Risk assessment of antimicrobial usage in Danish pig production on the human exposure to antimicrobial resistant bacteria from pork

PhD Thesis
Tina Struve
2011

DTU Food
National Food Institute

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National Food Institute
Technical University of Denmark
Department for Microbiology and Risk Assessment
Epidemiology and Risk Modeling Group
Mørkhøj Bygade 19, DK-2860 Søborg
Denmark

Supervisors:

Tine Hald, DVM, PhD, Research leader
Epidemiology and Risk Modeling Group, Department for Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark

Hanne-Dorthe Emborg, DVM, PhD, Senior scientist
Department of Epidemiology, Statens Serum Institut, Copenhagen, Denmark

Frank Aarestrup, DVM, PhD, Professor, Research leader
Antimicrobial Resistance Group, Department for Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark

Assessment committee:

Lars Bogø Jensen, cand. polyt., PhD, Assoc. Professor
Department for Microbiology and Risk Assessment, National Food Institute, Technical University of Denmark, Søborg, Denmark

Poul Bækbo, DVM, PhD, Dipl. ECPHM, Head of section
Veterinary Research and Development at Pig Research Centre, Danish Agriculture and Food Council, Kjellerup, Denmark

Dr. Matthias Greiner, Dr. med. vet., Dipl. ECVPH, M.Sc., Professor, Head of Department
Department for Risk Assessment, Federal Institute for Risk Assessment (BfR), Scientific Services, Berlin, Germany

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Preface

The past three years have been a journey for me, and I have evolved tremendously. For this I owe a special thanks to my supervisors. Hanne-Dorthe who helped me getting a great start on the study of epidemiology, your door was always open and I was so fortunate to have the office right next to you. Frank for always bringing inspiration to my work, and for setting things in a different perspective. Whenever input and idea generation was necessary Frank was always full of new ideas. Also a special thanks to my main supervisor Tine Hald, you have always been there for me. Whenever I felt I was in trouble you were never further away than an email. You have a lot of duties and responsibilities, but you always had the time to help me solve the problems. Thank you for believing in me.

Not long after my employment the Danish Zoonosis Centre was split in two groups, and I was enrolled as a PhD student in the group - Epidemiology and Risk Modeling. I have had some wonderful years in my group, the Zoonosis Centre and also in the Antimicrobial Resistance group where especially Yvonne, Hanne N. and Lisbeth have been very patiently answering all my stupid questions. To all my colleagues in the three groups, you guys have always been there for me and been able to put a smile on my face, and I very soon got caught in the enthusiasm and professional passion that was shown in the three groups. It has meant the world to me that you all made me feel so welcome and accepted. Even those of you who have taken the task of decorating my office to make me feel more at home have a special place in my heart (even though I do not need any more Christmas decorations, please).

I would like to appreciate the help from the veterinarians at Ø-vet for having me along on farm visits, and later on taking their time to discuss with me all my questions regarding the “real life” pig production. It has been an indispensable help to get a different view on my reflections and findings during the PhD.

Also, I would like to express a special thank to Håkan Vigre, you have learned me so much during the past three years and have patiently supervised my work while inspiring me to always have a logic approach to the tasks. Thank you for always taking your time to help me and for making sure that I was always on the right track.

Last but certainly not least I would like to thank Henrik, my close friends and my family for supporting me, and for being very patient with me during stressful periods. Also a special thanks to Louise with whom I have shared the office, you have become a very close friend during the past three years, and have contributed to make my time as a PhD student a period in my life that I will think back at as nothing less than fantastic.

Glostrup, December 2011

Tina Struve

Summary

During the last decades, bacteria with resistance to all commonly used antimicrobial agents have been detected, thereby posing a major threat to public health. In worst case, infections with resistant bacteria can lead to treatment failure and death of humans. The evolution of bacteria resistant to antimicrobials are influenced by the use of antimicrobial agents, and the prudence of antimicrobial use have been emphasized since the Swann report in 1969 recommended that antibiotics used in human medicine should not be used as growth promoters in food-producing animals. In 2007, the World Health Organisation (WHO) pronounced a list of the antimicrobial classes critically important for the treatment of infectious diseases in humans. On this list occurred among others the third and fourth generation cephalosporins.

Cephalosporins have been used increasingly worldwide throughout the recent years to treat various infections in veterinary and human medicine and the occurrence of resistance to this antimicrobial class have been detected with increasing frequency.

The purpose of this thesis was to perform a quantitative assessment of the association between the use of antimicrobial agents for treatment of slaughter pigs and the occurrence of extended-spectrum cephalosporinase (ESC) producing *E. coli* in pigs and pork. The thesis addresses this purpose by estimating the effect of the antimicrobial usage on the occurrence of resistance. By using the obtained results in a risk assessment model where the human exposure to cephalosporin resistance from pork purchased in retail shops was assessed, different scenarios were used for the amount of antimicrobial used in the primary production. Also, farm-related factors affecting the antimicrobial usage were investigated as a part of this thesis.

The thesis addresses this in the following sections:

Objective 1: Estimating the association between antimicrobial usage and the detection of ESC producing *E. coli*

Objective 2: Quantifying the effect of antimicrobial usage on the proportion of ESC producing *E. coli*

Exposure: Assessing the human exposure to ESC producing *E. coli* through the purchase of pork chops

Objective 3: Identification of management factors in the Danish slaughter pig production important for antimicrobial usage

In **Objective 1**, the occurrence (presence/non-presence) of ESC producing *E. coli* in samples from healthy pigs at slaughter was investigated using selective agar plates supplemented with ceftriaxone. The occurrence of ESC producing *E. coli* was used as the outcome in the data analysis, where the effect of using cephalosporins, extended spectrum penicillins and tetracyclines was estimated using regression analysis.

In **Objective 2**, the samples collected for **Objective 1** were diluted in 10 fold and spread on selective plates in two set of triplicates (one set containing three MacConkey agar plates, and one set containing three MacConkey agar plates supplemented with ceftriaxone). This

provided quantitative data for the number of ESC producing *E. coli* and total concentration of *E. coli* in each sample. The proportion of ESC producing *E. coli* was thereafter estimated using a Poisson regression adjusting for applied dilution factor. The resistance proportion was subsequently used as outcome in a regression model to estimate the effect of the antimicrobial usage on the proportion of ESC producing *E. coli*.

The prevalence, concentration and proportion of ESC producing *E. coli* obtained in **Objective 1** and **Objective 2** was used as input in a human exposure assessment model. In **Objective 2**, a significant effect on the resistance proportion was found from the quantitative use of tetracyclines one year prior to the sampling date. This effect was used in the exposure assessment model. This model also used data from additional sources to estimate the human exposure to ESC producing *E. coli* from the purchase of Danish pork chops. By using the ESC producing *E. coli* prevalence of 41 % (obtained in **Objective 1)**, the resulting prevalence in pork chops was found to vary from 19.70 % to 21.80 %. The prevalence of ESC producing *E. coli* was increasing as the usage of tetracyclines increased. However, this prevalence was estimated in pork chops originating from the study population, which was chosen based on their previous usage of cephalosporin.

In an attempt to check the validity of the model, the data from a national survey was used as input. This survey also used selective enrichment, but did not estimate the concentration of *E. coli* or the proportion of ESC producing *E. coli*, therefore the prevalence obtained from the healthy pigs at slaughter was used as input in the model, whereas the remaining steps of the model were not changed. The resulting effect on the estimated prevalence of ESC producing *E. coli* in 100,000 pork chops was compared to the observed prevalence from the national survey. This analysis estimated the prevalence to be 5.3 % ESC producing *E. coli,* which is 2.6 times the observed prevalence on 2 %. However, the data from the national survey was obtained at retail, whereas the model was not considering the growth or inactivation taking place under the transport and storage of the meat.

In **Objective 3**, the risk factors for occurrence of tetracycline resistance were investigated by assessing the effect of tetracycline usage on the occurrence of tetracycline resistance in pigs originating from three different production types. The effect of the tetracycline usage and the effect of the production type was estimated using logistic regression. The results obtained in this objective showed a highly significant effect of the production type, where the organic production had significantly lower occurrence of tetracycline resistance, and also had a much lower average usage of tetracycline. No significant difference in the tetracycline resistance could be found between the conventional and free range productions. When estimating the effect of the tetracycline usage in general using all the production types, a significant effect on the occurrence of resistance was found on the quantitative usage of tetracycline.

Data in this study unfortunately did not have enough power to point out single factors within the production types that could be responsible for the size of the tetracycline usage.

The overall conclusion of this thesis is that there is a significant effect of the quantitative antimicrobial usage (i.e. the amount of antimicrobial used) on the occurrence of ESC producing *E. coli*. A high antimicrobial usage gives an increased prevalence of resistance, but also an increased proportion of resistance. Furthermore, the occurrence of cephalosporin resistance appears to be influenced by a generic use of antimicrobial agents rather than the effect of a single antimicrobial class. The exposure assessment indicate that human exposure to ESC producing *E. coli* is to some degree affected by the generic use of antimicrobial agents in the primary pig production. However, this thesis also found big differences in the occurrence of resistance and antimicrobial usage, when comparing conventional and free range production to organic production. There seems to be a huge

potential to lower the generic antimicrobial usage in the conventional and free range productions. Future studies evaluating the effect of specific risk factors in the organic pig production could lead to useful recommendations on how to lower the antimicrobial usage in the other production types. However, welfare issues need to be investigated to rule out the possibility of untreated diseases in the organic production.

Sammendrag

På nuværende tidspunkt er der påvist resistens over for alle de almindeligt anvendte antibiotika, hvilket udgør en alvorlig trussel mod folkesundheden. I værste fald, kan infektioner med resistente bakterier føre til behandlingssvigt og død hos både dyr og mennesker. Selektionen af de resistente bakterier er bevist at være påvirket af anvendelse af antibiotika, og brug af antibiotika med omtanke har været i fokus siden Swann-rapporten i 1969 anbefalede, at antibiotika der anvendes i humanmedicin ikke bør anvendes som vækstfremmere til dyr. I 2007 udgav Verdenssundhedsorganisationen (World Health Organisation - WHO) en liste over de antibiotika klasser der er kritisk vigtige til behandling af infektionssygdomme hos mennesker. På denne liste findes blandt andet tredje og fjerde generations cephalosporiner. Gennem de seneste år har cephalosporiner været brugt i stigende grad over hele verden, til behandling af forskellige infektioner i veterinær- og humanmedicin, samtidig er forekomsten af resistens over for denne type af antibiotika blevet påvist med stigende hyppighed.

Formålet med denne afhandling var, at give en kvantitativ vurdering af sammenhængen mellem brugen af antibiotika til behandling af slagtesvin og forekomsten af extended spectrum cefalosporinase (ESC) producerende *E. coli* i svin og svinekød. Denne afhandling belyser denne sammenhæng ved at estimere effekten af antibiotikaforbrug på forekomsten af resistens. Derefter anvendes de opnåede resultater i en risikovurderingsmodel, hvor menneskers eksponering overfor ESC producerende *E. coli* via køb af svinekød blev vurderet ved hjælp af forskellige scenarier for antibiotikaforbrug. Desuden blev enkelt faktorer der antages at påvirke antibiotikaforbruget undersøgt som en del af denne afhandling.

Afhandlingen omfatter følgende delafsnit (Objektiver):

Objektiv 1: Estimering af sammenhængen mellem antibiotikaforbrug og påvisning af ESC producerende *E. coli*

Objektiv 2: Kvantificering af effekten af antibiotikaforbrug på proportionen af ESC producerende *E. coli*

Eksponering: Vurdering af den humane eksponering for ESC producerende *E. coli* ved køb af svinekoteletter

Objektiv 3: Identifikation af management faktorer i den danske svineproduktion af betydning for antibiotikaforbruget

I **Objektiv 1**, blev forekomsten (ja / nej) af ESC producerende *E. coli* i caecum prøver fra raske svin ved slagtning undersøgt vha. selektive agarplader suppleret med ceftriaxone (cephalosporin). Forekomsten af ESC producerende *E. coli* blev brugt som udfald i de efterfølgende dataanalyser, hvor effekten af cefalosporiner, udvidet spektrum penicilliner og tetracykliner blev estimeret ved hjælp af regressionsanalyser.

I **Objektiv 2**, blev prøverne der var indsamlet i forbindelse med **Objektiv 1** fortyndet via 10fold fortyndingsrækker og spredt på selektive plader i to sæt af tre eksemplarer (ét sæt, der indeholdt tre MacConkey agarplader, og ét sæt, der indeholdt tre MacConkey agarplader suppleret med ceftriaxone). Kvantitative data for koncentrationen af ESC producerende *E. coli* og den totale koncentration af *E. coli* blev derved indsamlet. I hver prøve blev

resistensproportionen derefter estimeret ved hjælp af en Poisson regression der tog højde for fortyndingsfaktoren. Resistensproportionen (og dens standard afvigelse) blev estimeret via denne Poisson model og efterfølgende anvendt i en regressionsmodel for at vurdere effekten af antibiotikaforbrug på proportionen af ESC producerende *E. coli*.

Prævalensen, koncentrationen og proportionen af ESC producerende *E. coli* blev estimeret i **Objektiv 1** og **Objektiv 2**, og blev anvendt som input i en risikovurdering af den humane eksponering af ESC producerende *E. coli* fra dansk svinekød. I **Objektiv 2**, fandtes en signifikant effekt på resistensproportionen af det kvantitative forbrug af tetracyklin et år forud for prøveudtagningen. Denne effekt af tetracyklin forbruget på resistensproportionen estimeret i **Objektiv 2**, blev anvendt i eksponeringsmodellen. Denne model anvendte også data fra yderligere kilder, for at vurdere den humane eksponering for ESC producerende *E. coli* fra køb af danske svinekoteletter. Ved at bruge en prævalens af ESC producerende *E. coli* på 41 % (estimeret i **Objektiv 1**), blev den estimerede forekomst i svinekoteletter, ved brug af 100.000 iterationer et sted mellem 19,69 % til 21,80 %. Forekomsten af ESC producerende *E. coli* var stigende i takt med stigningen i tetracyklin forbruget. Det skal dog bemærkes, at denne prævalens var fundet i svinekoteletter der stammede fra den undersøgte population, der var blevet valgt ud fra deres forbrug af cephalosporiner.

I et forsøg på at kontrollere nøjagtigheden af eksponeringsmodellen anvendtes data fra en national undersøgelse som input. Denne undersøgelse brugte ligeledes selektiv opformering, men indsamlede ikke data angående koncentration eller proportion af ESC producerende *E. coli*. Derfor er forekomsten hos sunde svin ved slagtning blevet brugt som input, og de resterende trin af modellen blev ikke ændret. Den resulterende estimerede forekomst af ESC producerende *E. coli* ud af 100.000 svinekoteletter blev sammenlignet med den observerede forekomst fra den nationale undersøgelse. Denne kontrol af modellen fandt en estimeret prævalens på 5,3 % ESC producerende *E. coli* hvilket var 2,6 gange højere end den observerede prævalens på 2 %. Det skal dog bemærkes at data fra svinekød i den nationale undersøgelse blev indsamlet i detailhandlen. Derudover tog eksponeringsmodellen ikke højde for den vækst eller inaktivering der finder sted i henhold til transport og opbevaring af kød.

I **Objektiv 3** blev risikofaktorerne for højt forbrug af tetracyklin undersøgt for at vurdere effekten af tetracyklinforbrug på forekomsten af tetracyklinresistens i svin fra tre forskellige produktionstyper. Effekten af tetracyklinforbruget og effekten af produktionstypen blev estimeret ved hjælp af logistisk regression. Resultaterne af dette Objektiv viste en stærkt signifikant effekt af produktionstypen; den økologiske produktion havde signifikant lavere forekomst af tetracyklinresistens, og havde også et meget lavere gennemsnitligt forbrug af tetracyklin. Ingen signifikant forskel i tetracyklinresistens kunne påvises mellem den konventionelle produktion og frilands produktionen. Ved estimering af effekten af tetracyklinforbruget generelt blandt samtlige produktionstyper, fandtes en signifikant effekt på forekomsten af resistens af det kvantitative forbrug af tetracykliner.

Data der indgik i **Objektiv 3** var desværre ikke tilstrækkelige til at udpege specifikke faktorer inden for hver produktions type, som kunne påvises at være af betydning for tetracyklinforbruget.

Den overordnede konklusion af denne afhandling er, at en betydelig effekt ses på forekomsten af resistens afhængig af det kvantitative antibiotikaforbrug. Et højt forbrug af antibiotika fandtes at medføre en øget forekomst af resistens, men også en øget proportion af resistens blandt tarmbakterien *E. coli*. Desuden tyder de opnåede resultater i denne afhandling på, at forekomsten af resistens er påvirket af et mere generelt forbrug af antibiotika end effekten af den enkelte klasse af antibiotika alene. En kvantitativ

risikovurdering viste, at den humane eksponering af ESC producerende *E. coli* påvirkes af det generelle forbrug af antibiotika i den primære svineproduktion. Ydermere påviste undersøgelserne i denne afhandling store forskelle i forekomsten af resistens og antibiotikaforbrug, når man sammenligner konventionel og frilands produktion med den økologiske produktion. Dette tyder på et potentiale for at sænke det generelle antibiotikaforbrug i konventionelle og frilands besætninger. Fremtidige undersøgelser bør vurdere effekten af de enkelte elementer i den økologiske produktion der kan antages at medføre et lavere antibiotikaforbrug i de øvrige produktionstyper. Dog bør der sideløbende udføres velfærdsundersøgelser for at udelukke muligheden for forekomsten af ubehandlede sygdomme i den økologiske svineproduktion.

1. Introduction and hazard identification

1.1 Public health impact of antimicrobial resistance in E. coli

Throughout history, infectious diseases have been a threat to animals as well as humans. The discovery of antimicrobial agents in 1928 by Alexander Fleming therefore had a major impact all over the world. However the effect of this usage has by the Darwinian principle of "survival of the fittest", selected for bacteria on which the antimicrobial agent is no longer working. This occurrence of resistant bacteria made the importance of new antimicrobial agents a necessity ever since the first reporting's of resistance to antimicrobial agents was made. At present, resistance has been detected for all the commonly used antimicrobial agents posing a major threat to public health. The selection of resistant bacteria are influenced by the use of antimicrobial agents, and the prudence of antimicrobial use have been emphasized since the Swann report in 1969 recommended that antibiotics used in human medicine should not be used as growth promoters in food animals (Swann et al., 1969).

Several studies have demonstrated an association between the use of antimicrobial agents in the production of food animals, and the occurrence of antimicrobial resistance among bacteria isolated from healthy animals (Wise et al., 1998; van den Bogaard and Stobberingh, 2000). Once resistance determinants have been acquired by bacterial populations, they may be retained for a long time after the termination of the selective pressure, particularly if the encoding genes are linked to other genes for which the selection pressure remains (Aarestrup et al., 2001; Maynard et al., 2003)

Based on the principle of prudent use strategies, the World Health Organization (WHO, 2007) made a recommendation in 2007, listing the antimicrobial agents according to their importance for treatment of human infectious diseases. In 2009 the U.S. Food and Drug Administration (FDA, 2009) supported this approach by making a similar statement regarding the prudent use of critically important antimicrobial agents. One of the antimicrobial classes listed as critically important is the 3rd and 4th generation cephalosporins which are used to treat infections with *Salmonella* and *E. coli* in humans. Cephalosporins have been used increasingly worldwide throughout the recent years to treat various infections in veterinary and human medicine (Liu et al., 2007). As a possible effect of this, the resistance to these antibiotics has also increased in many countries (Tragesser et al., 2006).

In this thesis risk assessment has been used as a tool to assess the link between antimicrobial use in the production of pigs and the emergence of cephalosporin resistant bacteria in the human population. The risk assessment is a component of risk analysis which is a formal process used to assess, communicate and manage risk. In antimicrobial resistance risk assessments there are two commonly used risk analysis frameworks, the Codex Alimentarius Commission framework and a slightly different approach defined by the World Organization for Animal Health (OIE). Under the Codex definition, risk analysis consists of three components; these are: risk management, risk communication and risk assessment, where the risk assessment has four components: hazard identification, hazard characterization, exposure assessment and risk characterization. The OIE framework is slightly different since it considers hazard identification as a separate component of risk analysis while the Codex framework considers it to be a part of risk assessment. Further the OIE framework differs by defining the risk assessment differently, since this consists of release assessment, exposure assessment, consequence assessment and risk estimation (Table 1 summarises the differences among the Codex and the OIE frameworks) (Guardabassi et al., 2008).

Table 1: Comparison of the Codex and World Organisation for Animal Health, OIE Risk Analysis framework

Codex Alimentary Commission	World Organisation for Animal Health (OIE)
	Hazard Identification: process of identifying the pathogenic agents (hazards) which could potentially cause adverse effects
Risk Assessment: *Hazard identification:* the identification of biological, chemical, and physical agents which may be present in food. For chemical agents, a dose-response assessment should be performed. For biological or physical agents, a dose-response assessment should *Hazard characterization:* the evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food. For chemical agents, a dose-response assessment should be performed. For biological or physical agents, a dose-response assessment should be performed if the data are obtainable. *Exposure assessment:* the evaluation of the likely intake of biological, chemical, and physical agents via food as well as exposures from other sources if relevant *Risk Characterization:* the estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment.	**Risk Assessment:** *Release assessment:* describes the biological pathway(s) necessary for an activity to 'release' (i.e. introduce) pathogenic agents into a particular environment, and estimates the probability of that complete process occurring *Exposure assessment:* describes the biological pathway(s) necessary for exposure of animals and humans to the hazards (in this case the pathogenic agents) released from a given risk source, and estimating the probability of the exposure(s) *Consequence assessment:* describes the potential consequences (direct or indirect) of a given exposure and estimates the probability of them occurring. *Risk Estimation:* Integrates the results from the release assessment, exposure assessment and consequence assessment to produce overall measures of risks associated with the hazards identified at the outset.
Risk Management: the process, distinct from risk assessment, of weighing policy alternatives, in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair trade practices, and, if needed, selecting appropriate prevention and control options.	**Risk Management:** the process of identifying, selecting and implementing measures that can be applied to reduce the level of risk.
Risk Communication: the interactive exchange of information and options throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions.	**Risk Communication:** interactive exchange of information on risk among risk assessors, risk managers and other interested parties.

Source: Guardabassi et al., 2008

1.2 Aim of the thesis

The aim of my thesis is to provide a quantitative assessment of the association between usage of antimicrobials in slaughter pigs, and the occurrence of extended-spectrum cephalosporinase (ESC) producing *E. coli* in pigs and pork. This focus was chosen due to the emerging importance of ESC producing *E. coli*, and due to the size and impact of the Danish pig production. The results may contribute to the fundament of future risk assessment focusing on the human risk for exposure to ESC producing *E. coli* due to consumption of pork products. Furthermore, the work presented, may be a part of future recommendations for possible risk management.

1.3 Outline of the thesis

The thesis follows the structure of risk assessment described by the Codex Alimentarius Commission, and uses data on antimicrobial usage and occurrence of antimicrobial resistance from the Danish production of slaughter pigs.

In the introduction (chapter 1) the potential hazard being investigated are identified. The hazard characterization (chapter 2) covers a description of the evolution and causal mechanisms involved in the occurrence and spread of ESC producing *E. coli* in pigs. The hazard characterization do not include a dose-response section since the hazard used is ESC producing *E. coli* which is an indicator for cephalosporin resistance, but in this study will not be investigated as a pathogen. A further impact of this approach is that the assessment will terminate at the human exposure and not assess the human health consequences. The material and methods chapter (chapter 3) describes the methods used in the collection and analysis of the data presented in Manuscript I and II. In chapter 4, I present the application of these data in a human exposure model. Chapter 5 describes a study of risk factors for occurrence of antimicrobial resistance in pig farms (Manuscript III). All results are presented in chapter 6, and discussed in detail in chapter 7. In the final chapter (chapter 8), I give a conclusion, where I am tying all the previous chapters together and setting them in perspective to the overall aim of the thesis.

2. Hazard characterization

2.1 Primary production

2.1.1 Danish Pig Production

Almost 28 million pigs are produced in Denmark every year (Danmarks statistik, 2011), this makes Denmark one of the largest pig producers in the world, and the export of live pigs and pork products has a considerable impact on the national economy. Around 90 % of the pigs produced in Denmark are used for export, making Denmark the world's largest exporter (DMA, 2009). The pig production primarily consists of three different pig production types, conventional, free range and organic production. The conventional production constitutes more than 99 % of the total pig production with around 5000 farms producing more than 27 million pigs. The organic and free range production constitutes the remaining 1 % of the total production and is considered a niche production, and this even though Denmark is the country in the world with the largest marked for organic products (Hjelmar & Sandøe, 2011). In 2007 the organic pig production consisted of 139 farms, producing around 80,000 pigs. The free range production consisted of 38 farms in 2007, which were producing around 100,000 pigs (Sørensen et al., 2011).

During the recent years, the number of pig farms in Denmark has decreased while the production of pigs has increased, this means that the number of pigs produced per farm is increasing dramatically.

2.1.2 Pig production types

The three production types have considerable differences regarding their farm management. Major differences include space requirements, feeding practices, weaning age and not least additional regulations to the legislation regarding the use of antimicrobial agents in the alternative productions. The scope of this thesis was not to investigate in detail the importance of all the management differences, however, this section will highlight some of the most important differences important for the interpretation of the results presented in Chapter 5.

In general, the organic produced pigs have considerable more space than the conventional pigs, the piglets are born outdoor, where they live with the sow in huts on pasture until the weaning age after which the pigs are moved to indoor pens. The weaned free range pigs can be kept in-door where soft bedding should be provided and access to outdoor areas is a requirement. Sows, gilts and boars are required to be kept on pasture or in outdoor pens. The slaughter pigs are housed in indoor pens in all three production types. However, the free range and the organic pigs have more space per pig than do the conventional. All animals in the free range and organic production are allowed to be kept in-door during winter time if provided free access to outdoor areas. The outdoor areas in connection to the barn can be covered.

Feeding practices are another area of great difference between the pig production types. Especially access to roughage is very different. In the conventional farms, straw is accepted as roughage, and regulations state that roughage or activation material should be provided in appropriate amounts. Sows and gilts, however, are exempt from this requirement. In the free range production, the access to roughage is recommended, but it is only demanded for the sows. Straw is not accepted for roughage in the free range production. In the organic production, the access to roughage is a demand for all age groups, and straw is not accepted

as roughage (could instead be silage, grass or hay). In addition, pigs in the organic production need to be fed with at least 95 % organic feed since 2010 (Dansk landbrug, 2011).

Another management difference between the production types are the weaning age. For conventional raised pigs, the piglets need to be at least 4 weeks old before they are allowed to be weaned, unless medical reasons contradicts this. In the free range production, the weaning age is required to be at least 5 weeks, and in the organic production the weaning age is required to be at least 7 weeks.

Table 2 summarizes the most important differences between the three production types regarding management that do not concern the use of antimicrobial agents.

Table 2: Major differences among the three production types (not considering regulations regarding space and antimicrobial usage)

Production type	Weaning age	Access to roughage
Conventional	>4 weeks	Access to roughage or activation material in appropriate amounts - straw is accepted as roughage, rule does not account for sows and gilts
Free Range	>5 weeks	Access is recommended, but only demanded for sows. Straw is not accepted as roughage
Organic	>7 weeks	Access to roughage is required for all age groups. Straw is not accepted as roughage

The rules for the use of antimicrobials in all three production types are set in the Danish legislation (Table 3). All farms need a prescription from a veterinarian in order to purchase antimicrobial drugs, and use of antimicrobials for growth promoting and prophylactic treatment is prohibited.

Table 3: Regulations regarding the use of antimicrobial agents in the Danish pig production types

Common for all production types:
* All use of antimicrobial agents requires prescription from a veterinarian
* Prophylactic treatment is not allowed
  - however it is allowed to prescribe antimicrobial agents to expected disease occurrence at the farms the next 35 days, if they have a HAC[a]

For the free range and organic production types:
* Double withdrawal time for slaughter

For the organic production alone:
* Only one treatment per pig (For a duration of up till 14 days)
* The diagnosis is specific for the single animal, which means that a veterinary consultation is required before every treatment is initiated
* Only medicine for ongoing treatment is allowed to be kept at the farm

Footnote:

[a] : *HAC (Health Agreement Contract)*

Common for the free range and conventional production is the Health Agreement Contract (HAC) with a veterinarian. In free range slaughter pig production, a HAC is mandatory, whereas for conventional farms, a HAC was optional until July 2010. Now a HAC is mandatory for all farms of any production type producing more than 3000 slaughter pigs annually. Having a HAC allows prescription of antimicrobials for treatment of expected disease at the farm for 35 days after the veterinary consultation. As a consequence of this, antimicrobial agents are allowed to be kept at the farm. Only very large organic producers are required to have a HAC, and in this case, the farm is not allowed to keep antimicrobial agents that are not used for ongoing treatment.

Besides these common rules, the free range and organic productions have additional regulations regarding the use of antimicrobial agents. The withdrawal times for slaughter after the usage of drugs are doubled in these two production types, thereby encouraging the use of drugs with a shorter withdrawal period. Specific for the organic production, is that slaughter pigs receiving antimicrobial treatment more than once in their lifespan lose their organic status; and that each antimicrobial treatment in organic pig farms must be based on an individual diagnosis and the prescription is specific for the actually diseased pigs.

2.2 Antimicrobial resistance

2.2.1 Occurrence and development of antimicrobial resistance

Antimicrobial resistance is either intrinsic, or acquired by mutation or transfer of resistance genes. Resistance acquired by mutation can be transmitted vertically, while resistance determinants located on mobile genetic elements as plasmids, transposons or integrons might be horizontally spread to other bacteria (Cavaco et al., 2008). The mutations potentially leading to resistance might arise during treatment with antimicrobial agents as a result of spontaneous mutations that are further selected by selective pressure conferred by the use of the antimicrobial agents (Cavaco et al., 2008). In *E. coli*, an example of resistance mediated by mutations is resistance to quinolones.

Resistance genes in Gram-negative bacteria are usually associated with the larger plasmids, most of which are conjugative (Chopra & Roberts, 2001). In *E. coli*, the main tetracycline resistance mechanisms are the extrusion of the agent from the cytoplasm via efflux, ribosome protection and enzymatic inactivation (Chopra & Roberts 2001). Beta-lactam antimicrobials act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. Resistance to beta-lactams is mainly mediated by inactivation caused by beta-lactamases, acquisition of penicillin binding proteins (PBPs) and decreased uptake of beta-lactamase due to permeability barriers or increased export by multidrug transporters.

The genes conferring resistance to antimicrobial agents might be located on the chromosome, but a large proportion of the resistance genes are located on mobile genetic elements which might be transferred between bacteria of the same or different species. Selective pressure caused by exposure to antimicrobial agents can induce a selection of resistant bacteria and may enhance the horizontal transfer of resistance genes between bacteria, and facilitate the spread of resistant clones. The mobile genetic elements such as plasmids, transposons, bacteriophages and integrons are therefore of major importance for the horizontal dissemination of antimicrobial resistance. The plasmids are probably the most important antimicrobial resistance mediators, as they are able to replicate independently of the chromosomal DNA (Aarestrup et al., 2006). Cross selection might occur between different drugs of the same class, but also co-selection of resistance due to location of different resistance mechanisms on the same genetic element can occur due to antimicrobial exposure.

Several studies have demonstrated an association between the use of antimicrobial agents in the production of food animals, and the occurrence of antimicrobial resistance among bacteria isolated from healthy animals (Wise et al., 1998; van den Bogaard and Stobberingh, 2000; Jensen et al., 2006; Emborg et al., 2007; Harada et al., 2008; Alali et al. $_{a}$, 2009; Jordan et al., 2009; Varga et al., 2009). Once resistance determinants have been acquired by bacterial populations, they may be retained for a long time after the termination of the selective pressure, particularly if the encoding genes are linked to other genes for which the selection pressure remains (Aarestrup et al., 2001; Maynard et al., 2003).

2.2.2 Measuring resistance

In general, three main sampling strategies can be used to measure antimicrobial resistance. The most common strategy used for detecting resistance is characterization of a single isolate per sample (Caprioli et al. 2000); this strategy is used in most national surveillance systems, including the Danish Integrated Antimicrobial Resistance Monitoring Programme (DANMAP, 2010).

The samples are randomly chosen and tested for susceptibility to a panel of antimicrobials and classified as resistant, susceptible or intermediate based on the susceptibility results. This method is easy to perform and incur low costs, but the sensitivity is low and detection of organisms occurring at very low concentrations cannot be expected (Caprioli et al. 2000).

Furthermore the strain diversity within a sample cannot be examined using this method and no information concerning co- or cross- resistance for the entire bacterial population investigated can be determined.

In order to determine inter-sample diversity a second sampling strategy can be applied, where multiple isolates taken from the same sample are tested (Brun et al., 2002). However, if most of the variation is attributed to the variation between animals, then sampling a single isolate from several animals would give similar results as sampling multiple isolates from fewer animals. Should the variation be attributed to the differences between isolates within an animal, then multiple isolates from each animal would give a better estimate of herd prevalence (Dunlop et al., 1999; Brun et al., 2002).

The third sampling strategy is to obtain a quantitative measure of the proportion of resistant bacteria within a sample. This is done by comparing the number of colonies that grow on a medium supplemented with the antimicrobial of interest with the number of colonies growing on a non-selective medium. Thereafter the resistance prevalence is estimated as the proportion of colonies resistant to the tested antimicrobial agent out of the total colonies within a sample (Nijsten et al., 1996). This quantitative method has a higher sensitivity of detecting emerging antimicrobial resistance and furthermore provides quantitative data, which is needed for quantitative risk assessment (Fegan et al., 2004). However the costs of this form of sampling and testing are considerably higher.

2.3.2 *E. coli* as an indicator

E. coli is a common habitant of the intestinal tract of humans and animals, and is widely disseminated in the environment (secondary habitat) through the contamination with faeces of humans and animals. The presence of *E. coli* in food is generally considered to indicate direct or indirect faecal contamination and the possible presence of enteric pathogens (Krumperman 1983).

Indicator *E. coli* is typically selected to represent the Gram-negative bacteria and monitoring antimicrobial resistance in this population is considered to provide insight into selective pressure on other bacteria. Indicator *E. coli* in animals, humans and surroundings can function as recipients and donors of exchanging antimicrobial resistance determinants with other bacteria, including those pathogenic to humans (Hammerum & Heuer, 2009). Currently, indicator *E. coli* is the standard organism in antimicrobial resistance monitoring programmes as they can be isolated from both healthy animals and humans, thus giving a more representative estimate of the occurrence of resistance in the entire animal or human population than would a pathogenic organism (Aarestrup, 2004). The tendency of *E. coli* to easily develop antimicrobial resistance, their ability to transfer resistance genes and their potential to work as a source or reservoir of antimicrobial resistance, leave *E. coli* among the most suitable organisms for epidemiologic studies regarding antimicrobial resistance among Gram negative bacteria in the food chain (Turnidge et al., 2004).

Even though *E. coli* most often occurs as a commensal indicator, infections are commonly reported in humans, where *E. coli* may cause urinary tract infection, abdominal infection and bloodstream infections (Decousser et al., 2003; Turnidge et al., 2004). In Denmark, 80 % of all urinary tract infections and 30-40 % of all bacteraemias in humans are caused by *E. coli* (SSI, 2011; Jacobsen et al., 2010). Furthermore, antimicrobial resistance is an increasing problem in *E. coli*, and multi-resistant strains causing infections are of major concern (DANMAP, 2009).

2.3.3 Cephalosporin resistance

Resistance of Gram-negative enteric bacteria such as *Salmonella* spp. and *Escherichia coli* to third and fourth generation cephalosporins are of major concern (WHO, 2007; EMA 2009). Resistance to cephalosporins occurs through a natural selection process of genetically mediated survivability in the presence of antibiotics (Lutz et al., 2011). The predominant cause of resistance towards cephalosporin in *E. coli* is due to the presence of plasmid-mediated extended spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases, also referred to as extended-spectrum cephalosporinase (ESC) (Giske et al., 2009). The majority of the ESCs are produced by variants of TEM, SHV and CTX-M genes (Bradford, 2001). In 1983, just two years after the introduction of the cephalosporins to the market, the first extended spectrum beta-lactamases were isolated in Germany from *Klebsiella pneumoniae* strains (Giamarellou, 2005). In the past 15 years, the occurrence of ESC mediated resistance has been reported worldwide in numerous outbreaks of infection with organisms resistant to cephalosporin (Paterson, 2001).

In the recent years, especially, the global development of resistance towards critically important antimicrobials such as cephalosporins has been addressed (Schwaber et al., 2006). Selection of cephalosporin resistance is dependent on the resistance genes present and the affinity that the produced beta-lactamases have towards the drugs used. Selection can lead to an increase in counts of the bacteria carrying resistance genes and/or increase the horizontal transfer of these resistance determinants to other bacteria, enhancing the spread of resistance. Previous findings indicate that selection of resistance to ESC producing *E. coli* in pigs is associated with the use of cephalosporins for treatment (Jørgensen et al., 2007). A recent study in finishing swine farms in USA showed increased odds of recovering ESC *E. coli* when the usage of ceftiofur increased (Lutz et al., 2011). In that study, Lutz et al. (2011) concluded that routine use of cephalosporin may influence the probability of recovering enteric ESC producing *E. coli*.

In Denmark, a recent study performed by Hammerum et al. (2011) found ESC producing faecal *E. coli* from six out of 84 healthy Danish army recruits (7 %), indicating a human reservoir in the community. Food of animal origin may be an important vehicle in the spread of ESC producing *E. coli* through the food chain, thereby causing a threat to human health (EFSA, 2011). Use of especially third and fourth generation cephalosporins in food animals is likely to select for the occurrence of resistance phenotypes in animal bacteria (Jørgensen et al., 2007; Agersø et al., 2011 In press). Furthermore, recent studies performed by Frye et al. (2011) found that 47 % of *E. coli* and *Salmonella* isolated from the same faecal sample shared resistance genes, suggesting that either resistance genes are horizontally exchanged between these genera or there may be a common pool of resistance genes in the swine environment (Frye et al., 2011).

The genes, encoding for ESC production, are often located on plasmids and/or transferable genetic elements, often linked to other resistance genes, and may therefore mediate resistance to other, unrelated antimicrobials (EMA, 2009; Winokur et al., 2000). Consequently, the selection pressure provided by the common use of non-β-lactam antimicrobial drugs such as tetracycline can lead to the dissemination of β–lactamase producing genes (co-selection) (Lutz et al., 2011).

2.3 Monitoring antimicrobial consumption and resistance in animals

2.3.1 Antimicrobial usage in Denmark

Data on antimicrobial usage are essential for risk assessment studies on resistance, antimicrobial resistance monitoring and for the control and prevention of antimicrobial resistance levels at the country, region or farm level (Nicholls et al., 2001). Currently, only few countries present programmes for continuous monitoring of non-human antimicrobial usage (notably Denmark, Sweden, Norway, Finland, Canada, France, The Netherlands, Great Britain, Germany, Czech Republic, Belgium and USA).

In Denmark, there is a unique registration on end user level of all veterinary prescriptions, including all sales of antimicrobial agents. All farms need a prescription from a veterinarian in order to purchase antimicrobial drugs and prophylactic treatment is prohibited. The information on each veterinary prescription is collected in the national database VetStat (Stege et al., 2003). The Danish Veterinary Medicines Statistics Programme (VetStat) was established in 2000, with the purpose of monitoring the antimicrobial usage for production animals. Furthermore, this database allows for the veterinarians to use the consumption data (with easy accessible comparisons to the national level) in their advisory service to their clients, and for researchers to study the effect of the antimicrobial usage (Stege et al., 2003). The VetStat database includes data on all antimicrobial usage in animals obtained from pharmacies, veterinary prescriptions and also from preparation of medicated feed from the feed mills. In the database, each prescription is recorded together with the information on the date of sale, source, identity of prescribing veterinarian, antimicrobial agent (product identity and quantity) and recipient (farm, animal species, age-group and disease category).

The antimicrobial usage can be measured in many ways, such as volume of active compound, number of prescriptions or number of doses, with each unit of measurement having its applications, advantages and limitations. In the Danish VetStat database, the Animal Defined Doses (ADD) is adopted as an attempt to standardize the measure for antimicrobial consumption to allow for comparison between different antimicrobial compounds and the age-group of treated animals. An ADD is, for each formulation, the daily dosage required to treat an animal of a certain weight. The ADD is usually defined per kg bodyweight (ADDkg), subsequently, an ADD is calculated by multiplication with a defined standard body weight for each age group (sows with suckling piglets, weaning pigs, and slaughter pigs) (Jensen et al., 2004). In this thesis, the usage of antimicrobial agents was measured in Animal Daily Doses 50 (ADD_{50}), where the “standard” body weight for a slaughter pig (50 kg, which is indicated by the suffix 50 in ADD_{50}) was used (Jensen et al., 2004).

2.3.2 The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP)

The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP) was established in 1995 and represents a close collaboration between veterinary, food and human health authorities in order to provide comparable data to investigate trends of antimicrobial resistance, monitor consumption of antimicrobials and explore associations between their usage and occurrence of resistance at national and regional level. The DANMAP report publishes annually the latest observed trends of antimicrobial resistance in bacteria from animals, food and humans in Denmark, as well as data on antimicrobial usage provided by VetStat and the Danish Medicine Agency (human sector).

Pig slaughterhouses included in the DANMAP programme slaughter 95 % of the total number of pigs slaughtered in Denmark each year. In order to obtain samples from a representative subset of all pigs in Denmark, the number of faecal samples taken by each slaughterhouse is proportional to the number of animals slaughtered at each site the previous year. The sampling process provides a stratified random sample representative of 95 % of the Danish pig population, and the prevalence of antimicrobial resistance detected in the isolates represents an estimate of the occurrence in the population (DANMAP, 2010). Only one isolate of each bacterial species included in the program per farm per year is susceptibility tested. This procedure ensures, as far as possible, that the samples are representative of the Danish pig population.

3. Materials and methods used to collect data for the exposure assessment

3.1 Project design in a farm to fork pathway

The aim of this thesis was, to estimate the human exposure to ESC producing *E. coli* from purchase of Danish produced pork chops. In order to address this issue, the risk pathway shown in Figure 1 was used to identify and characterize the path leading to the exposure of the human consumer to the ESC producing *E. coli* when purchasing one pork chop produced and sold in Denmark. The quantitative effect of antimicrobial usage (tetracyclines, extended spectrum penicillins and cephalosporins) in the farms on human exposure was estimated by varying the amount of antimicrobial usage in the risk model (scenario analysis).

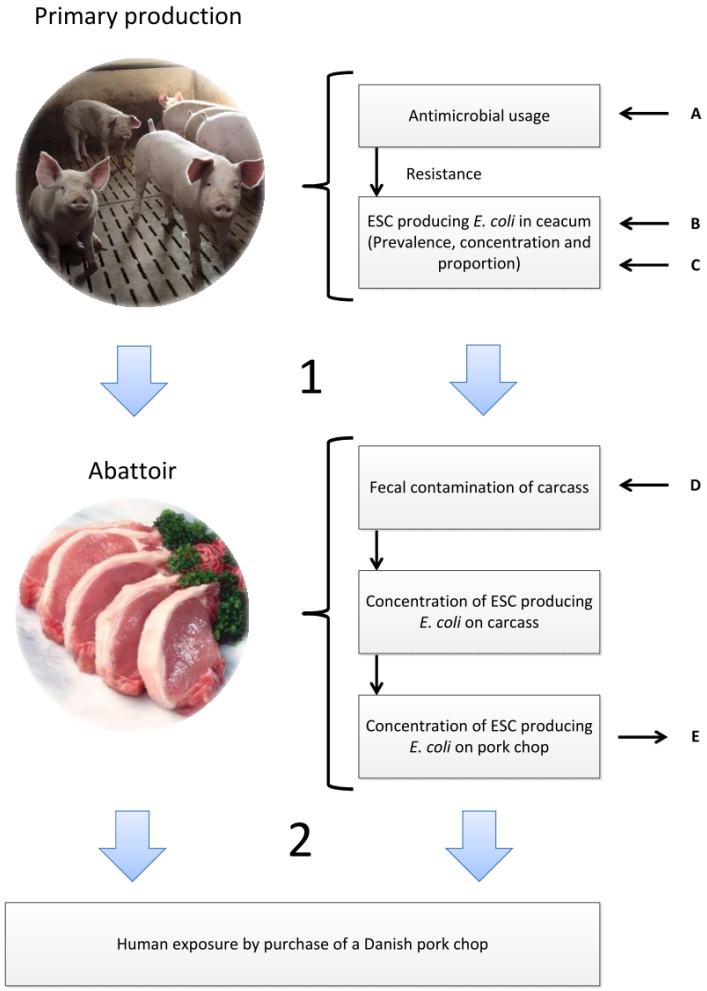

Figure 1: The risk pathway showing the human exposure to ESC producing *E. coli* from purchase of one pork chop

1: Assuming that the caecal content sampled at slaughter is representing the farm
2: Exposure before preparation and cooking, and assuming that the concentration of ESC producing E. coli is not influenced by transport and storage
A: Data input – usage of antimicrobial agents at each farm collected from VetStat
B: Data input – prevalence of ESC producing E. coli as obtained from ***Objective 1***
C: Data input – concentration and proportion of ESC producing E. coli collected in ***Objective 2***
D: Data input – faecal contamination of carcass (Barfod et al., In preparation)
E: Data validation – national prevalence of ESC producing E. coli (Agersø et al, In press; DANMAP, 2010)

The risk pathway starts at the primary production (Figure 1, top) where we have the opportunity to simulate different scenarios regarding the antimicrobial usage at the farms. The data on antimicrobial usage was collected for each farm included in the studies described in **Objective 1** which provided data on the prevalence of ESC producing *E. coli* (Figure 1, B). The proportion and concentration of ESC producing *E. coli* was estimated using count data in **Objective 2** (Figure 1, C). After slaughtering, the carcass is processed and dismembered. We assume that the proportion and concentration of ESC producing *E. coli* in the faecal material before processing at the abattoir is the same when the faecal matter is on the carcass (Figure 1, 1). The faecal contamination of the carcass (Figure 1, D) was estimated using the model described in the DECONT project (Barfod et al., In preparation). Data from a national survey (a CKL project) (Figure 1, E) on the prevalence of ESC producing *E. coli* in Danish pork was thereafter used to validate the exposure estimated in the assessment model. We assume that the concentration of ESC producing *E. coli* on the pork chop is not influenced by the transport and storage before reaching the human consumer (Figure 1, 2). Since we are usually not considering *E. coli* to be a pathogen, a dose response model was not included in the exposure assessment.

The identification of risk factors for the human exposure that can lead to management recommendations will be described in the results of **Objective 3**.

3.2 Data collection

3.2.1 CHR register

Danish farms are registered in the Central Husbandry Register (CHR) database managed by the Danish Ministry of Food, Agriculture and Fisheries. The CHR contains all holdings with cattle, pigs, sheep and other production animals, including information on each holding such as the unique holding code (CHR number), address, geographical position, data on the owner (name, address and contact number), number of animals present and veterinary related information. It also includes all animal movements and therefore allows traceability of all animals produced and raised within a farm. The CHR database is the backbone of the national surveillance programs on antimicrobial usage (VetStat) and resistance (DANMAP) in Denmark. In my studies the CHR register was used to extract information of the number of animals produced at each farm. This was used to standardize the amount of antimicrobial agent given at the farm thereby accounting for the size of the pig production on the total antimicrobial usage at the farm.

3.2.2 VetStat database

The data on antimicrobial usage was collected for each of the farms included in this thesis. The data on antimicrobial usage was extracted on the basis of the included farm's CHR numbers one year prior to the date of the collection of faecal samples. The following information was extracted for each sample: the date of sale, source, antimicrobial agent (product identity and quantity) and recipient (animal species, age-group and disease category).

3.2.3 The Danish Integrated Antimicrobial Resistance Monitoring and Research Programme – DANMAP

The data collected for all three objectives are totally or partly based on the DANMAP sampling and detection of resistance. A subset of the samples collected for **Objective 3** was

not a part of the DANMAP surveillance, the sampling procedure regarding these will be described in detail in Chapter 5. For the remaining samples included in this thesis they are all collected as a part of the national surveillance. In **objective 1** and **2**, the faecal samples were provided for further investigation. For **Objective 3** the DANMAP samples and the additionally collected samples *E. coli* were isolated and susceptibility tested by the Zoonosis laboratory (DTU, Zoonoselab, Søborg).

3.2.4 Isolation of *E. coli*

The generic *E. coli* investigated as a part of **Objective 3** was isolated by direct inoculation of the faecal material on to Drigalski plates (Statens Serum Institute, Denmark) and incubated at 37^{o}C overnight. Presumptive *E. coli* colonies were transferred onto CHROMAgar plates (CHROMAgar Microbiology, France), where red colonies were identified as *E. coli* after incubation at 37^{o}C overnight. Only one *E. coli* colony from each sample was subcultured and susceptibility tested using a commercially available minimum inhibitory concentration (MIC) technique (Sensititre; Trek Diagnostic Systems, UK). Only susceptibility test results for tetracycline were used in the following analysis. The isolate susceptibility status was based on the standardized MIC breakpoint adopted for DANMAP, intermediate isolates was considered susceptible (DANMAP, 2010).

3.3 Study design and data collection

3.3.1 Objective 1 - Estimating the association between antimicrobial usage and detection of ESC producing *E. coli*

The faecal samples from healthy Danish slaughter pigs was collected from May 2009 to May 2010, and investigated for ESC producing *E. coli.* The samples were selected based on the exposure to cephalosporin at the farm in 2008 (a retrospective cohort design). From the samples included in the DANMAP surveillance, we made a monthly collection of samples from four farms with exposure to cephalosporin (identified in the VetStat database) and samples from two non-cephalosporin exposed farms. If more than four farms sampled for the DANMAP surveillance had used cephalosporin in 2008, four farms were chosen randomly. The samples from farms that had not used cephalosporin in 2008 were randomly selected by picking at random from the bucket containing all the samples collected that month.

E. coli was isolated differently than the normal procedure for DANMAP sampling since we used selective enrichment and dilution rows in order to isolate the ESC producing *E. coli* and quantify the total concentration of *E. coli* in the faeces and the quantitative occurrence of ESC producing *E. coli* in the faeces. One gram of faeces was added to 9 ml of saline (9 % NaCl), after vortex mixing for 30 min, 100 uL was added to a MacConkey agar plate (Oxoid CM5a) and spread, furthermore 100 uL was added and spread on a MacConkey agar plate supplemented with 1 mg/L of ceftriaxone (Sigma C5793-1G) both plates was incubated overnight at 44^{o}C. Ceftriaxone (CRO) was chosen based on a study performed by Aarestrup et al. (2010) where ceftriaxone was identified as the best measure of ESC producing *E. coli* when compared to seven other cephalosporins. Presumptive *E. coli* colonies were counted and transferred onto orientation CHROMAgar plates (CHROMAgar Microbiology, Becton Dickinson a/s), and red colonies were identified as *E. coli* after incubation at 37^{o}C overnight. For all the farms sampled in the study, data on the use of the antimicrobials that may select for ESC producing *E. coli* - cephalosporins, extended spectrum penicillins (amoxicillin and ampicillin) and tetracyclines (due to possible cross-resistance) was retrieved from the VetStat database (Stege et al., 2003).

The occurrence (presence/non-presence) of ESC producing *E. coli* in samples from slaughter pigs was used as the outcome in the data analysis where the effect of the use of antimicrobial agents was estimated.

3.3.2 Objective 2 - Quantifying the effect of antimicrobial usage on the proportion of ESC producing *E. coli*

The data collected for this study was also used for the study mentioned in **Objective 1** (section 3.3.1), therefore the study design was the same as mentioned in that section. However the samples collected for **Objective 1**, was used in a quantitative study in this objective in order to estimate the proportion of ESC producing *E. coli* in the collected samples. Sampling and isolation of *E. coli* is described in section 3.3.1, additionally *E. coli* was enumerated visually in three replicates in the dilution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} on the two different substrates after all plates were incubated overnight at 44 ^{o}C. Total plate count was performed for colonies with characteristic *E. coli* morphology on MacConkey plates and MacConkey + CRO. One presumptive *E. coli* colony from each plate were transferred onto orientation CHROMAgar plates (CHROMAgar Microbiology, France), where red colonies were identified as *E. coli* after incubation at 37^{o}C overnight.

The quantitative measure of the ESC producing *E. coli* was used as data for the estimation of a resistance proportion. In each sample, the resistance proportion was estimated using a Poisson regression adjusting for dilution factor. The resistance proportion (and its standard error) estimated in this Poisson model was subsequently used in a regression model to estimate the effect of the antimicrobial usage on the proportion of resistance.

3.4 Statistical methods used

3.4.1 Introduction

This section will present and discuss some of the statistical methods used for the data analysis included in my thesis. Different statistical techniques were used throughout the work, ranging from data summaries and descriptive statistics to regression analysis for estimation of causal effects. For **Objective 1**, descriptive statistics was used to describe the differences in consumption of antimicrobial agents in the farms with and without detection of ESC producing *E. coli*. Furthermore regression analysis was used to assess the effect of using the antimicrobial agents investigated on the occurrence of ESC producing *E. coli*. In **Objective 2**, the proportion of ESC producing *E. coli* was investigated as the outcome of a linear mixed model where the antimicrobial usage was used as explanatory variables. In **Objective 3**, the effect on antimicrobial resistance of the production type and antimicrobial usage was investigated by multivariable regression analysis.

3.4.2 Regression analysis

The causal mechanisms analyzed in the regression analyses were based on initial descriptions of the potential biological relationship, which was described in a causal path model. Numerical estimates of the magnitude of the causal effect are determined using regression analysis, in simple models odds ratios may be used. The regression model is used to describe how the value of the outcome changes across population groups formed by the values of the predictor variable (Dohoo et al., 2007). In the case of antimicrobial resistance data, most sensitivity results are presented as MIC values, this value is thereafter used to dichotomize the outcome as resistant or susceptible. The relation between the dichotomous outcome and a continuous explanatory variable can be evaluated using logistic regression. This approach was used in **Objective 1** where the effect of antimicrobial usage

was investigated with the outcome being occurrence of ESC producing *E. coli*. In **Objective 3** the causal effect of different factors was investigated also with the outcome of detecting resistance. In that study, each factor was estimated using a mixed logistic regression model.

3.4.4 Assessing linearity

Before specification of the effect, it has to be investigated whether the effect of a continuous explanatory variable is linear or not in a regression model. If a particular variable should be kept in a model, the linearity in the logit between this variable and the outcome should be assessed. The linearity can be assessed in several ways, for instance a univariable plot (linear model) or a smoothed scatter plot on the logit of the scale (logistic model) or by the use of fractional polynomials. In **Objective 1** and **2** the linear relationship between the occurrence of resistance and the quantitative use of antimicrobial agents was assessed using designed variables based on the quantiles. In **Objective 3** a smoothed scatter plot showed that the assumption of linearity was not correct for the variable containing the antimicrobial usage therefore we used design variables based on the quantiles of the distribution.

3.4.5 Handling clustered data

The data used in **Objective 3** was an example of clustered data, with multiple isolates collected at the same farm on different days of sampling. The assumption underlying most methods of statistical analysis is that all observations are independent and unrelated to each other.

In **Objective 1** and **2**, the samples was clustered on slaughter house origin, the faecal contamination however was obtained from the study performed by Barfod et al. (*In preparation*), in which the effect of the slaughter house was accounted for. The great difference between slaughterhouses was found to be the faecal contamination of the carcass, therefore the effect of the slaughterhouse was not included in these two Objectives.

In studies where several samples are collected from the same farm, the isolates will generally be more similar to each other than expected under the assumption of independence (Reynolds et al., 2008). In the model used in **objective 3**, the clustering was accounted for by using mixed logistic regression, where the effect of farm was added as a random effect.

4. Exposure Assessment

4.1 The exposure model

A stochastic simulation model using 100,000 iterations was constructed in @Risk (Palisade Corporation), the stochasticity was incorporated into the model by using probability distributions for variable parameters. Since only variability is included in this relatively simple model, a second-order model separating the uncertainty from the variability was not found necessary (Nauta, 2000).

The data obtained in **Objective 1** and **Objective 2** was used as input in an exposure assessment, where the human exposure to ESC producing *E. coli* when purchasing Danish produced pork chops was estimated. The steps included in the model were described in the risk pathway (Figure 1).

A prevalence of ESC producing *E. coli* (Prev $_{ESC}$) of 41 % was estimated using data from **Objective 1**. In the first step of the model we assumed that all pigs were infected with ESC producing *E. coli*, the prevalence was accounted for later in order to optimize the iteration process. All samples included in **Objective 2** were used to estimate the faecal concentration of *E. coli*. The estimated log concentration ($C_{Log\ faeces}$) was fitted to a normal distribution (Figure 2), in many situations the frequency distribution of the contamination level across samples can be described as log-normal (Jarvis, 1989), i.e., having a normal distribution when expressed as log CFU values, and characterised by a mean log concentration and a standard deviation (Schothorst et al., 2009).

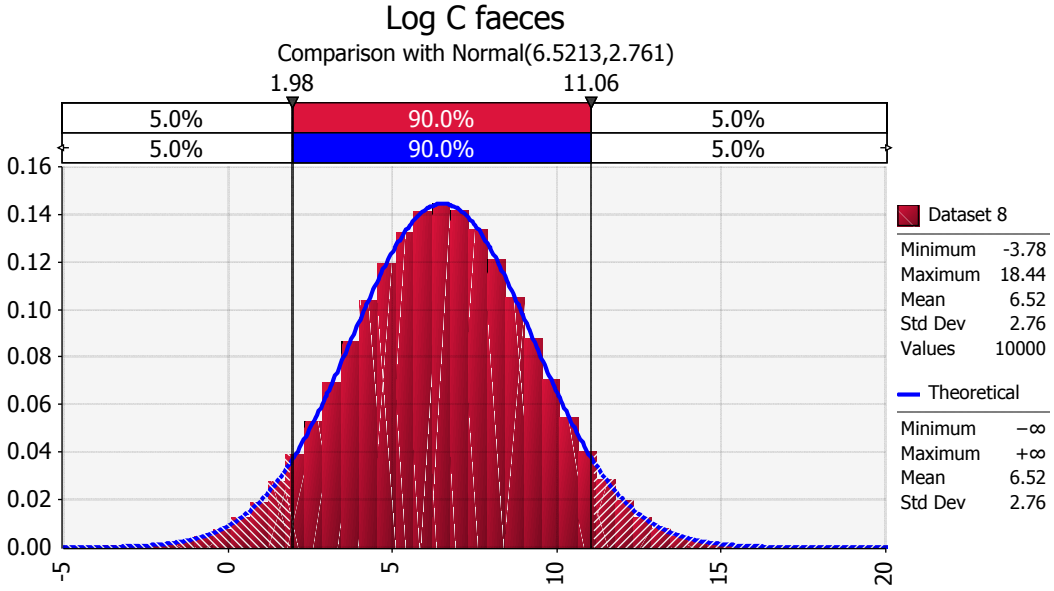

Figure 2: *The normal distribution fitted to the estimated log concentration of E. coli in faeces*

It was assumed that the initial concentration of *E. coli* in the faeces (C_{faeces}) was the same in all scenarios. The concentration of ESC producing *E. coli* in the faeces ($C_{ESC\ faeces}$) was estimated using the faecal concentration of *E. coli* and the in **Objective 2** estimated proportion of ESC producing *E. coli* (Prop $_{ESC}$) in the samples (Equation 1).

Equation 1: $C_{ESC\ faeces}$ (CFU/g) = C_{faeces} (CFU/g) * $Prop_{ESC}$ (%)

Where:

C_{faeces} = 10^ ($C_{log\ ESC\ faeces}$) (estimated in **Objective 2** using the data collected for **Objective 1**)

and:

$Prop_{ESC}$ = proportion of ESC producing *E. coli* in every faecal sample (estimated in **Objective 2**)

The proportions of ESC producing *E. coli* were assumed to be the same in the chosen scenarios since no significant differences could be found among the groups when split this way. Therefore the proportion was assumed to be the fixed value obtained in **Objective 2**, accounting for the effect of antimicrobial usage by adding the effect of increasing the usage by 1 ADD as obtained in **Objective 2**.

The effect of increasing the generic antimicrobial usage (ADD_{50}) was estimated in **Objective 2**, and included in the exposure assessment as an addition to the $Prop_{ESC}$. In order to investigate the uncertainty of this effect, the 95 % confidence limits were used as scenarios to estimate the upper and lower interval.

During the slaughter process the carcass may become contaminated with faeces from the pig itself, but also with faeces from other pigs from the farm of origin or even from other farms. In this model, we assumed that the carcass was only contaminated with faeces from the pig itself. Furthermore we assumed that the faecal contamination was evenly distributed on the carcass. The estimates obtained in Equation 1 were multiplied with the faecal contamination on the carcass, to estimate the concentration of ESC producing *E. coli* on the carcass surface (Equation 2).

Equation 2: $C_{ESC\ carcass}$ (CFU/g) = $C_{ESC\ faeces}$ (CFU/g)* W_{faeces} (g)

Where:

W_{faeces} = faecal contamination in gram faeces per carcass (Barfod et al., *In preparation*).

The number of ESC producing *E. coli* per carcass was assumed to be evenly distributed between the individual servings produced from a carcass. The number of ESC producing *E. coli* on a serving was modelled as the weight of a serving (200 gram) divided by the weight of the carcass (75 kilogram). The concentration of ESC producing *E. coli* on a pork chop ($C_{ESC\ pork\ chop}$) was estimated using Equation 3.

Equation 3: $C_{ESC\ pork\ chop}$ (CFU/g) = $\frac{W_{pork\ chop}(g)}{W_{carcass}(g) * C_{ESC\ carcass}(CFU/g)}$

Where: $W_{carcass}$ = the standard weight of a carcass (Barfod et al., *In preparation*.)

and $W_{pork\ chop}$ = the standard weight of a pork chop (Barfod et al., *In preparation*.)

In order to estimate the true concentration of ESC producing *E. coli* in the pork chop (True $C_{ESC\ pork\ chop}$), the $C_{ESC\ pork\ chop}$ was transformed to a normal scale and assumed to be Poisson distributed (Equation 4, Figure 3), thereby only considering a serving with at least 1 CFU/g as positive.

Equation 4: True $C_{ESC\ pork\ chop}$ = Poisson ($C_{ECS\ pork\ chop}$)

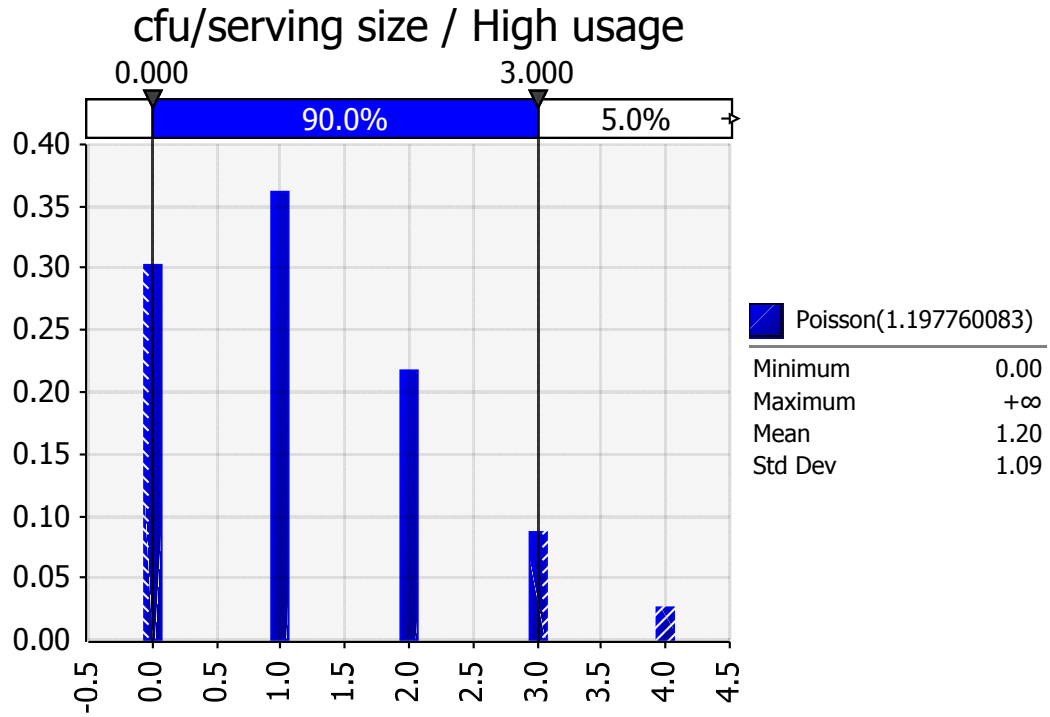

Figure 3: The Poisson distribution fitted for the true concentration of ESC producing *E. coli* in the pork

The prevalence of ESC producing *E. coli* on a pork chop when purchasing 100,000 pork chops (using 100,000 iterations) was estimated using the function Risk Target in @Risk, this function estimates the probability of obtaining the concentration 0. The prevalence of a serving contaminated with ESC producing *E. coli* ($Prev_{ESC\ serving}$) was estimated using Equation 5.

Equation 5: $Prev_{ESC\ serving}$ = 1- RiskTarget (True $C_{ESC\ pork\ chop}$, 0)

Accounting for the prevalence of resistance was done finally by multiplying the prevalence in a serving of one pork chop with the prevalence in the investigated pig population.

The model does not include the effect of packing, transportation, storing and handling by the consumer. The purpose of the model was to estimate the effect of changing the antimicrobial usage in the primary production on the human exposure to ESC producing *E. coli* when purchasing a pork chop. The growth and inactivation of the bacteria after leaving the abattoir was out of the scope of this study.

All parameters included in the exposure model are summarized in Table 4.

Table 4: Description and origin of data used in the exposure assessment

Parameter (unit)	Description	Distributions	Origin
C_{faeces} (log CFU/g)	The log CFU/ gram of *E. coli* in the faeces from healthy slaughter pigs	Normal: (6.5213,2.761)	Objective 1, Objective 2
$Prop_{ESC}$ (proportion)	The proportion of ESC producing *E. coli* out of the total concentration of *E. coli*	0.000263	Objective 1, Objective 2
ADD_{50}	The effect of increasing the tetracycline usage by one ADD_{50}	0.00003545 (CI 95: 0.00000227 - 0.00003545)	Objective 2
$C_{ESC\ faeces}$ (log CFU/g)	The concentration of ESC producing *E. coli* in the faeces	Calculated	$C_{ESC\ faeces} = C_{faeces} * Prop_{ESC}$
W_{faeces} (g)	The faecal contamination (g) on the surface of the carcass after slaughter	Normal: (-0.77, 0.78)	(Barfod et al., In preparation)
$C_{ESC\ carcass}$ (log CFU/g)	The concentration of ESC producing *E. coli* on the carcass	Calculated	$C_{ESC\ carcass} = Prop_{ESC} * C_{faeces} * W_{faeces}$
$W_{pork\ chop}$ (g)	The standard weight of one pork chop	Standard: 200 g	(Barfod et al., In preparation)
$W_{carcass}$ (g)	The standard weight of a carcass	Standard: 75 kg	(Barfod et al., In preparation)
$C_{ESC\ pork\ chop}$ (log CFU/serving size)	The concentration of ESC producing *E. coli* on a pork chop (log CFU/serving size)	Calculated	$C_{ESC\ pork\ chop} = (W_{pork\ chop}/W_{carcass}) * C_{ESC\ carcass}$
True $C_{ESC\ pork\ chop}$ (CFU/serving size)	The concentration of ESC producing *E. coli* on a serving size (CFU/serving size)	Poisson: (1.20, 1.09)	True $C_{ESC\ pork\ chop}$ = Poisson ($10\text{^}C_{ESC\ pork\ chop}$)
$Prev_{ESC\ serving}$ (proportion/serving size)	The prevalence of ESC producing *E. coli* in a serving size	Risk Target	$Prev_{ESC\ serving}$ = 1- RiskTarget (True $C_{ESC\ meat}$)
$Prev_{ESC}$ (proportion)	Prevalence of ESC producing *E. coli* in a): Study population and b): National survey	Calculated	a): Objective 1 and b): Agersø et al., In press; DANMAP, 2010

4.2 Validating the model

In order to validate the exposure model, data collected as a part of a national CKL project (Agersø et al., In press; DANMAP, 2010) was used to assess whether the estimated prevalence of ESC producing *E. coli* in pork chops was realistic. For this, the national prevalence of ESC producing *E. coli* was used in the model (Figure 1, B), thereafter the human exposure was calculated in the assessment model, the outcome being the prevalence and mean concentration in the retail pork. The estimated prevalence of ESC producing *E. coli* is thereafter held together with the actual prevalence found in the retail meat in the CKL project. The data for the CKL project was collected throughout 2009 and 2010 and included samples from 1205 pigs at slaughter used to estimate the national prevalence in the slaughter pigs, and 286 samples from pork, used to estimate the national prevalence of ESC producing *E. coli* in Danish pork.

The sampled pork products did not have a farm identification number therefore it was not possible to use these data to validate the effect of antimicrobial usage on the occurrence of ESC producing *E. coli* in the retail products.

5. Identification of management factors in the Danish slaughter pig production important for antimicrobial usage (Objective 3)

A cross sectional study design was used for this study. Originally the samples included in this study were a part of a larger study where the samples were collected to determine the prevalence of *Salmonella* in different pig production types. Therefore the sample size was calculated based on the expected *Salmonella* prevalence in the conventional and alternative productions. The occurrence of resistance was investigated using *E. coli* as an indicator.

Caecal samples were collected from healthy slaughter pigs from conventional, free range and organic farms (Figure 4). In this study, the samples were collected at ten different slaughter houses - nine slaughtering conventional pigs (slaughtering ~90 % of the pigs that is slaughtered in Denmark), and one primarily slaughtering free range and organic pigs (slaughtering ~80 % of the free range and organic pigs that is slaughtered in Denmark). The first sample from each conventional farm (also included in the DANMAP surveillance) was selected at random at the slaughter house by the technician - the two following pigs from the same farm were thereafter included in this study. For the free range and organic farms, the first sample was selected by the technician at convenience and the number of pigs successively sampled from the same farm was based on the proportion of pigs delivered to slaughter annually by that specific farm. In total, 2 % of the Danish conventional farms, 76 % of the Danish organic farms and 71 % of the Danish free range farms were included in the study. In order to make the sampling representative for the national level, the number of samples per farm was kept as low as possible in order to sample as many farms as possible.

	Production type	
	Conventional	Free range and Organic
Sampling week	1 and 6	2-5
Number of slaughterhouses sampled	9	1
Days of sampling per week	1	2-3
Selection of pigs for sampling	First animal selected by the technician at convenience	
	systematic sampling of the two following pigs from same farm	systematic sampling of the following *n* pigs[a]
Total number of samples in the sampling period	250	2 x 250
Number of samples susceptibility tested	250	2 x 125 [b]

Figure 4: Planned sampling procedure for the summer sampling

Footnotes:

a): The number of samples from organic and free range pigs was based on the number of animals delivered per year by the farm

b): The number of farms in the two alternative production types was relatively small, and many samples was collected from the same farms, due to financial reasons only half of these were susceptibility tested

Caecal samples were collected during two periods' October 2007 to January 2008 (fall/winter) and March 2008 to May 2008 (spring/summer). A total of 868 caecal samples were collected; 402 samples from conventional production, 228 samples from free range production and 238 samples from organic production. Due to the small number of active farms in the free range and organic production most of these were sampled in both rounds, only a few conventional farms were sampled in both sampling rounds.

E. coli was isolated from the samples and susceptibility tested using MIC determination classifying the isolates as resistant or susceptible to tetracycline.

The association between occurrence of resistance and i) the usage of antibiotics and ii) the production type (conventional, free range or organic) were analysed by logistic regression as described in **Manuscript III.**

6. Overview of results

6.1 Objective 1 - Estimating the association between antimicrobial usage and detection of ESC producing E. coli

Pig faecal samples from a total of 63 farms were included in this study. One year prior to the sampling, 33 farms (52 %) had reported usage of cephalosporin, 41 farms (65 %) had reported usage of extended spectrum penicillin (ESP) and 57 farms (90 %) had reported usage of tetracyclines (Table 5). Four farms (6 %) did not have reported usage of any of the three antimicrobial classes in the one year period prior to the sampling. The prescription of antimicrobial agents was more or less equally distributed among the three age groups.

Table 5: Use of antimicrobial agents at the farms, and the distribution of the antimicrobial use in the different pig agegroups

		Use of the antimicrobial agent at the farm in each agegroup				
Antimicrobial agent	No. of farms with usage	Unknown	Sows/piglets	Weaning pigs	Slaughterpigs	Cephalosporin resistance
Cephalosporins	33 farms (52 %)	1 (3%)	14 (42%)	10 farms (30%)	13 farms (40%)	13 farms (39%)
Tetracyclines	57 farms (90 %)	3 (5%)	18 (32%)	29 farms (50%)	44 farms (77%)	22 farms (38%)
Extended Spectrum Penicillins	41 farms (65 %)	2 (5%)	23 (56%)	12 farms (29%)	17 farms (41%)	15 farms (37%)

The average usage of cephalosporin was low in all the investigated age groups when compared to the average usage of the two other antimicrobial groups investigated. The amounts used per pig in the weaning and finisher section was similar, indicating a higher treatment incidence in weaning pigs, having a lower bodyweight, compared to finishing pigs. Twenty three farms (or 37 %) were reported as housing all three age groups (integrated production). ESC producing *E. coli* was found in slaughter pig samples from 41 % of the farms (the prevalence used in the exposure assessment ($Prev_{ESC}$)). The usage of antimicrobial agents stratified on susceptibility result is shown in Table 6. ESC producing *E. coli* was isolated in samples from two farms (non-integrated) with no reported usage of any of the three antimicrobial agents in the study period.

Table 6: Consumption of antimicrobial agents stratified by susceptibility to cephalosporin

		Antimicrobial usage			
	Farms with no usage*	Cephalosporins	Tetracyclines	Extended Spectrum Penicillins	Total no. of farms
Cephalosporin susceptible	2 farms (5%)	20 farms (54%)	35 farms (95%)	26 farms (70%)	37 farms (59%)
Cephalosporin resistant	2 farms (8%)	13 farms (50%)	22 farms (85%)	15 farms (58%)	26 farms (41%)

Footnote:

*: *no registered usage of the selected antimicrobial agents in the study period*

The association between the occurrence of ESC producing *E. coli* and antimicrobial usage in each age group was investigated for each antimicrobial class. Apart from the use of tetracyclines in weaning pigs, no significant association was found between the usage in any age group of any of the investigated antimicrobial classes and the occurrence of resistance in finishing pigs (Table 7). A significant association was found between the quantitative usage of tetracyclines in weaning pigs and the occurrence of resistance in finishing pigs ($p=0.03$, $OR=1.25$).

Table 7: Results of the univariable regression analysis with occurrence of ESC producing *E. coli* used as outcome

Antimicrobial class		OR	CI		Pr>|t|
All three classes					
	Sows/piglets	1.06	1.01	1.09	0.69
	Weaning pigs	1.05	1.04	1.16	0.26
	Finisher pigs	1.04	1.01	1.07	0.7
Tetracyclines					
	Sows/piglets	1.07	0.76	1.5	0.71
	Weaning pigs	1.25	1.08	1.44	0.03
	Finisher pigs	1.035	1.03	1.11	0.32
Cephalosporins					
	Sows/piglets	1.14	0.77	1.67	0.51
	Weaning pigs	0.13	0	7.11	0.32
	Finisher pigs	0.33	0.01	3.64	0.57
Extended spectrum penicillins					
	Sows/piglets	0.83	0.59	1.15	0.26
	Weaning pigs	1.41	0.25	7.92	0.69
	Finisher pigs	1.02	0.45	2.31	0.97

6.2 Objective 2 - Quantifying the effect of antimicrobial usage on the proportion of ESC producing E. coli

Among the 63 samples from the farms included in **Objective 1**, it was possible to quantify *E. coli* colonies in 59 samples. The four samples in which *E. coli* was not possible to count, was excluded due to growth of other bacteria (*Enterococci* and *Salmonella*). Only the antimicrobial usage for finishing pigs was included in this objective. 80 % (47 farms) of the investigated farms used one or several of the three antimicrobial classes included in the study, cephalosporins, tetracyclines and extended spectrum penicillins for finishers (Table 8). The remaining 20 % (12 farms) did not use any of the three mentioned antimicrobial classes for treatment of finisher pigs.

Table 8: Usage of antimicrobial agents for finishing pigs at the farms that did use the antimicrobial agents

		Quantitative usage[1]			
Antimicrobial class	No. Farms	Mean	Median	Min	Max
Usage of tetracyclines	44	5,202	3,037	0,04	44,93
Usage of cephalosporins	13	0,32	0,089	0,02	2,08
Usage of extended spectrum penicillins	17	1,068	0,397	0,08	3,48
Total use of the three antimicrobial classes	47	5,345	2,776	0,02	44,93

Footnote:

[1] *: Quantitative usage among farms using the antimicrobial classes (ADD_{50} per slaughterpig produced annually)*

The concntration and proportion of ESC producing *E. coli* was estimated using a Poisson regression, the output from this model was entered in the exposure assessment ($C_{ESC\ Faeces}$

and Prop $_{ESC}$). The distribution of the estimated and the observed concentration of ESC producing *E. coli* can be found as Figure 3 and Figure 4.

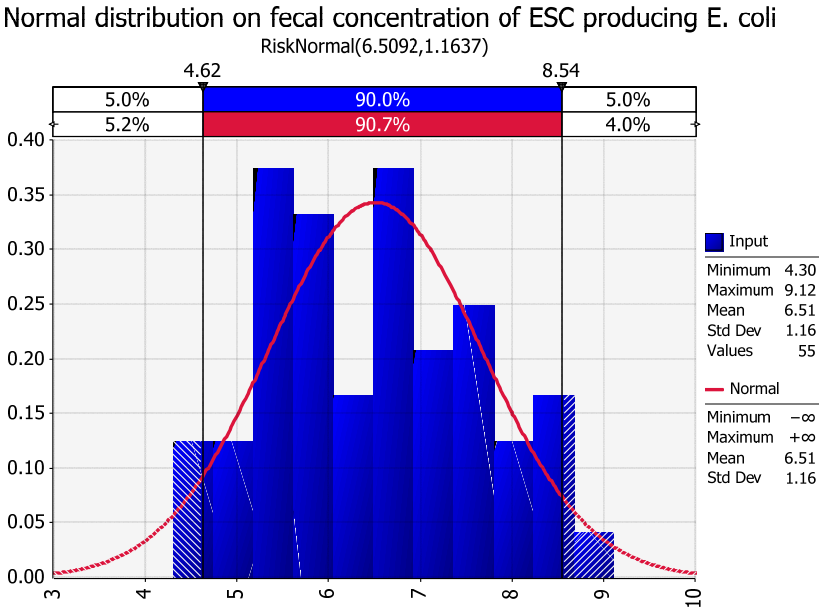

Figure 3*:* The distribution of the estimated concentration (log CFU/g) of ESC producing *E. coli*

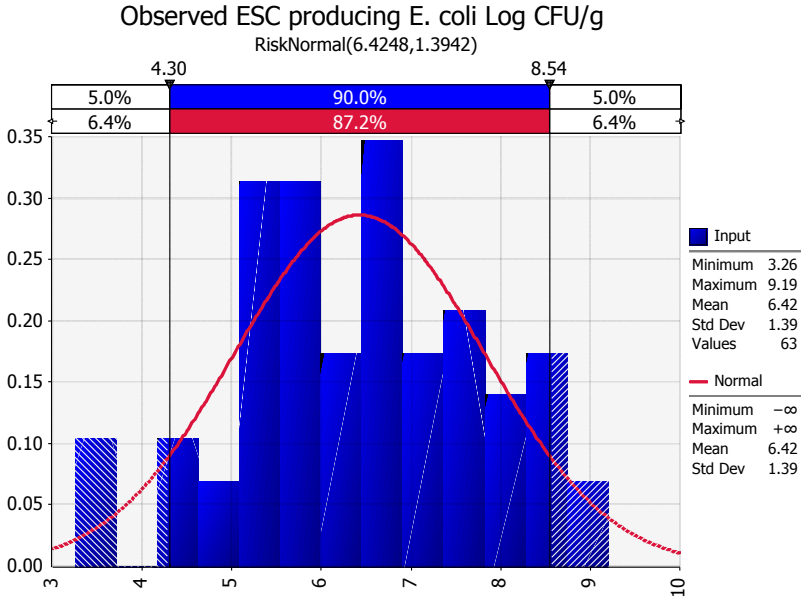

Figure 4: The distribution of the observed concentration (log CFU/g) of ESC producing *E. coli*

The effect of the use of antimicrobial agents on the resulting resistance proportion was investigated using regression analysis. The initial analyses, estimating the effect of both short term and long term usage did not show a clear pattern of the effect of antimicrobial usage, and no factor was found to be significant. When estimating the effect of the generic use of the

three antimicrobial classes, a significant effect of was found of the long term tetracycline use (p=0.04). Using the estimated effect of tetracycline (0.13, Table 9), backwards calculation in the linear model shows that an increased use of tetracycline by 1 ADD_{50}/ finishing pig produced will increase the proportion of resistance by 14 % (this effect and its 95 % CI was included in the exposure assessment).

Table 9: The association between the proportion of ESC producing *E. coli* and the use of cephalosporins, tetracyclines and extended spectrum penicillins

Model	Estimate	Std. Err.	Pr>|t|
1: Usage of cephalosporins			
Intercept	-7.95	0.26	<0.0001
Cephalosporin use - long term effect	3.75	2.31	0.11
Cephalosporin use - short term effect	-18.17	10.04	0.08
2: Usage of tetracyclines			
Intercept	-8.26	0.28	<0.0001
Tetracycline use - long term effect	0.16	0.11	0.15
Tetracycline use - short term effect	-0.14	0.42	0.75
3: Usage of extended spectrum penicillins (ESP)			
Intercept	-8.08	0.26	<0.0001
ESP use - long term effect	-0.02	0.48	0.97
ESP use - short term effect	1.01	0.85	0.24
4: Generic long term usage			
Intercept	-8.24	0.28	<0.0001
Cephalosporin use	-1.07	0.93	0.26
Tetracycline use	0.13	0.06	0.04
Extended Spectrum Penicillin use	0.39	0.43	0.36

6.3 Exposure assessment

The main purpose of this thesis was to investigate the quantitative association between the antimicrobial usage in pigs and the occurrence of ESC producing *E. coli* in pigs and pork. The data collected in **Objective 1** and **Objective 2** was used as input in an exposure assessment. This model used the effect of the antimicrobial usage (tetracyclines, extended spectrum penicillins and cephalosporins) on the human exposure to ESC producing *E. coli*. By using different scenarios in the exposure model, the effect of changing the usage was estimated (Table 10).

Table 10: Effect of the antimicrobial usage on the mean prevalence and concentration of ESC producing *E. coli* in a retail pork chop

	Effect of antimicrobial usage			
	0 ADD_{50}	0.1 ADD_{50}	1 ADD_{50}	10 ADD_{50}
Prevalence	19.69%	19.73%	19.97%	21.80%
Mean concentration (log CFU/serving) [1]	1.992	1.999	2.020	2.130
Standard deviation	1.698	1.695	1.706	1.769

Footnote:

[1] : *Mean concentration of resistance when ESC positive (zero's not included)*

The result of changing the antimicrobial usage from 0 ADD_{50} to 0.1 ADD_{50} (representing a low usage) was a 0.04 % increase in the prevalence of ESC producing *E. coli* on a random pork chop. The mean log concentration of ESC producing *E. coli* on the positive pork chop was higher (increased by 0.007 log) on the pork chop produced with a low antimicrobial usage. Changing the antimicrobial usage to 1 ADD_{50} (representing a moderate usage) resulted in a 0.24 % increase in the prevalence of ESC producing *E. coli* when compared with the prevalence given no antimicrobial usage. Furthermore the mean log concentration on an ESC positive pork chop increased by 0.028 log. By increasing the antimicrobial usage to 10 ADD_{50} (representing a high usage), the effect on the ESC prevalence was an increase of 2.11 % when compared to not using antimicrobial agents, in this scenario the mean log concentration increased by 0.138 log. The effect of the antimicrobial usage on the prevalence of ESC producing *E. coli* with confidence intervals is shown graphically in Figure 4.

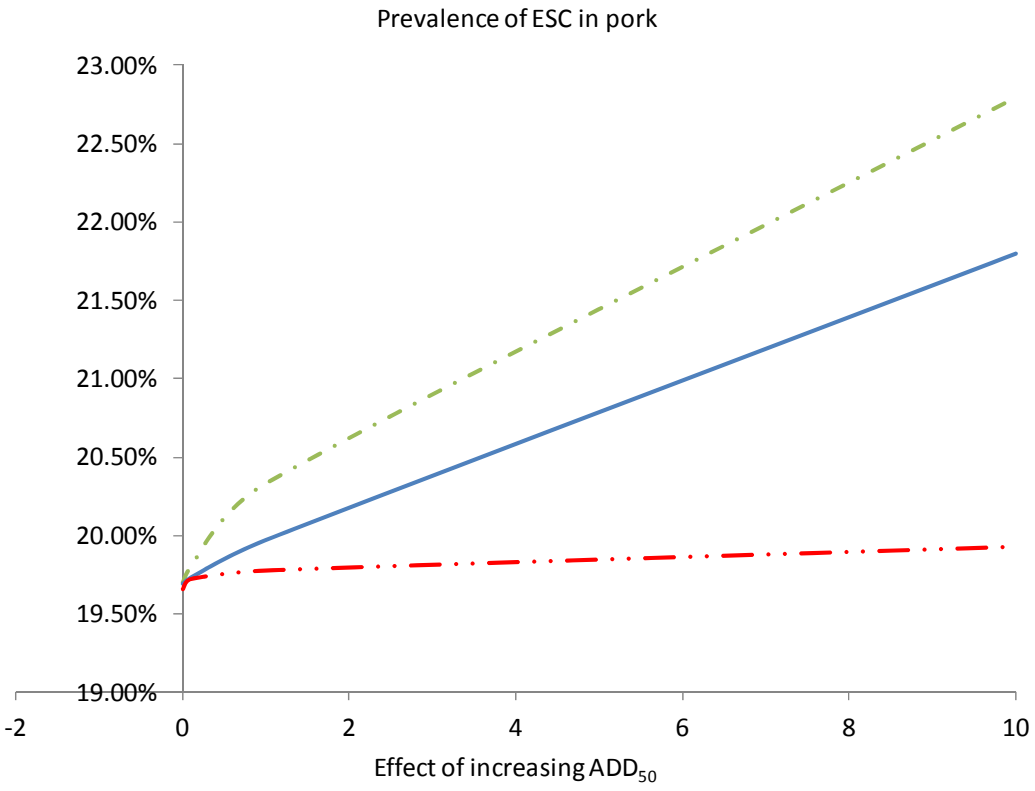

Figure 4: The effect of increasing the antimicrobial usage on the prevalence of ESC producing *E. coli* in a pork chop with upper (green line) and lower (red line) confidence limits.

The change in the log concentration estimated when changing the antimicrobial usage is depicted in Figure 5, where the percentile distribution of the log concentration is shown graphically.

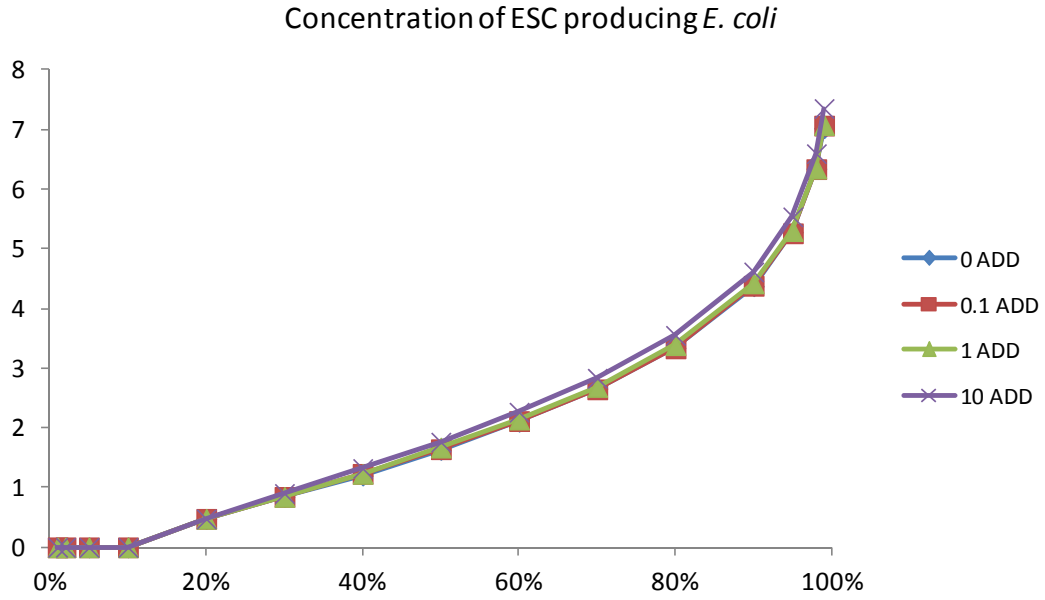

Figure 5: The effect of antimicrobial usage on the percentile distribution of the estimated mean concentration of ESC producing *E. coli* in a pork chop

In an attempt to validate the exposure model, we used data from a national CKL project of the prevalence of ESC producing *E. coli* in Danish pigs at slaughter and Danish pork at retail. When entering the prevalence in pigs observed in the national survey in the model, the output prevalence could be compared to the actual prevalence found in the CKL project. The exposure assessment model estimated the prevalence in Danish pork chops to be 5.3 % (with a standard deviation of 1.697) this was obtained using 100,000 iterations, in comparison the actual prevalence observed in the national survey was found to be 2 % (Agersø et al., In press; DANMAP, 2010).

6.4 Objective 3 – Identification of management factors in the Danish slaughter pig production important for antimicrobial usage

A total of 868 *E. coli* isolates were available from 224 farms (146 conventional, 27 free range, 51 organic (Table 11)). In total, 205 *E. coli* isolates were resistant to tetracycline (conventional: 32 % resistant, free range: 25 % resistant and organic: 8 % resistant). The organic farms had the lowest average usage of tetracycline in 2007 with 0.05 ADD_{50} per slaughter pig produced, followed by free range farms and conventional farms with 0.49 and 0.72 ADD_{50} per slaughter pig produced. In 38 % of the conventional farms, 33 % of the free range farms and 76 % of the organic farms no tetracycline was prescribed in 2007. Using chi square analysis, the occurrence of tetracycline usage (yes or no) was found to be significantly lower in the organic farms when compared to conventional farms (p=<0.0001). No significant difference was found between conventional and free range farms (p=0.19).

Table 11: Distributions of samples on production type and season and descriptive statistics on occurrence of resistance and consumption of tetracycline

	Production type			
	Conventional	Free range	Organic	Total
Number of farms	146	51	27	224
Number of E. coli isolates	402	228	238	868
Isolates in spring/summer	203	123	122	448
Isolates in fall/winter	199	105	116	420
Tetracycline resistance (%)	32%	25%	8%	24%
Farms using tetracycline in 2007 (%)	62%	67%	24%	54%
Mean ADD_{50} tetracycline/pig consumed in 2007	72%	49%	5%	42%

Using logistic regression, we found significant effects of both production type and the quantitative amount of tetracycline used on the occurrence of tetracycline resistance in *E. coli* (Table 12). No significant interactions between these effects were revealed in the analysis.

Table 12: Factors associated with the occurrence of tetracycline resistance investigated by multivariable regression analysis.

Explanatory variable	Odds ratio	95 % CI	Pr>z
Effect of the production type			
Conventional (Reference)	.		
Organic	0.27	0.16-0.47	<0.01
Free range	1.71	0.48-1.05	0.09
Quantitative effect of the tetracycline usage			
No usage (ADD_{50}/ per pig produced = 0) (Reference)	.		
Low usage (0 < ADD_{50}/ per pig produced < 0.1)	1.19	0.68-2.08	0.55
Medium usage (0.1 ≤ ADD_{50}/ per pig produced < 0.4)	1.78	1.07-2.96	0.03
High usage ((0.4 ≤ ADD_{50}/ per pig produced < 1.2)	2.43	1.47-4.03	<0.01
Very high usage (1.2 ≤ ADD_{50}/ per pig produced)	1.86	1.11-3.12	0.02

The odds ratio of getting a resistant isolate was significantly lower in organic farms (OR= 0.27, p=<0.0001) when comparing to the conventional farms. No significant difference was found between the conventional farms and the free range farms. The probability of occurrence of resistant *E. coli* increased successively by an increased usage of tetracycline going from an OR=1.19 at a low usage to an OR=1.86 for farms with a very high usage.

7. Discussion

7.1 General discussion of the results

7.1.1 Objective 1

In **Objective 1**, the prevalence of ESC producing *E. coli* in slaughter pigs from a selected study population was determined. Also, the association between the occurrence of ESC producing *E. coli* in slaughter pigs and the usage of three selected antimicrobial groups were investigated. We chose to investigate the effect of using the three antimicrobial classes tetracyclines, extended spectrum penicillins and cephalosporins due to our hypothesis that the development of resistance is a product of not only using the antimicrobial agent to which resistance is investigated, but also the usage of antimicrobial classes expected to cause co-selection. Therefore the occurrence of cephalosporin resistance was not only thought to be associated with the use of cephalosporin but also with the use of agents with the same mechanism of action (in this study represented by the extended spectrum penicillins) and also the generic use of antimicrobial agents (in this study represented by tetracyclines which have been the most frequently used antimicrobial class in Denmark for several years). According to DANMAP (2010) the usage of cephalosporin for pigs was 99 kg active compound in 2009, while the usage of extended spectrum penicillins was 8,977 kg active compound and the usage of tetracyclines was 35,254 kg active compound.

The choice of focusing on these three antimicrobial classes has recently been supported by the publication of a scientific opinion from the BIOHAZ panel of EFSA where it was concluded, that since most ESBL/AmpC are often connected to co-resistance mechanisms the full human impact of the extended spectrum cephalosporinases can only be assessed by looking at other antimicrobial groups than only cephalosporin (EFSA, 2011). Furthermore, the BIOHAZ panel concluded that “the generic antimicrobial use is a risk factor for occurrence of ESBL/AmpC mechanisms” (EFSA, 2011).

The results obtained in this objective found a prevalence of ESC producing *E. coli* in the study population of 41 % which was later used as input in the human exposure assessment (Figure 1, B).

Also the results of this objective showed no significant association between the usage of cephalosporin or extended spectrum penicillin for any of the three pig age groups investigated. Only the quantitative amount of tetracyclines used for weaning pigs in a period up to one year prior to sampling seemed to be significantly increasing the risk of detecting ESC producing *E. coli* in slaughter pigs from these farms. Previous studies have demonstrated an association between the usage of antimicrobials and the occurrence of resistance (Emborg et al., 2007; Alali et al$_b$., 2009; Varga et al., 2009; Harada & Asai., 2010). Agersø et al., (In Press 2011) also suggest an association between the presence of ESC producing *E. coli* obtained after selective enrichment and the usage of 3rd or 4th generation cephalosporins in slaughter pigs although the number of farms with usage were few (Agersø et al., in press 2011).

In order to study the complete association between occurrence of cephalosporin resistance and generic antimicrobial usage, more antimicrobial classes could have been investigated. However, the sample size in this objective did not allow enough power to estimate the association between the ESC producing *E. coli* and the less frequently used antimicrobial groups, which is also reflected in the results of the analysis made on the cephalosporin usage.

The results obtained as a part of this objective, suggest that associations among the antimicrobial usage and occurrence of cephalosporin resistance do exist. In order to further investigate this, future studies should be made where the sample size should be increased, but also the sampling should include sows/piglets and weaning pigs. Data from a yet unpublished Danish study found a significant association between the occurrence of cephalosporin resistance in sows and piglets and the usage of cephalosporin in that same age group ($Anon_a$., 2011).

7.1.2 Objective 2

In **Objective 2** the proportion and concentration of ESC producing *E. coli* (Figure 1, C) for the human exposure assessment were estimated. Furthermore the quantitative effect of the usage of tetracyclines, extended spectrum penicillins and cephalosporins in slaughter pig production on the proportion of ESC producing *E. coli* was investigated.

In this objective we investigated the effect of antimicrobial usage in slaughter pigs in relation to the usage of cephalosporin in the farm of origin. It is important to consider, that 89 % of the cephalosporin used in the pig production are used for sows and piglets. The resulting effect of this usage is difficult to correlate to the occurrence in slaughter pigs, since the usage in the piglets and sows are not traceable directly to the slaughter pigs in that same farm, due to transport, export and purchase of pigs making it uncertain whether the slaughter pigs at the farm are actually the same pigs that were treated as piglets. Therefore, the generic antimicrobial usage in the slaughter pig section (in this case represented by the three mentioned antimicrobial classes) is expected to better represent the antimicrobial selection pressure occurring in the slaughter pig environment.

In **objective 2**, the quantitative measures of the proportion and concentration of resistant bacteria within a sample were estimated. This was done, by making a serial dilution and obtaining quantitative measures in replicate numbers for each sample. This type of sampling is more resource demanding than the sampling strategies typically used to measure antimicrobial resistance. The most common strategy used for detecting resistance is phenotypic testing of a single isolate per sample (Caprioli et al. 2000). This strategy is used in most national surveillance systems, including the national surveillance system in Denmark (DANMAP, 2011). However this single isolate sampling could not have provided the quantitative data used in the human exposure assessment. Furthermore the selective enrichment used in the chosen study design, resulted in a higher sensitivity; which is found to be appropriate in the case of emerging resistance types.

The quantitative information on the concentration of ESC producing *E. coli* obtained in this objective was used in a Poisson model to estimate the concentration and proportion from the observed data but also accounting for the effect of dilution on the concentration. The comparison of the observed and estimated concentrations (Figure 3 and Figure 4) show that the mean concentration was not differing substantially.

No significant associations were found between the proportion of ESC producing *E. coli* and the short term or long term use of each of the three antimicrobial classes when investigated separately. However, a significant effect on the proportion of ESC producing *E. coli* when using tetracycline was found when investigating the long-term use of all three antimicrobial classes in the same model, which corresponds well with other studies and recommendations (EMA, 2009; Lutz et al., 2011; EFSA, 2011).The most likely reason for this effect is that the tetracyclines are used in such large quantities in Danish slaughter pigs, and therefore constitutes the majority of the generic usage on the farm. In this study we found a very low proportion of ESC producing *E. coli,* however a significant effect on the ESC producing *E. coli* proportion was found when the use of tetracycline in the farms increased. Whether this effect is due to co-selection need to be investigated in detail in future studies.

The estimated effect from the regression model was used as an input in the exposure assessment, also the 95 % confidence limits of this effect was used as alternative scenarios in the exposure model.

7.1.3 Exposure Assessment

In **Objective 1** and **Objective 2**, the association between antimicrobial usage and the occurrence of ESC producing *E. coli* were investigated. The results indicated an effect of the tetracycline consumption for slaughter pigs on the occurrence of ESC producing *E. coli*. The quantitative results obtained in these two objectives were used in a human exposure assessment, where the effect of antimicrobial consumption in the primary production (represented by the tetracycline usage) on the human exposure to ESC producing *E. coli* was estimated.

In this assessment model, the prevalence of ESC producing *E. coli* in slaughter pigs was assumed to be 41 % (as found in **Objective 1**). However, it should be noted, that the farms investigated in **Objective 1** are not representative of the Danish population of slaughter pigs. The farms included in **Objective 1** and **Objective 2** were selected based on their usage of cephalosporin in 2008. Thereby we selected a high proportion of farms using cephalosporin for slaughter pigs when compared to the overall national usage. This is most likely the reason for the relatively high prevalence of ESC producing *E. coli* found in meat (pork chops) by the exposure model, when compared to the results obtained by applying data from a national survey (Agersø et al., *In Press*) of ESC producing *E. coli* in pigs at slaughter and meat at retail collected throughout a one year period.

In the human exposure assessment model, we used a positive linear effect of the tetracycline usage on the proportion of ESC producing *E. coli*, as investigated in **Objective 2**. Unfortunately, the results in **Objective 2** are based on an insufficient sample size to allow for an investigation of the true pattern of this effect. Most likely the effect of the quantitative usage will at some point reach a threshold wherefore the positive linear effect is not reliable when reaching levels of very high antimicrobial usage. However, in order to have adequate power to investigate this, further studies with increased sampling are needed to show whether this assumption is correct.

In the exposure model, the concentration of *E. coli* in pig faeces as investigated in **Objective 2** was used as an input in order to estimate the concentration of ESC producing *E. coli* in the faeces of the pig. The faecal concentration of total *E. coli* was picked from a distribution, and was considered to be the same irrespective of the antimicrobial usage. This is considered to be likely, since the gut flora of warm blooded animals are normal expected to be colonized by *E. coli*. The concentration of ESC producing *E. coli* in faeces was then estimated using the faecal concentration of *E. coli* and the estimated proportion of ESC producing *E. coli*

accounted for the effect of the tetracycline usage. When picking from a probability distribution the model allows a range of possible values to be considered, which integrates the uncertainty/variability. In most cases it is recommended to separate these entities, by using second order modelling (Guardabassi et al., 2008). However, the model used in this thesis was very simple, and the data included did not allow for such a separation. In order to construct a model accounting for the uncertainty/variability while assessing the quantitative effect of the antimicrobial usage, data collection need to harmonised in respect to microbiological and sampling methods due to the need for quantitative data on the occurrence of the bacteria investigated, the occurrence of resistance, the proportion of resistance and the effect attributed to the antimicrobial agent. Future data collection including these factors could allow the application of a more sophisticated model.

The amount of faecal contamination on the carcass used as an input in the exposure model was obtained from Barfod et al. (*in preparation*), who describes the quantitative contamination of carcasses with faeces after slaughter as a normal distribution.

In our model, we assume that the estimated faecal contamination was spread evenly on the carcass thereby making all servings contaminated, and causing the human exposure to be the same in every serving originating from a contaminated carcass. The carcass was assumed to consist entirely of pork chops. Therefore the actual servings in contact with the carcass surface will be less than was included in our model, causing the prevalence of contaminated pork chops to be less than estimated, and the concentration on those contaminated pork chops to be higher.

Furthermore, it was assumed that the ESC producing *E. coli* was behaving the same way as the non ESC producing *E. coli* throughout the production chain. This assumption has been contradicted by the study performed by Wu et al. (2009), who found that the tetracycline susceptible *E. coli* probably persisted better than the resistant ones during the carcass processing. However, the data obtained in this thesis did not allow us to account for this fact in the model, and the data presented in Wu et al. (2009) was not providing a measure of the observed effect that could be included in the model.

An attempt was made to validate our model by comparing with data obtained in a national survey; the results indicated that the model was overestimating the human exposure by approximately 2.6 times. However the input used in the model was an estimation of the CFU/serving, which was then compared to observed data from the survey. The observed data is dependent on the detection limit and the sensitivity of the analysis, and can therefore be expected to lead to a lower detection than would an estimated occurrence which includes the quantitative occurrence that would be below the detection limit.

The potential growth and inactivation of the bacteria after leaving the abattoir was unfortunately out of the scope of this thesis, but is very likely to be influencing the human exposure.

7.1.4 Objective 3

In **Objective 3**, the effect of certain risk factors, including the farm production type and tetracycline usage on the occurrence of tetracycline resistant *E. coli* was estimated. The results showed that the probability of isolating tetracycline resistant *E. coli* from slaughter pigs increased significantly by an increased usage of tetracycline in slaughter pigs a year before sampling. Several other studies have shown an association between the usage of antimicrobials and the occurrence of antimicrobial resistance (Jensen et al., 2006; Jordan et al., 2009; Harada and Asai 2010), but studies regarding the quantitative usage in different

production types have not been made previously. This study found a significant difference between the occurrence of resistance in *E. coli* when comparing organic production to conventional and free range production, which is in accordance to previous studies, where the occurrence of resistance in organic produced pigs also was found to be significantly lower than the occurrence in the conventional pigs (Hoogenboom et al., 2008; Miranda et al., 2008; Young et al., 2009; Rollo et al., 2010). Studies have suggested that antimicrobial use practices on conventional farms are more selective of resistance and multi-drug resistance than usage practices on organic farms (Kijlstra et al., 2009; Young et al. 2009; Rollo et al., 2010).

The management of antimicrobial usage in a pig farm can be expected to be strongly associated with the general health of the animals and the attitude among the individual pig producers. The pig producer is acting under law regulations. According to the regulations in Denmark, organic productions are not allowed to keep antimicrobials at the farm that are not prescribed for a specific ongoing treatment of a sick animal, and the producer has to consult a veterinarian every time an animal needs treatment. This single animal treatment differs from the conventional and free range production where medication of groups of animals through food or water is often used. Furthermore, pigs cannot keep their organic status if treated more than once (defined as one specific animal being diagnosed and following being treated with antimicrobials in up to 14 days). However, it can be speculated whether the organic regulations are causing the pig producers to be more reluctant to start treatment in case of sick animals. Thereby pigs in need of treatment can possibly remain untreated, or may start the appropriate treatment after an unnecessary long time of infection.

Besides the effect on resistance by usage of tetracycline, we also saw an effect of organic production, where the occurrence of tetracycline resistance was lower in organic farms compared to conventional and free range. The causal mechanisms for how the management of organic pigs can cause a lower occurrence of resistance compared to management of pigs in free range and conventional farms can be discussed. One possibility is that the use of other antimicrobials can select for the occurrence of tetracycline resistance in *E. coli* and this coupled with the fact that other antimicrobials are generally used more frequently in non-organic farms, this may explain the observed effect of the production type. Though, a detailed discussion of co- and cross resistance mechanisms influencing the occurrence of tetracycline resistance is out of the scope of this study. However the results obtained in this thesis suggests that the generic usage may have a substantial effect on the development of resistance, suggesting a great effect of co- and cross resistance in respect to the effect contributed to the antimicrobial usage.

Though the organic production has special rules regarding use of antimicrobial agents, they are allowed to use zinc in the feed. However, recent studies have suggested that the use of zinc might have contributed to the emergence of multi-resistance in *Staphyloccus Aureus* (MRSA) (Cavaco et al., 2011). The effect of zinc on the antimicrobial resistance in other organisms needs to be further investigated, as need the effect of antimicrobial usage in addition to the use of zinc. The actual amount of zinc used in any of the production types have not been investigated, but the effect of zinc could be investigated in future studies comparing the occurrence of resistance between the production types.

Another management factor that may influence the usage of antimicrobial agents is the weaning age, which differs between the three production types. In the organic production, piglets are weaned at almost twice the age than are the conventional piglets, which might allow for a more mature intestinal flora and intestinal villi, thereby making the pigs more robust to the huge impact the change of feed during the weaning period will cause. The feeding of the pigs also differs greatly. Organic produced pigs and to some degree also the

free range pigs get more access to roughage, which induces the production of saliva and might also stimulate the intestinal development of villi. However, the very high costs related to the purchase of organic feed might also influence the choice of protein source in the feed, using cheap options like peas that might cause enteritis after the weaning of the organic piglets.

However, we need to perform more research to reveal knowledge that can be applied in pig production to reduce the occurrence of antimicrobial resistance in pigs, irrespectively of production type. There seem to be a huge potential to lower the antimicrobial usage in the conventional production by looking at the management in the organic farms, although, the welfare in the organic farms needs to be thoroughly investigated to rule out the problems with undertreated diseases. A possible way of measuring these welfare parameters could be using slaughter house data investigating the occurrence of disease and meat percentages. Also, the mortality rate could be an important contribution in the welfare characterization of the production types. Finally, the usage of a questionnaire to the Danish vets working at the organic farms could reveal the occurrence of under treatment.

7.2 Overall discussion of the thesis

In the objectives included in this thesis we have investigated the association between antimicrobial usage and the occurrence of resistance in slaughter pigs. However, in **Objective 1** we have also investigated the effect of the antimicrobial usage in the two other pig age groups on the occurrence of resistance in slaughter pigs. The results obtained in this Objective showed an effect of using tetracycline in weaning pigs on the occurrence of ESC producing *E. coli*. Though showing a significant association between antimicrobial usage and occurrence of resistance, the results obtained in this objective was found to not fully explain the antimicrobial usage related to the slaughtered pig. By estimating the effect of the usage for slaughter pigs, there is a more straight forward correlation between the slaughtered pig and the antimicrobial usage. However, future studies should investigate the relation between the use of antimicrobial agents in other age groups with the occurrence of resistance in that corresponding age group. Especially, since sows and piglets are the main consumers of cephalosporins, the true effect of this usage is possibly better described by relating the resistance to the usage in this age group.

In **Objective 1** and **Objective 2** of this thesis, the effect of antimicrobial usage on the occurrence and proportion of ESC producing *E. coli* was investigated. During the production of this thesis, the Danish pig producers agreed on a voluntary discontinuation of the use of cephalosporins in the pig production (July 2010). Besides this initiative the Danish government introduced a so-called “yellow card” intervention in June 2010 where pig farmers having the 20 % highest usage of antimicrobials per pig produced receives a letter urging them to bring down the consumption. The latter intervention was initiated in December 2010 ($Anon_{b.}$, 2011). The overall effect of both initiatives is likely to have greatly affected the overall consumption of antimicrobials in Denmark, which decreased by 3 % in 2010 as compared to 2009, and the use of cephalosporins decreased by 48 % in that period (DANMAP, 2011). However, while the voluntary ban will most likely reduce the primary selection for ESC producing genes, the results of this thesis shows, that the effect of the yellow card intervention is more likely to have an effect on the occurrence of ESC producing *E. coli* and probably also the antimicrobial resistance in general by reducing the generic antimicrobial use. Future studies of the association between the antimicrobial use and the occurrence of resistance should investigate the effect of co- and cross-resistance by including the genes coding for the resistance. Also the possible synergy of the antimicrobial use and the use of

zinc and copper should be further investigated in order to fully understand the complexity of the selection of resistant bacteria.

The results obtained in this thesis, supported the assumptions from WHO (2007) and EMA (2009), regarding the effect of the generic usage of antimicrobial agents on the occurrence of resistance. A better estimation of the effect of the generic usage of antimicrobial agents could be made by including all relevant antimicrobial agents in the investigation. Also the effect on the gram positive bacteria needs to be investigated in order to estimate the full effect on the human exposure to resistant bacteria from pork. However further data collection is needed in order to improve the exposure model and fill some of the many data gaps occurring in the model.

8. Conclusions and future studies

The results of this thesis found an association between usage of tetracyclines for weaning pigs and the occurrence of cephalosporin resistance in slaughter pigs. Furthermore, the results suggest an association between the proportion of ESC producing *E. coli* and the long-term generic use of the three antimicrobial classes: cephalosporin, tetracycline and extended spectrum penicillin (in this thesis mostly driven by the high usage of tetracycline).

Estimation of the quantitative effect of the tetracycline usage on the occurrence of ESC producing *E. coli* in pork indicates that human exposure to the resistant *E. coli* is affected by tetracycline usage. The exposure estimated, given as the prevalence and concentration in pork chops at retail was found to be higher than what was observed in a national survey of pork products at retail and what is reported by the DANMAP programme. However, the estimates fall within an acceptable range when taking into consideration the data applied and the assumptions made in the framework of the model as discussed previously. Also, the model was found useful for giving indications of differences with regard to antimicrobial usage and the possible effects on human exposure to ESC producing *E. coli*. However, future and more extensive studies evaluating the quantitative effect of the antimicrobial usage are needed to provide more data and thereby increased strength to the exposure assessment model. Additionally, the model could be developed to handle more steps in the farm to fork pathway, such as the consumer phase and the effect of the transport and storage of the pork.

This thesis also investigated the effect of the tetracycline usage, the farm size, season and the production type on the occurrence of resistance. The result of these investigations indicated that an increased usage of tetracycline in the pig production lead to an increased probability of the occurrence of resistance in *E. coli* irrespectively of production type. Also, it was found that organic production had a lower occurrence of resistance when compared to conventional and free range production. However, with the data at hand it was not possible to show which specific management factors were leading to this difference. In order to separate this multicollinearity, future studies could be made in the conventional production where the effect of the single factors considered important for the occurrence of antimicrobial use (i.e. weaning age or feeding) could be investigated.

The overall conclusion of this thesis is that the occurrence of resistance is highly dependent on the use of antimicrobial agents. However the mechanisms underlying this resistance development and spread are complex and the discontinued use of one antimicrobial class alone is not considered sufficient to reduce the selective pressure for development of resistance if the generic usage of antimicrobial agents is not reduced simultaneously. Future studies should investigate the role of co-resistance and generic antimicrobial usage. Also, there seem to be a huge potential to lower the antimicrobial usage in production types that are not organic. However welfare studies should be made in all production types simultaneously in order to ascertain that the decreased antimicrobial usage will not lead to under treatment and increased morbidity and mortality.

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10. Manuscripts

Manuscript I:
Cephalosporin resistance in Danish slaughter pigs from farms with a registered use of antimicrobials selecting for cephalosporin resistance.
T. Struve, Agersø, Y., Vigre, H. & Hald, T.

Manuscript II:
Quantitative effect of antimicrobial usage on the proportion of ESC *E. coli* in Danish finishing pigs.
T. Struve, Agersø, Y., Hald, T. & Vigre, H.

Manuscript III:
The effect of tetracycline usage on the occurrence of tetracycline resistance in Danish conventional, free range and organic slaughter pig farms.
T. Struve, Vigre, H., Emborg, H-D., Sørensen, A., Hald, T. & Wingstrand, A.

Manuscript I:

Cephalosporin resistance in Danish slaughter pigs from farms with a registered use of antimicrobials selecting for cephalosporin resistance.

T. Struve, Agersø, Y., Vigre, H. & Hald, T.

Cephalosporin resistance in Danish slaughter pigs from farms with a registered use of antimicrobials selecting for cephalosporin resistance.

Authors: T. Struve[1], Agersø, Y.[1], Vigre, H. [1] & Hald, T. [1]

[1]: National Food Institute, Technical University of Denmark, 2860 Søborg, Denmark

Corresponding author:

Tina Struve,

Department of Microbiology and Risk Assessment

Phone: +4535887103 Fax: +4535887028

Mail: tstr@food.dtu.dk

Abstract

The occurrence of cephalosporinase producing (ESC) producing *E. coli* is a major concern worldwide. Not only due to the complications implicated with an infection with ESC producing *E. coli*, but also because of the risk of spreading the resistance mechanisms to other human pathogenic bacteria such as *Salmonella*. We investigated the occurrence of ESC producing *E. coli* in Danish pig at slaughter originating from farms with a registered usage of antimicrobial agents. ESC producing *E. coli* was isolated from fecal samples by direct plating on MacConkey agar supplemented with 1 mg/L ceftriaxone.

The association between the occurrence of ESC producing *E. coli* and the use of the three antimicrobial groups: tetracyclines, extended spectrum penicillin and cephalosporin respectively, were investigated using regression analysis. The usage was stratified on three swine age groups, as reported in the veterinary medicine consumption database (Vetstat).

Apart from the consumption of tetracyclines for weaning pigs, no significant associations were found among the antimicrobial usage and the occurrence of ESC producing *E. coli* in slaughter pigs. For the tetracyclines usage in weaning pigs, the relative odds of occurrence of cephalosporin resistance increased by 0.25 for every additional Animal Daily Dose (ADD_{50}) used in the farms. Even though the statistical association between the use of antimicrobial agents and the occurrence of ESC producing *E. coli* was weak, the study still revealed a larger prevalence of ESC producing *E. coli* in slaughter pigs from farms with a selective pressure due to antimicrobial usage than was expected from data collected as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP).

Introduction

Even though *E. coli* most often occurs as a commensale, referred to as an indicator organism, infections are commonly reported in humans, where *E. coli* may cause urinary tract infection, abdominal infection and bloodstream infections (Decousser et al., 2003; Turnidge et al., 2004). In Denmark, 80% of all urinary tract infections and 30-40% of all bacteraemias in humans are caused by *E. coli* (SSI, 2011; Jacobsen et al., 2010). Furthermore, antimicrobial resistance is an increasing problem in *E. coli*, and multi-resistant strains causing infections are of major concern (DANMAP, 2009). In the recent years especially, the global development of resistance towards critically important antimicrobials such as cephalosporins has been addressed (Schwaber et al., 2006). The predominant cause of resistance towards cephalosporin in *E. coli* is due to the presence of plasmid-mediated extended spectrum β-lactamases (ESBLs) and AmpC-type β-lactamases, also referred to as extended-spectrum cephalosporinases (ESCs) (Giske et al., 2009). Resistance mediated by enzymes able to hydrolyse β-lactams has been increasingly reported in many areas around the world (Batchelor et al., 2008; Guardabassi et al., 2008). In Denmark, a recent study performed by Hammerum et al. (2011) found extended-spectrum cephalosporinases in faecal *E. coli* from six out of 84 healthy Danish army recruits (7%), indicating a human reservoir in the community. Food of animal origin may be an important vehicle in the spread of extended-spectrum cephalosporinase producing *E. coli* through the food chain, thereby causing a threat to the human health (BIOHAZ, 2011). Use of especially third and fourth generation cephalosporin in food animals is likely to select for the occurrence of resistance phenotypes in animal bacteria (Jørgensen et al., 2007; Agersø et al., 2011 in press). Furthermore, recent studies performed by Frye et al. (2011) found that 47% of *E. coli* and *Salmonella* isolated from the same fecal sample shared resistance genes suggesting

that either resistance genes are horizontally exchanged between these genera or there may be a common pool of resistance genes in the swine environment (Frye et al., 2011).

In Denmark there is a unique registration of the veterinary prescriptions, including all sales of antimicrobial agents. This means, that all farms need a prescription from a veterinarian in order to purchase antimicrobial drugs and prophylactic treatment is prohibited. All the information on each veterinary prescription is collected in the national database VetStat (Stege et al., 2003).

The objective of the present study was to investigate the association between the usage of antimicrobial drugs selecting for cephalosporin resistance (cephalosporin, tetracycline and extended spectrum penicillin) and the prevalence of ESC producing *E. coli* in healthy slaughter pigs using regression analysis.

Materials and methods

Sampling

From May 2009 to May 2010, ESC producing *E. coli* was investigated in samples from Danish slaughter pigs collected as a part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme, DANMAP. Through this programme, 80 caecal samples are randomly collected from healthy slaughter pigs every month. Only one sample per farm is included. The abattoirs participating in the DANMAP programme slaughter around 90% of the total number of pigs processed in Denmark and are consequently considered to represent a stratified random sample of the Danish slaughter pig population (DANMAP, 2010).

We used a retrospective cohort design, where the samples were selected based on the exposure to cephalosporin at the farm in 2008. From the samples included in the DANMAP surveillance, we made a monthly collection of samples from four farms with exposure to cephalosporin (identified in the VetStat database) and samples from two non-cephalosporin exposed farms. If more than four farms sampled for the DANMAP surveillance had used cephalosporin in 2008, four farms were chosen randomly. The samples from farms that had not used cephalosporin in 2008 were randomly selected by picking at random from the bucket containing all the samples collected that month.

Isolation of *E. coli*

E. coli was isolated by adding one gram of faeces to 9 ml of saline (9% NaCl), after vortex mixing for 30 min, 100 uL was added to a MacConkey agar plate (Oxoid CM5a) and spread, furthermore 100 uL was added and spread on a MacConkey agar plate supplemented with 1 mg/L of ceftriaxone (Sigma C5793-1G) both plates was incubated overnight at 44^{o}C. Ceftriaxone was chosen based on a study performed by Aarestrup et al. (2010) where ceftriaxone was identified as the best measure of ESC producing *E. coli* when compared to seven other cephalosporins. Presumptive *E. coli* colonies were counted and transferred onto orientation CHROMAgar plates (CHROMAgar Microbiology, Becton Dickinson a/s), and red colonies were identified as *E. coli* after incubation at 37^{o}C overnight.

Antimicrobial consumption

For all the farms sampled in the study, data on the use of the antimicrobials that may select for ESC producing *E. coli* - cephalosporin, extended spectrum penicillin (amoxicillin and ampicillin) and tetracyclines (due to possible cross-resistance) was retrieved from the VetStat database (Stege et al., 2003) including the following

information: farm identification number, animal species, age group, antimicrobial agent identity, ordination group, amount of drug, authorisation number of the veterinarian, and the date of prescription (Jensen et al., 2004). Consumption of the three antimicrobial groups one year prior to the sampling date was extracted for each farm based on the farm identification number and the date of prescription. Consumption for three swine age groups (sows/piglets, weaning pigs and slaughter pigs) on each farm was included. The usage of the antimicrobial agent was measured in Animal Daily Doses (ADD_{50}), defined as the assumed average maintenance dose per day for the main indicated disease in a specified species and "standard" body weight for the species and age group (Jensen et al., 2004).

Data set

Data on the occurrence of cephalosporin resistance and data on usage of cephalosporin, tetracyclines and extended spectrum penicillin were merged by farm identification number. The number of slaughter pigs, weaning pigs and sows/piglets on each farm in 2008 was extracted from the Central Husbandry Register (CHR). This resulted in a dataset containing the following variables available for each isolate: *farm identification number, sampling date, result of the detection of ESC producing E. coli (1 if detected, and 0 if not detected), number of slaughter pigs produced at the farm in 2008, number of sows at the farm in 2008, number of weaning pigs at the farm in 2008,* and *the total amount of consumed antimicrobial agent (ADD_{50}) for the three antimicrobial classes separated on each of the age groups for all prescriptions one year prior to the sampling of the pig.*

The association between the detection of ESC producing *E. coli* and the antimicrobial usage was analysed for each drug group used in each of the three

different age groups. The antimicrobial consumption was measured on farm level and divided by the number of pigs in the corresponding age group thereby adjusting for the size of the production at each farm. The ADD_{50} per slaughter pig produced at the farm was estimated using the average weight for the pig throughout its time in the slaughter pig section (50kg). Farms where housing all three age groups was reported was considered to have integrated production.

Data analyses

The occurrence of ESC producing *E. coli* in the sample from one slaughter pig at each farm was used as the outcome in the data analysis. Associations were investigated using logistic regression with the following variables as explanatory: farm size (size 1: 1-800 slaughter pigs, size 2: 801-1250 slaughter pigs, size 3: 1251-2150 slaughter pigs and size 4: 2151-13000 slaughter pigs), integrated production (integrated production vs. slaughter pig production), and when usage was registered, the linear effect of the quantitative usage of cephalosporin, tetracyclines or extended spectrum penicillin (ADD_{50} per animal in each age group) one year prior to sampling. Logistic regressions were performed using the procedure GENMOD in SAS, significance of variables in the models was tested using the likelihood ratio test with a significance level of 5%.

Results

Pig faecal samples from a total of 63 farms were sampled from May 2009 to May 2010 were included in this study. One year prior to the sampling, 33 farms (52%) had a reported usage of cephalosporin, 41 farms (65%) had reported usage of extended spectrum penicillin (ESP) and 57 farms (90%) had reported usage of tetracyclines (Table 1). Four farms (6%) did not have reported usage of any of the three antimicrobial classes in the one year period prior to the sampling. The prescription of

antimicrobial agents was more or less equally distributed among the three age groups. In the case of cephalosporin and extended spectrum penicillin, only a minor part of the farms (four and 11 respectively) received the antimicrobial agent for more than one age group at the same farm.

The average usage in ADD_{50}/animal was stratified by age group (Table 2) which revealed that the usage of all three antimicrobial agents were considerably higher in sows/piglets, reflecting the higher bodyweight of the individual pig in this section. The average usage of cephalosporin was low in all three age groups when compared to the average usage of the two other antimicrobial groups investigated. The amounts used per pig in the weaner and finisher section was similar, indicating a higher treatment incidence in weaning pigs, having a lower bodyweight, compared to slaughter pigs.

Twenty three (37%) farms were reported as housing all three age groups (integrated production). No significant effect was found of farm size or production type.

ESC producing *E. coli* was found in slaughter pig samples from 26 farms (41%). The usage of antimicrobial agents stratified on susceptibility result is shown in Table 3. ESC producing *E. coli* was isolated from samples from two farms (non-integrated) with no reported usage of any of the three antimicrobial agents in the study period. No significant association was found between the occurrence of ESC producing *E. coli* and the usage of all three antimicrobial agents as a total.

The association between the occurrence of ESC producing *E. coli* and antimicrobial usage in each age group was investigated for each antimicrobial agent. Apart from the use of tetracyclines in weaning pigs, no significant association was found

between the usage in any age group of any of the investigated antimicrobial agents and the occurrence of resistance in slaughter pigs (Table 4). A significant association was found between the quantitative usage of tetracyclines in weaning pigs and the occurrence of resistance in slaughter pigs (p=0.03, OR=1.25).

Discussion

In this study the association between occurrence of cephalosporin resistance in slaughter pigs and the usage of three selected antimicrobial groups were investigated. The choice of these three classes were recently supported by the publication of the scientific opinion from the BIOHAZ panel where it was concluded, that since most ESBL/AmpC are often connected to co-resistance mechanisms, the full human impact of the extended spectrum cephalosporinases can only be assessed by looking at other antimicrobial groups than only cephalosporin (BIOHAZ, 2011). This recommendation from the BIOHAZ panel further supports our decision to investigate the two most commonly used groups of beta-lactam antibiotic – cephalosporin (3^{rd} and 4^{th} generation) and the extended spectrum penicillins (ampicillin and amoxicillin). Furthermore, also the usage of tetracyclines was included, which also became the recommendation of the BIOHAZ panel when they concluded that “the generic antimicrobial use is a risk factor for occurrence of ESBL/AmpC mechanisms” and the tetracyclines are the most commonly used antimicrobial class in the Danish pig production (DANMAP, 2011).

The antimicrobial consumption collected in VetStat is published annually in the national surveillance programme of antimicrobial consumption and resistance in animals, food and humans (DANMAP). According to DANMAP (2011) the usage of cephalosporin for pigs was 49 kg active compound in 2010, which was a reduction of 48% when compared to 2009. Of all the cephalosporin prescribed, 89% is prescribed

for sow herds (primarily used for piglets). In 2010 the Danish pig industry decided to voluntary stop consuming cephalosporin for pigs (DANMAP, 2011).

In this study we used the average weight for the pig throughout its time in the slaughter pig section (50kg), the standardisation of the weight of the pigs might have been a possible bias throughout the analyses since this standard is underestimating the weight of the sows, but also overestimate the weight of the weaning pigs. However, in order to estimate these age groups more accurate we would have needed reliable information on the number of sows, piglets and weaning pigs at the farms, and previous studies have shown that this information was not validated in the CHR register at the time of this study. However, this information has later been updated since the yellow card agreement is using these data also.

The results of this study showed no significant association between the usage of cephalosporin or extended spectrum penicillin for any of the three pig age groups investigated. Only the quantitative amount of tetracyclines used for weaning pigs in a period up to one year prior to sampling seem to be significantly increasing the risk of detecting cephalosporin resistance in slaughter pigs from these farms. Previous studies have demonstrated an association between the usage of antimicrobials and the occurrence of resistance (Alali et al., 2009; Emborg et al., 2007; Harada et al., 2008; Varga et al., 2009). Agersø et al., (In Press 2011) also suggested an association between the presence of ESC producing *E. coli* obtained after selective enrichment and the usage of 3rd or 4th generation cephalosporins in slaughter pigs although the number of farms with usage were few (Agersø et al, in press 2011). In order to study the complete association between occurrence of resistance and antimicrobial usage more antimicrobial groups could have been investigated. However the sample size in this study did not allow enough power to estimate the

association between the cephalosporin resistance and the less frequently used antimicrobial groups which is also reflected in the results of the analysis made on the cephalosporin usage. The study revealed a larger prevalence of ESC producing *E. coli* in slaughter pigs (41% of the farms) in farms with a selective higher use of cephalosporins, compared to the national average (7% ESC producing *E. coli*) (DANMAP, 2010).

The results of this study suggest that associations among the antimicrobial usage and occurrence of resistance do exist. In order to further investigate this, future studies should be made where the sample size should be increased, but the sampling should also include sows/piglets and weaning pigs. Data from a yet unpublished Danish study found a significant association between the occurrence of cephalosporin resistance in sows and piglets and the usage of antimicrobial agents in that same age group ($Anon_a$., 2011).

As mentioned previously the Danish pig producers agreed on a voluntary discontinuation of the use of cephalosporin in the pig production in 2010. In addition to this initiative, the Danish government introduced the “yellow card” intervention in June 2010 with a letter to those pig farmers representing the 20% highest usage of antimicrobials per pig produced. The intervention was initiated in Dec 2010 ($Anon_b$., 2011), and the overall effect of the announcement and the initiation is likely to have greatly affected the overall consumption of antimicrobials in Denmark, which actually decreased by 12% in 2^{nd} half year of 2010 as compared to the same period in 2009 (DANMAP, 2011). Therefore more studies in the near future is highly relevant in order to assess the effect of the overall decreasing usage of antimicrobial agents and especially also the discontinuation of the cephalosporin usage.

Conclusion

The results of this study indicate an association between usage of tetracyclines for weaning pigs and the occurrence of cephalosporin resistance in slaughter pig samples. Further studies should investigate the effect of antimicrobial usage on the detection of resistance when sampling in the two other age groups.

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Transparency declarations:

None to declare

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Table 1 : Consumption of antimicrobial agents at the farms, and their consumption in the different agegroups

		Consumption of agent at the farm in agegroup					
Antimicrobial agent	*No. of farms with usage*	*Unknown*	*Sows/piglets*	*Weaning pigs*	*Slaughterpigs*	*Uses for more than one agegroup*	*Cephalopsorin resistance*
3. and 4 Gen. Cephalosporins	33 farms (52 %)	1 (3%)	14 (42%)	10 farms (30%)	13 farms (40%)	4 farms	13 farms (39%)
Tetracyclines	57 farms (90 %)	3 (5%)	18 (32%)	29 farms (50%)	44 farms (77%)	31 farms	22 farms (38%)
Extended Spectrum penicillin	41 farms (65 %)	2 (5%)	23 (56%)	12 farms (29%)	17 farms (41%)	11 farms	15 farms (37%)

Table 2: Quantitative consumption of antimicrobial agents at the farms that used the antimicrobial group

		Usage (ADD $_{50}$/pig)		
Antimicrobial agent	*No. of farms*	*Mean*	*Median*	*Std Deviation*
3. and 4. Gen. Cephalosporins				
Sows/piglets	11	3.53	2.9	3.09
Weaning pigs	7	0.42	0.38	0.44
Slaughterpigs	13	0.32	0.09	0.57
Tetracylines				
Sows/piglets	16	9.23	3.08	11.87
Weaning pigs	21	7.74	7.16	6.8
Slaughterpigs	44	5.2	3.04	7.8
Extended spectrum penicillin				
Sows/piglets	20	4.18	3.26	2.93
Weaning pigs	11	0.81	0.7	0.89
Slaughterpigs	17	1.07	0.4	1.26

Tabel 3: Consumption of antimicrobial agents stratified by susceptibility to cephalosporin

		Antimicrobial usage			
	Farms with no usage*	Cephalosporin	Tetracycline	Extended Spectrum Penicillin	Total no. of farms
Cephalosporin susceptible	2 farms (5%)	20 farms (54%)	35 farms (95%)	26 farms (20%)	37 farms (59%)
Cephalosporin resistant	2 farms (8%)	13 farms (50%)	22 farms (85%)	15 farms (58%)	26 farms (41%)

*Footnote: *no registered usage of the selected antimicrobial agents in the period investigated*

Table 4: Results of the univariable regression analysis with occurrence of cephalosporin resistance as outcome

Parameter	OR	CI		p
Farm size				
size 1: 1-800 pigs	1.5	0.32	7.05	0.60
size 2: 801-1250 pigs	1.77	0.33	9.72	0.51
size 3: 1251-2150 pigs	3.51	0.72	17.09	0.12
size 4: 2151-13000 pigs (ref)				.
Production type				
Non-integrated productior	1.03	0.32	3.28	0.96
Integrated production (ref)				
Sows/piglets	1.06	1.01	1.09	0.69
Weaning pigs	1.05	1.04	1.16	0.26
Finisher pigs	1.04	1.01	1.07	0.70
Tetracyclines				
Sows/piglets	1.07	0.76	1.50	0.71
Weaning pigs	1.25	1.08	1.44	0.03
Finisher pigs	1.04	1.03	1.11	0.32
Cephalosporins				
Sows/piglets	1.14	0.77	1.67	0.51
Weaning pigs	0.13	0.00	7.11	0.32
Finisher pigs	0.33	0.01	3.64	0.57
Extended spectrum penicillins				
Sows/piglets	0.83	0.59	1.15	0.26
Weaning pigs	1.41	0.25	7.92	0.69
Finisher pigs	1.02	0.45	2.31	0.97

Manuscript II:

Quantifying the effect of antimicrobial usage on the proportion of ESC producing *E. coli* in Danish finishing pigs

T. Struve, Agersø, Y., Hald, T. & Vigre, H.

Quantitative effect of antimicrobial usage on the proportion of ESC *E. coli* in Danish finishing pigs

Authors: T. Struve[1], Agersø, Y. [1], Hald, T. [1] & Vigre, H. [1]

[1]: National Food Institute, Technical University of Denmark, 2860 Søborg, Denmark

Corresponding author:

Tina Struve,

Department of Microbiology and Risk Assessment

Phone: +4535887103 Fax: +4535887028

Mail: tstr@food.dtu.dk

Summary

Cephalosporins have been used increasingly worldwide to treat various infections in veterinary and human medicine. A possible effect of this is the occurrence of resistance to these antibiotics, which have also increased in the recent years. The cephalosporins of third or fourth generation are considered of critical importance in the treatment of severe and invasive infections in humans, and therefore the emerging resistance to cephalosporins is a major threat to the public health.

In this study we estimated the effect of antimicrobial usage on the proportion of extended spectrum cephalosporinase (ESC) producing *E. coli* in finisher pigs delivered to Danish abattoirs for slaughter. The study population included 59 finishing swine farms in Denmark, and the study design used, was a retrospective cohort study. The samples were collected monthly throughout a year to account for seasonal variation.

The effect of using the three classes of antimicrobial agents, cephalosporin, tetracyclines and extended spectrum penicillins on the ESC producing *E. coli* proportion was estimated in a two step process: in the first step a Poisson model was fitted to the count data obtained from each of the dilutions with the purpose of generating an ESC producing *E. coli* proportion. In the second step a linear model estimated the effect of antimicrobial usage on the ESC producing *E. coli* proportion in the *E. coli* population in finishers.

The results of the study indicate an association between the proportion of ESC producing *E. coli* and the long-term generic use of the three antimicrobial classes: cephalosporins, tetracyclines and extended spectrum penicillins. However future studies are needed in order to determine the proportion of ESC producing *E. coli* and concurrent effect of using the antimicrobial classes in other age groups than finishing pigs

Introduction

Cephalosporins have been used increasingly worldwide throughout the recent years to treat various infections in veterinary and human medicine (Liu et al., 2007). As a possible effect of this, the resistance to these antimicrobial agents has also increased in many countries (Tragasser et al., 2006). Cephalosporins of the third and fourth generation are considered of critically importance in the treatment of severe and invasive infections in humans which was recognized by WHO in 2007 (WHO, 2007; EMA 2009). A few years later, the U.S. Food and Drug Administration also followed this policy (FDA, 2009). Therefore resistance of Gram-negative enteric bacteria such as *Salmonella* spp. and *Escherichia coli* to third generation cephalosporins are of major concern (WHO, 2007; EMA 2009). Resistance to third generation cephalosporin occurs through a natural selection process of genetically mediated survivability in the presence of antibiotics (Lutz et al., 2011). The genes carrying this resistance are typically located on horizontally transferable plasmids (Tragasser et al., 2006). The predominant cause of resistance towards cephalosporin in *E. coli* is due to the presence of plasmid-mediated extended spectrum β-lactamases (ESBLs) or AmpC-type β-lactamases, also referred to as extended-spectrum cephalosporinases (ESC) (Giske et al., 2009). Selective pressure on bacterial populations when β–lactams are present might have the potential to select for the widely disseminated extended spectrum β-lactamase leading to ESC producing *E. coli.* ESC producing *E. coli* is reported to act as a reservoir of ESBL that may spread into other closely related pathogenic enteric bacteria for example *Salmonella* (Sunde et al., 1998; Winokur et al., 2000; Hammerum et al., 2011). *E. coli* including ESC producing strains is also reported as the cause of urinary tract infection or blood stream infections (Jacobsen et al., 2010).

The use of third or fourth generation cephalosporins can influence resistance in two ways: either by favoring the evolution of new variants of cephalosporin resistance genes by selecting for emerging mutants or by selecting for genes that are already present or that have been introduced from other sources into the exposed population (EMA, 2009). In cows the use of the veterinary drug ceftiofur (a third generation cephalosporin) has shown to create a selection pressure resulting in the detection of ESC producing *E. coli* in the fecal flora of treated animals (Singer et al., 2008; Alali et al., 2009). A recent study in finishing swine farms in USA has shown increased odds of recovering ESC *E. coli* when the usage of ceftiofur increased (Lutz et al., 2011). In this study Lutz et al. (2011) conclude that routine use of cephalosporin may influence the probability of recovering enteric ESC producing *E. coli*.

The genes, encoding for ESC, are often encoded by genes present on plasmids and/or transferable genetic elements, and are often linked to other resistance genes thereby mediating resistance to other, unrelated antimicrobials (EMA, 2009). The gene *Bla*$_{CMY}$, which is one of the genes mediating cephalosporin resistance, is often located on large plasmids that carry additional resistance genes (Winokur et al., 2000). Therefore, the selection pressure created by the common use of non-β-lactam antimicrobial drugs such as tetracycline can lead to the dissemination of β–lactamase genes (co-selection) (Lutz et al., 2011). A possible role of co-selection in the dissemination of *Bla*$_{CMY}$ and other β–lactamase resistance genes in bacterial flora have not yet been reported (Lutz et al., 2011). However, mass medication of large groups of animals with various antimicrobials in animal husbandry seems to contribute to the occurrence and dissemination of resistance in exposed populations (EMA, 2009).

A recent American study, investigated the prevalence of third generation cephalosporin resistance in commensal *E. coli* and *Salmonella enterica* obtained from 1000 different packages of fresh meat products (Mollenkopf et al., 2011). Over 8% of all retail meat packages contained *E. coli* with bla_{CMY} whereas 4% contained *Salmonella* and only 0.5% contained *Salmonella* with bla_{CMY}. *E. coli* with bla_{CMY} was more frequently recovered from retail pork products than from beef products (Mollenkopf et al., 2011). This study supports the conclusion made by the EMA (2009), which states, that attention need to be paid to the potential role of community reservoirs of animal origin such as food of different origins (EMA, 2009).

The objective of the present study was to investigate the quantitative association between the proportion of ESC producing *E. coli* and the usage of the three antimicrobial classes: cephalosporin, tetracycline and extended spectrum penicillin at the farm level.

Materials and methods

Sampling

From May 2009 to May 2010, ESC *E. coli* was investigated among samples from Danish slaughter pigs collected as a part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP). Caecal samples from healthy slaughter pigs were collected at abattoirs providing a total of 80 samples for the DANMAP surveillance every month (the selected abattoirs account for 88-95 % of the total number of pigs slaughtered in Denmark and the collected samples are considered to be a representative stratified random sample of the Danish slaughter pig population (DANMAP, 2010)). One sample per farm was randomly collected by

the technician at the abattoir and included in the DANMAP surveillance. Among these monthly samples, samples from four farms with a reported consumption of cephalosporin in 2008 and samples from two farms without reported usage of cephalosporin in 2008 were selected and included in the study. Information on cephalosporin usage per farm was extracted from the VetStat database (see below). If more than four farms were included in the DANMAP sampling per month were using cephalosporin, four farms were chosen randomly. In a similar manner, samples from two farms not using cephalosporin were randomly selected. Every farm included in the study was sampled only once.

Enumeration of E. coli

E. coli was isolated by adding one gram of faeces to 9 ml of saline (9% NaCl), thereafter 10-fold serial dilutions was made in saline (9% NaCl). 100 µl of all dilutions were added to MacConkey agar plates and spread (Oxoid CM5a), furthermore 100 µl of all dilutions was added to a MacConkey agar plate supplemented with 1 mg/L of ceftriaxone (CRO) (Sigma C5793-1G). The plates were incubated overnight at 44 ^{o}C. *E. coli* was enumerated visually in triplicates in the dilution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, depending on the colony number. Total plate count was performed for colonies with characteristic *E. coli* morphology on MacConkey plates and MacConkey + CRO from one to 400 colonies were counted. One presumptive *E. coli* colony from each plate were transferred onto orientation CHROMAgar plates (CHROMAgar Microbiology, France), where red colonies were identified as *E. coli* after incubation at 37^{o}C overnight.

Estimating the proportion of ESC E. coli

The occurrence of ESC producing *E. coli* was measured as the proportion of ESC *E. coli* of the total *E. coli* count within each faecal sample. The ESC producing *E. coli*

proportion was calculated as the maximum likelihood estimate in a Poisson model. The estimation procedure was implemented with a linear model using the procedure NLMIXED in SAS.

$$\text{Log (CFU)} = \beta_0 + \text{dilution}*\log(10) + \beta_{\text{resistance}} * (\text{presence of CRO in the agar})$$

Where:

β_0 = log(number of CFU per gram feces)

$\beta_{\text{resistance}}$ = log(ESC *E. coli* proportion)

Antimicrobial usage

Antimicrobial usage for the sampled farms was provided by the VetStat database (Stege et al., 2003). Based on the farm identification number and the sampling date, data on cephalosporin, extended spectrum penicillin (amoxicillin and ampicillin) and tetracycline usage were extracted in a one year period prior to the sampling date for each of the farms included in the study. The following information was available for each prescription: farm identification number, animal species, age group, antimicrobial agent identity, ordination group, amount of drug. The usage of the antimicrobial agent was measured in Animal Daily Doses (ADD_{50}), defined as the assumed average maintenance dose per day for the main disease indication in a specified species and a standard weight of 50 kg (Jensen et al., 2004). Only antimicrobial usage for finishing pigs was included.

The use of antimicrobial agents for finishing pigs was measured on farm level and adjusted for the number of finishing pigs annually produced at each farm. The

number of finishing pigs produced annually on each farm in 2008 was extracted from the Central Husbandry Register “CHR”.

Data analyses

The objective of the data analysis was to estimate the quantitative effect of antimicrobial usage in the farm on the proportion of ESC *E. coli* isolated from the pigs from the farm. The effect was estimated in a linear regression model (equation 1) with the log (ESC *E. coli* proportion) as the outcome.

The effect of using the antimicrobials was estimated using a continuous scale for the amount of antimicrobial agent used (ADD_{50}/finishing pig produced) 1-3 months prior to the sampling (short-term effect) and 1-12 months prior to the sampling (long term effect). The three months period represent the time where the actual pig sampled was in the finishing section, whereas the 12 months period represent a long-term usage at the farm.

Initially, the effects of the short and long-term usage was estimated using equation 1, for each antimicrobial class (cephalosporin, extended spectrum penicillin and tetracycline) separately (model 1-3).

Equation 1: log (ESC *E. coli* proportion) = β_0 + #Antib_long term*$\beta_{\text{Antimicrobial long term use}}$ + #Antib_short term*$\beta_{\text{Antimicrobial short term use}}$

Where:

#Antib_long term = Quantitative usage (ADD_{50}/finishing pig produced) of each antimicrobial class 1-12 months prior to sampling

#Antib_short term = Quantitative usage (ADD50/finishing pig produced) of each antimicrobial class 1-3 months prior to sampling

Secondly, the effect of a generic long term usage of all three antimicrobial classes was estimated in a mixed model containing all three antimicrobial classes (model 4).

The estimation procedure was implemented using the procedure GLM in SAS. To adjust the analysis for the fact that the ESC producing *E. coli* proportion was determined with different accuracy (due to varying number of CFU counts in the samples), the analysis was weighted by the reciprocal of the standard error of each estimated $\beta_{resistance}$.

Results

From May 2009 to May 2010 a total of 63 farms were sampled. Among these, useful *E. coli* were obtained from 59 farms. Samples from four farms were excluded due to overgrowth by other bacteria (*Enterococci* and *Salmonella*). One year prior to the sampling, 80 % (47 farms) of the investigated farms used one or more of the three antimicrobial classes included in the study, cephalosporins, tetracyclines and extended spectrum penicillins for finishers (Table 1). The remaining 20% (12 farms) did not use any of the three mentioned antimicrobial classes for treatment of finisher pigs in the period one year prior to the sampling date. The most commonly used antimicrobial class was the tetracyclines, which was used by 75% of the farms. This class also had the highest average usage with 5.2 ADD_{50} per produced finishing pig. The extended spectrum penicillins were used by 29% of the farms with an average use of 1.07 ADD_{50} per produced finisher pig. Twenty two percent of the farms were using cephalosporin for finishing pigs, were the average use per produced finishing pig was 0.32 ADD_{50}.

The descriptive measures for the concentration and proportion of ESC producing E. coli estimated in the Poisson model, is shown in Table 2. This table also contain the descriptive measures for the original raw data to allow comparison.

The 33 percentile distribution of concentration and proportions of ESC producing *E. coli* is summarised in Table 3, information on estimated results from the Poisson model and raw data is included.

The results from the regression analyses are presented in Table 4. The initial analyses, estimating the effect of both short-term and long-term usage of each of the

antimicrobial classes did not show a significant effect of antimicrobial use and proportion of ESC producing *E. coli*. When estimating the effect of the generic use of the three antimicrobial classes, a significant effect was found of the long term tetracycline use (0.13, p=0.04, Table 4). Backward calculation in the linear model applying the parameter estimate of tetracycline usage suggest that an increased use of tetracycline by 1 ADD_{50}/ finishing pig produced will result in a 14% increase in the proportion (an absolute increase of 0.000035) of ESC producing *E. coli.*

Discussion

In this study the association between the proportion of ESC producing *E. coli* and the usage of three antimicrobial classes were investigated.

Three main sampling strategies are typically used to measure antimicrobial resistance. The most common strategy used for detecting resistance is phenotypic testing of a single isolate per sample (Caprioli et al. 2000). This strategy is used in most national surveillance systems, including the national surveillance system in Denmark (DANMAP, 2010). The isolates are randomly chosen and tested for susceptibility to a panel of antimicrobials and classified resistant, susceptible or intermediate based on the susceptibility results. This method is easy to perform and has a low cost, but the sensitivity is low and detection of organisms occurring in low concentration cannot be detected (Caprioli et al. 2000). Furthermore the strain diversity within a sample cannot be detected using this method. In order to determine this inter-sample diversity a second sampling strategy can be applied, where multiple isolates are taken within each sample (Brun et al., 2002). The third sampling strategy, which was used in this study, is to obtain a quantitative measure of the proportion of resistant bacteria within a sample. This was done, by making a serial dilution and obtaining quantitative measures in replicate numbers for each sample.

In Denmark, all farms need a prescription from a veterinarian in order to purchase antimicrobial drugs, and the information on each veterinary prescription is collected in the national database VetStat (Stege et al., 2003). The antimicrobial usage collected in VetStat is published annually in the national surveillance of antimicrobial resistance (DANMAP), where the occurrence of resistance is also reported. According to DANMAP (2010) the usage of cephalosporin for pigs was 99 kg active compound in 2009, in 2010 this usages was reduced considerably. The occurrence of cephalosporin resistance seems to be influenced not only by the usage of cephalosporins, but also by the generic use of antimicrobial agents. Therefore the effect of using extended spectrum penicillin and tetracycline was also included in the present study, we chose to include the extended spectrum penicillins (the usage of extended spectrum penicillins in 2009 was 8977 kg active compound) because they are very similar to the cephalosporins (both containing the β-lactam ring) and the tetracyclines because they are the most commonly used class of antimicrobial agents in Denmark (the usage of tetracyclines in 2009 was 35.254 kg active compound).

The selection of the three antimicrobial classes were supported by the recently published scientific opinion from the BIOHAZ panel which concluded, that since most ESBL/AmpC are often connected to co-resistance mechanisms, the full human impact of the extended spectrum cephalosporinases can only be assessed by also looking at other antimicrobial groups than cephalosporin (BIOHAZ, 2011). Based on a statement from EMA (2009), and supported later by the BIOHAZ panel (2011), we decided to investigate the two most commonly used groups of beta-lactam antibiotic – cephalosporin (3th and 4th generation) and the extended spectrum penicillins (ampicillin and amoxicillin). Furthermore, the usage of tetracycline was included

since both EMA (2009) and the BIOHAZ panel (2011), also concluded that “the generic antimicrobial use is a risk factor for occurrence of ESBL/AmpC mechanisms”.

The farms included in this study were selected on the basis of their usage of cephalosporin in 2008 thereby we selected a high proportion of cephalosporin using farms when compared to the reported national average. Only the use of the antimicrobial classes for finishing pigs was included in the study, since that the sampling was made on the abattoir and the effect of the usage was measured on finishing pigs. Valuable additional information could be obtained by sampling other age groups and investigating the effect of the corresponding antimicrobial use in that same age group.

The proportions of farms in the study using the three antimicrobial classes do not resemble the national situation, and therefore corresponds well with the selection of farms that did have a higher usage of especially cephalosporin. Furthermore, very different usage patterns were found between the three antimicrobial classes investigated. The farms in average used five times as many tetracyclines per finishing pig produced when compared to extended spectrum penicillins. The average use of cephalosporin per finishing pig at the farms was 16 times lower than was the average use of tetracycline. However most of the farms included in this study were sampled based on their usage pattern of cephalosporin therefore the national use of cephalosporin expected to be even lower than what we have observed in this study. This could be a plausible explanation of why usage of tetracyclines is providing more power to the analysis than the two other investigated antimicrobial classes.

I our study, no significant associations were found between the ESC producing *E. coli* proportion and the short term or long term use of each of the three antimicrobial classes when investigated separately.

However a significant effect on the ESC producing *E. coli* proportion of using tetracycline was found when investigating the long-term use of all three antimicrobial classes in the model, which corresponds well with other studies (EMA, 2009; Lutz et al., 2011; BIOHAZ, 2011). Still, others have reported that the use of tetracyclines and other non-β-lactam drugs are not associated with the frequency of cephalosporin resistance in the enteric flora of pigs (Wagner et al., 2008).

The antimicrobial class driving of the significant effect was tetracyclines. This finding however should not be used to make the conclusion that tetracycline usage is more important in the respect of cephalosporin resistance. The most likely reason is that the tetracyclines are used in such large quantities in Danish finishing pigs, and therefore constitute the majority of the generic usage on the farm. In this study we found a very low proportion of ESC producing *E. coli*, however a very strong effect on the ESC producing *E. coli* proportion was found by increasing the use of tetracycline in the farms. Whether this effect is due to co-selection, need to be investigated in detail.

According to EMA (2009) usage of cephalosporin at the farms is the cause of the natural selection of ESC genes, which is also supported by other sources (BIOHAZ, 2011; Lutz et al., 2011).

In this study we investigated how usage of three antimicrobial classes selected for ESC *E. coli* in the farms. It is important to note that the introduction of the ESC genes to a farm is either by natural mutation in present *E. coli*, or that the genes are introduced by e.g. purchase of animals carrying the genes. Thereafter the generic use of other antimicrobial classes may increase the selection pressure for the

resistance genes already introduced in the farm. This possible co-resistance mediated by the generic antimicrobial usage rather than one specific antimicrobial class, highlights the importance of community reservoirs of resistance genes as also reported by other studies (Sunde et al., 1998; Hammerum et al., 2010).

Danish pig producers agreed on a voluntary discontinuation of the use of cephalosporins in the pig production in July 2010. Besides this initiative the Danish government introduced a so-called “yellow card” intervention in June 2010 where pig farmers having the 20% highest usage of antimicrobials per pig produced received a letter urging them to bring down the consumption. The latter intervention was initiated in December 2010 (Anon., 2011). The overall effect of both initiatives is likely to have greatly affected the overall consumption of antimicrobials in Denmark, which decreased by 3% in 2010 as compared to 2009, the use of cephalosporins decreased by 48% in this period (DANMAP, 2011). The voluntary ban will most likely reduce the primary selection for ESC genes. When taking into account the findings in the present study, an overall reduction of the generic use of antimicrobial agents (in this study represented by tetracyclines and extended spectrum penicillins), may in addition to the voluntary ban of cephalosporins probably result in a decreasing occurrence of ESC *E. coli*.

Conclusion

The results of this study suggest an association between the proportion of ESC *E. coli* and the long-term generic use of the three antimicrobial classes: cephalosporin, tetracycline and extended spectrum penicillin. The data obtained in this study

provides a sound base for future investigations and can be useful in future risk assessments.

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Tables.

Table 1: The usage of antimicrobial agents for finishing pigs at the farms that did use the antimicrobial agents

		Quantitative usage[1]			
Antimicrobial class	No. Farms	Mean	Median	Min	Max
Usage of tetracycline	44	5.202	3.037	0.04	44.93
Usageof cephalosporin	13	0.320	0.089	0.02	2.08
Usage of extended spectrum penicillin	17	1.068	0.397	0.08	3.48
Total use of the three antimicrobial classes	47	5.345	2.776	0.02	44.93

Foodnotes:

[1] : Quantitative usage among farms using the antimicrobial classes

Table 2: Descriptive statistics for the concentration and proportion of *E. coli* and ESC producing *E. coli*

	Mean	Mode	Min	Max
Raw data				
E. coli	6.30E+07	2.49E+06	1.82E+03	1.57E+09
ESC producing E. coli	1.27E+05	3.03E+02	1.52E+02	6.14E+06
Resistance proportion	1.55%	0.03%	nc	20.11%
Estimated data				
E. coli	5.79E+07	3.31E+06	2.00E+04	1.30E+09
Resistance proportion	1.493%	0.195%	0.005%	16.226%

Foodnote:

nc= not countable (<100 CFU/ml)

Table 3: Distribution for the concentration and proportion of *E. coli* and ESC producing *E. coli*

		Percentiles		
		33%	66%	99%
Raw data				
	Susceptible	1.82E+03 - 6.21E+05	7.10E+05 - 8.72E+06	9.60E+06 - 1.57E+09
	Resistant	nc - 2.27E+02	2.27E+02 - 1.11E+04	5.38E+03 - 6.14E+06
	Proportion	0.000% - 0.011%	0.012% - 0.192%	0.259% - 20.100%
Estimated data				
	Estimated concentration	2.00E+04 - 6.21E+05	7.10E+05 - 8.72E+06	9.60E+06 - 1.30E+09
	Estimated proportion	0.003% - 0.063%	0.074% - 0.581%	0.646% - 16.226%

Foodnote:

nc= not countable (<100 CFU/ml)

Table 4: The association between cephalosporin resistance ratio in *E. coli* and the use of cephalosporin, tetracycline and extended spectrum penicillin

Model	Estimate	Std. Err.	Pr>|t|
1: Usage of cephalosporin			
Intercept	-7.95	0.26	<0.0001
Cephalosporin use - long term effect	3.75	2.31	0.11
Cephalosporin use - short term effect	-18.17	10.04	0.08
2: Usage of tetracycline			
Intercept	-8.26	0.28	<0.0001
Tetracycline use - long term effect	0.16	0.11	0.15
Tetracycline use - short term effect	-0.14	0.42	0.75
3: Usage of extended spectrum penicillin (ESP)			
Intercept	-8.08	0.26	<0.0001
ESP use - long term effect	-0.02	0.48	0.97
ESP use - short term effect	1.01	0.85	0.24
4: Generic long term usage			
Intercept	-8.24	0.28	<0.0001
Cephalosporin use	-1.07	0.93	0.26
Tetracycline use	0.13	0.06	0.04
Extended Spectrum Penicillin use	0.39	0.43	0.36

Manuscript III:

The effect of tetracycline usage on the occurrence of tetracycline resistance in Danish conventional, free range and organic slaughter pig farms.

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Authors: T. Struve[1], Vigre, H. [1], Emborg, H-D.[2], Sørensen, A.[1], Hald, T. [1] & Wingstrand, A[1].

[1]: National Food Institute, Technical University of Denmark, 2860 Søborg, Denmark

[2]: Department of Epidemiology, Statens Serum Institut, 2300 Copenhagen, Denmark

Corresponding author:

Tina Struve, Phone: +4535887103 Fax: +4535887028

Mail: tstr@food.dtu.dk

Abstract

Antimicrobial resistance is a public health problem, and the main selective pressure driving the development of resistance is the use of antibiotics. In this study we compared the tetracycline usage among three different types of slaughter pig production, and investigated the quantitative effect of tetracycline usage in pigs to the occurrence of tetracycline resistance in generic *E. coli*.

By using data from 2007 and 2008 we investigated the usage of tetracycline in 224 farms producing slaughter pigs. The farms represented three different production types, conventional production (146 farms), free range production (27 farms) and organic production (51 farms). The usage of tetracycline per produced pig was measured as the average Animal Defined Dosage per slaughter pig produced at the farm one year prior to the sampling date (ADD_{50}/per slaughter pig). The occurrence of tetracycline resistance was determined in indicator *E. coli* from healthy pigs at slaughter. The quantitative effect of the tetracycline usage on the occurrence of resistance was investigated by logistic regression.

The organic farms had an average usage of 0.05 ADD_{50} tetracycline per slaughter pig produced. The free range and conventional farms respectively had an average usage of 0.49 ADD_{50} and 0.72 ADD_{50} per slaughter pig. The study showed that the occurrence of resistance was lower in organic (OR=0.27, p=<0.0001) and free range farms (OR=0.71, p=0.08) compared to the conventional farms, and that an increase in the quantity of tetracycline usage increases the odds of detecting tetracycline resistant *E. coli* at the farm irrespectively of the production type.

Introduction

Consumer habits are changing in industrialized countries, thereby creating a larger market for organic and free range products (Kijlstra et al., 2009). To support and strengthen the sustainability of the Danish food production, a research project was initiated with the aim of investigating how the usage of tetracycline for slaughter pigs in conventional, free range and organic pig production influenced the occurrence of tetracycline resistance in caecal *E. coli* from pigs at slaughter.

Several studies have demonstrated an association between the use of antimicrobial agents in the production of food animals, and the occurrence of antimicrobial resistance among bacteria isolated from healthy animals (Wise et al., 1998; Bogaard, van. den. and Stobberingh, 2000). Once resistance determinants have been acquired by bacterial populations, they may be retained in the environment for a long time after the termination of the selective pressure, particularly if the encoding genes are linked to other genes for which the selection pressure remains (Aarestrup et al., 2001; Maynard et al., 2003).

Rules for the use of antimicrobials in Danish pig production are set in Danish legislation. All farms need a prescription from a veterinarian in order to purchase antimicrobial drugs, and use of antimicrobials for growth promoting and prophylactic treatment is prohibited. In free range slaughter pig production a Health Agreement Contract (HAC) with a veterinarian is mandatory. A HAC was optional for conventional farms until July 2010 but is now mandatory for all farms of any production type producing more than 3000 slaughter pigs annually. Having a HAC allows prescription of antimicrobials for treatment of expected disease at the farm for 35 days after the veterinary consultation. Among the farms included in this study,

98% of all conventional, 96% of all free range and 30% of all organic farms had a HAC (Sørensen et al., 2011).

In addition to the legislation, free range and organic pig productions follow specific restrictive rules for the use of antimicrobial drugs: i) in organic production, slaughter pigs receiving antimicrobial treatment more than once in their lifespan lose their organic status; ii) each antimicrobial treatment in organic pig farms must be based on an individual diagnosis and the prescription is specified for the actually diseased pigs; iii) the withdrawal times for slaughter after the usage of drugs are doubled in the free range and organic production types, thereby encouraging the use of drugs with a shorter withdrawal period. In 2010 the Danish usage of antimicrobials in pig production was 100,527 kg active compound. The most frequently used drug in the pig production was tetracycline, which accounts for one third of all antimicrobial agents used for pigs. Therefore, this study was based on the occurrence of tetracycline resistance and the association of this with the usage of tetracycline.

The objective of this study was to i) investigate the differences in tetracycline usage between the conventional, free range and organic slaughter pig productions and ii) estimate the effect of tetracycline usage in slaughter pigs in the farm in relation to occurrence of resistance in intestinal bacteria from healthy slaughter pigs. In this study we used the occurrence of tetracycline resistance in *E. coli* as an indicator for occurrence of resistance in the healthy animal.

Materials and methods

Sample size considerations

The samples included in this study were originally a part of a larger study where the samples were collected to determine the prevalence of *Salmonella* in the different pig production types. Therefore the sample size was calculated based on the expected *Salmonella* prevalence in the conventional and alternative productions. These calculations estimated a sample size of 500 isolates from each production to be significant enough to detect a difference in the *Salmonella* prevalence of 50 %.

The investigation of the occurrence of resistance in the three production types was later added as a subproject, and due to financial reasons 1000 samples could be susceptibility tested. In order to sample as many farms as possible, the number of samples per farm was in average three samples. Since the total number of free range and organic farms in Denmark is very low when compared to the conventional production, more samples per farm had to be collected in these in order to achieve 500 samples within each production type. Since many samples per farm were submitted from the free range and organic production types, it was chosen to limit the number of samples being susceptibility tested to half. Thereby the number of samples to be submitted to susceptibility testing was 1000.

Retrospective sample size analysis for the isolates tested for resistance was performed using EpiINFO statcalc (version 3.5.3, 2011). If assuming a tetracycline resistance prevalence of 30% in farms using tetracycline and a tetracycline resistance prevalence of 10% in farms without tetracycline usage - 112 farms with tetracycline usage and 112 farms without tetracycline usage need to be sampled in order to obtain a power level of 95%. If we are assuming a tetracycline resistance prevalence of 28 % (national average in 2006 (Anon., 2007)) for farms with a tetracycline usage, and a tetracycline resistance prevalence of 0.1% in farms without a tetracycline usage 103 farms need to be sampled in each group to obtain a power

level of 95%. At the time of sampling 50% of the farms in Denmark was using tetracycline, therefore the number of farms in the groups with and without tetracycline usage was approximately the same.

Selection of slaughter pig farms

Caecal samples were collected from slaughter pigs from conventional, free range and organic farms (figure 1). In this study, the samples was collected at ten different slaughter houses - nine slaughtering conventional pigs (slaughtering ~90 % of the pigs that is slaughtered in Denmark), and one primarily slaughtering free range and organic pigs (slaughtering ~80 % of the free range and organic pigs that is slaughtered in Denmark). The first sample from each conventional farm (also included in the DANMAP surveillance) was selected at the slaughter house by the technician at random - the two following pigs from the same farm were thereafter included in this study. For the free range and organic farms, the first sample was selected by the technician at convenience and the number of pigs successively sampled from the same farm was based on the proportion of pigs delivered to slaughter annually by that specific farm. In total 2% of the Danish conventional farms, 76% of the Danish organic farms and 71% of the Danish free range farms were included in the study.

Caecal samples from slaughter pigs

Caecal samples were collected from the selected farms during two periods' October 2007 to January 2008 (fall/winter) and March 2008 to May 2008 (spring/summer).

A total of 868 caecal samples from healthy pigs were collected; 402 samples from conventional production, 228 samples from free range production and 238 samples from organic production. Due to the small number of active farms in the free range and organic production most of these were sampled in both rounds, only a few conventional farms were sampled in both sampling rounds.

Isolation and sensitivity testing of caecal *E. coli*

In accordance with the DANMAP sampling, the generic *E. coli* was isolated by direct inoculation of the material on to Drigalski plates (Statens Serum Institute, Denmark) and incubated at 37^{o}C overnight. Presumptive *E. coli* colonies were transferred onto CHROMAgar plates (CHROMAgar Microbiology, France), where red colonies were identified as *E.coli* after incubation at 37^{o}C overnight. Only one *E. coli* colony from each sample was subcultured and susceptibility tested using a commercially available minimum inhibitory concentration (MIC) technique (Sensititre; Trek Diagnostic Systems, UK). Only susceptibility test results for tetracycline were used in the following analysis. The isolate susceptibility status was based on the standardized MIC breakpoint adopted for DANMAP, intermediate isolates was considered susceptible (Anon., 2010).

Antimicrobial usage

For all farms, data on tetracycline usage (all types of administration have been included) was achieved from the VetStat database (Stege et al., 2003). The total usage of tetracycline for slaughter pigs for year 2007 (used for descriptive purposes)

and the total usage of tetracycline on the farm one year prior to each sample date (used for regression analysis) were extracted for each farm.

The usage of tetracycline was measured in Animal Daily Doses (ADD_{50}), defined as the assumed average maintenance dose per day for an indicated disease in a specified species and "standard" body weight (50 kg for slaughtering pigs, which is indicated by the suffix 50 in ADD_{50}) for the species and age group (Jensen et al., 2004).

In the combined dataset, the occurrence of resistance and the tetracycline consumption were merged by the farm identification number. The following variables were available for each isolate: *farm identification number, sampling date, production type, result of the susceptibility test (1 if resistant, and 0 if susceptible), number of slaughter pigs produced in 2007,* and *total amount of consumed tetracycline (ADD_{50}) for slaughter pigs in 2007 and one year prior to sampling.*

In order to describe the usage of tetracycline between farms the tetracycline usage in each farm (ADD_{50}/per slaughter pig) was calculated for 2007. Since multiple samples was collected at different sampling dates, the quantitative association between each sample and the tetracycline usage was investigated by creating a variable for the tetracycline usage (ADD_{50}/per slaughter pig) one year prior to the sampling date. This created two variables describing the tetracycline usage, one for each farm used for descriptive purposes, and one for each sample used for analysing the effect of the quantitative usage of tetracycline on the occurrence of tetracycline resistance.

Statistical analysis

The aim of the statistical analysis was to estimate the effect of tetracycline usage in slaughter pigs in the farm on the occurrence of tetracycline resistance in caecal

E.coli isolates from healthy pigs. The effect of the production type on the usage of tetracycline (yes or no) was analysed using simple chi-square statistics.

All additional statistical analysis was done using logistic regression where the outcome was presence or absence of tetracycline resistance in each individual sample. For many farms, we have several *E. coli* isolates, and the occurrence of resistance in samples from the same farm cannot be assumed to be independent. To account for the correlation between samples from the same farm, we used a mixed logistic regression, where the effect of farm were added as a random effect ($N(0,\sigma^2)$). The analyses were performed in the GLIMMIX procedure in SAS 9.1.

The quantitative effect of tetracycline usage was analysed by categorising the farms into one of five categories – no usage (0 ADD_{50}/per slaughter pig); usage in the 1st quartile (0< – 0.1 ADD_{50}/per slaughter pig), usage in the 2nd quartile (0.1≤ – 0.4 ADD_{50}/per slaughter pig), usage in the 3rd quartile (0.4≤ – 1.2 ADD_{50}/per slaughter pig), and usage in the 4th quartile (≥1.2 ADD_{50}/per slaughter pig).

First, the association between occurrence of resistance and i) the usage of antibiotics and ii) the production type (conventional, free range or organic) were analysed in separate models. Next, to adjust the effect estimate of usage for a potential confounding effect of production type a logistic model including both the usage of antibiotics and the production type was fitted (model 1). Finally, the presence of interaction between usage and type of production was assessed by adding an interaction-term between the variables to the model (model 2). The significance of each fixed variable was tested by the Type III *F*-test. For the fixed effect parameter estimates, t-type confidence intervals were computed.

Results

A total of 868 *E. coli* isolates were available from 224 farms (146 conventional, 27 free range, 51 organic (table 1)). On average 4 isolates were collected per farm (range 1 to 22 isolates). In total, 205 *E.coli* isolates were resistant to tetracycline (conventional: 32% resistant, free range: 25% resistant and organic: 8% resistant). The organic farms had the lowest average usage of tetracycline in 2007 with 0.05 ADD_{50} per slaughter pig produced, followed by free range farms and conventional farms with 0.49 and 0.72 ADD_{50} per slaughter pig produced. In 38% of the conventional farms, 33% of the free range farms and 76% of the organic farms no tetracycline was prescribed in 2007.

The occurrence of tetracycline usage (yes or no) was found to be significantly lower in the organic farms when compared to conventional farms (p=<0.0001). No significant difference was found between conventional and free range farms (p=0.19).

In the logistic regression we found significant effects of both production type and the quantitative amount of tetracycline used on the occurrence of tetracycline resistance in *E. coli* (table 2). No significant interactions between these effects were revealed in the analysis. The odds ratio of getting a resistant isolate was significantly lower in organic farms (OR= 0.27, p=<0.0001) when comparing to the conventional farms. No significant difference was found between the conventional farms and the free range farms. The probability of occurrence of resistant *E. coli* increased successively by an increased usage of tetracycline going from an OR=1.19 at a low usage to an OR=1.86 for farms with a very high usage (table 2).

Discussion

The results of this study showed that the probability of isolating tetracycline resistant *E. coli* from slaughter pigs increased significantly by an increased usage of tetracycline in slaughter pigs a year before sampling. Several studies have shown an association between the usage of antimicrobials and the occurrence of antimicrobial resistance (Jordan et al., 2009; Harada and Asai 2010; Jensen et al., 2006), but studies regarding the quantitative usage in different production types have not been made previously. This study found a significant difference between the occurrence of resistance in *E. coli* when comparing organic production to conventional and free range production, which is in accordance to previous studies, were the occurrence of resistance in organic produced pigs also was found to be significantly lower than the occurrence in the conventional pigs (Hoogenboom et al., 2008; Miranda et al., 2008; Young et al., 2009; Rollo et al., 2010). In a meta-analysis, Young et al. (2009) found eight studies describing a significantly higher occurrence of multi drug resistance in zoonotic and indicator bacterial isolates from conventional compared to organic broilers, broiler meat, swine and pork. These studies suggest that antimicrobial use practices on conventional farms are more selective of resistance and multi drug resistance than usage practices on organic farms (Young et al. 2009; Rollo et al., 2010; Kijlstra et al., 2009).

The management of antimicrobial usage in a pig farm can be expected to be strongly associated with the general health of the animals and the attitude among the individual pig producers. The pig producer is acting under law regulations. According to the regulations, organic productions are not allowed to keep antimicrobials at the farm that is not meant for the ongoing treatment of a sick animal, and the producer has to consult a veterinarian every time an animal needs treatment. Furthermore the pigs cannot keep their organic status if treated more than once (defined as one

specific animal being diagnosed and following being treated with antimicrobials in up to 14 days). This single animal treatment differs from the conventional and free range production where food and water treatments of groups of animals are often used.

In our study, besides the effect on resistance by usage of tetracycline, there was also a “direct” effect of organic production, where the occurrence of tetracycline resistance was lower in organic farms compared to conventional and free range. It can be speculated whether the casual mechanisms for how the management of organic pigs can cause a lower occurrence of resistance compared to management of pigs in free range and conventional farms. One possibility is that besides the use of tetracycline, also use of other antimicrobials can select for the occurrence of tetracycline resistance in *E.coli* and because other antimicrobials are generally used more frequently in non-organic farms, this can explain the observed effect of the production type. Though, a detailed discussion of co- and cross resistance mechanisms influencing the occurrence of tetracycline resistance is out of the scope of this study.

However, we need to perform more research to reveal knowledge that can be applied in pig production to reduce the occurrence of antimicrobial resistance in pigs, irrespectively of production type.

Conclusion

The result of this study indicates that an increased usage of tetracycline in pig production leads to an increased probability of occurrence of resistance in *E. coli* irrespectively of production type. We also found that organic farms had a lower occurrence of resistance when compared to conventional and free range production.

However, in this study we did not obtain sufficient information to show which management factors was leading to this difference.

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Figures

	Production type	
	Conventional	Free range and Organic
Sampling week	1 and 6	2-5
Number of slaughterhouses sampled	9	1
Days of sampling per week	1	2-3
Selection of pigs for sampling	First animal selected by the techinician at convenience: systematic sampling of the two following pigs from same farm	First animal selected by the techinician at convenience: systematic sampling of the following *n* pigs[a]
Total number of samples in the sampling period	250	2 x 250
Number of samples susceptibility tested	250	2 x 125 [b]

Foodnotes:

a): The number of samples from organic and free range pigs was based on the number of animals delivered per yea

b): The number of farms in the two alternative productiontypes was relatively small many samples was collected from the same farms, due to financial reasons only half of these were susceptibility tested

Fig. 1. Planned sampling procedure for the summer sampling

Tables

Table 1
Distributions of samples on production type and season and desriptive statistics on occurrence of resistance and consumption of tetracycline

	Production type			
	Conventional	Free range	Organic	Total
Number of farms	146	51	27	224
Number of *E. coli* isolates	402	228	238	868
Isolates in spring/summer	203	123	122	448
Isolates in fall/winter	199	105	116	420
% res to tetracycline	32%	25%	8%	24%
% farms using tetracycline in 2007	62%	67%	24%	54%
Mean ADD tetracycline/pig consumed in 2007	0.72	0.49	0.05	0.42

Table 2

Factores associated with the occurrence of tetracycline resistance investigated by multivariable regression analysis.

Explanatory variable	Odds ratio	95 % CI	Pr>z
Effect of the production type			
Conventional (Reference)	.		
Organic	0.27	0.16-0.47	<0.01
Free range	1.71	0.48-1.05	0.09
Quantitative effect of the tetracycline usage			
No usage (ADD50/ per pig produced = 0) (Reference)	.		
Low usage (0 < ADD50/ per pig produced < 0.1)	1.19	0.68-2.08	0.55
Medium usage (0.1 ≤ ADD50/ per pig produced < 0.4)	1.78	1.07-2.96	0.03
High usage ((0.4 ≤ ADD50/ per pig produced < 1.2)	2.43	1.47-4.03	<0.01
Very high usage (1.2 ≤ ADD50/ per pig produced)	1.86	1.11-3.12	0.02

National Food Institute
Technical University of Denmark
Mørkhøj Bygade 19
DK - 2860 Søborg

Tel. 35 88 70 00
Fax 35 88 70 01

www.food.dtu.dk

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