pens allowed snout contact.

5.1.1 Sample collection, part one

As described in Manuscript 1[8], 20 samples were collected from a farm on Funen. The section of the pig farm from which the samples were collected consisted of eight pens with a total of 53 pigs. The number of pigs sampled per pen was decided based on the number of pigs per pen, ensuring that at least one and a maximum of five pigs per pen was sampled (Fig. 5.1). The samples were collected from the approachable pigs by digital manipulation of the rectum. The farmer informed that the sampled pigs were approximately 25 weeks old and they were the pigs closest to slaughter at the farm. The section was partly empty as pigs from the section were continuously sent to slaughter.

5.1.2 Sample pooling, part one

The samples were pooled, dissolved in Phosphate Buffer Solution (PBS), and DNA was extracted according to the scheme shown in Figure 5.2 and described below:

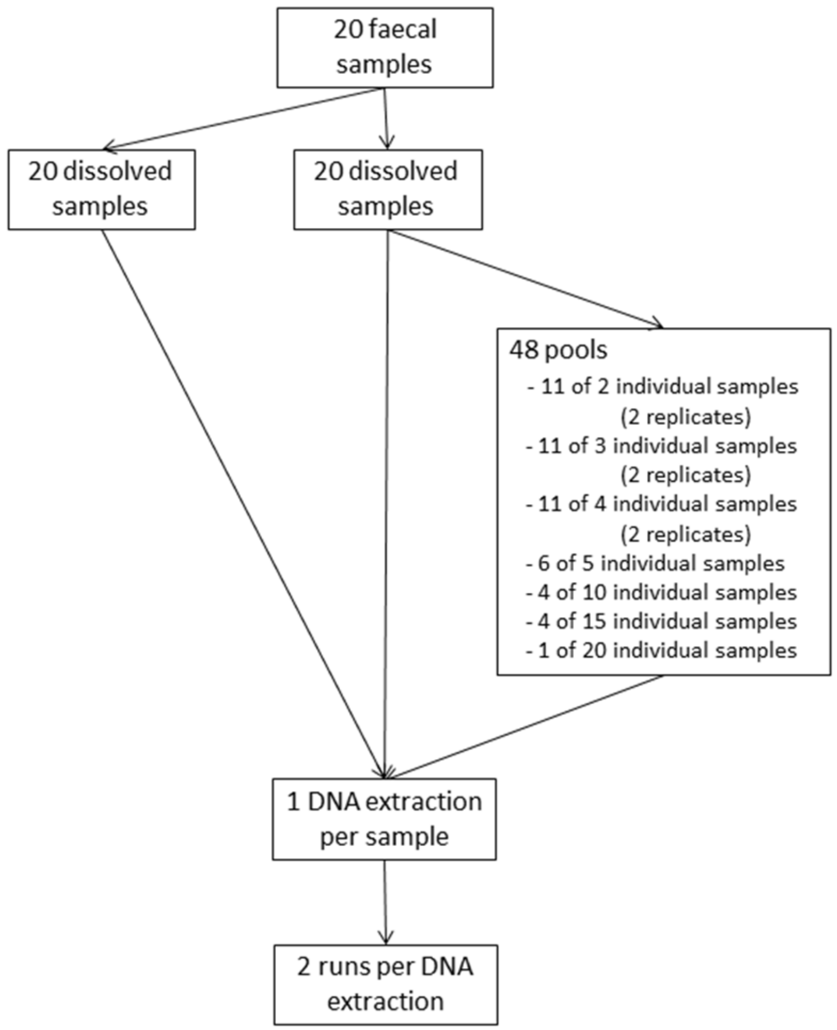


Figure 5.2 Pooling scheme. Scheme showing how samples were pooled and processed.

One gram of the faecal material was taken from each of the 20 sample and dissolved in 9 mL PBS. The process was repeated. The pools were made from one set of the dissolved samples by taking 0.5 mL from each of the dissolved individual samples contributing to the pools (Fig. 5.3 “pooling method 1”). DNA was extracted from each set of dissolved individual samples and the pools. Each DNA extraction was run twice on the qPCR. There were 88 DNA extractions and 176 run on the qPCR. Each run on the qPCR had two technical replicates. The RQ values for the qPCR runs were subsequently calculated as described in Manuscript 1[8]. The replicates of the dissolved samples and replicates of the qPCR runs were made to test the agreement between them. Furthermore, replicates of some of the pools were made to test their agreement (Fig. 5.2).

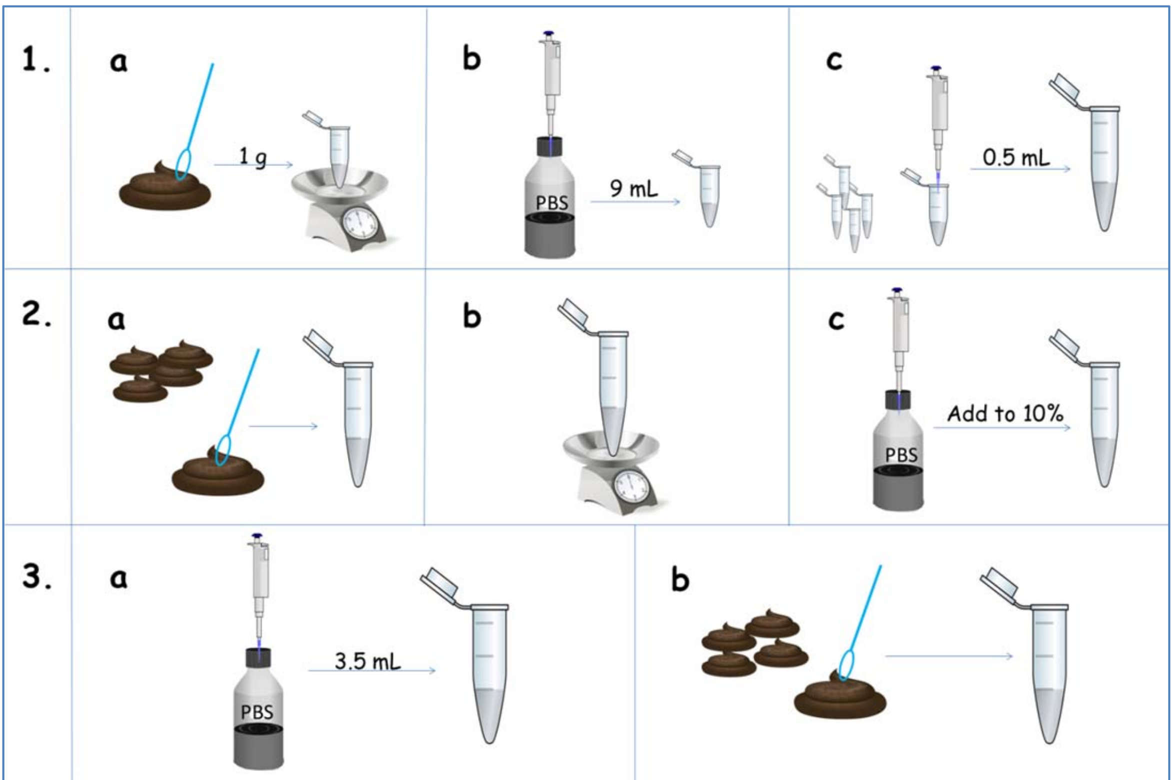


Figure 5.3 Pooling methods. Pooling method 1: a) 1 g of faeces was transferred to a sample vial, b) 9 ml PBS was transferred to the sample vial and the faeces were dissolved in the PBS, steps a-b were repeated for five samples, c) 0.5 ml from each of the five dissolved sample was transferred to a new vial. Pooling method 2: a) a small amount of faeces (enough to fit the eye of an inoculation needle) from each of the five samples was transferred to a sample vial, b) the vial was weighed, c) PBS was added to a 10% solution and transferred to the tube. Pooling method 3: a) 3.5 ml PBS was transferred to a sample vial, b) a small amount of faeces (enough to fit the eye of an inoculation needle) from each sample was transferred to the sample vial. The 3.5 ml PBS used in pooling method 3 was calculated based on the mean amount of PBS used in pooling method 2.

5.1.3 Optimising the pooling method, part two

To optimize the pooling method a new set of samples were collected in January 2015. This was done after finalizing the first part of the study. Five samples from five farms were collected at an abattoir in Jutland (Manuscript 1[8]).

The aim of the sampling was twofold:

1) Assessing whether sampling at the lairage was possible and the time needed to collect samples from five pigs
2) To find the least time-consuming pooling method that provided equivalent results to that of the previous pooling method[68].

For the first part of the aim, it was found out that sampling in the lairage was possible. However, for the cross-sectional study, this sampling method was replaced by collecting samples from slaughtered pigs at the slaughter line following recommendations from a abattoir manager. The suggested method had the advantages that the pigs would not be stressed by handling prior to slaughter and it would require much less time to collect samples.

For the second part of the aim, three different pooling methods were used and compared in terms of the time required to complete 10 pools. The pooling methods are presented in Figure 5.3. An overview of how the samples were pooled in the second part of the pooling study can be found in the appendix.

5.2 Sampling from the finisher farms

A finisher farm was defined throughout this thesis as a farm with finishers. A finisher farm can also have other age groups (sows and/or weaners).

The term target population is defined as the population that the researcher want to draw conclusion upon[13]. For the sampling from the finisher farms, the target population was all Danish pig farms with a conventional production of finishers registered in the CHR on the 19th of January 2015[14]. The term study population refers to the part of the target population that includes the pig farms that will be selected for sampling [13]. This is also called the source population[127]. For the sampling from the finisher farms, the study population was farms with finishers slaughtered at the sampling sites[14].

The sampling was planned, conducted, and evaluated as described in Manuscript 2[14] and 4-6[9–11]. Here, the planning is elaborated on and the evaluation expanded.

5.2.1 Planning the sampling

The sampling process was planned to ensure spatial randomness and representativeness with regard to the target population. Furthermore, the criterion for the sampling process was that the sampling period should be short. The strict quarantine rules of most Danish pig farms made it difficult to sample from many farms in a short period of time. Therefore it was decided to sample pigs at the slaughter line in abattoirs in order to reduce the sampling time and disturbance of the pigs. Sampling at the abattoir is also used in DANMAP[20]. However, in order to prevent damaging the

intestine, sampling from the rectum was chosen instead of sampling from the caecum as done in DANMAP[20].

Five of the seven largest Danish owned abattoirs for finisher pigs in Denmark were chosen as sampling sites to ensure that the entire country (except Bornholm) should be covered by the sample[14]. The reason for leaving out one abattoir was that it primarily slaughters pigs from free range and organic farms which were not part of the target population. Another was left out due to the location at the island Bornholm.

The number of farms to sample at each abattoir was calculated based on the mean number of farms sending pigs for slaughter during five weeks in November 2014 and five weeks in February 2014 through March 2014. The total number of farms targeted for sampling was 800 Danish pig farms, with 140, 300, 160, 120, and 80 farms per abattoir.

The sampling was planned to be carried out during the period of 2nd of February to 3rd of March 2015, both dates included. The sampling period was kept short as it is known that the level of AMR in pig farms might change with the seasons of the year[105] and furthermore to meet one of the requirement of the cross-sectional studies, which is to provide a snapshot of the population[13].

5.2.2 Evaluating the sampling

In Manuscript 2[14], the farms with the sampled finishers were compared to all farms with finishers, that were not included in the sample. However, other studies have only considered farms with more than 200 to be production farm. Therefore, the farm size for farms with sampled finishers was compared to those without sampled finisher excluding all farms with less than 200 finishers.

The sampling procedure used to collect samples from the finisher farms was evaluated in Manuscript 2[14] in order to assess whether the sampled farms were representative of the target population in terms of size and geographical location. However, this sample was also used to estimate the quantitative relationship between antimicrobial exposure and AMR genes. Therefore, it was also relevant to assess whether the sampled farms were representative of the target population in terms of antimicrobial exposure. Hence, the antimicrobial exposure was calculated using the LEA algorithm for the sampled and non-sampled farms, as described in Manuscript 3[15]. The antimicrobial exposure estimates were then compared using boxplots and a Wilcoxon signed-rank test.

5.2.3 Repeated samples

Due to the sampling method one farm could be sampled more than once. The repeated samples occurred because it was not known to the sampling technician which farms were sampled on other days or at other abattoirs. Repeated samples from individual farms were used to evaluate the consistency of AMR gene levels using Bland-Altman plots[128]. When multiple samples were obtained from a farm, samples were compared pairwise, e.g. farms sampled three times (sample A, B, and C) were compared using three pairs of samples (A-B, A-C, and B-C).

5.3 Sampling from the sow farms

A sow farm is defined as a farm that has sows. Nevertheless, a sow farm can also have other age groups (finishers and/or weaners).

5.3.1 Planning the sampling of the sow farm

The sampling process for the sow farms is briefly described in Manuscript 6[11]. The sampling from the sow farms were done by two approaches. The first approach took place at an abattoir only for sows in the same period as the sampling from the finisher farms. The goal was to sample sows from 200 farms. However, the sampling was slow and due to miscommunication and logistical challenges the yield of the sampling was too low. Therefore, a more focused sample approach was chosen. This approach was to invite sow farms that had delivered pigs to the finisher farms to take part in the study as explained below:

The finisher farms were divided into three categories:

1) integrated farms, i.e. finisher farms that also had sows and weaners with no delivery of pigs from other farms
2) farms only receiving pigs from one sow farm,
3) farms receiving pigs from more than one sow farm.

Data from the PMD from the period between 1st September 2014 and 1st January 2015 was used to assess the categories of the farms. In total, 60 finisher farms from each category were chosen randomly by using the “sample”-function in R[129]. Subsequently, the sow farms that had delivered pigs to the finisher farms were identified. The resulting list consisted of 128 different sow herds. In

total, 108 owners of the sow farms were invited to take part in the project, the remaining 20 farms were not contacted due to logistical reasons. The sampling process is described in details in Manuscript 6[11].

5.3.2 Comparison of farms with and without sampled sows

It was desirable to know whether or not the sampled sow farms were representative of the total population of sow farms in terms of yearly antimicrobial consumption and number of sows registered in the CHR.

Antimicrobial consumption was calculated per antimicrobial class for the sampled sow farms and compared with non-sampled sow farms with more than 50 sows registered in the CHR. It was calculated for a year prior to the first sampling date and divided by the number of sows registered in the CHR.

Both antimicrobial consumption and the number of sows were compared for sampled and non-sampled sow farms using a Wilcoxon signed-rank test.

5.4 LEA

5.4.1 Manual changes to the LEA algorithm

The LEA algorithm was run as described in Manuscript 3[15] for the farms with finishers sampled in the cross-sectional study. The result was used to evaluate the quantitative relationship between antimicrobial exposure and AMR described in Manuscript 5[10]. For the smoothing of the amount of antimicrobial product purchased a 180 days period was used. As described in Manuscript 3[15], the algorithm did not work for all farms due to a lack of data input in the algorithm, or because the actual production system at the farm differed from what had been assumed. Manual changes were made in order to increase the number of farms for which the algorithm could be run. Four sets of manual changes were made:

1) Adjustments of the number of pigs calculated, as described in Manuscript 3[15].
2) For finisher units with no identified weaner or sow units, the transfer window was expanded, or cut-offs were changed. The transfer window and the cut-offs (cut-off$_{weaner}$ and cut-off$_{sow}$), are defined in Manuscript 3[15].

3) Further tracement of the finisher batch. The online version of the PMD[130] was checked for delayed registrations of movements.
4) Changing the number of age groups present. It was assumed that the majority of active farms would use medication (including vaccines intended for veterinary use) within a period of one year. Therefore, weaner units, and sow units with no registrations of purchases in VetStat for an age group registered in the CHR data from May 2014 to May 2015 were each checked to assess whether the age group was actually present at the farm, using the following approach:
   a. Sows: any movements of dead sows, or movements of pigs to an abattoir that slaughtered sows were investigated.
   b. Weaners: any movements of containers were investigated.

   If no registrations were found, it was assumed that the age group was not present and the number of pen places for the farm was set to zero. If the checked age group was the only one on the farm, no corrections were made.

If it was still not possible to trace the batch back after step 3 and 4, the batch was excluded.

5.4.2 Estimating the effect of using standard treatment weights

In the LEA algorithm, the antimicrobial exposure is not divided by the standard treatment weight as done when antimicrobial consumption has to be compared between age groups and to benchmark the farms in the yellow card initiative[62]. In Denmark the following standard treatment weights are used for standardization of the antimicrobial consumption: sows: 200 kg, weaners: 15 kg, and finishers: 50 kg[131]. In Manuscript 5[10], the regression analyses were used to assess the association between categories of antimicrobial exposure and the levels of AMR genes. In that study, the LEA algorithm is used and hence the standard treatment weights are not used for standardization of the antimicrobial exposure. In order to investigate if the use of the standard treatment weights would have altered the results of the regression analyses reported in Manuscript 5[10], the LEA was recalculated using the standard weight as presented in the equation beneath and the regression analyses were re-run.

$$Lifetime\ exposure_{standardized} = \frac{exposure_{piglet}}{200} + \frac{exposure_{weaner}}{15} + \frac{exposure_{finishers}}{50}$$

The categorisation for the antimicrobial exposure in the piglet, weaner, and finisher periods will not be altered if the standard treatment weight were used as the same factor would be used for all farms.

However, the lifetime categorization could be influenced by whether the standard treatment weights are used or not. Therefore, the potential effect of using the standard weights was investigated as explained above.

Subsequently, the LEA categories was recalculated using the same approach as in Manuscript 5[10]. Antimicrobial exposure (measured in ADD_{kg}) was categorised according to the quartiles for the total exposure of antimicrobials for the standardized lifetime exposure. The following categories were used[10]:

1) No exposure (a value of zero)
2) Very low exposure (>0 - 25th percentile)
3) Low exposure (>25th percentile - Median)
4) High exposure (>Median - 75th percentile)
5) Very High exposure (>75th percentile).

The categories found with or without standardized LEA were compared farm-wise. For those antimicrobial classes where differences were found between the two calculations, the regression analyses investigating the association between AMR genes and antimicrobial exposure were re-run with the new categories.

5.4.3 Comparison of LEA and movement

Movements of pigs in to a farm can result in introduction of new diseases and/or increased risk of infection due to both mixing of new pigs and stress caused by the movement. An increased risk of infection may increase disease occurrence, which may result in a higher level of antimicrobial consumption. The LEA algorithm can be used to test whether movements of pigs may have been associated with higher antimicrobials exposure. In the LEA algorithm, it is calculated how many different weaner units, sow units, and farms in total the finishers in the batch altogether have been in. These numbers are proxies for the frequency of movement of the pigs and the degree of mixing of pigs from different farms, and were compared to the total LEA (measuring the total amount of ADD_{kg} the finishers in a batch have been exposed to).

5.5 Evaluating the spatial patterns of antimicrobial resistance genes

Spatial randomness of the sampled farms can affect the validity of spatial analyses. Therefore, the spatial randomness of the sampled farms in the cross-sectional study (assessed in Manuscript 2[14]) was compared to the result of the spatial cluster analyses of the AMR genes (assessed in Manuscript 4[9]). This was done by drawing the clusters from the two analyses on the same map of Denmark. This allowed assessing whether clusters were found in the same area, and consequently validate the AMR gene clusters.

6. RESULTS NOT INCLUDED IN THE MANUSCRIPTS

The primary results of this PhD study are presented in the manuscripts and summarized in Chapter 7. This chapter discusses the results of the additional analyses presented in Chapter 5.

6.1 Sampling from the finisher farms

6.1.1 Sampling

The sampling was planned so that samples were collected at one or two sampling sites per day (Table 6.1). No samples were collected on Fridays or at weekends. Sampling time was 4-7 hours per day.

Table 6.1 Number of farms sampled per day per abattoir A-E

	DAY OF SAMPLING PERIOD															
Abattoir	1	2	3	7	8	9	10	14	15	16	17	21	22	28	29	30
A				24	44	48							24			
B		24	49					55	67	52	53					
C								30	30	30	30	20	20			
D	30	30	30				30									
E														20	30	30

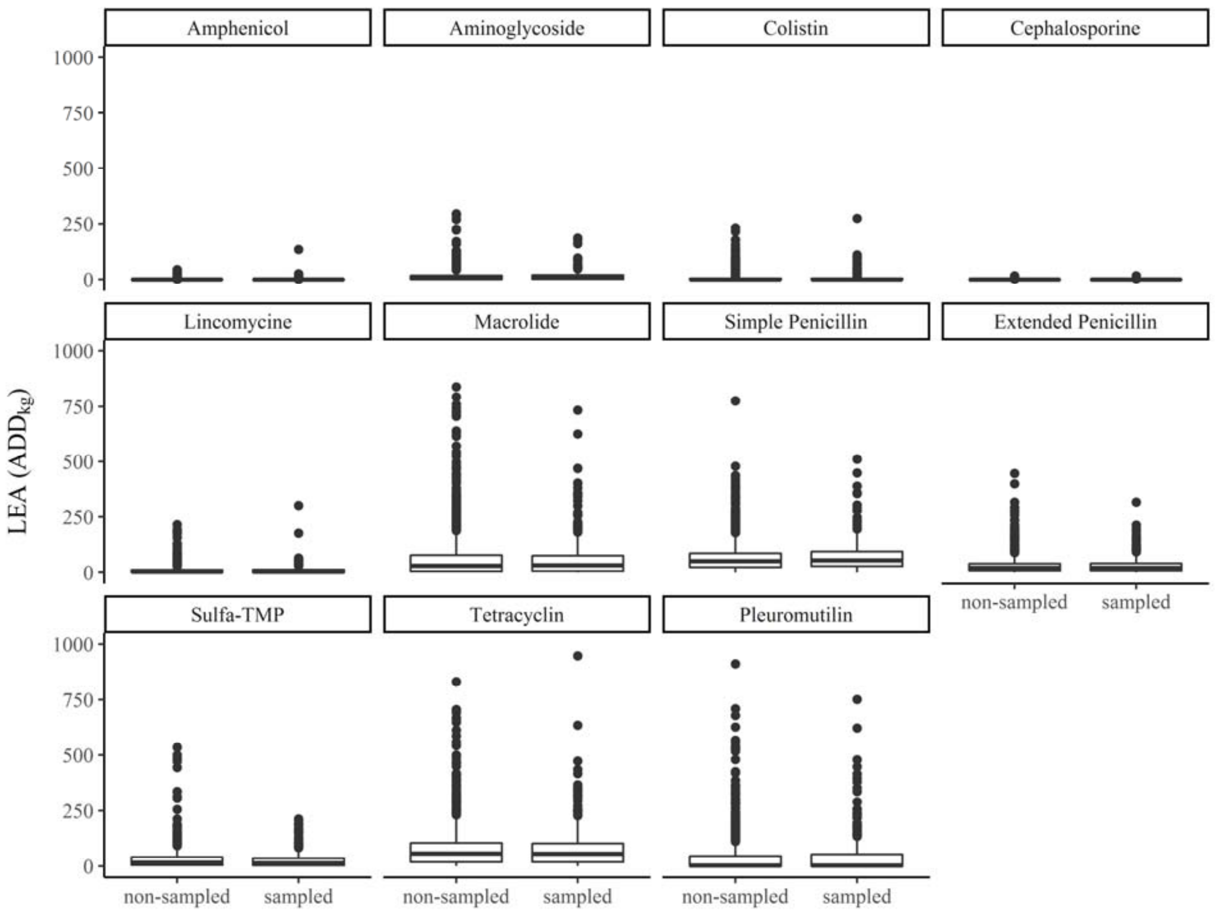


Figure 6.1 Lifetime exposure to antimicrobials in the sampled and non-sampled finisher farms.

6.1.2 Evaluating the representativeness of sampled farms

In Manuscript 2[14], farms with and without sampled finishers were compared according to farm size. Two parameters for farm size were used, the 26 week delivery of finishers to slaughter (the farm production of finishers) and the number of finishers registered in the CHR. In this thesis, the farms with and without finishers were compared once more, but excluding the farms with less than 200 finishers. Again, a significant difference in farm size between farms with and without sampled finishers was found, both in terms of 26 week delivery and number of finishers registered on the farm.

The lifetime exposure of 11 antimicrobial classes was compared. There was no significant difference in antimicrobial exposure between the sampled farms and the non-sampled farms except for amphenicol and simple penicillins (Fig. 6.1).

6.1.3 Repeated samples

A total of 95 farms were sampled two to four times (82 farms were sampled twice, 12 farms were sampled three times, and one farm was sampled four times), either at the same abattoir on the same day, at the same abattoir on different days, or at several abattoirs. Repeated samples at the same abattoir were due to the same farm having more than one delivery number, or due to an error by the sampler. The delivery numbers were not checked against those already sampled on previous days which enabled sampling of the same farm on several days. Eight of the farms had two delivery numbers and one had three.

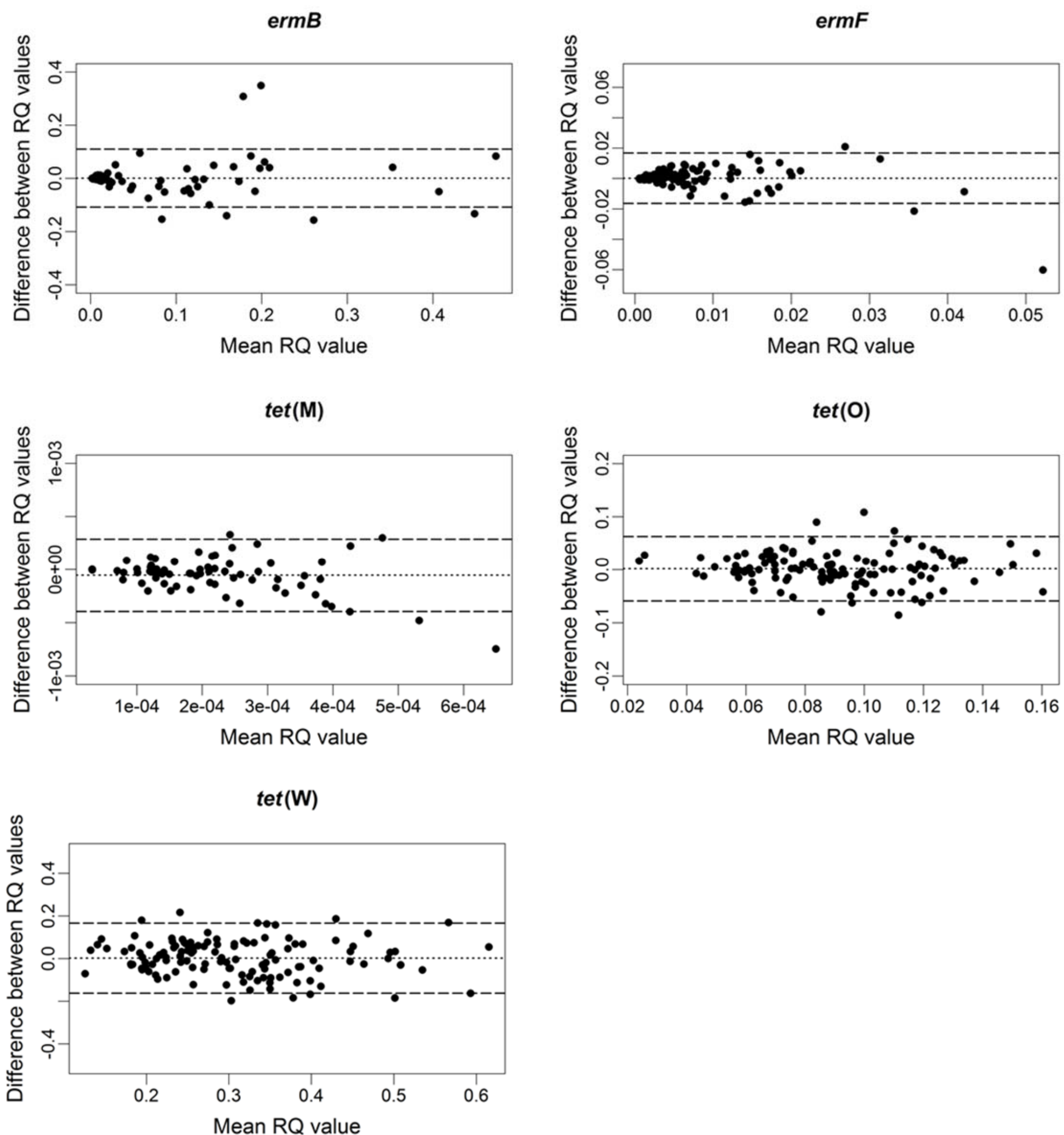


Figure 6.2 Bland-Altman plots comparing the AMR gene levels in farms that were sampled multiple times. Each pair of samples is shown separately, e.g. farms sampled three times were compared using three pairs of samples.

The repeated samples were used to assess the agreement using Bland-Altman plots[128]. These showed that the gene levels of the *erm* and *tet* genes were comparable between repeated samples from the same farms (Fig. 6.2). The *sul* genes were dichotomised as either absent or present. For the *sul* genes, 50 of the 95 farms had a change in the *sulI* gene status and 23 farms had a change in the *sulII* gene status.

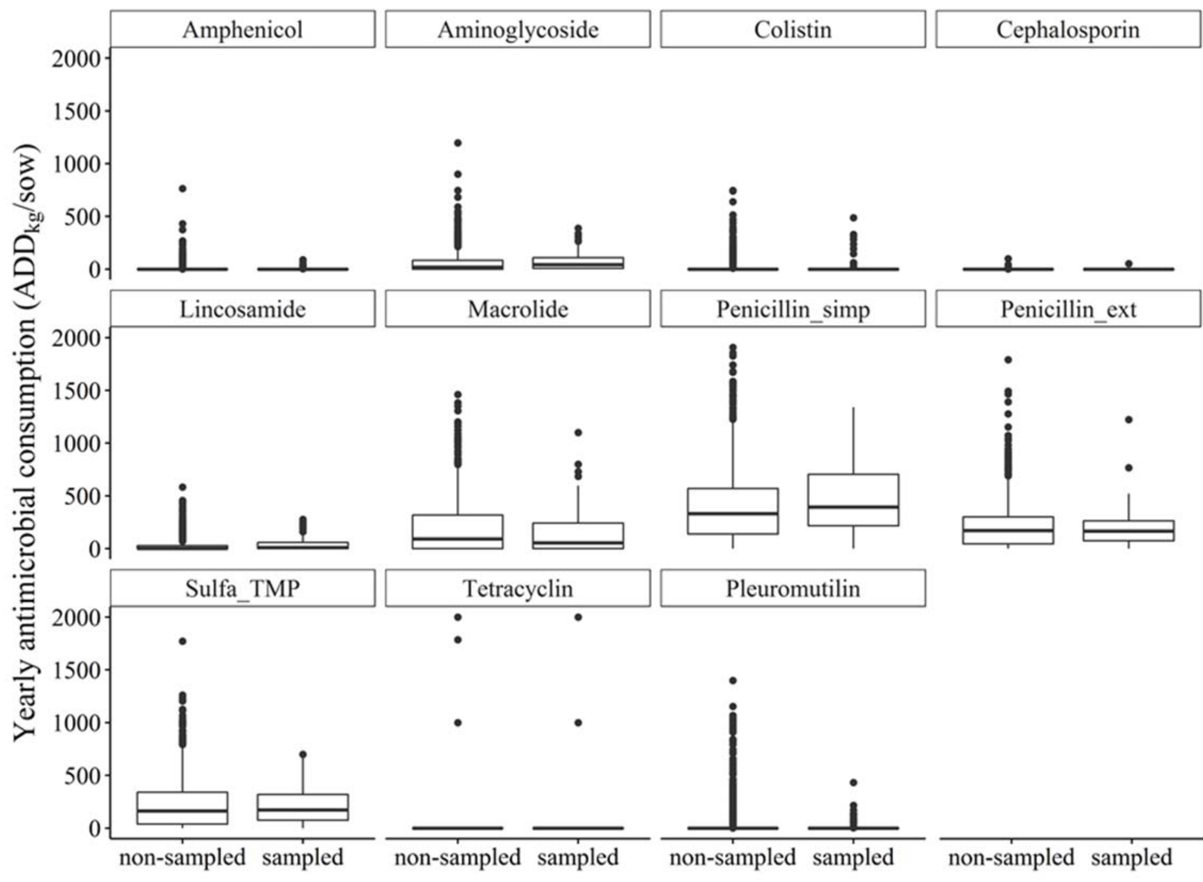


Figure 6.3 Boxplots comparing the antimicrobial consumption in sampled and non-sampled sow farms. The antimicrobial consumption was calculated as the total amount of ADD per antimicrobial class per sow registered in the CHR. Simp: simple, ext: extended.

6.2 Sampling from the sow farms

Comparable levels of antimicrobial consumption were found in non-sampled and sampled sow farms for most antimicrobial classes (Fig. 6.3). There were no significant differences between the consumption levels of the two groups for the antimicrobial classes except aminoglycosides ($p = 0.003$), simple penicillin ($p = 0.03$), and tetracycline ($p = 0.0001$). There was a significant difference in the number of sows ($p = 0.0004$), with a median of 700 sows for the sampled farms and 500 sows for the non-sampled farms.

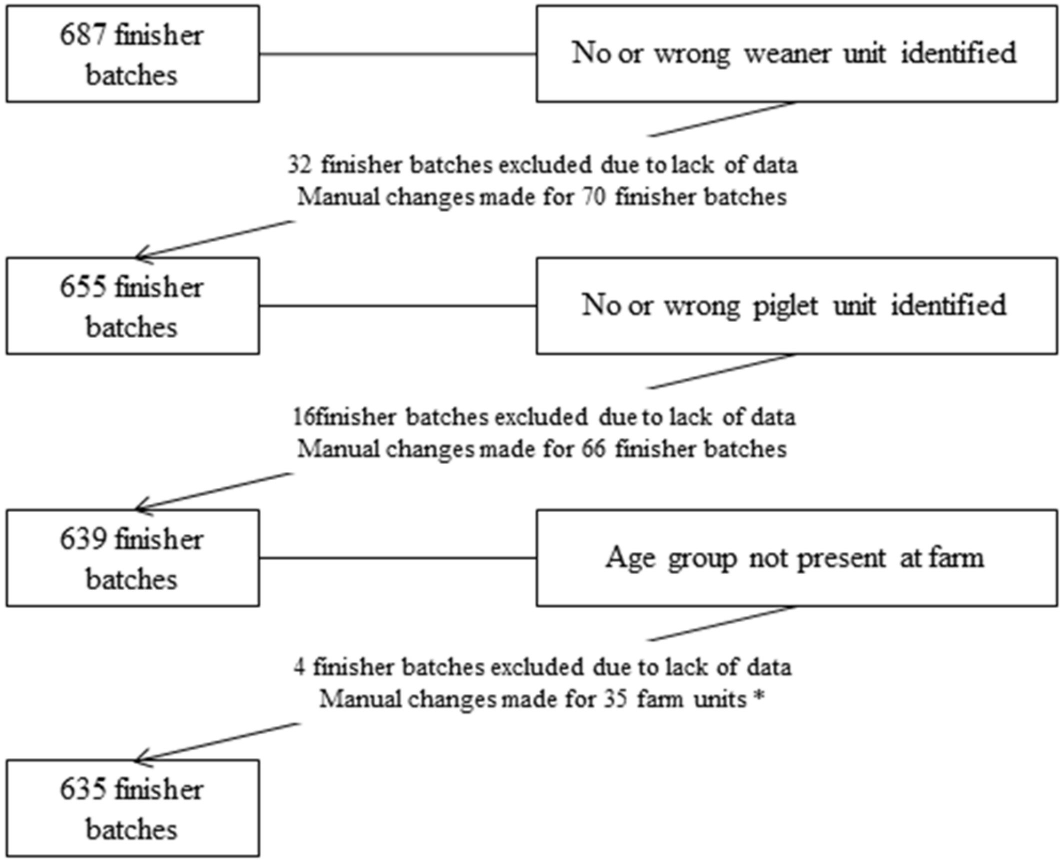


Figure 6.4 Manual changes made to optimise the number of farms for which the LEA algorithm worked. *the 35 farms were a mix of weaner units and sow units

6.3 LEA algorithm

6.3.1 Manual changes to the LEA algorithm

Manual changes were made to the LEA algorithm for some of the sampled finisher batches and their corresponding weaner and sow units (Fig. 6.4), with 52 finisher batches being excluded because it was not possible to trace the pigs back to the corresponding weaner and sow units.

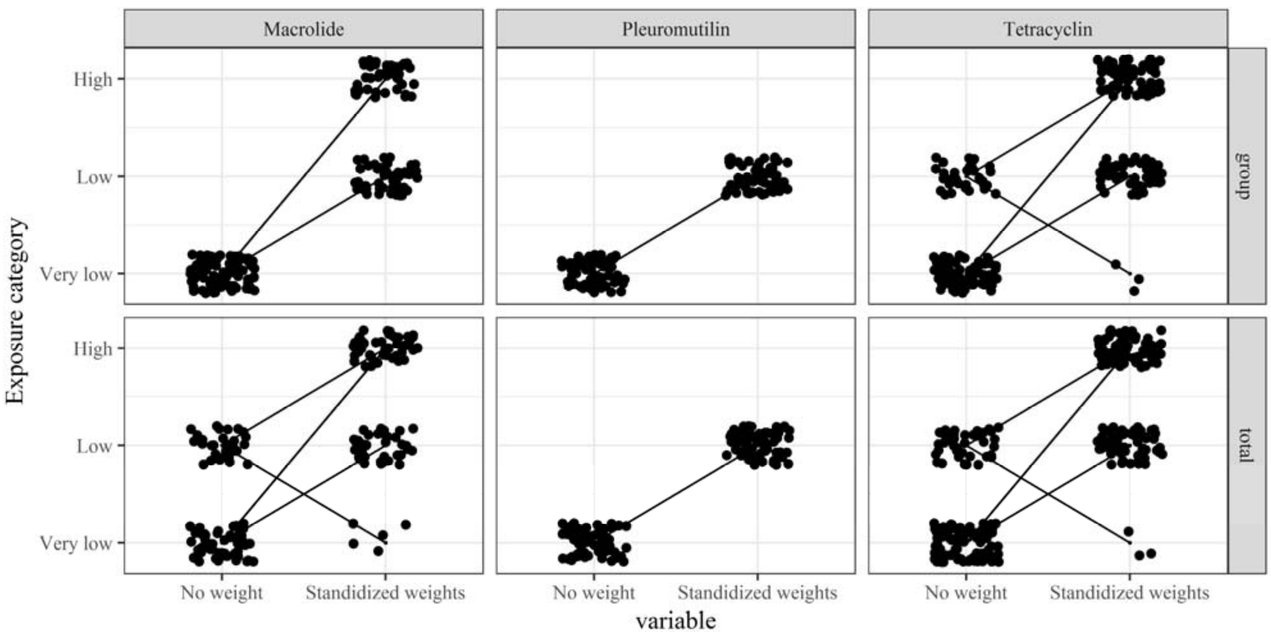


Figure 6.5 Changes in exposure categories. The antimicrobial exposure estimates was calculated using and not using standardized weights. The jittered data points indicate the exposure category of the two calculation methods and the lines indicate the change in categories between the calculation methods. Only farms with a change in exposure category are included in the plot.

6.3.2 Estimating the effect of using standard treatment weights

The LEA categories were recalculated using standard treatment weights. For one farm, the exposure category changed from 1 to 2 for sulfonamides. There where changes in the categories for tetracyclines (114 for group treatment and 131 for total exposure), macrolides (89 for group treatment and 88 for total exposure), and pleuromutilins (156 for group treatment and 63 for total exposure). The pattern of the changes is illustrated in Figure 6.5.

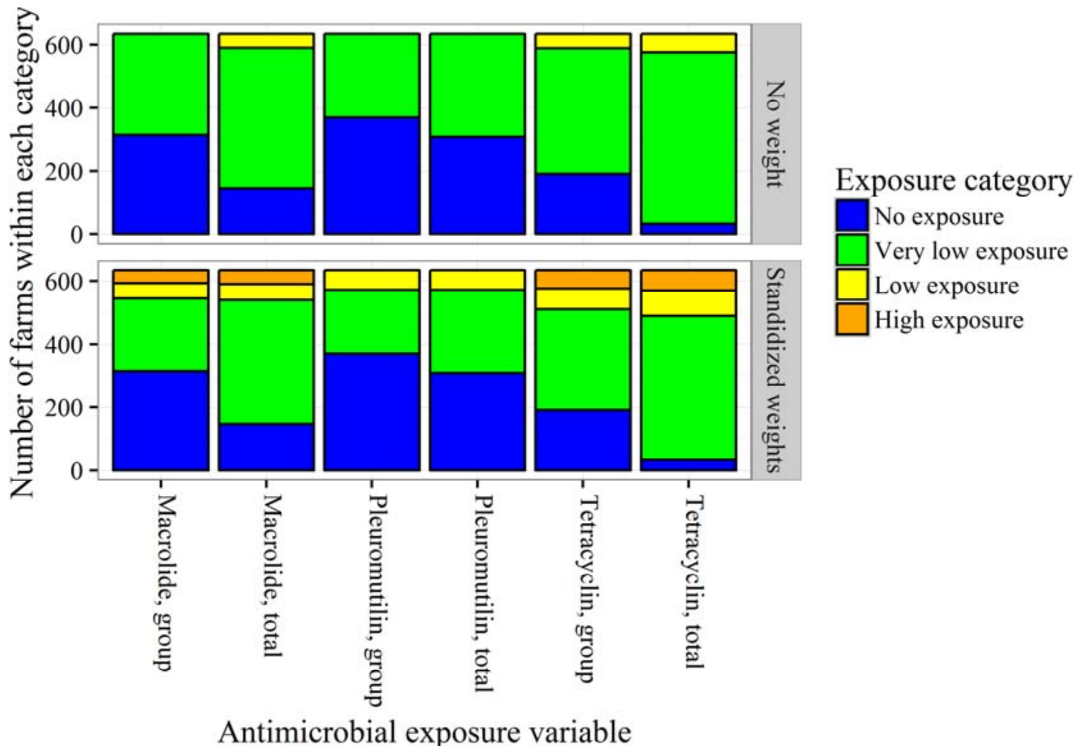


Figure 6.6 Comparison of number of antimicrobial exposure categories with and without the use of standardized weights. There were only changes in the number of categories for macrolides, pleuromutilins, and tetracycline. Hence, these are the only ones shown here.

Figure 6.6 shows the distribution of farms with or without using the standardized weights for the antimicrobial classes where a number of farms had a change in the category. The regression analyses for the seven AMR genes described in Manuscript 5[10] were redone using the categories obtained with use of the standard treatment weights. There were no changes to the results of the regression analyses.

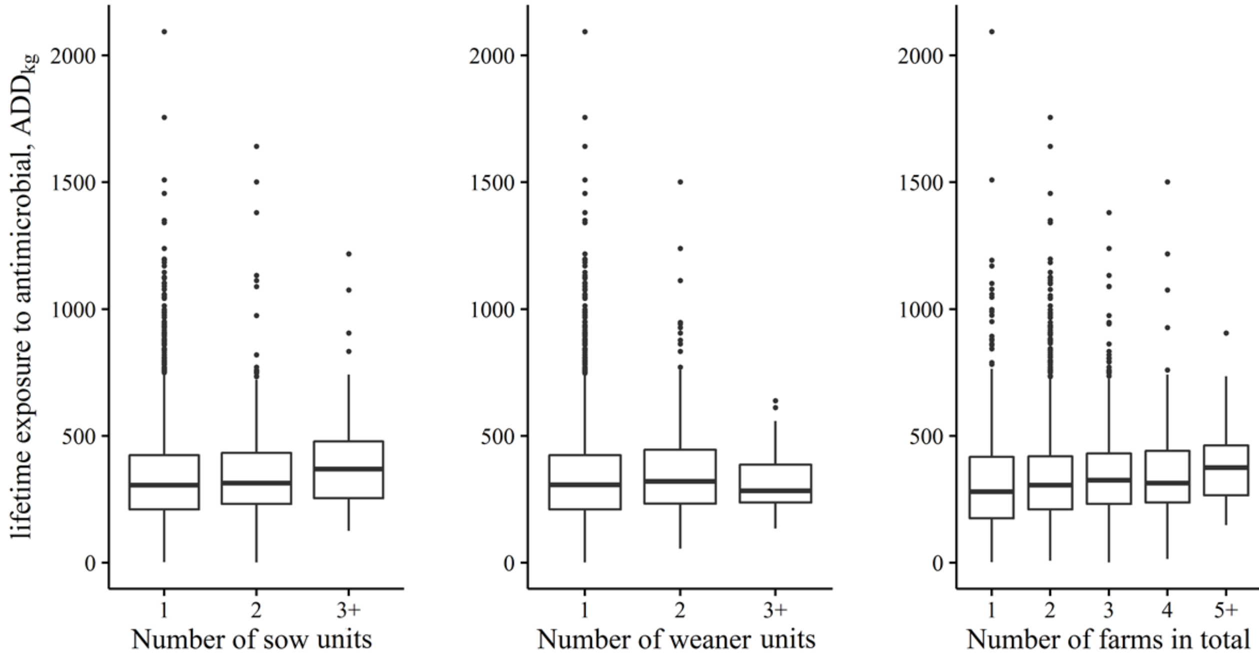


Figure 6.7: Comparison of movement patterns and antimicrobial consumption. The figure shows the distribution of LEA in farms with the same number of The group of farms with the same number of sow units, weaner units or farms in total accounting for less than 5% were collapsed into a single group. 3+: three or more units; 5+: five or more farms. Lower and upper hinges describe the 25th and 75th percentiles and the middle hinge describes the median. The lower and upper whiskers extend from the lower and upper hinges to +/-1.5 times the distance between the 25th and 75th percentiles. Values below the lower whiskers or above the higher whiskers are considered outliers, and are plotted as dots [132].

6.3.3 Comparison of LEA and movements

In Figure 6.7 is seen a comparison of movement patterns and antimicrobial consumption. The movement pattern is measured in three variables: 1) how many different weaner units, 2) how many different sow units, and 3) how many different farms in total the finishers in the batch have been situated in during their lifetime.

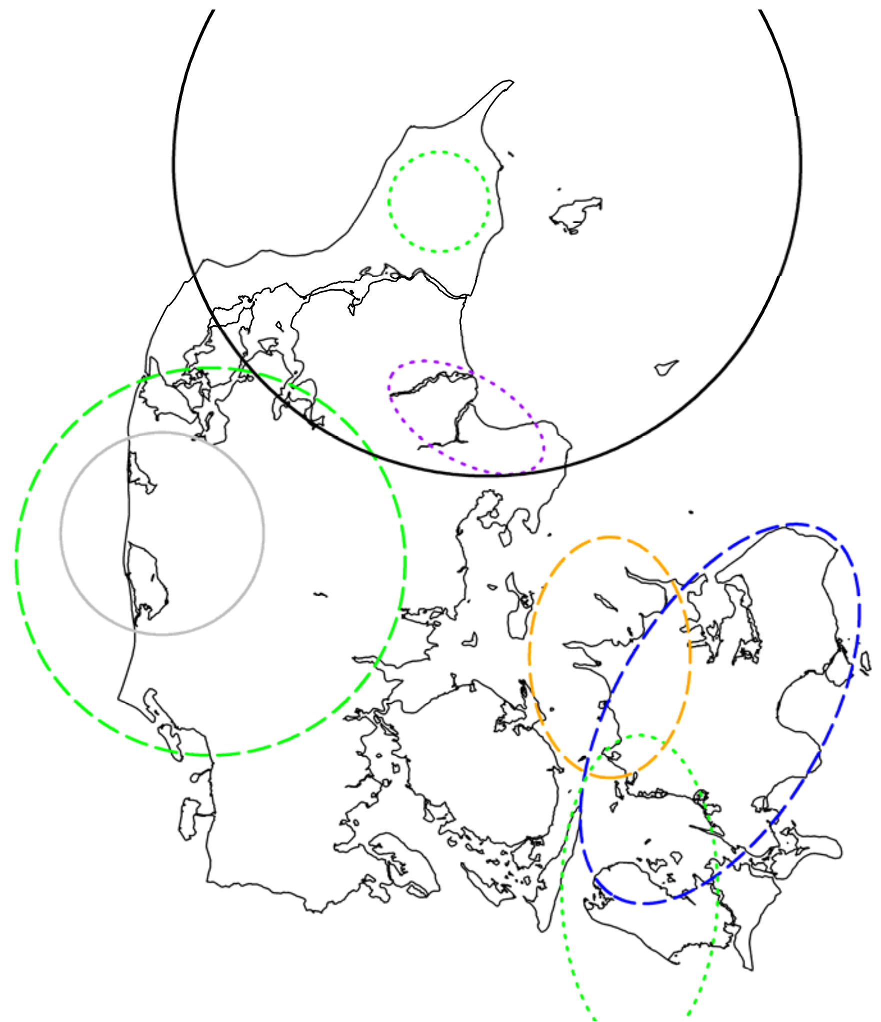


Figure 6.8 Location of spatial clusters of AMR genes and of under- and oversampled areas. A map of Denmark, excluding Bornholm, showing the locations of undersampled (grey circle) and oversampled (black circle) areas found when comparing the cross-sectional sample with the target and study populations. The oversampled area was only found when the sampled farms were compared to the target population. The coloured circles and ellipses show areas with higher (long dashed lines) or lower (short dashed lines) levels of AMR genes than farms outside of the clusters. The colours indicate the gene associated with the cluster - blue: *ermB*, green: *ermF*, purple: *sulI*, and orange: *tet*(W).

6.4 Evaluating the spatial patterns of antimicrobial resistance genes

The map combining the results from Manuscripts 2[14] and 4[9] shows that three clusters of AMR genes are located within the under- or oversampled areas (Fig. 6.8). These were clusters of farms with higher or lower levels of *ermF* and *sulI* genes compared to other farms with sampled finishers.

7. SUMMARY OF THE RESULTS IN THE MANUSCRIPTS

The main goal of this PhD thesis was to provide new knowledge about factors that may be associated with the levels of specific AMR genes. To accomplish this goal, the thesis had four main objectives as mentioned in Chapter 1. In this section, a summary of the main results of Manuscripts 1-6 is presented.

7.1 Objective 1 – Optimizing the data collection

The first objective was to ascertain how the data should be collected in order to study, which factors may be associated with AMR. The answers to the following questions were provided:

7.1.1 How many pigs should be sampled to obtain a representative estimate of the farm level of antimicrobial resistance genes?

It was found that five individual faecal samples pooled together resulted in a representative farm-level quantification of AMR genes in a Danish pig farm (Manuscript 1[8]). Therefore, five pigs per farm were sampled in the cross-sectional study.

During the setup of the pooling study, it was decided that the agreement should be tested using Bland-Altman plots[128]. The agreement was tested for two buffer solutions of the same sample; two pools of the same individual samples; and two runs on the qPCR of the same DNA extraction. Good agreement was found for all three. Furthermore, a pooling method was identified that was less time-consuming than the previously used method, but with comparable results[8].

7.1.2 Were the sampled farms representative of the target population?

It was found that collecting faecal samples at the abattoir resulted in a potential bias towards larger farms (Manuscript 2[14]). Furthermore, a simulation study showed that sampling five pigs per farm would inevitably result in an overrepresentation of larger farms. It was concluded that the sampling at the abattoirs resulted in a study unit with some non-randomness in the spatial distribution[14]

7.1.3 How can exposure to antimicrobials be estimated?

Calculating the LEA was essential in order to study the relationship between antimicrobial consumption and AMR. The LEA algorithm was therefore developed and optimized for larger data registers as described in Manuscript 3[15]. Combining data from three registers with published data on different production parameters and several assumptions enabled the development of the LEA algorithm. The LEA algorithm was validated through a reasonable correlation between real farm usage data and the LEA algorithm. However, the results indicate that some antimicrobials are purchased for one age group, but are used in another, which cannot be detected using the LEA algorithm[15].

7.2 Objective 2 – The association with the geographical location of pig farms

One of the objectives in the overall goal of this PhD study was to assess if the geographical location of a pig farm was associated with the AMR gene levels in Denmark. The result reported in Manuscript 4[9] shows that there was some non-randomness in the spatial distribution of the levels of AMR genes within the population of sampled farms. However it was concluded that the geographical location of the farm had a minor effect on the AMR genes.

7.3 Objective 3 – The association with exposure to antimicrobials

It was found that the relationship between antimicrobial exposure and the levels of the selected AMR genes is very complex. The quantitative effect of antimicrobial exposure on AMR genes depends both on the specific gene and on the specific antimicrobial class in question. Furthermore, antimicrobial exposure during the period prior to finishing had an effect on the levels of the AMR genes found at slaughter[10]. Hereby the LEA algorithm provided a useful estimate of antimicrobial exposure to study the association between antimicrobial exposure and the level of AMR genes.

7.4 Objective 4 – The association with trade patterns

Significant correlations with correlation coefficients ranging from 0.33 to 0.47 were found between the pairs of finishers and sows from their birth farm for *ermB* (p-value = 0.002), *ermF* (p-value = 0.03), and *tet*(O) (p-value = 0.04). Non-significant correlations with correlation coefficients of 0.18 and 0.06 were found for *tet*(M) and *tet*(W), respectively. Furthermore, a

significant difference was shown between AMR gene levels for sow and finisher farm populations for *ermB*, *ermF*, *sulI*, *sulII*, *tet*(O), and *tet*(W), but not for *tet*(M)[11].

8. DISCUSSION

This chapter includes a separate discussion for each objective, followed by a general discussion across the objective, discussion of the limitations, and the perspectives for future studies.

8.1 Objective 1 – Optimizing the data collection

The first objective was to ascertain how the data should be collected in order to study which factors may be associated with AMR. The following three questions had to be answered:

8.1.1 How many pigs should be sampled to obtain a representative estimate of the farm level of antimicrobial resistance genes?

As described in Manuscript 1[8], samples from five pigs were needed to obtain a representative estimate of the farm level of AMR genes. The answer was based on a small pilot study with collection of samples from only one farm. This could mean that the result was not valid for other farms. The variation in AMR genes within a farm could differ from one farm to another, which might affect the results of this study. However, even though the variation between the AMR genes was already very large within the selected farm, the result of the pooling was the same for all farms: adding extra individual samples to the pool (beyond five samples) did not cause any significant change to the result. This could indicate that the results with the five pigs per farm would not differ if samples from more farms had been obtained.

There were also collected samples from five pigs per sow farm. However, it is unknown whether AMR gene dynamics in sow farms are similar to those in finisher farms. Sows live longer. Therefore, it could be expected that they have time to develop a uniform intestinal microflora. On the other hand sows are more often treated individually, which could result in a more differentiated AMR gene population.

Pooling method 3 (Fig. 5.3) was found to be the most time efficient method and was therefore used to acquire the data that was used in the subsequent studies.

8.1.2 Were the sampled farms representative of the target population?

The post-sample evaluation described in Manuscript 2[14] was necessary because sampling at the abattoir meant that it could not be predicted which farms would be sampled on a specific day. The sampled farms where compared to the non-sampled farms in order to found out if they were randomly spatially distributed. Both under- and oversampled areas were found. However, they were not found consistently in the same areas of the country when the sampling method was reproduced in a simulation study. For the sampled farms in the cross-sectional study, there was an undersampled area in the western part of Jutland and an oversampled area in the northern part of Jutland, while the sampled farms on Funen and on Zealand was randomly distributed among the non-sampled farms[14]. The latter cluster was only found through a comparison with the target population of all pig farms with indoor non-organic production of finishers[14].

It has been suggested that true production farms can be defined as those with more than 200 finisher pigs[10]. Farms with fewer than 200 finishers registered in CHR were included in the target and study populations which potentially biased the results. However, repeating the comparison with exclusion of farms with less than 200 finishers did not alter the conclusions. Larger farms have a larger production of slaughter pigs and thereby constitute a higher risk to human AMR genes than smaller farms. Consequently, it is important to focus the sampling on these larger farms.

Manuscript 2[14] did not evaluate whether the sampled farms were representative in terms of the LEA estimates. The sampled and non-sampled farms had comparable LEA estimates (Fig. 6.1). However, this comparison only included farms with more than 200 finishers.

8.1.3 How can exposure to antimicrobials be estimated?

An estimate of the exposure to antimicrobials throughout the whole life of the pig for a large number of farms was important for the study investigating the association between antimicrobial exposure and the levels of AMR genes. This was relevant because it has been shown that the antimicrobial exposure in early rearing stages is of importance to the AMR levels in later production stages[16–18]. Therefore, the LEA algorithm was developed[15]. However, including the antimicrobial exposure in previous periods posed a challenge as many of the pigs in Denmark are moved to another farm during their lifetime. The inclusion of data from the PMD and some assumption regarding movement patterns in Danish pig farms helped overcoming this challenge. It was shown that pigs in 80% of the batches had been moved at least once[15].

It is possible to return purchased, but unused, antimicrobials to the pharmacy. This is entered as a negative value in the VetStat data. This occurs due to the yellow card initiative, where all farmers are benchmarked by their use of antimicrobials based on VetStat data[62]. Therefore, it can be assumed that antimicrobial purchases registered in the VetStat data can be regarded as being used in the farm. However, it is not possible to ascertain when and to which pigs the antimicrobials are used. As described by Dupont et al.[126], this is difficult to establish based on data in VetStat, as data are collected for a different purpose. The question regarding when the antimicrobials were used was, in the LEA algorithm, estimated through smoothing of the purchased antimicrobials. It was indicated when comparing the smoothed data to actual farm records that the algorithm smoothing was a good estimation of when the antimicrobials were used[15]. Furthermore, the question of which pigs have been exposed to the antimicrobials was solved using the exposure approach. In the LEA framework, it is estimated that antimicrobials consumed by one pig in one age group will indirectly affect the AMR gene levels of the other pigs within the same age group. This effect is through excretion of bacteria harbouring AMR genes and through excretion of excess antimicrobials. The hypothesis was that all antimicrobials used at a farm can influence the level of AMR genes in the pigs present at the farm. Thus, the estimated exposure merely describes the amounts of antimicrobial used for the production a given pig. However, if the antimicrobial is used for another age group, it would not be possible for the algorithm to correct for the resulting mistake. If the farm receives a prescription for a herd diagnosis, the antimicrobial will be registered to the age group in which the majority of the antimicrobials is used[126].

The LEA estimate is an estimate of the most likely events. Pigs that are moved during their lifetime will be traced back to the weaner and sow units using register data. This complicates the estimation of the LEA. However, most farms only receive pigs from one or two farm and the proportion of pigs coming from each farm is included in the calculation. Therefore, it is estimated that for the majority of the sampled pigs, the LEA estimate will be close to the reality. Furthermore, using register data enabled the inclusion of a considerably larger number of farms in the analyses than if actual farm data on antimicrobial usage should had been collected.

The data registrations on the purchase of antimicrobials for use in piglets could not be distinguished from those for use in sows. However, it has been shown that antimicrobial exposure to sows can affect AMR levels in piglets[33,34]. Therefore, all antimicrobials estimated as being used in sows were also considered to contribute to the antimicrobial exposure of piglets

If farm-level productivity data were incorporated into the LEA algorithm, it would increase the validity. Productivity data is not currently available for all farms, and can therefore not be integrated into the LEA algorithm.

8.2 Objective 2 – The association with the geographical location of pig farms

It was found that the geographical location of the farms was vaguely associated with the AMR gene levels. Furthermore, comparing the results of Manuscripts 2[14] and 4[9] showed that three of the six observed clusters with either low or high levels of *ermF* and *sulI* genes were found in areas where the sampled farms were not randomly distributed among non-sampled farms (Fig. 6.8). This indicates that there might not truly be a difference in the levels of the genes in these areas. In the evaluation of the sampling method, described in Manuscript 2[14], the non-random spatial distribution of the sampled farms was inevitable.

8.3 Objective 3 – The association with antimicrobial exposure

Antimicrobial exposure is considered to be the most important risk factor for AMR. Therefore, one of the objectives of the study was to quantify the association between AMR genes and antimicrobial exposure. To my knowledge, this has not previously been done for AMR genes in total community DNA using such a large number of farms. Antimicrobial exposure was calculated using the LEA algorithm[15].

In the study described in Manuscript 5[10], it was found that some antimicrobials decrease the prevalence of some genes, e.g. the effect of tetracycline exposure on *ermB*. This might be because a decrease in the use of one antimicrobial class will normally be accompanied by an increase in another[133], for example, farms using few tetracyclines might use more macrolides. Therefore, the decreasing effect of tetracycline on *ermB* could have been a hidden effect of macrolide use.

A new yellow card scheme was implemented in 2016, which encourages antimicrobial usage away from tetracycline and towards simple penicillin, pleuromutilins, and sulpha-TMP. However, it was shown that increasing levels of simple penicillin exposure was associated with an increase in the *tet*(W) gene levels[10]. These are genes that are found in both humans and pigs and are thus also important in the consideration of the human risk of getting AMR.

8.4 Objective 4 – The association with trade patterns

The study described in Manuscript 6[11] indicates that the AMR gene levels in sows can influence the AMR gene levels in their offspring at the time of slaughter (at approximately 6 months of age). Many of the samples collected from the sows were collected after the sampling from the finisher farms were carried out. This could be a problem as it was the intention to study the initial level of AMR genes in the finishers which the level in the sow was a proxy for. As the samples of the sows were taken after the samples of the finishers this relationship could not really be establish because the timeline was invers. However, the correlations were not significant if unrelated finisher and sow pairs were compared.

It could be speculated that pigs mixed from many farms could have a more diverse AMR gene distribution. Due to the limited number of genes, this hypothesis could not be tested. However, it was shown that these pigs have a higher level of lifetime exposure (Fig. 6.7).

8.5 General discussion

It is generally accepted that the use of antimicrobial agents is the main risk factor for developing new AMR genes. However, the *erm* and *tet* genes were present in all sampled farms regardless of the antimicrobial exposure status[9–11]. This is in accordance with previous studies that have shown the presence of *ermB*, *ermF*, *tet*(M), and *tet*(O) in pigs not directly exposed to antimicrobial agens[78,95–97]. This is probably a result of a long tradition of using antimicrobial products in the pig production. This hypothesis is supported by a study by Österblad et al[134] that found almost no AMR genes in faecal bacteria in wild life from remote areas. Österblad et al discussed that this was in contrast to another study by Gilliver finding higher levels of AMR genes in rodents in closer proximity to humans and farm life[134,135]. The difference in the findings was explained by the difference in the distance to the anthropogenic use of antimicrobials[134]. Gilliver et al discussed that decreasing the use of antimicrobials leads to decreasing levels of these genes, but it does not eliminate AMR[135] which has also been reported in other studies[136]. This might be due to the low fitness cost of the bacteria associated with the AMR genes[137]. Furthermore, some antimicrobial products remain in the environment for a long time due to low biodegradability, and some strains of bacteria produce antimicrobials[42,43] that select for certain populations of resistant bacteria[42,43,138,139]. There is considered to be a constant background level of AMR genes[140], which might explain why

the factors studied in Manuscripts 4-6[9–11] (geographical location of pig farms, antimicrobial exposure, and trade patterns) were unable to explain the majority of the variation in the AMR genes.

The presence of the *erm* and *tet* genes in all sow and finisher farms indicates that these have become environmental genes present in practically all pig farms and found in high levels in both sow and finisher farms. Furthermore, it might be speculated that the *sul* genes are established during the piglet period, and that different factors influence the presence of these genes at the time of slaughter, as *sul* genes are present in all sow farms, but have a significantly lower prevalence in finisher farms[11]. However, there was little consistency in the present/absent status of the *sul* genes in the finisher farms, indicating that the results for the *sul* genes might be unreliable. The poor consistency in the presence/absence state could explain why only a small percentage of the variation could be explained by the regression analyses in Manuscript 5[10].

It is clear from the studies that different genes encoding resistance against the same antimicrobial classes behave differently[9–11]. However, there are some similarities between *ermB* and *ermF* genes, *sulI* and *sulII* genes, while the three *tet* genes all behave differently. The *tet*(M) gene was the only gene found at the same levels in both sow and finisher farms[11]. Furthermore, *tet*(M) was the only *tet* gene to be unaffected by tetracycline exposure during any of the rearing periods[10]. The two findings for *tet*(M) correspond well, as the differences in AMR gene levels between sow and finisher farms for the other genes were explained by the different antimicrobial exposure patterns in the sow and finisher farms[11]. The *tet*(W) levels in finishers were not correlated with the levels of the same gene in sows from the farms where the finishers were born[11]. However, *tet*(W) was strongly associated with tetracycline exposure in weaner and finishers[10] indicating that the *tet*(W) levels of the finishers may be determined in these periods.

Trade patterns were associated with antimicrobial exposure (Fig. 6.7) and some AMR genes[11,15]. Laanen et al[141] found a significant correlation between the use of antimicrobials and the biosecurity score in 95 finisher pig farms, where the movement of pigs into the farm was one of the major contributors to the score. In data from 2013 (results not shown), the median distance between trade partners was 11.76 km. Furthermore, the frequency of movements and the total number of pigs moved in 2013 were higher for trade partners within a range of 11.76 km than for those located further apart. The range of influence was calculated to be 2 km - 10 km for the genes included[9], which might simply be an effect of the pig movements.

8.6 Limitations

The cross-sectional study approach was chosen as the study design for collection of the samples. Cross sectional study designs are the most frequently chosen design for studies in veterinary epidemiology as they are cheaper and quicker to conduct than many other study designs. However, there are disadvantages and limitation to this study design. Cross-sectional studies are best suited for time-invariant exposures where it is known that the exposure should precede the outcome. If this is not true, the reverse-causation problem exists, which indeed may exist in the studies described in this thesis. Therefore, results obtain through a cross-sectional study cannot be used to determine causality[13,127]. However, they can be used to create hypotheses that can then be tested using an appropriate study design.

In this study, AMR gene levels in total community DNA from porcine faecal samples were considered. This implies that it is not known, if the genes are silent or active. Furthermore, it is not known which bacteria are hosting the genes and the importance of these genes.

In all studies where register data are used, an evaluation of the data quality is crucial[142,143]. It became obvious while using the various national registers that even though the Danish data are known to be good – they are not perfect. The data quality of the registers should be improved if the LEA algorithm is to be further optimised. These improvements could include registering the age groups in the PMD, regular updates of the CHR when the farmer change production considerably (with thresholds), registration of movements by both receiver and sender as done in cows, and more control by the authorities of the databases.

9. PERSPECTIVES FOR FUTURE STUDIES

In this thesis, AMR gene levels in finishers were compared with AMR gene levels in sows. However, it could be of paramount interest to study AMR gene levels in weaners as well, since the largest quantity of antimicrobials are used in this age group. If the age or weight of the weaners at sampling were known, it would be possible to estimate their antimicrobial exposure using the LEA algorithm and to include the antimicrobial exposure in the piglet period. It would be interesting to know whether the quantitative relationship between antimicrobial exposure and AMR was higher for weaners than it was shown to be for the finishers.

Surprisingly, it was found that both antimicrobial exposure in the finisher farms and the levels of AMR in the sow farm of origin could explain similar amounts of variation in AMR gene levels in finisher farms. In a future study, it would be interesting to combine information on the AMR gene levels in the sow farm of origin with information on antimicrobial exposure. This was not possible in this project as the data were not sufficient.

It was found that some AMR genes were associated with the levels of other AMR genes, which might indicate co- or cross-resistance[10]. However, it could also be a result of the impact of the bacterial composition of the intestinal microflora on AMR gene levels[144]. If this is the case, it could be hypothesised that feeding practices and management factors might have a substantial influence on AMR levels. This would also be an interesting subject to further investigate.

Finally, it would be interesting to assess whether a clinical breakpoint or the zoonotic threshold could be determined. The clinical breakpoint could be measured as the level of AMR genes that would impair the treatment options for the pigs, whereas the zoonotic threshold could be determined by the level of AMR genes at which a zoonotic hazard is posed. Further studies could then focus on finding factors that keep the level of the genes under the specified clinical breakpoint, for which data from the cross-sectional study could be used.

10. CONCLUSIONS

The goal of this thesis was to acquire knowledge about the association between AMR genes and the three factors as described in Chapter one. The conclusions for the studies of these associations were:

1. The geographical location of the pig farm had a weak association with the AMR gene levels

2. Exposure to antimicrobials was associated with the AMR gene levels to different extents, depending on the antimicrobial class and the AMR gene

3. The trade patterns were associated with the AMR gene levels for certain genes

Furthermore, it was concluded that exposure to antimicrobials, and trade patterns only partly explained the observed variation in AMR gene levels.

11. FINAL REMARKS

Terry Pratchett, one of my favourite authors, wrote in the novel A Hat Full of Sky, "Coming back to where you started is not the same as never leaving". I think that this describes my feelings after completing this thesis very well. We (my supervisors and I) started this project with the optimistic hope that we would find factors that could explain a large part of the variation in AMR gene levels, but this was not possible with the data available. However, along the way, we learned a lot about AMR genes, antimicrobial exposure, and sampling procedures.

I have attempted to cover the most relevant peer-reviewed articles concerning antimicrobial resistance. However, not all peer-reviewed papers have been read, as the topic is so wide. For example, a quick literature search ([TS=(antimicrobial*) OR TS=(antibiotic*)] AND [TS=(pig*) OR TS=(swine)] per 2/11-16) resulted in a list of more than 9,000 references. This was also described in a review article on AMR by Davies and Davies in 2010[145], "We apologize to the authors of many significant papers in the field for not citing their work. There have been upwards of 200,000 references on antimicrobial resistance since the 1950s, and our choice, though selective, was not intended to be exclusive." More have been added since, and I would like to repeat the apology.

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APPENDIX

Pooled sample	Pooling method	Sample number*	Weight	Amount of PBS+
1	1	1-5	0.10 g	0.90 ml
2	1	6-10	0.10 g	0.90 ml
3	1	11-15	0.10 g	0.90 ml
4	1	16-20	0.10 g	0.90 ml
5	1	21-25	0.10 g	0.90 ml
6	1	1-6-11-16-21	0.10 g	0.90 ml
7	1	2-7-12-17-22	0.10 g	0.90 ml
8	1	3-8-13-18-23	0.10 g	0.90 ml
9	1	4-9-14-19-24	0.10 g	0.90 ml
10	1	5-10-15-20-25	0.10 g	0.90 ml
11	2	1-5	0.26 g	2.34 ml
12	2	1-5	0.28 g	2.52 ml
13	3	1-5	-	3.50 ml
14	3	1-5	-	3.50 ml
15	2	6-10	0.45 g	4.05 ml
16	2	6-10	0.46 g	3.50 ml
17	3	6-10	-	3.50 ml
18	3	6-10	-	3.50 ml
19	2	11-15	0.35 g	3.15 ml
20	2	11-15	0.35 g	3.15 ml
21	3	11-15	-	3.50 ml
22	3	11-15	-	3.50 ml
23	2	16-20	0.42 g	3.78 ml
24	2	16-20	0.32 g	2.88 ml
25	3	16-20	-	3.50 ml
26	3	16-20	-	3.50 ml
27	2	21-25	0.37 g	3.33 ml
28	2	21-25	0.46 g	4.14 ml
29	3	21-25	-	3.50 ml
30	3	21-25	-	3.50 ml
31	2	1-6-11-16-21	0.40 g	3.60 ml
32	2	1-6-11-16-21	0.49 g	4.14 ml
33	2	2-7-12-17-22	0.42 g	3.78 ml
34	2	2-7-12-17-22	0.36 g	3.24 ml
35	2	3-8-13-18-23	0.33 g	2.97 ml
36	2	3-8-13-18-23	0.36 g	3.24 ml
37	2	4-9-14-19-24	0.41 g	3.69 ml
38	2	4-9-14-19-24	0.41 g	3.69 ml
39	2	5-10-15-20-25	0.35 g	3.15 ml
40	2	5-10-15-20-25	0.46 g	4.14 ml

* Sample number 1-5 was from Farm A, 6-10 was from Farm B, 11-15 was from Farm C, 16-20 was from Farm D and 21-25 was from Farm E. +for a 10% dilution

Anna Camilla Birkegård
PhD thesis 2017

DTU Vet
National Veterinary Institute
Technical University of Denmark

Kemitorvet, Bygning 202
DK-2800 Kgs. Lyngby
www.vet.dtu.dk