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Development of methods for Genetic Assessment of Antibiotic Resistance In Animal Herds

A PhD thesis by
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Copenhagen, 2014

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Development of Methods for Genetic Assessment of Antibiotic Resistance in Animal Herds

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To my family

Preface

The work included in this PhD thesis was supported by a grant from the Strategic Research Council, Ministry for Food Agriculture and Fisheries and the Faculty of Life Sciences, University of Copenhagen for which I am grateful. The research has primarily been conducted in Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark, in collaboration with Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen.

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Anders Folkesson
Øystein Angen
John Olsen

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Copenhagen, February 2014

Gunilla Veslemøy Schmidt

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Summary (English)

Antibiotic drugs are important in treating bacterial infectious diseases in humans and animals. There are severe consequences when infectious bacteria become resistant to antibiotics such as treatment failure and even death. Since antibiotics were discovered, their use has been associated with a parallel selection for resistant bacteria. Since the hazards related to antibiotic resistance development have been recognized, the prudent use of antibiotics has been in focus, especially concerning their use in animal production. For many years antibiotics have been, and still are, recklessly used in the animal production especially in the form of growth promoters. Due to the associated risks of resistant zoonotic bacteria transmission from animals to humans, it is of interest to keep antibiotic use and antibiotic resistance under strict surveillance.

This PhD study was based on the development of real-time PCR (qPCR) assays that supply an easy and rapid method for quantifying antibiotic resistance levels in animal herds. The pig production is accountable for a large portion of the antibiotics used for food producing animals in Denmark. Therefore, the antibiotic resistance genes included in this study had previously been described in association with pig herds, and they encoded resistance to antibiotics used in the Danish pig production.

The **first objective** had emphasis on the qPCR assays' design and development. The goal was to design 10-20 qPCR assays representing different antibiotic classes that ultimately would be tested in a swine herd. A total of 14 assays were developed, representing the following antibiotic classes: Tetracycline (*tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W)), β-lactam (bla_{SHV} family, $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CMY\text{-}2}$), sulphonamide (*sulI*, *sulII*), macrolide, lincosamide, and streptogramin B (*ermB*, *ermF*), and glycopeptide (*vanA*).The glycopeptide *vanA* gene was included as a follow-up to the avoparcin growth promoter ban implemented in Denmark in 1995. Besides the 14 antibiotic resistance gene qPCR assays, a 16S rDNA assay was also included.

Manuscript I was an investigation of the affects PCR conditions had on the diversity and prevalence of antibiotic resistance genes detected in swine manure. This work was carried out in Dr. Zhongtang Yu's laboratory at The Department of Animal Science, The Ohio State University, Columbus, Ohio. At this point of the first objective, decisions were being made concerning qPCR chemistry (probe vs. DNA-binding dye) and mastermix composition. In this study, three cycle numbers and 4 $MgCl_2$ concentrations were evaluated for their effect on the diversity and prevalence of ribosomal protection proteins (RPPs) in a 3 x 4 factorial design. Significant differences in genetic diversity and prevalence of *tet* genes were found amongst the cycle number and $MgCl_2$ combinations, and suggested that 35 PCR cycles and 7 mM $MgCl_2$ enabled optimal detection of RPP genes in swine manure using the Ribo2_new_FW/Ribo2_RV primer pair. The results emphasized the importance of the PCR conditions when performing studies involving *tet* gene prevalence, and when results are interpreted.

Upon completion of the qPCR assay development and optimization the project progressed to the **second objective.** The second objective was to establish if the qPCR assays could quantify antibiotic resistance genes in swine herds by comparing this principle to culture dependent antibiotic resistance detection. In order to do so, fecal samples in a swine herd were collected using different sampling methods that were also pooled at different levels. The antibiotic resistance levels were then determined both by the qPCR assays and coliform colony forming unit (CFU) estimates. Furthermore, the different sampling and pooling methods were evaluated. This established the qPCR assays' capacity to quantify antibiotic resistance genes in a swine herd (*Manuscript II*). In order to compare the qPCR principle of antibiotic resistance quantification, 20 of the individually

sampled animals were randomly selected and analyzed by qPCR, coliform CFU counts, and colony hybridization using probes that correspond to the fragments amplified by the qPCR assays' primers (*Manuscript III*). This study showed that it is important to define which bacterial population is relevant in achieving the specific goal of the antibiotic resistance quantification, and the method chosen for antibiotic resistance quantification has a large influence on the results obtained.

The first and second objectives established that the qPCR assays could be utilized in quantifying antibiotic resistance genes in total DNA extracted from swine feces. It was also confirmed that qPCR and culture dependent antibiotic resistance estimates represent two completely different populations, and cannot be compared directly. Furthermore, pen floor sampling (pooled at stable level or not pooled), shoe cover samples (not pooled), and slurry tank samples were evaluated and are promising sampling methods when determining antibiotic resistance at herd level.

The **third objective** involved the application of the qPCR assays in an animal population that was completely distant from the Danish pig production. Fecal samples from wildlife and Massai cattle in Tanzania were screened for the presence of the 14 antibiotic resistance genes using the qPCR assays. The wildlife and cattle samples were collected in the Ngorongoro Conservational Area (NCA) (wildlife and cattle interaction), and wildlife samples from the Mikumi National Park (MNP) (cattle are prohibited). Antibiotic resistant coliform bacteria estimates were also determined. This study constitutes *Manuscript IV* and the findings were surprising. The antibiotic resistance genes that were found in the cattle were also detected in the wildlife samples, regardless of the sampling site. Eight of the antibiotic resistance genes were detected in the samples, the most prevalent being *tet*(W) and $bla_{\text{CMY-2}}$. Due to the nature of the $bla_{\text{CMY-2}}$ antibiotic resistance spectrum, and the finding of this gene in 10 of 12 screened samples gives rise to concern. However, the finding of the $bla_{\text{CMY-2}}$ gene in the wildlife further substantiates the qPCR assay as this gene was not detected in any of the pig samples collected and described in *Manuscript II*. Nevertheless, further studies should be conducted to study the antibiotic resistance gene pool among the wildlife in northern Tanzania.

In conclusion, the 14 qPCR assays developed here successfully quantified antibiotic resistance in pig herds, where pen floor sampling (pooled at stable level or non-pooled), shoe cover sampling (non-pooled), and slurry tank sampling are promising sampling collection methods. The qPCR assays were also capable of detecting antibiotic resistance genes in Tanzanian wildlife and cattle samples representing a completely different population than the Danish pig production. Also, a gene not detected in the Danish pigs was detected in the Tanzanian wildlife and cattle samples further validating the qPCR assay. Generally, our results indicate that there is a large variation in the antibiotic gene abundance, regardless of animal species or sampling method. Furthermore, the level of antibiotic resistance detected at herd level largely depends on the method used for resistance detection. Additional studies evaluating the sampling methods in several animal herds should be tested in order to assist in understanding the antibiotic resistance gene variation. This study illustrates the immensity of the antibiotic resistance problem and the necessity for systematic surveillance of antibiotic consumption and resistance development at global, national, and local scales.

Resumé (Dansk)

Antibiotika er vigtig i behandlingen af bakterielle infektiøse sygdomme hos mennesker og dyr. Det kan have store konsekvenser som utilstrækkelig behandling og død når infektiøse bakterier bliver resistente imod antibiotika. Siden antibiotika blev opdaget, har brugen af antibiotika været associeret med en parallel selektion for resistente bakterier. Siden de farer som er relateret til udviklingen af antibiotika resistens blev anerkendt, har forsigtig brug af antibiotika været i fokus, især når det angår brugen hos produktionsdyr. I mange år har antibiotika været brugt, og bliver stadigvæk, brugt hensynsløst i produktionsdyr især i form af vækstfremmere. På grund af de associerede risici for smitte med zoonotiske resistente bakterier fra dyr til mennesker, er det af interesse at overvåge antibiotika forbruget og antibiotikaresistens meget nøje.

Dette PhD projekt var baseret på udviklingen af "real-time PCR" (qPCR) assays førende til en let og hurtig metode til at kvantificere niveauet antibiotikaresistens på besætningsniveau. Svineproduktionen står for en stor andel af det antibiotika der anvendes til produktionsdyr i Danmark. Derfor omfatter antibiotikaresistensgener der er inkluderet i dette studie nogle som tidligere har været beskrevet i sammenhæng med svinebesætninger. Disse gener koder for resistens imod antibiotika der bruges i den Danske svineproduktion.

Det første formål i dette studie havde vægt på qPCR assays design og udvikling. Målet var at designe 10-20 qPCR assays, der repræsenterer forskellige antibiotikaklasser, som efterfølgende skulle afprøves i en svinebesætning. I alt 14 assays blev udviklet og repræsenteres af følgende antibiotika klasser: Tetracyklin (*tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W)), β-laktam (bla_{SHV} familie, $bla_{CTX\text{-}M\text{-}1}$ gruppe, $bla_{CMY\text{-}2}$), sulphonamid (*sulI*, *sulII*), macrolid, lincosamid, og streptogramin B (*ermB*, *ermF*), og glykopeptid (*vanA*). Glykopeptid *vanA* genet var inkluderet som en opfølgning på det avoparcin vækstfremmerforbud der blev implementeret i Danmark i 1995. Udover de 14 antibiotika resistensgener qPCR assays, er et 16S rDNA assay også inkluderet.

Manuskript I beskriver en undersøgelse af de effekter PCR opsætningen havde på diversiteten og forekomsten af antibiotikaresistensgener, der blev fundet i svinegylletank. Dette arbejde blev udført i Dr. Zhongtang Yus laboratorium på "The Department of Animal Science, The Ohio State University, Columbus, Ohio". På dette tidspunkt i forløbet var beslutninger omkring qPCR kemiparametre (probe vs. DNA-bindende farve) og mastermix sammensætning ved at blive truffet. I dette studie, blev tre cyklus antal og fire $MgCl_2$ koncentrationer undersøgt for deres påvirkning af diversiteten og forekomsten af "ribosomale protection proteiner" (RPPs) i en 3 x 4 faktorial design. Signifikante forskelle i gendiversitet og forekomsten af *tet* gener blev fundet blandt cyklus antal og $MgCl_2$ kombinationerne. Femogtredve PCR cyklusser og 7 mM $MgCl_2$ tillod optimal detektering af RPP gener i svinegylle ved brug af Ribo2_new FW/Ribo2_RV primer parret. Resultaterne understreger vigtigheden af PCR opsætningen når studier, der involverer *tet* gen forekomsten udføres, og når resultaterne fortolkes.

Efter at qPCR assay udviklingen og optimeringen var afsluttet, fortsatte projektet mod **det andet formål**. Det andet formål var at fastlægge hvorvidt qPCR assaysne kunne kvantificere antibiotikaresistensgener i svinebesætninger ved at sammenligne dette princip med dyrkningsafhængig kvantificering af antibiotikaresistens. Til det brug blev fæces prøver opsamlet i en svinebesætning ved at anvende forskellige prøveopsamlingsmetoder, der også blev poolet på forskellige niveauer. Antibiotikaresistensniveauer blev derefter fastlagt både ved qPCR assays og coliforme "colony forming unit" (CFU) estimater. Desuden, blev de forskellige prøveopsamlings- og poolingsmetoder evalueret. Dette fastslog qPCR assaysnes, evne til at kvantificere

antibiotikaresistensgener i en svinebesætning (*Manuskript II*). For at sammenligne qPCR antibiotikaresistents kvantificerings princippet blev, 20 af enkeltdyrsprøverne tilfældigt udvalgt og analyseret ved qPCR, coliforme CFU estimater og kolonihybridisering. Til kolonihybridisering blev der benyttet prober som modsvarede DNA-fragmenter amplificeret af primerne fra qPCR assaysne (*Manuskript III*). Dette studie har vist vigtigheden i, at definere hvilken bakteriel population der er relevant for at opnå det specifikke mål af antibiotikaresistenskvantificeringen, og at den metode man vælger til at kvantificere antibiotikaresistens har en stor indflydelse på de opnåede resultater.

Det første og andet formål fastslog at qPCR assaysne kunne anvendes til at kvantificere antitiobikaresistensgener i total DNA oprenset fra svinefæces. Det blev også her bekræftet at qPCR estimater af antibiotikaresistensgenniveauer og dyrkningsafhængig kvantificering af antibiotikaresistens repræsenterer to vidt forskellige bakterielle populationer, som ikke kan sammenlignes direkte. Derudover er stibundsprøver (poolet ved sektionsniveau eller ikke poolet), sokkeprøver (ikke poolet), og gylletanksprøver lovende opsamlingsmetoder, når antibiotikaresistens skal bestemmes på besætningsniveau.

Det tredje formål omhandler anvendelsen af qPCR assaysne i en dyrepopulation som var fjern fra den Danske svineproduktion. Fæces prøver fra vildedyr og Massai kvæg i Tanzania blev screenet for tilstedeværelsen af de 14 antibiotika resistensgener ved brug af qPCR assaysne. Fæcesprøverne fra vildedyr og kvæg blev opsamlet i ”Ngorongoro Conservational Area” (NCA), hvor vildedyr og kvæg har kontakt, og der blev opsamlet prøver fra vilde dyr fra Mikumi National Parken (MNP) hvor der er kvæg forbud. Antibiotikaresistente coliforme bakterieestimater blev også fastlagt. Dette studie udgør *Manuskript IV*, og resultaterne var overraskende. De antibiotikaresistensgener der blev påvist i kvæg blev også påvist i vildedyrs prøverne, uanset prøveopsamlingstedet. Otte af de 14 antibiotikaresistensgener blev påvist i prøverne. De hyppigste var *tet*(W) og $bla_{\text{CMY-2}}$. På grund af at både $bla_{\text{CMY-2}}$ antibiotikaresistens spektrumet og at dette gen blev fundet i 10 af de 12 undersøgte fæcesprøver fra vilde dyr, er der grund til bekymring. Dog er fundet af $bla_{\text{CMY-2}}$ genet i prøverne fra vilde dyr en yderligere validering at qPCR assayen, da dette gen ikke blev påvist i nogle af svinefæcesprøverne der blev undersøgt og beskrevet i *Manuskript II*. Omfattende undersøgelser af antibiotikaresistensgenpoolen blandt vildedyr i det nordlige Tanzania bør derfor udføres.

Det konkluderes, at det med de 14 qPCR assays som er udviklet i dette projekt lykkedes at kvantificere antibiotikaresistens i svinebesætninger. Stibundsprøver (poolet på sektionsniveau eller ikke poolet), sokkeprøver (ikke poolet), og gylletanksprøver viser sig som lovende metoder til indsamling af fæces prøver. qPCR assaysne var også i stand til at påvise antibiotikaresistensgener i vildedyr og kvæg fra Tanzania. Disse prøver repræsenterer en anden population end den danske svineproduktion. Ydermere, blev et gen, der ikke blev fundet i de Danske svin fundet i de Tanzaniske vildedyr og kvæg. Dette validerer det tilsvarende qPCR assay yderligere. Generelt indikerer vores resultater, at der er en stor variation i mængden af antibiotikaresistensgener, uanset dyreart eller prøveopsamlingsmetode. Derudover afhænger det detekterede niveau af antibiotikaresistens på besætningsniveau meget af den metode der anvendes til resistensbestemmelse. Yderligere studier der evaluerer prøveopsamlingsmetoder i flere dyrebesætninger bør testes for at bidrage til forståelsen for antibiotikaresistensgenvariationen. Dette studie illustrerer omfanget af antibiotikaresistensproblemet og nødvendigheden for systematisk overvågning af antibiotikaforbrug og resistensudvikling på globalt, nationalt og lokalt plan.

List of abbreviations

β – beta

CFU – colony forming units

CIA – critically important antibiotics

DANMAP - The Danish Integrated Antimicrobial Resistance Monitoring and Research Program

DHFR - dihydrofolate reductase

DHPS - dihydropteroate synthase

$DiBAC_4$ - bis-(1,3-dibutylbarbituric acid) trimethineoxonol

DVFA - Danish Veterinary and Food Association

ESBL- extended spectrum β-lactamase

EU – European Union

FDA- fluorescein diacetate

MBC - minimal bactericidal concentration

MBL – metallo-beta-lactamase

MIC – minimum inhibitory concentration

MLS_B – macrolide, lincosamide, streptogramin B

MLSKO – macrolide, lincosamide, streptogramin B, ketolide, oxazolidinones

NMC-A - nonmetallocarbepenemases

PBB - penicillin binding protein

PCR – polymerase chain reaction

qPCR – real time PCR/quantitative PCR

RPP – ribosomal protection protein

WHO – world health organization

Manuscripts

I. Gunilla Veslemøy Schmidt, Jill Stiverson, Øystein Angen, Zhongtang Yu, 2014. Number of PCR cycles and magnesium chloride concentration affect detection of *tet* genes encoding ribosomal protection proteins in swine manure.

Submitted to *FEMS Microbiology Letters*

II. Gunilla Veslemøy Schmidt, John Elmerdahl Olsen, Lasse Engbo Christiansen, Marie Ståhl, Anders Mellerup, Øystein Angen, 2014. Comparing Antibiotic Resistance Gene and Antibiotic Resistant Bacteria Estimates in Fecal Samples Collected Using Different Sampling and Pooling Methods in a Swine Herd.

Submitted to *Journal of Applied Microbiology*

III. Gunilla Veslemøy Schmidt, Øystein Angen, John Elmerdahl Olsen, 2014. Quantitative measurement of antibiotic resistance in swine feces using qPCR, colony hybridization, and coliform CFU estimates.

Manuscript in preparation

IV. Gunilla Veslemøy Schmidt, Abdul Ahmed Selemani Katakweba, John Elmerdahl Olsen, Øystein Angen, 2014. Eight antibiotic resistance genes including the $bla_{\text{CMY-2}}$ gene detected in wildlife grazing with and without cattle in Tanzania.

Target Journal *Letters in Applied Microbiology*

Introduction

Bacterial resistance towards antibiotics is a global problem as there is an ongoing battle between development of new drugs and subsequent resistance among the target bacteria. Despite attempts in outsmarting the bacteria, e.g. by developing novel drugs with different targets, the bacteria eventually succeed due to mutations leading to antibiotic resistance and consequent survival. Commensal bacteria may become a reservoir of resistance genes for pathogenic bacteria, where antibiotic resistance may consequently spread to zoonotic bacteria such as *Escherichia coli*, *Salmonella* spp., and *Campylobacter* spp. possibly complicating disease and challenging treatment, if humans become infected [1]. Furthermore, resistant bacteria can both cause production and economic loss in food producing animals and negatively affect animal welfare. Surveillance of antibiotic resistance can be a powerful tool in providing information for antibiotic resistance containment and for evaluating the effects of an intervention [2].

In 2012, 112.3 tons of antibiotics were used in animals in Denmark where the pig production was responsible for 76% of the antibiotic consumption [3]. It is therefore of interest to evaluate the risk of antibiotic resistance spread within food producing animal herds, and to find a method that rapidly can give an estimate of the antibiotic resistance at the herd level. Proper detection and surveillance of antibiotic resistance enables precaution when choosing a drug for disease treatment. This makes it possible to help avoid further resistance development and treatment complications thus both increasing animal welfare and decreasing the risk of resistance development in pathogenic bacteria. In this project, swine herds are used as a prototype for antibiotic resistance quantification in animal herds, and the antibiotic resistance genes are included based on the extent of the antibiotic use in the Danish swine industry (For an overview of the included genes see Table S1).

Aim of the PhD study

The aim of this thesis is to develop real-time PCR (qPCR) assays that quantify antibiotic resistance genes ultimately functioning as a quantitative measure for determining antibiotic resistance at herd level. The qPCR assays will be compared to traditional methods for determining antibiotic resistance including cultivation of coliform indicator bacteria on selective and indicative media.

The qPCR assays may be used in attaining a rapid and inexpensive documentation of antibiotic resistance levels within animal herds, enabling precise recommendations concerning the use of antibiotics in food producing animals. The present thesis focuses on the development and validation of the qPCR assays and the proof-of-concept for antibiotic resistance level quantification in swine herds using qPCR.

The project was divided into three main objectives:

- To develop and optimize qPCR assays that can be used to quantify antibiotic resistance genes in a swine herd.
- To establish if the qPCR assays could quantify antibiotic resistance genes in swine herds by comparing this principle to culture dependent antibiotic resistance detection.
- To apply the qPCR assays in another animal population than swine herds.

This thesis contains a chapter providing a general insight in detection and surveillance of antibiotic resistance, antibiotic usage and monitoring in Denmark, and a brief description of the pig production types in Denmark (Chapter 1). The next section (Chapter 2) describes the mechanisms of antibiotic resistance for tetracyclines, β-lactams, sulphonamides, glycopeptide, and macrolide, lincosamide, and streptogramin B antibiotics which are those included in the PhD study. Finally (Chapter 3) the research activities are provided and the main results and conclusions of this PhD study are described and discussed, and future perspectives are summarized.

1 CHAPTER 1 – Background

1.1 Culture dependent detection of antibiotic resistance in animal herds

The present methods for quantifying the degree of antibiotic resistance within animal herds include culture of indicator bacteria that are present in all healthy animals, e.g. *Escherichia coli*, and *Enterococcus faecalis*, and characterization of these isolates with respect to antibiotic susceptibility [1,4]. Fecal samples are attained from a random sample of animals, cultured on selective and indicative media and susceptibility is determined as Minimum Inhibitory Concentration (MIC) value of different antibiotics, or by disk diffusion test (Bauer-Kirby Procedure) [5]. Alternatively, the amount of colonies of a given bacterial species that grow at a given concentration of antibiotic can be counted giving the number of colony forming resistant units (CFU) per gram feces. Multiple plates can be used to enable multiple drug testing.

Colony hybridization is a growth dependent genotypic method that enables detection of antibiotic resistance determinants both in phenotypically resistant bacteria and bacteria not exhibiting phenotypic resistance [6]. In principle, the collected samples are homogenized, diluted, and spread on indicative and selective plates each containing an antibiotic of interest. The bacterial colonies that grow are then replica plated onto a filter where they are lysed with subsequent DNA fixation to the membrane. The resulting bacteria colony DNA-prints are then hybridized to a labeled probe complimentary to the antibiotic resistance DNA sequence of interest. The results of the hybridization are then analyzed by autoradiography [7].

These methods only enable investigation of the cultivable fraction of the gut microflora which is further narrowed down to the denoted indicator species. This may introduce bias by underestimating the antibiotic resistance levels in the true bacterial population and consequently the animal herd [8]. The relative numbers of resistant bacteria ((number resistant bacteria/total number of bacteria (both resistant and susceptible)) can also change due to changes in the number of resistant bacteria and/or changes in the number of total bacteria [8].

1.2 Culture independent detection of antibiotic resistance in animal herds

Due to the cultivable limitations and potential interpretation errors involved in phenotypic antibiotic resistance determination, alternative DNA-based methods are increasingly in focus [9]. The advantage of these alternative methods is the capability of detecting antibiotic resistance in slow-growing or non-cultivable bacteria reflecting the community's entire gene pool [8].

1.2.1 Real-time polymerase chain reaction (qPCR)

Polymerase chain reaction amplification (PCR) duplicates the amount of DNA after each round of amplification where both products and negative and positive controls can be visualized after agarose gel electrophoresis [10,11]. In real-time PCR (qPCR) the amount of DNA is measured after each cycle of amplification by means of a fluorescent marker so gel electrophoresis is not necessary. The increase of fluorescent signal is directly proportional to the number of PCR product amplicons generated in each PCR cycle. The use of standard curves enables calculation of the initial quantity of genetic material. There are two types of qPCR principles. The first principle entails DNA amplification by primers that are complementary to the DNA target, thereafter a fluorescent

dye binds to all double stranded DNA products and fluorescence can be detected. Here, it is necessary to check for primer dimers and contamination by means of a melting curve analysis as the fluorescent dye binds all double-stranded DNA.

The second principle utilizes a fluorescent probe, which is complementary to the target DNA amplified by the primers. The probe binds specifically to the target DNA and once the sequence is amplified by the primers and DNA polymerase the fluorescent marker is released actively emitting fluorescence. The probe chemistry is more specific than the fluorescent dye as there are three specific DNA sequences (two primers and one probe) that must complementarily bind to the target DNA in order for fluorescence to be emitted and detected.

qPCR has shown useful in quantification of antibiotic resistance genes in fecal samples and fecal contaminated environments [11-13] and is the selected culture independent method in this study. Due to the high similarity between several of the genes, probe based chemistry was used in order to heighten the specificity. Ultimately, qPCR may become an alternative e tool in objectively measuring the degree of resistance development in the gut microflora and certifying the presence or absence of specific antibiotic resistance genes at herd level. While qPCR quantification of DNA entails information on the presence and quantity of certain genes, using cDNA may provide further information giving an impression of gene activity.

1.2.2 Metagenomics

Metagenomics is the study of the genetic material of bacterial populations that is directly isolated from their natural environment circumventing both isolation and culture of individual bacterial species [14]. DNA sequencing techniques such as “sequence-based metagenomics” enables detection and characterization of both known and unknown antibiotic resistance genes [15-17]. In sequence-based metagenomics DNA is directly extracted and randomly sequenced from e.g. feces [18]. The randomly sequenced DNA is assumed to represent a fraction of the sample’s entire bacterial community. Resistance genes and/or mutations that are known to lead to resistance are subsequently identified by comparing the metagenomic sequences to known reference sequences found in available databases [18].

A recent study used the sequenced-based metagenomic approach to track changes in microbial membership and encoded functions within swine intestinal microbiomes [19]. The metagenomes revealed that the swine harbored several antibiotic resistance genes in the absence of selective pressure. However, antibiotic administrations lead to a clear increase in resistance gene abundances, which became homogenous over time [19]. So-called “collateral” effects of antibiotic treatment e.g. increases in *E.coli* populations, and genes encoding functions that may be involved in enhanced stability and spread of resistance genes within a microbial community, were also demonstrated [19].

Functional metagenomics entails total DNA extraction from an e.g. fecal sample. After DNA extraction, a smaller DNA fragment between 1-3 kilobases is cloned into an indicator bacteria strain, such as *E.coli*, that is susceptible to antibiotics. The successfully transformed bacteria harbor the cloned DNA fragment and are consequently resistant to antibiotics. They will therefore grow on the antibiotic-containing agar plates. The resistant colonies are then selected and their DNA inserts are amplified and sequenced in order to identify the antibiotic resistance genes [15-17]. This method enables rapid insight in novel resistance gene development as well as an understanding of the general resistance gene profile of a bacterial community.

1.2.3 Flow cytometry

Flow cytometry can be utilized to count, detect or sort individual fluorescent labeled cells passing in a stream through a laser beam. Individual cells can be marked by pre-treatment with specific monoclonal antibodies labeled with fluorescent dyes, or they can be incubated with different types of dyes that are fluorescent e.g. after intracellular metabolization. This can be utilized in antibiotic susceptibility testing. In principle, bacteria that are grown in enrichment broth can then be incubated in broths containing a panel of test antibiotics. At the end of the antibiotic incubation period a fluorescent dye can then be added followed by flow cytometric analysis.

In one study, fluorescein diacetate (FDA) (Sigma) was utilized in antibiotic susceptibility testing of *Mycoplasma tuberculosis* [20]. FDA can freely diffuse across cell walls and membranes of *M. tuberculosis* where it is hydrolyzed to free fluorescein. Inactive and nonviable bacteria will have a decreased metabolism resulting in less FDA hydrolyzation and less fluorescein than viable cells when detected by flow cytometry. A determination of susceptibility can thus be made when comparing the fluorescence from control bacteria incubated in broth only to bacteria incubated in a panel of test antibiotics. The same principle was also used to test the antibiotic susceptibility in 67 bacterial strains [21]. Here, another dye was used, namely bis-(1,3-dibutylbarbituric acid) trimethineoxonol (DiBAC4) (Invitrogen/Life technologies). DiBAC4 accumulates within the cytoplasm of depolarized bacteria binding intracellular proteins or membranes resulting in an increase in fluorescence compared to viable bacteria [21]. The advantage with flow cytometry is that it is rapid and besides testing standard susceptibility it is also possible to investigate morphological changes in individual bacterial cells or surface protein expression [21].

1.3 Surveillance of antimicrobial resistance

1.3.1 Reasons for surveillance

Exposure of a bacterial population to antibiotics increases the potential risk for a selection favoring bacteria with antibiotic resistance genes. If these bacteria cause disease, treatment can be compromised leading to increased mortality and morbidity with economic consequences associated with increased care, diagnostic-, and treatment- costs [22,23]. Preventing and controlling infections caused by antibiotic resistant bacteria can be facilitated by monitoring not only the distribution of the actual infection, but also the antibiotic resistance patterns. Together, this information may support control of disease and prevent further emergence of resistance [23]. Surveillance of antibiotic resistance and use can be beneficial in providing the necessary information for antibiotic resistance containment by assisting in the choice of antibiotics and treatment regimes. Furthermore, long term surveillance is important when evaluating the effects of an intervention [2].

1.3.1.1 Surveillance at national level

Surveillance of antibiotic resistance at national level can facilitate in prioritizing during epidemiological implementations concerning diseases caused by antibiotic resistant bacteria and can assist in containing antibiotic resistance development. A national surveillance plan involves a

national reference laboratory with close epidemiological collaboration [23]. National surveillance is based on monitoring antibiotic resistance in chosen indicator bacteria and pathogenic bacteria isolated from clinical specimens that are relevant for the particular country [23]. Indicator bacteria, veterinary and/or human pathogens, and zoonotic bacteria are systematically isolated from food producing animal samples such as cattle, pigs, and poultry [2,3]. The indicator bacteria samples are taken from the intestines at the slaughter house, for example one sample per group of animals coming at slaughter [2]. The number of strains to collect depends on the resistance prevalence, but minimum 60 isolates of each bacterial species and preferably up to 250 isolates should be collected from each food producing animal type [2]. The veterinary/human pathogenic bacteria samples are included from routine testing in diagnostic laboratories. This data can be included in the national surveillance given that the laboratory methods used for bacterial identification and susceptibility testing are standardized [2]. The samples used for surveillance of antibiotic resistance in zoonotic bacteria are collected at primary production sites i.e. herds and flocks, where the survey is focused on the 5 most frequently isolated serotypes from human infections within the given country [2]. Generally, it is important that the data on antibiotic resistance is consistent in quality where the methods used should be based on standardized protocols both at national and local levels. Optimally, national antibiotic resistance programs in different countries should be harmonized in order to facilitate international surveillance [2]. The data collected is summarized in a report and is presented as percentages of resistant, intermediate or sensitive strains along with the quantitative data from inhibition zones or minimum inhibitory concentration (MIC) values [2].

1.3.1.2 Surveillance at herd level

Local antibiotic resistance surveillance is essential as individual herds may not have the same antibiotic resistance problems. The data obtained from food producing herds can be used in the practicing veterinarians' decision making concerning both recommendations for optimal antibiotic therapy and for management of current resistant problems [24,25] .

1.3.3 Detection of antibiotic resistance development in individual animals

When assessing the development of antibiotic resistance in individual animals it is common to monitor the phenotypic resistance development in chosen indicator and/or pathogenic bacteria, depending on the purpose of the study [26-28]. Samples may be collected from e.g. saliva, skin, nasal mucosal membranes or feces prior to, during, and after the antibiotic treatment period. This increases the likelihood of gaining sufficient knowledge of the antibiotic resistance status before, during, and after treatment.

1.4 Sampling methods for antibiotic resistance determination

Estimating herd antibiotic resistance levels may facilitate in suitable choice of drug for disease treatment ultimately minimizing resistance development and treatment complications. The choice of sampling method is crucial because it can introduce bias but is often compromised due to cost and time limits. Optimally, sampling should be simple and efficient yet yield accurate and reproducible results representative of the target population. Several studies have demonstrated the relevance of

the sampling method's effect on the outcome [29-31]. Theoretically, when investigating a factor at herd level sampling each individual animal within the herd is ideal. In practice this is strenuous and time consuming. Due to the cost of collecting, transporting, storing, and analyzing the amount of samples attained when all individuals within a herd are sampled it is common to collect fewer defined composite samples. Pooled samples are a more cost-efficient alternative, especially when testing for low prevalence diseases [32]. However, pooled samples also have their disadvantages that must be taken into consideration when defining the amount and type of samples that are to be included in each pool. It is important to be aware of false negative tests, for example if an antibiotic resistance gene has a low prevalence and there are sufficient numbers of negative samples included in the pool then the concentration of the gene is diluted potentially resulting in levels under the specific assay's detection limit. The sensitivity is therefore dependent on the prevalence of the gene, the number of samples per pool, the concentration of the gene in samples from positive animals, and the detection limit of the assay [32]. There can also be false positive test results due to cross-contamination during the sample collection, handling or processing [32]. It is therefore important to test and optimize the specific sampling strategy for the determination of herd antibiotic resistance.

In the present study, different sampling methods were evaluated for antibiotic resistance detection at pen, stable, and herd levels (shoe cover sampling, pen floor sampling, laboratory pooling at different levels, and slurry tank sampling). The samples were collected at two separate time points four months apart (sampling 1 and sampling 2). During sampling 1, a single stable in a pig herd was sampled in order to establish an understanding of the antibiotic resistance gene levels in the individual animals, pens, and stable. The samples from sampling 1 were individual animal samples, pen floor samples, and shoe cover samples (Figure 1). Individual animal sampling was the "gold standard" to which the other sampling methods were associated.

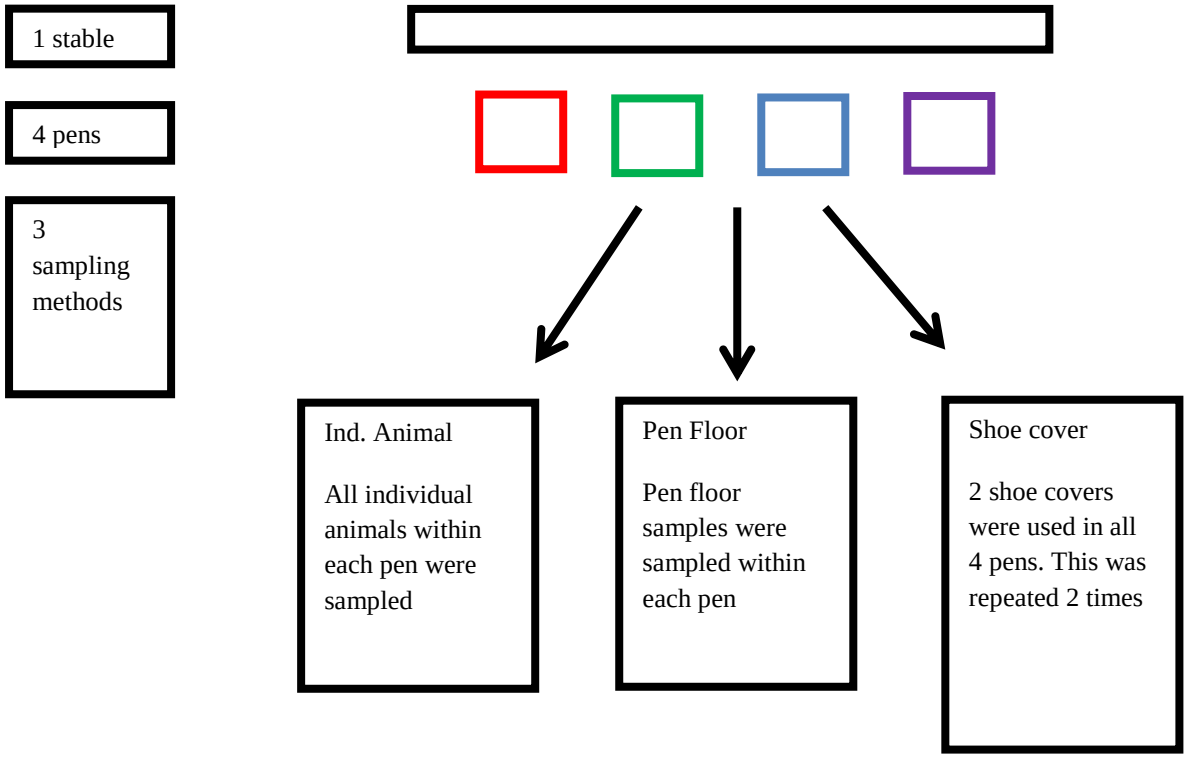

Figure 1 Overview of sampling 1. Three sampling methods were used, Individual animal sampling (Ind. Animal), pen floor sampling, and shoe cover sampling. Four pens in 1 stable were sampled.

During sampling 2, all stables were sampled in the same pig herd as sampling 1 in order to gain an understanding of the antibiotic resistance gene levels in the stables and the herd. The samples from sampling 2 include pen floor samples, shoe cover samples, and slurry tank samples (Figure 2).

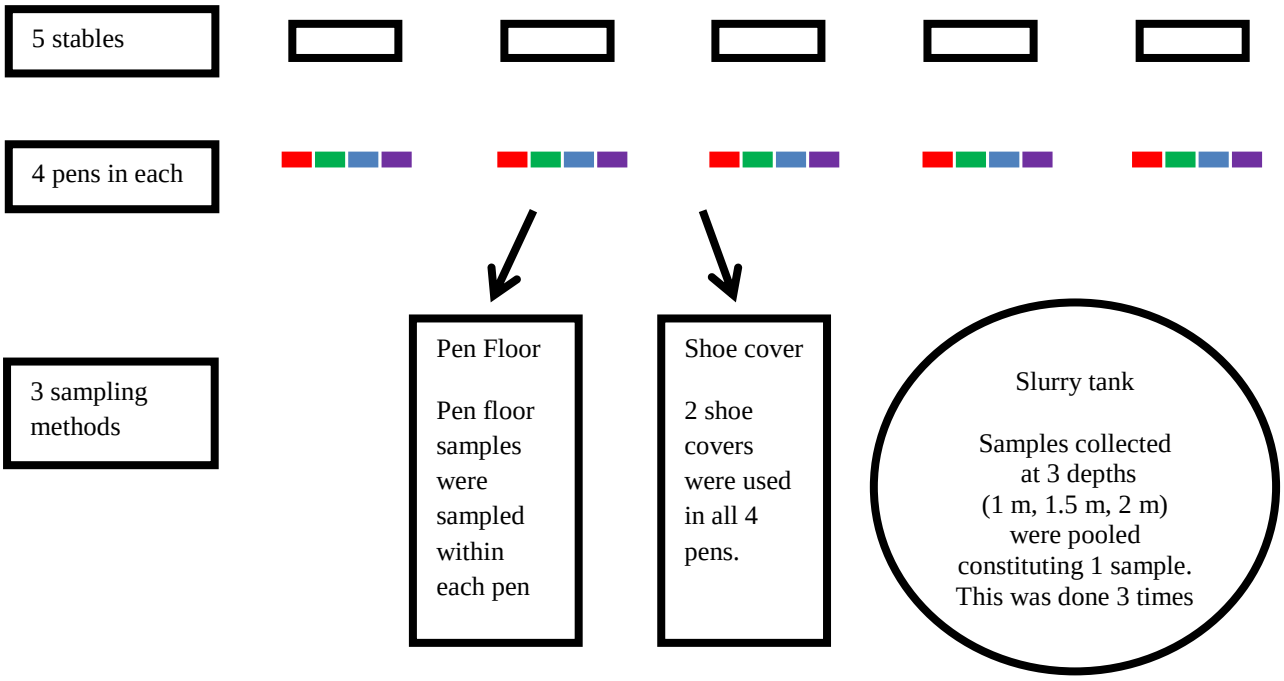

Figure 2 Overview of sampling 2. Three sampling methods were used, pen floor sampling, shoe cover sampling, and slurry tank sampling. Four pens were sampled per stable, and five stables were sampled.

1.4.1 Shoe cover sampling

In Denmark, shoe cover sampling (also called sock sampling) is used in the national *Salmonella* control program in broiler flocks. Shoe cover sampling is simple and literally entails pulling a sock over a shoe and collecting samples. The samples are collected by walking about in the area to be sampled until as much area is covered as possible. The number or sock samples to collect can vary. This method is straight forward, cheap and has been shown to be feasible in broiler flocks [31].

1.4.2 Pen floor sampling

Pen floor sampling is currently used in the Danish *Salmonella* control program in pig herds [33,34] and entails collecting 5 different fecal samples of about 5 g each within the same pen. The 5 samples are then mixed in a single plastic container that is sealed with a tight lid. Theoretically, each of the 5 subsamples that are collected come from 5 different pigs. The number of pens sampled can be varied.

1.4.3 Laboratory pooling

Laboratory pooling of samples enables collection of the desired amount of samples that are thereafter composited in the laboratory thus reducing the number of necessary analyses. Often, a 1:10 dilution of each sample is made to facilitate subsequent handling and thereafter the desired amount of each 1:10 dilution is taken from each sample and then mixed. Samples that were pooled and tested in the present study included those from individual animals, pen floor samples, and shoe cover samples all of which were pooled to infer resistance levels at stable and/or herd level (Table 1).

Table 1 Overview of laboratory pools at pen, stable, and herd levels

Pool name	Samples included in laboratory pool	Number of samples (n)
All animals	All individual animals from sampling 1 (digital extraction from rectum)	n=84
Individual animal pool pen	Pool of individual animals within each pen (digital extraction from rectum)	Pen 1 n=22 Pen 2 n=20 Pen 3 n=22 Pen 4 n=20
Pen floor samples	Not pooled	Sampling 1 n=4 Sampling 2 n=20
Pen floor pool stable	Pool of pen floor samples from pens 1-4 in each stable	Sampling 1 n=1 Sampling 2 n=5
Pen floor pool herd	Pool of pen floor samples from each stable (1-5)	Sampling 2 n=1
Shoe cover samples	Not pooled	Sampling 1 n=4 Sampling 2 n=10
Shoe cover pool stable	Pool of the shoe cover samples in each stable	Sampling 1 n=1 Sampling 2 n=5
Shoe cover pool herd	Pool of shoe cover samples from each stable (1-5)	Sampling 2 n=1
Slurry tank samples	Each sample was a pool from 3 depths collected at the same spot (1m, 1.5m, and 2m)	n=3
Pool slurry	Pool of slurry tank samples 1-3	n=1

1.4.4 Slurry tank sampling

Slurry tank sampling is also interesting as the slurry itself may represent a pool of the entire herd, potentially representing the antibiotic resistance levels for the past six months as they are often emptied twice a year. It has been demonstrated that levels of tetracycline and erythromycine resistance genes in slurry tanks from swine farms are not reduced over time [12,13]. Slurry tank samples were also collected in the present study, and compared to the herd pools of the shoe cover and pen floor samples.

1.5 Antibiotic usage and monitoring in Denmark

Denmark is known for pursuing initiatives that minimize antibiotic use in order to reduce resistance development and spread through the food chain. For example, in 1995 there was a national ban against the use of the antimicrobial growth promoter avoparcin and veterinarians were prohibited from profiting off the direct sale of drugs [35,36]. In 1998, Danish pig and poultry producers voluntarily ceased use of all antibiotic growth promoters in finisher pigs and broiler chickens. Since year 2000, no antibiotics have been used for growth promotion [35,36]. Danish interventions will be covered in the following sections, including the veterinary advisory service contract; the yellow card initiative; flouroquinolone and cephalosporin use; the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP).

1.5.1 Veterinary advisory service contract

Since July 2010, farmers housing more than either 300 sows, gilts or boars in stables, 3000 feeder pigs or 6000 piglets[37] are obligated to have a veterinary advisory service contract where the veterinarian must visit the farm minimum 12 times yearly [38]. The intentions of this contract entail improving animal welfare, reducing the risk of infectious disease, and optimizing antibiotic usage in order to minimize resistance development [37]. When there is a veterinary advisory service contract, the veterinarian can prescribe antibiotics for up to 35 days of treatment for pigs less than one year of age [37]. The criteria are that, the veterinarian must find it necessary to continue treatment during a control visit and the reason is described, plus there must be a mutual agreement between the veterinarian and farm owner about the revised treatment plan. The attained information must be recorded and registered.

Typically, a veterinarian issues a prescription and the farmer collects the drug at the pharmacy. The pharmacy then registers the drug sale, including the information from the prescription, to an official register Vetstat. In Vetstat, 98% of the antibiotics prescribed to pigs are from pharmacy registrations. The veterinarians only report to Vetstat directly when they have treated animals during a herd visit or if the veterinarian has distributed the drug personally. The information that the veterinarian is obligated to record is the veterinarian responsible for the treatment (including authorization number), the person responsible for the herd (name and address), the prescribed drug (amount, dose, administration, treatment period, withdrawal period and indication), diagnosis, date for drug use, prescription or distribution, and identification of the herd (CHR number) and species to be treated (which animals and how many) [37,39] . Likewise, the farmer must also register the animals that are treated, the drug used, date and reason for treatment, dose, administration route,

person responsible, and origin of the drug (if not received by a veterinarian directly) [37,40]. These registrations must be kept for up to 5 years.

Any drugs that must be administered by intravenous injection (IV) or fluoroquinolones for injection can only be administered by the veterinarian, and it is strictly prohibited to prophylactically treat with antibiotics.

1.5.2 The yellow card initiative

About 76% of the antibiotics prescribed in Denmark can be ascribed to the pig industry with a 35% increase between 2001 and 2009 [3,41]. Therefore, in July 2010, the Danish Veterinary and Food Association (DVFA) enforced the yellow card initiative which has the following three ranks: yellow card, increased supervision, and red card (Figure 3). The initiative is based on threshold levels for antibiotic consumption calculated as an average for the preceding 9 months in weaners (7-30 kg), finishers (30 kg – slaughter), and finally sows and piglets (including gilts, and boars).

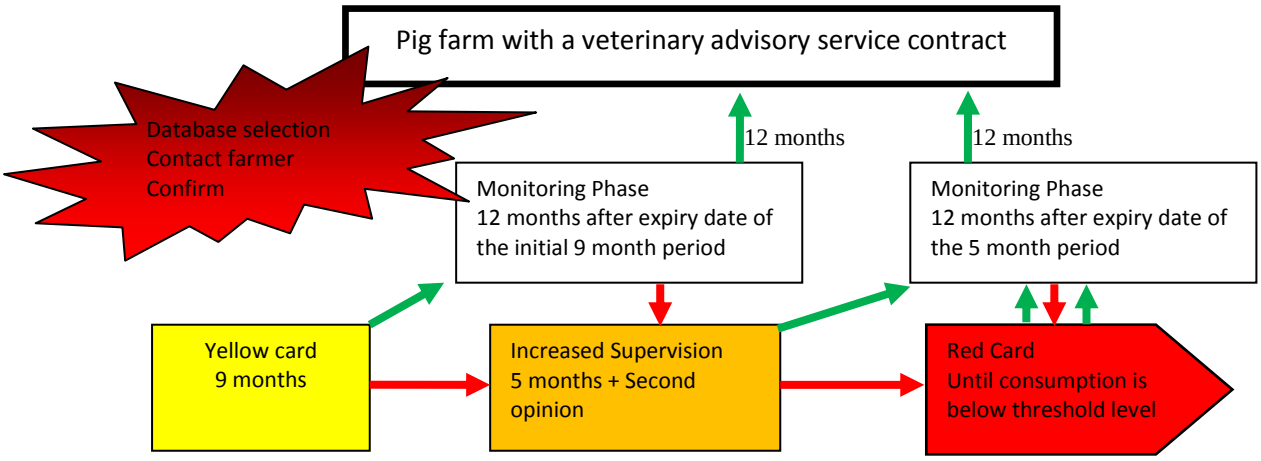

Figure 3 The yellow card initiative. The green arrows indicate the flow when antibiotic levels are below threshold, and the red arrows indicate when the antibiotic levels are above threshold. Modified from www.foedevarestyrelsen.dk [41]

A yellow card is issued if the average antibiotic consumption exceeds the given threshold for one or more of the age groups within a nine month period [41]. Consequently, the farm owner has nine months to reduce the antibiotic consumption below the threshold. The DVFA can also forbid any use or storage of any antibiotics at the farm during these months if the specific antibiotic has been prescribed more than once and is to be administered via feed or drinking water. Finally, the DVFA may carry out up to several unannounced inspection visits during the time period the issue is in effect.

The increased supervision stage is reached if the farmer has not managed to decrease the antibiotic consumption levels below the set threshold by the end of the yellow card nine month period [41]. The increased supervision lasts five months, and enables the DVFA to implement third party veterinary advice to the farmer on how to reduce the antibiotic consumption levels within a subsequent nine month period. Increased supervision can also be issued if the antibiotic

consumption that has been reduced to below the set threshold within the first nine month period crosses the set threshold again within 12 months after the expiration of the first nine month yellow card period. During increased supervision a concrete action plan including initiatives to decrease antibiotic consumption must be made.

The red card stage is reached when the farmer does not manage to reduce antibiotic consumption levels to below the set threshold within a five month period following the second issuing's nine month period [41]. The farmer must then implement the action plan initiatives, or as a last resort, reduce the stocking density consequently reducing the antibiotic consumption. The yellow card stage prohibitions regarding antibiotic storage and administration also apply to the increased supervision and red card stages.

1.5.3 Flouroquinolones and Cephalosporins

For the first time in 2007, the World Health Organization (WHO) defined several antibiotics that are important for treating certain infections in humans, namely the critical important antibiotics (CIA). Among the CIAs listed in 2011 are fluoroquinolones and cephalosporins [42].

The veterinary use of fluoroquinolones in Denmark is kept at a minimum due to special guidelines. Flouroquinolones may be used, handed out or prescribed for production animals for a maximum of 5 days and only if a laboratory can confirm that the disease causing agent is resistant to all other permitted antibiotics. If there is an acute onset of a disease that demands fluoroquinolone use, the treatment can be initiated before the laboratory results are known. However, if the laboratory results show that the disease causing agent is sensitive to any other permitted antibiotics, then another drug must be used. The Regional Veterinary Officer must be informed about any use of fluoroquinolones within production animals within two weeks following treatment termination [37].

In June 2010, the Danish Meat Association announced a voluntary two year discontinuation of cephalosporin antibiotics. This resulted in a 50% reduction in cephalosporin consumption between 2009 and 2010 [43].

1.5.4 The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP)

The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP) is a close collaboration between veterinary, food and human health institutes and was established in 1995. The objectives are to monitor the consumption of antimicrobials in humans and animals; the occurrence of antimicrobial resistance in bacteria isolated from food animals, food of animal origin and humans; to study associations between antimicrobial consumption and resistance development and to identify antimicrobial resistance transmission routes and initiate joint research [3,44]. The DANMAP report is published annually describing current national and regional trends in antimicrobial use and resistance occurrence.

The pig samples used in DANMAP 2012 were collected from slaughterhouses that accounted for 98% of the total number of pigs slaughtered in Denmark during 2012 [44]. The samples were caecum samples and the number collected from each slaughterhouse was proportional to the number of pigs annually slaughtered at the specific slaughter house. The isolated bacteria included: *Escherichia coli*, *Enterococcus faecium*, *Enterococcus faecalis*, *Campylobacter coli*, *Campylobacter jejuni* and

Salmonella spp. where one isolate per farm of each species was included in the DANMAP 2012 report [3]. The bacterial isolates from the pigs may be regarded as representing a stratified random sample of 98% of the Danish pig population where the prevalence of antimicrobial resistance in the bacterial isolates represents an estimate of the true occurrence of resistance in the population.

After sampling, the bacteria are isolated and identified using selective and indicative media. The bacterial isolates are then susceptibility tested (one isolate per bacterial species per farm) using the minimum inhibitory concentration (MIC). Multi-resistance is defined as resistance to three or more of the antimicrobial classes included in DANMAP 2012 and isolates fully sensitive if susceptible to all the antimicrobial agents in the test panel [3].

1.6 Pig production in Denmark

Globally, Denmark is one of the largest pig meat exporters producing approximately 29 million pigs on about 4100 farms in 2012 [45,46]. About 90% of the pigs are used for export emphasizing the significance of the pig production to the national economy [47].

1.6.1 Pig production types in Denmark

There are different pig production types, namely conventional, organic, free range, or special-pig production. The main differences between the four reside within the housing system, general management practices including feeding and weaning, and rules concerning the use of medicine.

1.6.1.1 Conventional pig production

The conventional pig production type is the most common in Denmark where the production must occur according to the Danish legislation regarding housing of pigs. All pigs must have access to roughage such as straw and that roughage or activation material must be available in the appropriate amounts [48,49]. Conventionally produced pigs are normally weaned at four weeks of age, and can be weaned up to seven days earlier if they are moved to special stables that are emptied, cleaned, and disinfected before a new group of pigs is introduced and that are separated from stables that house sows [50]. Generally, the space demands for the conventional pig production are less in comparison to the organic and free range production types.

1.6.1.2 Organic pig production

The organic pig production sows live in huts on pasture. The piglets are born on pasture and weaned at seven weeks of age where they are moved into housing [51]. The pigs are housed in stables with an outdoor yard and free access to roughage ad libitum. There are strict rules concerning organic feed and the use of antibiotics and other medicine [47]. Until December 31, 2014 it is allowed to use non-organic protein feed only if it is not possible to attain strictly organic feed. The amount is 5% per animal per calendar year and an obligatory food journal ensures that the limit is not exceeded [52].

After use of medicine, the required withdrawal period is twice as long as the minimum for conventional production [52,53]. Slaughter pigs receiving antibiotic treatment more than once lose their organic status where both the prescription and treatment with antibiotics must be for the diseased animals only [52]. Additionally, tail-amputation and tooth cutting/filing is prohibited.

1.6.1.3 Free range pig production

Free range pigs are born in huts on pasture and are weaned and moved to indoor housing at five weeks of age [54]. The stables are open with access to plenty of straw and outdoor areas at all times. It is prohibited to amputate tails and cut/file teeth. The withdrawal time after medicine administration is twice that for conventional production.

1.6.1.4 Special-pig production

This production type entails pigs that are specially produced for the Danish market and for export such as Antonius, “Bornholmergrisen”, “Den Go’e gris”, and “Grambogårdsgrisen”. These pigs are not organic or free range, but their production has several exceptions compared to the conventional production type.

2 CHAPTER 2 – Antibiotic resistance

2.1 Mechanisms of antibiotic resistance and spread

Bacterial resistance towards antibiotics can be caused by several mechanisms [9]:

(i) Production of an enzyme that can inactivate the antibiotic

(ii) The production of an enzyme that functions as an alternative to the antibiotic target

(iii) Mutation(s) in the antibiotic target that lead to a reduction in the antibiotic binding

(iv) Reduced uptake of the antibiotic

(v) Active efflux of the antibiotic

(vi) Overproduction of the antibiotic target

(vii) Mechanisms that are not yet recognized

Not only are there plenty of mechanisms the bacteria utilize in circumventing the effects of antibiotics, but once developed, the resistance can spread horizontally to other bacteria [55].

Tetracycline, β-lactam, sulphonamide, macrolide, lincosamide, and streptogramin B (MLS_B), and glycopeptide drugs and the respective bacterial resistance mechanisms will be discussed. The tetracycline, β-lactam, sulphonamide, and MLS_B antibiotics were included as they are used in the swine production in Denmark [3,56]. The antibiotic classes of choice for different indications in Danish sows/piglets, weaner pigs, and finisher pigs in 2008 are illustrated in Figure 4 [56]. The glycopeptide is included because avoparcin was used as a growth promoter in the pig industry, and has been banned since 1995. Therefore glycopeptide resistance surveillance may be of special interest as the levels are expected to be low. The above mentioned antibiotics also are included because of the feasibility of developing qPCR assays for the specific antibiotic resistance genes.

The genes included in this study are summarized in Table S1 (Appendix I) along with their phenotypic resistance, resistance mechanism, mobility, gene linkage, “other”, and references. Furthermore, a summary of the antibiotic resistance genes and the bacteria in which they have been described is also included (Table S2 Gram positive bacteria, Table S3 Gram negative bacteria (Appendix I)).

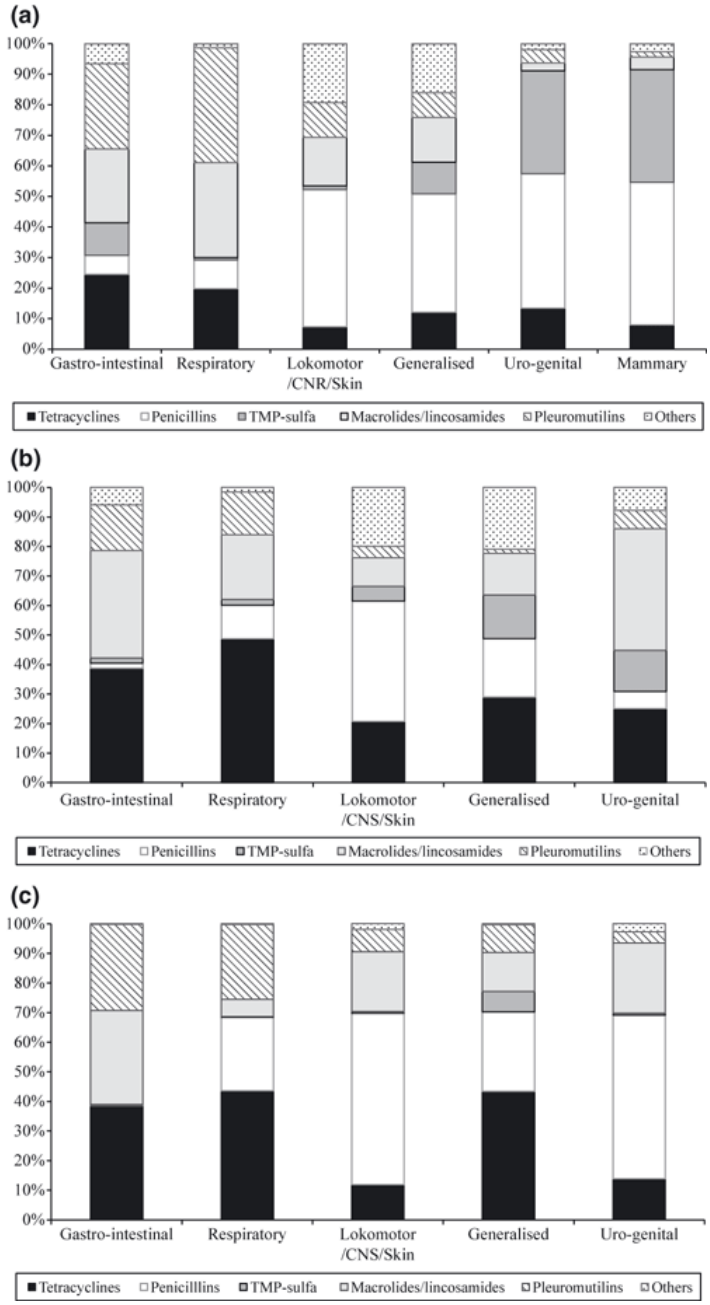

Figure 4 From Jensen *et al.* (2011) The antibiotic classes of choice for different indications in Danish sow/piglets (a), weaner pigs (b), and finisher pigs (c) in 2008. The indication is given as an organ system treated [56].

2.2 Resistance to tetracyclines

2.2.1 Tetracycline drugs and mode of action

In 1948, chlortetracycline was described from *Streptomyces aureofaciens* being the first discovered tetracycline antibiotic [57] . Since the discovery of chlortetracycline several other tetracycline antibiotics have been discovered in *Streptomyces* species and even more semi-synthetically produced. The tetracycline antibiotics can be grouped into three generations based on when they were developed, namely first generation (1948-1963), second generation (1965-1972), and third generation (1993) (Table 2).

Tetracycline antibiotic's working mechanism is binding to the bacterial 30S ribosomal subunit consequently disrupting protein synthesis. The tetracycline resistance genes either code for efflux proteins, ribosomal protection proteins (RPP), inactivating enzymes, or have unknown mechanisms [57]. Usually, tetracycline resistance in bacteria arises through acquisition of resistance genes by means of conjugative plasmids or transposons [58]. The tetracycline resistance genes are often associated with other antibiotic resistance genes, especially *erm* genes that confer resistance towards MLS_B [59]. Generally, tetracycline resistance genes are considered unique if there is ≤ 79% similar amino acid identity to other genes. There are currently 45 known tetracycline resistance genes not including recently described mosaic genes such as *tet*(O/W) and *tet*(W/O/W) [60-62]. The different resistance types will be discussed, except for the unknown mechanism, which is due to *tet*(U) [63]. An updated overview of the tetracycline resistance genes can be found at http://faculty.washington.edu/marilynr/ [62].

Table 2 Overview of the principle members of tetracycline antibiotic class, modified from Chopra *et al.* (2001)[57]

Generation	Naturally occurring	Semi-synthetic	Action and spectrum bacteriostatic
1 (1948-1963)	Tetracycline (humans)	Lymecycline (humans)	Broad spectrum:
	Chlortetracycline (animals)	Rolitetracycline	Gram positive bacteria
	Oxytetracycline (animals and humans)		Gram negative bacteria
	Demeclocycline		Chlamydia
			Rickettsia
2 (1965-1972)		Methacycline	Mycoplasma
		Minocycline	Protozoa
		Doxycycline (animals and humans)	
3 (1993)		Glycylcycline (humans)	

2.2.2 Tetracycline resistance mechanisms

2.2.2.1 Drug efflux

The *tet* efflux genes code for proteins that actively pump tetracycline out of the cell and generally confer resistance to tetracycline, but not minocycline or glycylcyclines [57]. There are exceptions, however, including *tet*(K) and *tet*(L) that encode proteins mainly associated with resistance to tetracycline and chlortetracycline [57] and *tet*(B) that confers resistance to tetracycline and minocycline but not glycylcyclines. There are 29 known efflux resistance genes which are all designated *tet* genes except for two *otr* genes and a single *tcr* gene [62]. The tetracycline efflux *tet* genes are widely dispersed and have been described in various Gram negative, Gram positive, aerobic and anaerobic bacteria (Table S1, Table S2) [63]. The *tet* efflux genes have a broad distribution where generally the Gram negative *tet* efflux genes are found on transposons inserted into plasmids or integrons often associated with other antibiotic resistance genes, heavy metal resistance genes, and/or pathogenic factors [57,64]. The Gram positive *tet* efflux genes are associated with small plasmids [64].

2.2.2.2 Ribosomal protection proteins (RPP)

The ribosomal protection proteins (RPP) are proteins that protect the ribosomes from the action of tetracycline conferring resistance to doxycycline and minocycline [57]. There are currently 12 known RPP genes [62]. The *tet*(M) gene was originally found in *Streptococcus* [65] and is found in both Gram positive and Gram negative bacteria, also having the widest host range of all *tet* genes [13,58,63]. Furthermore, *tet*(M) has been found associated with conjugative transposons such as Tn*916* and Tn*1545*, the latter also containing genes that encode resistance towards erythromycin and kanamycin [66]. The *tet*(O) gene has been associated with conjugative plasmids [58,67]. Other RPP encoding genes are *Otr*(A), and *tetB*(P). *tetB*(P) is a RPP gene consisting of the RPP encoding gene *tetB*(P) and an efflux protein encoding gene *tetA*(P) [57]. *tetA*(P) has been found independent of *tetB*(P), but *tetB*(P) has not been found independent of *tetA*(P) [57]. The final RPP genes are *tet*(Q) and *tet*(T). The *tet*(Q) gene is often associated with a large conjugative transposon carrying the *ermF* gene conferring erythromycin resistance [57]. The *tet*(W) gene has the second widest host range within the RPP genes, and has been associated with the conjugative transposon Tn*B1230* [58,68]. Three more RPP genes have recently been described, all having ≤ 79% similar amino acid identity to the previously described RPP genes, namely *tet*(32), *tet*(36), and *tet*(44) [69-71]. Generally, the RPP encoding genes are often found with conjugative or non-conjugative transposons integrated into the chromosome, only rarely are they associated with plasmids [64].

2.2.2.3 Enzymes that inactivate tetracycline and other resistance mechanisms

The following genes all encode enzymes that inactivate tetracycline; *tet*(X), *tet*(37), and *tet*(34) [57,58]. So far, these genes have only been described in Gram negative bacteria [63].

2.3 Resistance to β-lactams

2.3.1 β-lactam drugs and mode of action

Penicillin was accidentally discovered in the late 1920's by scientist Alexander Flemming being the first antibiotic described [72]. β-lactam antibiotics work by binding the penicillin binding proteins (PBBs) which are involved in the bacterial cell wall synthesis. The β-lactam antibiotics consist of penicillins, which include the original penicillins, aminopenicillins, penicillinase resistant penicillins, carbapenems, monobactams, β-lactam inhibitors (clavulanic acid, tazobactam, sulbactam), and the cephalosporins (Table 3).

Resistance is caused by mutations in PBPs resulting in reduced affinity, reduced uptake due to changes in the cell wall, active efflux, or β-lactamase production [9].

The Ambler class β-lactamase classification is a molecular classification and is based on amino acid sequences, dividing the β-lactamases into classes A-D [9]. Classes A,C and D utilize serine in hydrolyzing β-lactams and class B requires zinc ions for hydrolysis of β-lactams [73]. There are currently >1250 known β-lactamases, with the OXA, TEM, SHV, CTXM, and CMY types being the most abundant (http://www.lahey.org/Studies/) [74].

2.3.2 β-lactam resistance mechanisms

2.3.2.1 Class A β-lactamases

In general, the class A β-lactamases consists of the class A serine β-lactamases, extended spectrum β-lactamases (ESBL), and the serine carbapenemases. The class A serine β-lactamases includes the TEM and SHV types which can only degrade penicillin substrates [75]. The first plasmid mediated β-lactamase was identified in *E.coli* in 1963 (reported in 1965) and was called R_{TEM} (TEM-1) [76]. TEM-2 (encoded by $bla_{\text{TEM-2}}$) is the first derivative of TEM-1, and has a single amino acid substitution from the original β-lactamase [77]. TEM-3 was the first TEM-type β-lactamase to display the ESBL phenotype [78]. The SHV β-lactamase is derived from the chromosomal β-lactamase of *Klebsiella pneumonia* and is now a common plasmidic β-lactamase [79]. The ESBLs are β-lactamases that can hydrolyze penicillins, 3rd and 4th generation cephalosporins and monobactams, but are susceptible to β-lactamase inhibitors [80,81]. ESBLs often arise as a result of amino acid substitution(s) in the existing β-lactamases. The most common ESBLs encountered are TEM, SHV, and CTX-M [79]. Typically, the TEM-1, TEM-2, and SHV-1 spectrums are widened by point mutations resulting in single amino acid changes in the β-lactamase, altering the active site of the enzyme [80]. The CTX-M ESBLs probably arose by plasmid transfer from preexisting chromosomal ESBL genes from *Kluyvera* spp., which typically are non-pathogenic organisms [80]. The CTX-M encoding plasmids often contain other multiple resistance determinants, including bla_{TEM} genes, genes encoding resistance to aminoglycosides, chloramphenicol, sulphonamides, trimethoprim, and tetracyclines [75]. The serine carbapenemases compose a diverse group of β-lactamases including IMI, KPC, SME, and nonmetallocarbepenemases (NMC-A) [79].

Table 3 Overview of the principle members of the β- lactam antibiotic class

	Antibiotic agents	Action and spectrum bactericidal
PENICILLINS		
The original Penicillins	Benzylpenicillin, phenoxymethylpenicillin	Gram positive bacteria incl. *Staphylococcus aureus*
Aminopenicillins	Amoxicillin, Ampicillin	Broad spectrum: Gram positive bacteria Gram negative bacteria
Penicillinase resistant penicillins	Methicillin, cloxacillin, dicloxacillin, oxacillin	Gram positive bacteria incl. *Staphylococcus aureus*
Broad spectrum penicillins (carboxypenicillins)	Carbenicillin, ticarcillin	Broad spectrum: Gram positive bacteria Gram negative bacteria incl. *Pseudomonas*
Combination of penicillins incl. β-lactamase inhibitors	Amoxicillin/clavulanate (animals)	
MONOBACTAMS	Azetreonam (humans)	Gram negative bacteria incl. *Pseudomonas*
CARBAPENEMS	Meropenem (humans), ertapenem (humans), imipenem/cilastin (humans)	Gram negative bacteria
CEPHALOSPORINS/ CEPHAMYCINS	1. generation: Cephapirin, cefadroxil, cephalexin 2. generation: Cefuroxime, Cefoxitin, cefmetazole 3. generation: Cefoperazone, ceftiofur, cefovecin, cefotaxime, ceftriaxone 4. generation: Cefquinome	1. generation: Strong activity against Gram positive bacteria, moderate activity against Gram negative bacteria. 2. generation: Effective against Gram positive and Gram negative bacteria that are resistant to 1. Generation cephalosporins 3. generation: Moderate activity against Gram positive bacteria and strong activity against Gram negative bacteria. Some are also active against *Pseudomonas*

2.3.2.2 Class B β-lactamases

The class B metallo-β-lactamases (MBLs) are zinc ion dependent and therefore have a different hydrolytic mechanism than the class A, C, and D serine β-lactamases. The MBLs include: IMP, VIM, SPM, and GIM degrading penicillins, cephalosporins and carbapenems also conferring resistance to clavulanic acid. The IMP and VIM-families are found mostly associated with integron structures and are found in Enterobacteriaceae, *Serratia marcescens*, *Pseudomonas aerogenosa*, and *Actinobacter baumanii* species [82].

2.3.2.3 Class C β-lactamases

The class C cephalosporinases include AmpC, CMY-2, P99, ACT-1, and DHA-1 and degrade cephamycins and 3rd generation cephalosporins but not 4th generation cephalosporins or monobactams and also confer resistance to β-lactam inhibitors[80]. In many Enterobacteriaeceae, AmpC expression is low but inducible in response to β-lactam exposure [83]. In *E.coli*, the *ampC* gene is chromosomal and normally expressed in low amounts not causing clinical resistance. However, *E.coli* can hyper produce AmpC β-lactamases either through mutations in the promoter or acquisition of plasmid mediated *amp*-like genes such as $bla_{\text{CMY-2}}$ [84].

2.3.2.4 Class D β-lactamases

Class D oxacillinases can hydrolyze oxacillin at a rate that is much more rapid compared to the hydrolysis of oxacillin by Ambler classes A and C also conferring resistance to cloxacillin[80]. The OXA-1 enzyme (encoded by $bla_{\text{OXA-1}}$) is the most common type in enterobacteria [79,81]. Furthermore, amino acid substitutions in the OXA genes can also confer the ESBL phenotype [85].

2.4 Resistance to sulphonamides

Sulphonamides are synthetic antibiotics that target the enzyme dihydropteroate synthase (DHPS) which is involved in the folate biosynthetic pathway, and is required for thymine production and bacterial cell growth (Table 4) [86]. Since 1968, sulphonamides have often been used in combination with trimethoprim, targeting dihydrofolate reductase (DHFR) that is involved in the same cellular pathways as DHPS [86].

Table 4 Overview of the principle members of sulphonamide antibiotic class

	Antibiotic agents	Action and Spectrum bacteriostatic (combination with trimethoprim → bactericidal due to synergy)
Sulphonamides	Sulfadimidine, sulfadimethoxine, sulfamerazine, Sulfadiazin, Sulfamethizole	Broad spectrum: Gram positive and Gram negative bacteria Also active against many protozoa

Marked resistance to sulphonamides has been seen in *E.coli*, *Shigella* spp., *Staphylococcus aureus*, and *Salmonella* spp. [86]. Chromosomal resistance to sulphonamides is mediated by mutations in the DHPS encoding *folP* gene leading to an amino acid alteration in the wild-type enzyme [72,86]. Plasmid mediated sulphonamide resistance in bacteria is conferred by production of an alternative sulphonamide resistant DHPS enzyme encoded by *sulI* or *sulII* [86]. *sulI* is often linked to other resistance genes and is located on transposons of the Tn21 family and *sulII* has been found both on small plasmids of the IncQ family (RSF1010) and pB1 plasmids [86,87]. Finally, there is *sulIII* which is a variant of *sulI* that has been identified in *Mycobacterium fortuitum* and in *E.coli*, and has approximately 40% amino acid similarity with *sulI* and *sulII* [86,88].

2.5 Resistance to glycopeptides

Vancomycin was the first glycopeptide, described in the late 1950's originally isolated from the soil bacterium *Streptomyces orientalis* [72,89]. The Glycopeptide antibiotics exert their effect by binding to the D-alanyl-D-alanine side chains of the cell wall peptidoglycan precursor, thus inhibiting peptidoglycan chain cross-linking and causing cell death. Their activity is principally limited to the Gram positive bacteria as the glycopeptide molecules cannot gain access to the Gram negative peptidoglycan layer (Table 5) [9].

Table 5 Overview of the principle members of glycopeptide antibiotic class.

	Antibiotic agents	Action and Spectrum bactericidal
Glycopeptides	Avoparcin*, vancomycin, teicoplanin	Gram positive bacteria

* avoparcin animal growth promoter ban implemented in Denmark in 1995

The primary glycopeptide resistance mechanism is rebuilding of the peptidoglycan using alternative precursors resulting in a lower glycopeptide affinity. The phenotypic VanA resistance is due to a different ligase type, D-alanyl-D-lactate, and is caused by the *vanA* gene cluster [9,90]. The VanH and VanX proteins are also products of the *vanA* gene cluster, and are necessary for VanA phenotypic resistance expression which confers resistance to vancomycin and teicoplanin [9]. Furthermore, the *vanA* gene cluster has been associated with the Tn*1546* transposon and has also been associated with a conjugative plasmid [90-92]. The phenotypic VanB resistance is due to vancomycin induction of the *vanB* gene cluster that confers resistance to vancomycin while susceptibility to teicoplanin is maintained [9]. The VanA and VanB resistance phenotypes are primarily described in *Enterococcus faecalis* and *Enterococcus faecium*, but the more widely distributed *vanA* gene has also been found in other enterococci including *Corynebacterium* spp., *Arcanobacterium haemolyticum*, and *Lactococcus* spp. [90].

2.6 Resistance to macrolide, lincosamide, streptogramin B (MLS_B)

Erythromycin A was the first macrolide and was discovered in the early 1950s [72]. Macrolide antibiotics include erythromycin and tylosin and act by binding the ribosomal 50S subunit ultimately inhibiting protein synthesis (Table 6). Macrolide, lincosamide, and streptogramin B antibiotics (MLS_B) share overlapping binding sites and it is therefore common that resistance is conferred to more than one MLS drug.

Table 6 Overview of the principle members of macrolide, lincosamide, streptogramin B (MLS_B) class antibiotics

	Antibiotic agents	Action and Spectrum bacteriostatic
General MLS_B		Primarily Gram positive bacteria Some Gram negative bacteria and few anaerobic bacteria
Macrolide	Spiramycin, tylosin, tilmicosin, erythromycin, roxithromycin, clarithromycin	Tylosin: campylobacter and mycoplasma Tilmicosin: campylobacter, mycoplasma, actinobacillus pleuropneumoniae
Lincosamide	Clindamycin, lincomycin	Clindamycin: Especially anaerobes, Toxoplasmosis
Streptogramin B	Virginiamycin	

Macrolide resistance can be caused by rRNA methylases, rRNA methyltransferases, efflux systems, or antibiotic inactivation [59]. The rRNA methylase encoding *erm* genes confer resistance to MLS_B

antibiotics by inhibiting antibiotic binding to the ribosome [12,59,93]. Many of the *erm* genes are associated with transposons where the tetracycline resistance gene *tet*(Q) is often linked to *erm*(F) and *tet*(M) to *erm*(B) [59,66,94]. There are currently 36 known methylase genes, one methyltransferase gene, 21 efflux genes, and 22 inactivating enzyme encoding genes (http://faculty.washington.edu/marilynr/ermwebA.pdf) [95]. The recently developed synthetic ketolides and oxazolidinones can also be grouped with MLS_B antibiotics, thus resulting in the macrolide, lincosamide, streptogramin B, ketolide, and oxazolidone family (MLSKO) family [93] .

3 CHAPTER 3 – Research activities in this PhD Project

3.1 General discussion of the results of PhD project

The overall aim of the PhD project was to develop qPCR assays for simple and rapid quantification of antibiotic resistance levels in animal herds. In order to accomplish this goal, the project was divided into three main objectives, which are separately discussed in the following sections.

3.1.1 Objective 1: *Manuscript I and II*

The first objective was to design and develop qPCR assays to quantify antibiotic resistance genes in animal herds with pig herds as a prototype. The antibiotic classes used in the Danish pig production aided the choice of genes to include. A total of 14 qPCR assays were successfully developed, these included: tetracycline *tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W); β-lactam bla_{SHV} family, bla_{CMY-2}, $bla_{CTX-M-1}$ group; sulphonamide (*sulI*, *sulII*); MLS_B (*ermB*, *ermF*); glycopeptide (*vanA*). A 16S rDNA assay was also included for comparative purposes.

Primer design, DNA quality, thermal profile, and master mix all play an important role in the specificity and quality of the qPCR product, and sensitivity of the assay. The primers and probes had to be specific for each gene while detecting the antibiotic resistance genes in as many bacteria as possible. Therefore, the intended gene sequences were queried in GenBank and those residing in pig intestinal commensals used for alignments. The conserved regions were then used for primer and probe design. Some antibiotic resistance genes are extremely similar, making it impossible to distinguish particular genes using qPCR. For example, the $bla_{CTX-M-1}$ group contains >30 variants, with the bla_{CTX-M} family containing >190 genes [96] (Table S1). Therefore, the entire group or family for the $bla_{CTX-M-1}$ and bla_{SHV} were included, respectively. Due to the similarity between genes, probe based chemistry was preferred in order to increase the assay specificity.

PCR optimization is required for each untested PCR assay and includes the buffer system and cycling parameters [97]. The buffer system consists of a mastermix containing magnesium chloride ($MgCl_2$), deoxynucleotide triphosphates (dNTPs), primers, DNA polymerase, and the DNA template. The effects varying PCR cycle numbers and $MgCl_2$ combinations had on the class and proportion of RPP *tet* genes detected in swine manure using the Ribo2_new_FW/Ribo2_RV degenerate primer pair was investigated (*Manuscript I*). The tested combinations yielded significant differences in the diversity of RPP *tet* genes detected; there was a marked increase in both diversity and number of RPP *tet* genes with increasing cycle number and $MgCl_2$ concentrations. The results from this study emphasize the importance of optimizing each untested PCR assay. Both melting and annealing temperatures of the primers and template are affected the amount $MgCl_2$ in the PCR reactions, and the *Taq* DNA polymerase utilizes Mg^{2+} for activity [10,97,98]. It is important to test the $MgCl_2$ concentration for each PCR assay because increased concentrations of $MgCl_2$ may improve the efficiency of the PCR amplification but can also reduce the specificity, and too little $MgCl_2$ can result in no PCR product [10,97]. The optimal cycle number for the specific PCR reaction depends on the amount of starting material in the sample, the primers used, and the efficiency of the PCR assay [98]. If the cycle number is too low, then there will be no amplified products, but if it is too high non-specific products may appear [97,98]. It is therefore important to verify the specificity of any optimized qPCR assay. In the current project, this was accomplished by running each qPCR assay against a panel of 16 different antibiotic resistant gene amplicons at a concentration of $2x10^4$ copies μl^{-1}. There were no cross-reactions from any of the qPCR primers (*Manuscript II*). Ideally, the amplicons derived from the qPCR in swine feces should be sequenced in order to confirm the product, but this was not possible due to the short amplicon lengths. Therefore, the amplicon

lengths were confirmed using the High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Walbronn, Germany). Each PCR assay contained an internal control for inhibition, and both positive- and negative- DNA extraction controls were included for each DNA extraction.

The standard curve enables absolute quantification of gene copy number per gram feces, and were created using PCR derived amplicons serially diluted in water. Feces contain inhibitors such as cell debris, bacterial proteases and nucleases, and bile salts all of which can affect the sensitivity of a PCR assay [99]. Additionally, it has been previously shown that, for each qPCR assay, matrix and extraction method, a separate standard curve should be used to ensure meaningful quantitative measurements in specific samples[100]. Therefore, numerous attempts were made to find swine feces with low levels of antibiotic resistance genes to use for spiking and standard curve generation. Swine fecal samples from conventional pig herds, miniature pigs that had never received antibiotics and swine feces that had been treated with gamma radiation as earlier described [101] were screened for the presence of antibiotic resistance genes. Unfortunately, the levels of antibiotic resistance genes in the screened samples were not low enough to enable spiking for standard curve use, and the gamma radiation treated feces inhibited almost all the tested assays. As a consequence, water was used for standard curve generation and was considered satisfactory as comparable standard curves have previously been described [8,13,102,103].

In order to investigate the effect the pig fecal environment had on the quantification of the antibiotic resistance genes, template serial dilutions in water were run parallel to pig fecal DNA spiked with antibiotic resistance gene template serial dilutions. Using template assures confidence of the number of amplicons that are added to start with when preparing the serial dilutions and assumptions of gene copy number / positive control bacteria are avoided, which may introduce bias if the bacteria lose their plasmid, have different plasmid copy numbers, or shed the antibiotic resistance gene during the extraction process [8]. Discrepancies between the spiked pig fecal DNA and the serial dilutions in water have been described [104,105]. In the present study, the efficiency in the spiked DNA from pig feces remained between [0.90;1.10] and R^2 above 0.99 for all but four

assays. The lowest efficiency was 0.84 (16S rDNA and *tet*(M)) and R^2 was 0.87 for *tet*(M), and therefore not of great concern. Varying inhibition was also observed in the spiked pig fecal DNA extracts (1-4 Cq value increases) however, the dynamic ranges remained linear over a measurement range >4 orders of magnitude which is sufficient for genomic DNA. The qPCR performance in quantifying antibiotic resistance genes in pig fecal samples was therefore not regarded considerably altered.

3.1.1.1 Conclusion

The qPCR assay design procedure including the primers and corresponding probes, the internal controls, DNA extraction controls, and reaction mix all ensured a thorough qPCR assay design and verification. There were not cross reactions when each assay was tested against a panel of 16 different antibiotic resistance gene amplicons and the qPCR product lengths from pig feces were accurate. We believe that the developed qPCR assays possess the characteristics necessary for application to antibiotic resistance gene quantification in pig fecal samples. In addition, *Manuscript I* reports the first study that systematically evaluated the effect of PCR cycle numbers and magnesium chloride concentrations on detection of *tet* genes in swine manure samples. We showed

that these two parameters can significantly affect the detection of *tet* genes in terms of classes detected and their proportion. The results emphasize the importance of taking the PCR conditions into consideration and optimizing each untested PCR assay. Furthermore, PCR conditions should also be taken into consideration upon result interpretation because the PCR conditions can significantly affect the analysis results.

3.1.2 Objective 2: *Manuscript II, Manuscript III*

The second objective was to establish if the qPCR assays could quantify antibiotic resistance genes in swine herds by comparing this principle to culture dependent antibiotic resistance detection. First, swine fecal samples were collected in a single herd using different sampling methods which also were pooled at different levels. Second, antibiotic resistance levels were determined by the qPCR assays and coliform CFU counts. Third, the different sampling and pooling methods were evaluated (*Manuscript II*). Finally, the principle of qPCR quantification of antibiotic resistance was compared to culture dependent methods including coliform CFU counts and colony hybridization using probes corresponding to the fragments amplified by the qPCR primers (*Manuscript III*).

The swine fecal samples were collected from a single pig herd at two separate time points that were four months apart. Samples were collected from four pens within a single stable in sampling 1 and were subsequently pooled at different levels. The samples collected included individual animal samples, pen floor samples, and shoe cover samples. During sampling 2, samples were collected from four pens in each of the five sampled stables. The samples collected were pen floor samples, shoe cover samples, and slurry tank samples. These were also pooled at different levels after collection. Twenty fecal samples collected from the individually sampled animals were randomly selected for analysis with the qPCR and culture dependent methods for antibiotic resistance quantification.

3.1.2.1 Antibiotic resistant coliform CFU counts and qPCR gene copy number assessment

When assessing the coliform CFU counts from individually sampled animals within each pen from sampling 1, there was a significant difference between pens for the ampicillin resistant coliform CFUs ($P<0.05$). Furthermore, the CFU counts of the individual animal pool pen samples did not represent an average of the non-pooled individual animal samples demonstrating that, at pen levels, the pooled samples from individual animals were not representative of the individual animals (*Manuscript II*, Figure 1, top). Figure 2 (*Manuscript II*) shows the relative standard deviations of the fecal estimates of the coliform CFU counts and qPCR gene copy numbers. The coliform CFU estimates had large relative standard deviations, explaining the variation observed within each sampling method. This variation complicated the comparison of different sampling methods between pens using coliform CFU counts (*Manuscript II*, Figure 1, bottom).

Regarding the qPCR gene copy number estimates from sampling 1, *ermB*, *ermF*, *tet*(C), *tet*(O), and *tet*(W) differed significantly between pens ($p<0.05$ for *ermB*, *tet*(C), *tet*(W); $p<0.0001$ for *ermF* and *tet*(O)). The $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CMY-2}}$, bla_{SHV} family, and *vanA* antibiotic resistance genes were not detected in any samples during sampling 1 and sampling 2. In contrast, the coliform CFU counts differed significantly between pens for ampicillin and not tetracycline nor erythromycin. These results illustrate major shortcomings of both methods i.e. the CFU counts represent only the coliform bacteria which are a fraction of the intestinal population, and the qPCR assays only detect

the specific genes that are included. There was no correlation between the total coliform CFU counts and the total number of bacteria in the population represented by the 16S copy number (R^2=0.1). This emphasizes how these two methods represent each their population and cannot be directly compared. However, the qPCR gene copy numbers had lower relative standard deviations compared to the coliform CFU counts meaning that there is less variation in the qPCR gene copy estimates compared to the coliform CFU counts (*Manuscript II*, Figure 2). Therefore, only qPCR gene copy number g^{-1} feces were used to assess the different sampling and pooling methods.

3.1.2.2 Sampling method and pooling assessment

For the majority of the genes during sampling 1, the pen floor samples had higher copy number estimates compared to the individual animal pen pools (*Manuscript II*, Figure 3 and Figure S1). This could either be due to the dilution effect of pooling 20 ≥ individual samples together, or to the pen floor samples being more concentrated due to liquid run-off and/or evaporation. Furthermore, the individual animal pen pools were considerably more constant between pens than the individual animal samples illustrating how high- and low-level samples can balance each other in a pool [32]. Unfortunately, shoe cover sampling was not conducted at the pen level (one shoe cover per pen) but at stable level (one shoe sample in all four pens x4 shoe cover samples). It is therefore not possible to compare the shoe cover sampling method with the individual animal sampling or pen floor sampling conducted in the four pens included in sampling 1.

During the second sampling (sampling 2) four pens in five stables were sampled in the same herd as sampling 1. The shoe cover samples varied in being higher or lower than the pen floor samples for certain genes, which was observed for the same genes in sampling 1 and sampling 2. A lower gene copy number per gram feces may occur if there is excess roughage collected during sampling due to roughage weight that is attributed as feces. On the other hand, the higher shoe cover sample gene copy estimates may reflect that the shoe covers collect fractions of feces that the individual animal samples or pen floor samples cannot, thus different bacteria are represented. Moreover, the entire pen floor is included during shoe cover sampling, consequently increasing the chances of collecting a sample positive for a given gene.

The sensitivity of a specific assay is dependent on the gene prevalence, the number of samples included in the pool, the gene concentration in samples collected from positive animals, and the quantification limit of the assay [32,106]. This is illustrated by samples that were positive originally, but became negative after pooling (sampling 1 *sulI*, *sulII*, *tet*(A) ; sampling 2 *tet*(A), *tet*(B), *tet*(C), *sulII*). In contrast, there was apparent consistency in gene copy numbers per gram feces between the slurry tank samples, pen floor and shoe cover herd pools for the majority of the assays implying stability between the parameters affecting assay sensitivity.

3.1.2.3 Method comparisons for quantitative measurement of antibiotic resistance in swine feces

The principle of culture independent antibiotic resistance quantification using the developed qPCR assays was compared to the culture dependent principles. The culture independent methods included coliform CFU counts on plates with antibiotics (sulphonamide, tetracycline) and without

antibiotics, and colony hybridization using the qPCR amplicons as hybridization probes for antibiotic resistance genes. The colony hybridization was performed without antibiotics added to the agar enabling comparison with the qPCR estimates i.e. neither bacteria populations were subjected to an antibiotic selection pressure. On the other hand, the coliform estimates are based on an antibiotic selection pressure in order to select for phenotypic resistance.

Twenty of the 84 fecal samples from individually sampled animals were randomly selected and used for antibiotic resistance determination (*Manuscript III*). The following antibiotic-resistance genes were investigated by qPCR: tetracycline resistance *tet*(A),*tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W); sulphonamide resistance *sulI*, *sulII*. The gene copy number estimates were then compared to corresponding resistant coliform bacteria CFUs. Finally, colony hybridization was used to quantify the same tetracycline and sulphonamide antibiotic resistance genes in bacteria using the qPCR primers' amplicons as hybridization probes.

The antibiotic resistant coliform estimates are phenotypically based as only the antibiotic resistant coliforms that grow on the MacConkey plates containing antibiotics are counted. In contrast, the colony hybridization enables detection of antibiotic resistance genes in a larger bacterial population, namely those that grow on MacConkey without antibiotics and blood agar (BA) anaerobically incubated. Despite there being a smaller bacterial population represented in the coliform estimates compared to the colony hybridization, there was a higher level of tetracycline resistance observed in the coliform bacteria CFU counts compared to the colony hybridization ($P<0.0001$) (*Manuscript III* Table 2). This suggests that the majority of the tetracycline resistant bacteria are not detected by colony hybridization because they do not carry the specific genes that were used as probes, illustrating how the method chosen for antibiotic resistance detection has an influence on the results obtained. The *tet* genes detected by qPCR were not detected using colony hybridization on blood agar (anaerobic growth) as seen by low BA (anaerobic growth) CH:qPCR ratios (*Manuscript III*, Figure 3a). Either the bacteria containing the genes detected with qPCR are not present on the BA plates (anaerobic growth), or a single bacterium may contain more than one of the resistance genes.

There were also differences in the sulphonamide resistance estimates obtained by the different methods (*Manuscript III*, Table 3), however the qPCR estimates were only significantly different from the CFU estimates from plates without antibiotics. The *sulI* and *sulII* genes detected by qPCR are also represented in the culture dependent methods as seen by the BA (anaerobic) CH:qPCR ratios and sulphonamide resistant coliform CFU:qPCR ratios that did not significantly differ ($P=0.2010$) (*Manuscript III*, Figure 3b). This indicates that when fewer genes cause an antibiotic resistance phenotype (sulphonamide vs tetracycline), then there is less discrepancy between the resistance genotype and phenotype.

3.1.2.4 Conclusion

Great variation within each sampling method was observed for the coliform CFU counts making it difficult to compare different sampling and pooling methods. This variation can be explained by the large relative standard deviations seen for each coliform CFU count, indicating that coliform bacteria are not a good representative for the general resistance level. The qPCR and culture dependent for estimating antibiotic resistance represent each their bacterial population and cannot be directly compared. Furthermore, the results show that the method chosen for quantification can have an influence on the results obtained. Whether a phenotypic antibiotic resistance estimate is

signified by the genotype or not may depend on the number of corresponding resistance determinants. The qPCR gene copy estimates in swine feces had reduced relative standard deviations compared to coliform CFU counts, and were therefore used to assess the different sampling and pooling methods. Furthermore, the qPCR assays allowed simultaneous quantification of key antibiotic resistance determinants in total microbiomes of pig fecal samples. We recommend either pen floor samples or shoe cover sampling for antibiotic resistance estimation at herd level by qPCR. The slurry tank samples were also promising; their gene copy levels were consistent with those quantified in the pen floor and shoe cover samples. To our knowledge, this is the first study to test different sampling and pooling strategies for antibiotic resistance surveillance using qPCR estimation of antibiotic resistance levels in total DNA extracted from swine feces [29-31].

3.1.3 Objective 3: *Manuscript IV*

The third objective was to apply the qPCR assays in another animal population than swine herds. Wild animals have been associated as potential reservoirs of resistant bacteria, and might assist in the dissemination of resistant bacteria throughout the environment [107]. Fecal samples collected from wildebeest, zebra, and buffalo in Tanzania were screened for the presence of antibiotic resistance genes using the developed qPCR assays; the level of resistant coliform bacteria was also determined. The first sampling site was the Ngorongoro Conservation Area (NCA) where the Massai shepherds migrate with their short horned zebu cattle, interacting with the wildlife through grazing and at water holes [108,109]. Further south from NCA is the Mikumi National Park (MNP) where the Massai are prohibited to migrate with their cattle so the wildlife does not interact with cattle.

Eight of the 14 antibiotic resistance genes were detected in the wildlife and cattle samples. No wildlife samples from NCA or MNP were positive for antibiotic resistance genes not detected in the cattle. The cattle were all positive for *tet*(W), *ermF*, *sulI*, and $bla_{\text{CMY-2}}$. *tet*(A), *tet*(M), *tet*(O), and *sulII* were also detected in minimum one of the four cattle samples (*Manuscript IV*, Table 2). On the other hand, the *tet*(A), *tet*(M), *tet*(O), genes were detected the cattle but not wildlife samples. The Buffalo M13 sample had a relatively low amount of 16S rDNA implying that there may not have been sufficient DNA for qPCR gene detection as this was the only sample that was negative for all the tested antibiotic resistance gene determinants.

There were few samples displaying phenotypic resistance without having a corresponding antibiotic resistance gene encoding the resistant phenotype. For example, the Buffalo M15 sample had phenotypic sulphametizole resistance, but neither *sulI* nor *sulII* were detected. This can be explained by another sulphonamide resistance encoding gene causing the resistant phenotype for example, *sulIII* or a mutated DHPS encoding *folP* gene [86,87].

The most concerning finding was the presence of the $bla_{\text{CMY-2}}$ cephalosporinase encoding gene in 10 of the 12 screened samples. A study in Denmark revealed that the $bla_{\text{CMY-2}}$ was present in samples collected from broiler flocks where the administration of cephalosporins had been banned for 10 years [110]. This illustrates the persistence of the $bla_{\text{CMY-2}}$ gene within a population despite the absence of cephalosporin selection pressure. Furthermore, $bla_{\text{CMY-2}}$ is found on plasmids associated with transposons and multiple antibiotic resistance genes that spread both clonally and horizontally [75,110]. This emphasizes the role of $bla_{\text{CMY-2}}$ in the spread of resistance.

3.1.4.1 Conclusion

The detection of *bla*$_{CMY-2}$ was of great importance for two reasons. First, the nature of the *bla*$_{CMY-2}$ antibiotic resistance spectrum itself and the finding of this gene in 10 of 12 screened wildlife samples gives rise to concern. Second, this gene was not detected in any of the pig samples collected previously during this project. Therefore the finding of the *bla*$_{CMY-2}$ gene in the wildlife further substantiates the qPCR assay. Based on these results, further studies should be conducted to study the antibiotic resistance gene-pool among the wildlife in northern Tanzania in depth. It is important to minimize excessive antibiotic use in order to reduce selection of antibiotic bacteria both among humans, livestock, and wildlife.

3.3 Future perspectives

This PhD thesis mainly focuses on the development of qPCR assays, and their initial test in order to establish a principle of qPCR quantification of antibiotic resistance genes in animal herds. Future studies must be conducted in order to investigate the qPCR method's ability to quantify fluctuations in antibiotic resistance.

The assays from this project are in the process of being used in a larger study including five pig herds in Denmark that have been subjected to different antibiotic treatment regimes. Here a better understanding of the antibiotic resistance gene pool fluctuations may come to light, as these pigs were sampled prior to, during, and after antibiotic treatment. However, the quantified antibiotic resistance gene levels need to be understood in order to define for example if certain levels are a potential risk. In order to do so, it may be necessary to conduct initial studies that include parallel analysis of antibiotic resistance using several methods.

If the qPCR assays prove to be sensitive enough, it may be possible to certify herds free for certain types of antibiotic resistance or at least to categorize them according to their antibiotic resistance levels. This could be valuable information when considering animal trade but may also aid the choice of antibiotics for disease treatment. Nevertheless, more assays should be developed in order to ensure as many genes can be detected as possible.

Despite the global focus on antibiotic resistance and the associated hazards, there is a lack of epidemiological studies concerning the antibiotic resistance gene pools in animal herds. Future studies could include monitoring the antibiotic resistance genes in piglets from their birth (including a sample from the sow) and throughout their progression in the herd. This may enlighten when the antibiotic genes are acquired and their fluctuations can be monitored over time. The finding of antibiotic resistance genes both in pigs that had never received antibiotics and wildlife in Tanzania emphasizes the need for an understanding of antibiotic resistance gene dynamics not only among animal herds but also wildlife.

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APPENDIX I – Supplementary tables (Table S1, Table S2, Table S3)

Table S1 An overview of the antibiotic resistance genes included in the present study, and their phenotypic resistance, resistance mechanism, mobility, gene linkage, "other" information, and references.

Antibiotic resistance genes	Relevant resistance	Resistance mechanism	Mobility	Linked to other genes	Other	Ref.
tet(A)	tetracycline	efflux protein	Gram negative bacteria: transposons inserted in plasmids or integrons i.e. RP1 and Tn*1721*	Gram negative bacteria: antibiotic resistance genes heavy metal resistance genes pathogenic factors		57,63,64,72,111-115
tet(B)	tetracycline minocycline	efflux protein	Gram negative bacteria: transposons inserted in plasmids or integrons i.e. pSC101 and Tn*10*	Gram negative bacteria: antibiotic resistance genes heavy metal resistance genes pathogenic factors		63,72,112,116
tet(C)	tetracycline	efflux protein	Gram negative bacteria: transposons inserted in plasmids or integrons i.e. pRAS3 and Tn*1403*	Gram negative bacteria: antibiotic resistance genes heavy metal resistance genes pathogenic factors		63,72,117,118
tet(M)	doxycycline minocycline	ribosomal protection	Conjugative transposons i.e. Tn*916* Tn*1545*	Tn*1545*: erythromycin and kanamycin resistance genes often linked to *ermB*		59,63,66,72, 112
tet(O)	doxycycline minocycline	ribosomal protection	Conjugative plasmids i.e. piP1433, pUA466	Other antibiotic resistance genes		63,72,112,119,120
tet(W)	doxycycline minocycline	ribosomal protection	Conjugative transposon i.e. Tn*B1230*	Other antibiotic resistance genes		63,68,72,112

ermB	macrolide lincosamide streptogramin B ketolides oxazolidinones	rRNA methylase	Plasmids i.e.pSE20, pTE44 and associated with transposon Tn*917*	often associate with other antibiotic resistance genes encoding for i.e. resistance to Chloramphenicol, streptomycin, aminoglycoside, and tetracycline genes especially *tet*(M)		59,72,93,95, 121,122
ermF	macrolide lincosamide streptogramin B ketolides oxazolidinones	rRNA methylase	Plasmids i.e. pBF4 and associated with transposon Tn*4352*	often associate with other tetracycline resistance genes especially *tet*(Q)		59,72,93,95, 121,122
*sul*I	sulphonamide	alternative DHPS	Plasmids often associated with transposons i.e. Tn*21* or integrons	other antibiotic resistance genes encoding resistance to i.e. streptomycin, trimethoprim, ampicillin, kanamycin, chloramphenicol, and tetracycline		123-127
*sul*II	sulphonamide	alternative DHPS	small non-conjugative plasmids i.e.pBP1, p9123 and RSF1010 or large conjugative plasmids i.e. pGS05	other antibiotic resistance genes encoding resistance to i.e. streptomycin, trimethoprim, ampicillin, kanamycin, chloramphenicol, and tetracycline		87,124,128
$bla_{\text{CTX-M-1}}$ group	β-lactams ESBL*	β-lactamase	Conjugative plasmids and non-self-transmissible plasmids: 7-430kb size range i.e. IncFII, IncN, IncI1, and IncL/M chromosomal in *Kluyvera* associated with Insertion sequences i.e. IS*Ecp1*	bla_{TEM} genes, genes encoding resistance to aminoglycosides, chloramphenicol, sulphonamides, trimethoprim, and tetracyclines	CTX-M-1 group >30 variants: 1,3,10,12,15,22, 23,28,29,32,33, 42… CTX-M family > 119 types	74,96,129-131

bla$_{CMY-2}$	β-lactams**	AmpC-like β-lactamase	Conjugative or non-conjugative plasmids often associate with transposons/integrons	other antibiotic resistance genes encoding resistance to i.e. chloramphenicol, tetracycline, streptomycin, gentamicin and tobramycin		75,132-136
bla$_{SHV}$ family	β-lactams *bla*$_{SHV-1:}$ penicillins *bla*$_{SHV->1}$: ESBL*	β-lactamase	chromosomal in *Klebsiella* conjugative plasmids i.e. p453, pKOX105 often associated with transposons	antibiotic resistance genes encoding resistance to i.e. quinolones, aminoglycosides and trimethoprim	SHV family > 175 types	74,137-141
vanA	vancomycin teicoplanin	alternative peptidoglycan precursor	conjugative plasmids i.e. pIP816 often associated with transposons i.e. Tn*1546*	other antibiotic resistance genes encoding resistance to i.e. erythromycin, ampicillin, and tetracycline	*vanA* cannot confer resistance to vancomycin alone, other genes in the *vanA* operon must also be acquired to produce the substrate for VanA	90,92,142-147

* ESBL: β-lactamases that can hydrolyze penicillins, 3rd and 4th generation cephalosporins and monobactams, but are susceptible to β-lactamase inhibitors.

** AmpC Cephalosporinase: Degrade cephamycins and 3rd generation cephalosporins but not 4th generation cephalosporins or monobactams and also confer resistance to β-lactam inhibitors.

Table S2 The antibiotic resistance genes included in the present study and their Gram positive bacterial host spectrum found in current literature (*tet*(A)[57,63,64,72,111-115], *tet*(B)[63,72,112,116], *tet*(C)[63,72,117,118], *tet*(M)[59,63,66,72,112], *tet*(O)[63,72,112,119,120], *tet*(W)[63,68,72,112], *ermB*[59,72,93,95,121,122], *ermF*[59,72,93,95,121,122], *sulI*[123-127], *sulII*[87,124,128], $bla_{\text{CTX-M-1}}$ group[74,96,129-131], bla_{SHV} family[74,137-141], $bla_{\text{CMY-2}}$[75,132-136], *vanA*[90,92,142-147]). The black boxes denote the given gene has been described in the corresponding bacteria.

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1}}$ group	bla_{SHV} family	$bla_{\text{CMY-2}}$	*vanA*
Abiotrophia				■										
Actinomyces				■		■		■						
Aerococcus				■	■		■		■	■				
Arcanobacterium						■	■							■
Arthrobacter				■					■	■				
Bacillus				■		■	■		■	■				■
Bacterionema				■										
Bifidobacterium (anaerobic)				■	■	■								
Brevibactium										■				
Carnobacterium									■					
Catenibacterium (anaerobic)				■										
Caullulomonas														■
Clostridium (anaerobic)				■		■	■	■						

Table S2 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1}}$ group	bla_{SHV} family	$bla_{\text{CMY-2}}$	*vanA*
Corynebacterium				■			■	■		■				
Enterococcus				■	■		■	■		■				■
Erysipelothrix				■										
Eubacterium				■	■		■	■						
Fusobacterium (anaerobic)						■								
Gardnerella				■				■						
Gemella				■	■		■							
Granulicatella				■										
Lactobacillus				■	■	■	■	■						
Lactococcus				■										
Listeria				■										
Microbacterium				■										
Micrococcus							■							
Mobiluncus (anaerobic)				■	■			■						

Table S2 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1}}$ group	bla_{SHV} family	$bla_{\text{CMY-2}}$	*vanA*
Mycoplasma				■										
Paenibacillus				■										■
Planococcus									■	■				
Pediococcus							■							
Peptostreptococcus (anerobic)				■	■		■	■						
Roseburia						■								
Rothia							■							
Ruminococcus							■	■						
Staphylococcus				■	■	■	■	■						■
Streptococcus				■	■	■	■	■						
Streptomyces				■		■								
Ureaplasma				■			■							
Weisella									■	■				

Table S3 The antibiotic resistance genes included in the present study and their Gram negative bacterial hosts found in current literature (*tet*(A)[57,63,64,72,111-115], *tet*(B)[63,72,112,116], *tet*(C)[63,72,117,118], *tet*(M)[59,63,66,72,112], *tet*(O)[63,72,112,119,120], *tet*(W)[63,68,72,112], *ermB*[59,72,93,95,121,122], *ermF*[59,72,93,95,121,122], *sulI*[123-127], *sulII* [87,124,128], $bla_{\text{CTX-M-1}}$ group[74,96,129-131], bla_{SHV} family[74,137-141], $bla_{\text{CMY-2}}$[75,132-136], *vanA*[90,92,142-147]). The black boxes denote the given gene has been described in the corresponding bacteria.

Genera \ **Antibiotic resistance gene**	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1 group}}$	bla_{SHV}	$bla_{\text{CMY-2}}$	*vanA*
Acidaminococcus (anaerobic)						■								
Acinetobacter	■	■		■			■		■	■				
Actinobacillus		■			■									
Aeromonas	■	■	■	■					■					
Aggregatibacter		■					■	■						
Anaerovibrio (anaerobic)					■									
Afipia				■										
Agrobacterium										■				
Bacteroides (anaerobic)				■		■	■	■						
Bordetella	■		■						■					
Brachybacterium				■										
Brevundimonas		■												
Butyrivibrio (anaerobic)					■	■								
Burkholderia												■		

Table S3 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1 group}}$	bla_{SHV}	$bla_{\text{CMY-2}}$	*vanA*
Campylobacter					■									
Capnocytophaga (anaerobic)								■						
Chlamydia			■											
Chryseobacterium	■													
Citrobacter	■	■	■	■			■				■	■	■	
Comamonas										■				
Edwardsiella	■			■					■					
Eikenella				■										
Enterobacter	■	■	■	■			■							
Erwinia		■												
Escherichia	■	■	■	■			■		■	■	■	■	■	
Flavobacterium	■			■										
Fusobacterium (anaerobic)				■	■		■	■						
Francisella			■											
Haemophilus		■		■			■	■		■				

Table S3 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1 group}}$	bla_{SHV}	$bla_{\text{CMY-2}}$	*vanA*
Halomonas			■											
Kingella				■										
Klebsiella	■	■	■	■			■		■	■		■	■	■
Kluyvera											■	■		
Kurthia				■										
Laribacter	■													
Mannheimia		■												
Megasphera (anaerobic)					■	■								
Mitsuokella (anaerobic)						■								
Moraxella		■												
Morganella									■			■		
Neisseria		■		■	■	■	■	■						
Pantoea		■		■			■							
Pasteurella		■		■						■				
Photobacterium		■		■										

Table S3 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1 group}}$	bla_{SHV}	$bla_{\text{CMY-2}}$	*vanA*
Psychrobacter					■									
Plesiomonas	■	■	■											
Porphyromonas (anaerobic)					■		■	■						
Prevotella (anaerobic)				■	■			■						
Proteus	■	■	■				■						■	
Providencia		■												
Pseudoalteromonams				■										
Pseudomonas	■	■	■	■			■		■	■	■	■		
Psychrobacter									■	■				
Ralstonia				■										
Roseobacter		■	■											
Salmonella	■	■	■						■	■	■	■	■	
Selenomonas (anaerobic)				■	■			■						
Serratia	■	■	■	■			■				■	■		
Shewanella		■		■										

Table S3 continued

Genera \ Antibiotic resistance gene	*tet*(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	$bla_{\text{CTX-M-1 group}}$	bla_{SHV}	$bla_{\text{CMY-2}}$	*vanA*
Shigella	■	■	■	■				■	■	■				
Stenotrophomonas									■	■	■	■	■	
Subdoligranulum (anaerobic)					■									
Treponema		■					■	■	■					
Variovorax	■													
Veillonella (anaerobic)	■			■	■			■						
Vibrio	■	■	■	■						■				
Wolinella							■	■	■					
Yersinia		■												

APPENDIX II – Manuscripts

MANUSCRIPT I

Gunilla Veslemøy Schmidt, Jill Stiverson, Øystein Angen, Zhongtang Yu, 2014.

Number of PCR cycles and magnesium chloride concentration affect detection of *tet* genes encoding ribosomal protection proteins in swine manure.

Submitted to *FEMS Microbiology Letters*

Title

Number of PCR cycles and magnesium chloride concentration affect detection of *tet* genes encoding ribosomal protection proteins in swine manure.

Running Title:

Effect of PCR Conditions on Detection of *tet* Genes in Swine manure.

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Keywords:

Porcine, antibiotic resistance, assay, optimization, reaction-mix.

Abstract

PCR is routinely used in detection of antibiotic resistance genes including different classes of *tet* and *erm* genes. It remains unknown how PCR conditions affect detection of resistance genes in terms of genetic diversity and prevalence. In this study, numbers of PCR cycles and $MgCl_2$ concentrations were evaluated for their effect on the diversity and prevalence of the *tet* genes that encode ribosomal protection proteins (RPPs) in composted swine fecal samples using the degenerate Ribo2_new_FW/Ribo2-RV primer pair. Three cycle numbers and 4 $MgCl_2$ concentrations were tested in a 3 x 4 factorial design. A clone library was constructed for each PCR condition combination, and randomly selected clones were sequenced to determine the genetic diversity and relative distribution of RPP *tet* genes. Significant differences in genetic diversity and prevalence of *tet* genes were found among the tested cycle numbers and $MgCl_2$ concentration combinations. The results suggest that 35 PCR cycles and 7 mM $MgCl_2$ allow for optimal detection of the *tet* genes in swine feces using the Ribo2_new_FW/Ribo2-RV primer pair. These results suggest that PCR conditions should be taken into consideration when PCR conditions are chosen for ecological studies of *tet* genes and when the results are interpreted.

Introduction

Polymerase chain reaction (PCR) has been the choice of method to specifically and rapidly detect the presence and genetic diversity of genes of interest, including genes encoding antibiotic resistance in microbiome samples (Manuzon *et al.* 2007, Alali *et al.* 2009). Because it allows for detection of target genes carried by both culturable and nonculturable microbes, PCR can provide more accurate detection of the target genes than cultivation-based methods. However, a number of factors, such as primer design, amount and quality of the template DNA, PCR thermal profile, and reaction mix, can affect the specificity and quality of the PCR product, and thus sensitivity of the PCR assay. Most PCR assays are optimized to amplify the target gene producing the specific product, and the essential components of the PCR optimization include the buffer system and cycling parameters (Harris and Jones 1997). The buffer system consists of a reaction mix of magnesium chloride ($MgCl_2$), deoxynucleotide triphosphates (dNTPs), primers, DNA polymerase, and the DNA template. Additional components such as bovine serum albumin which attenuates amplification inhibition, or dimethyl sulfoxide (or glycerol, betaine, etc.) which reduce formation of secondary structures are often added to the reaction mix to improve PCR efficiency. $MgCl_2$ concentration plays an important role in PCR, as the melting and annealing temperatures of the primers and template are affected by Mg^{2+}. Furthermore, *Taq* DNA polymerase utilizes Mg^{2+} for activity (Williams 1989, Innis *et al.* 1990, Harris and Jones 1997). It is important to consider and test the $MgCl_2$ concentration for each specific PCR assay, as increased concentrations of $MgCl_2$ may increase the efficiency of the PCR amplification but can also reduce the specificity, while too little $MgCl_2$ can result in little or no PCR product (Williams 1989, Harris and Jones 1997). The optimal cycle number for specific PCR depends on the amount of template used, the primers used, and the efficiency of the PCR assay (Innis *et al.* 1990). For a given amount of DNA

template, if the cycle number is too low, then there will be little or no amplified products, but if it is too high non-specific products may form (Innis *et al.* 1990, Harris and Jones 1997).

When a class or family of related genes is detected together using PCR and a 'pair of ‘universal’ primers, the detection of different genes within that class or family can be skewed. In a previous study, it was noted that *Fibrobacter succinogenes*, a species of major cellulolytic bacteria in the rumen, could not be detected by PCR using a pair of universal bacterial primers that matches the 16S rRNA gene of that species perfectly (Larue *et al.* 2005). In a recent study, we also repeatedly failed to detect *Methanobacterium* in samples collected from anaerobic digesters by using a pair of Archaea domain-specific primers though the primers matched the 16S rRNA gene sequences of this genus, and qPCR using genus-specific primers revealed high abundance of this genus (Li, Chen and Yu 2014). Class- and group-specific primers have been commonly used in detecting the presence and diversity of antibiotic resistance genes, including *tet* genes and *erm* genes ((Aminov, Garrigues-Jeanjean and Mackie 2001, Aminov *et al.* 2002, Smith *et al.* 2004, Aminov and Mackie 2007, Peak *et al.* 2007, Knapp *et al.* 2010). We hypothesized that PCR conditions, particularly numbers of PCR cycles and $MgCl_2$ concentrations, can affect detection of individual antibiotic resistance genes, skewing characterization of the resistome actually present in a microbiome. To test this hypothesis, we evaluated the effect of numbers of PCR cycles and $MgCl_2$ concentration on the detection of *tet* genes that encode ribosomal protection proteins (RPPs). The previously published degenerate primer pair Ribo2-FW/Ribo2-RV detects 7 classes of RPP *tet* genes, namely *tet*(B(P)), *tet*(M), *tet*(O), *tet*(Q), *tet*(S), *tet*(T), and *tet*(W), and they have successfully been used in detecting RPP *tet* genes in various samples (Huys *et al.* 2005, Yu *et al.* 2005, Sharma *et al.* 2009). In the present study, the forward primer (Ribo2-FW) was altered so that the primer pair can detect the *tet*(32), *tet*(36), and *tet*(44) RPP genes which were discovered after the original Ribo2-FW/RV primers were designed (Melville *et al.* 2001, Whittle

et al. 2003, Abril, Brodard and Perreten 2010). Because this primer pair allows 10 different classes of RPP *tet* genes to be detected simultaneously, it can be useful in targeted metagenomic analysis of RPP *tet* genes. In the present study, the potential effects of different $MgCl_2$ concentrations and PCR cycle numbers on diversity and proportional distribution of RPP *tet* genes were systematically examined using a factorial design. Our results showed that both of the parameters can have significant effect on the RPP *tet* genes that can be detected. The findings of the present study may help design future studies and aid in interpretation of results.

Materials and Methods

Samples

Swine manure samples were collected from the swine farm of Agricultural Technical Institute of The Ohio State University, which is a conventional research farm utilizing tetracyclines for growth promotion, disease prevention or therapy. The swine manure samples were composted as previously described and have been used in a previous study to investigate the successions of *tet* and *erm* genes during composting (Wang *et al.* 2012). Briefly, a total of 3.3 kg swine manure was compost treated for 48 days. Fifty gram compost samples were collected on days 0, 17, and 48 and subsequently stored at -80°C prior to further analysis. Metagenomic DNA was extracted using the repeated bead-beating plus column purification (RBB+C) method as described previously and the DNA integrity was confirmed by agarose (0.8%) gel electrophoresis (Yu and Morrison 2004, Yu *et al.* 2005, Wang *et al.* 2012). In the present study, all the samples were pooled to create an 'average' sample with diverse classes of tetracycline genes for PCR detection.

Primers

The primer sequences, targets, annealing temperatures, and amplicon lengths are given in Table 1. Since the original primers Ribo2-FW and Ribo2-RV were published, three new classes of RPP *tet* genes, *tet*(32), *tet*(36) and *tet*(44), have been discovered. To expand the inclusiveness of the original Ribo2 primers, the sequences used to design the original Ribo2-FW and Ribo2-RV primers (Aminov, Garrigues-Jeanjean and Mackie 2001) were aligned with the *tet*(32), *tet*(36), and *tet*(44) sequences (respective accession numbers AJ295238, AJ514254, and FN594949) using ClustalX (Thompson *et al.* 1997). Then the new alignment of the RPP *tet* genes was aligned with the original Ribo2 primers. The Ribo2-RV matched the three new classes of RPP *tet*, but 2 of the 3 bases at the 3' end of the Ribo2-FW primer did not match the *tet*(32), *tet*(36), and *tet*(44) sequences. Thus, the Ribo2-RW was modified by introducing two degenerate bases at the 3' end. The modified Ribo2-FW primer (referred to as Ribo2_new_FW) was evaluated for specificity using BLASTn and analyzed for formation of secondary structure and primer dimers using the Integrated DNA Technologies SciTools Oligoanalyzer (Integrated DNA Technologies, Inc., Coralville, IA). Both RPP *tet* primers, Ribo2_new_FW and Ribo2-RV, were synthesized by Sigma-Aldrich (St. Louis, MO, United States).

PCR amplification of RPP *tet* genes

All PCR was performed using a 50 µl reaction volume with the same conditions except for varying $MgCl_2$ concentrations and thermal cycle numbers. All the PCR contained the same amount (4.83 ng per reaction) of the same pooled DNA samples. Four $MgCl_2$ concentrations (1.75 mM, 3 mM, 5 mM, and 7 mM) and three numbers of PCR cycles (20, 30 and 35) were tested in a 4 x 3 factorial design that resulted in 12 different combinations of PCR cycles and $MgCl_2$ concentrations. Each PCR was run in duplicate along with a single non-template control with water replacing the DNA template. The

following cycling conditions were used (Yu *et al.* 2005): Initial denaturation for 4 minutes (min) at 94°C, followed by 5 cycles of touchdown PCR consisting of 30 sec at 94°C, 30 sec at 57°C with 1°C $cycle^{-1}$ decrement, and 60 sec at 72°C. Thereafter, there were 20, 30 or 35 cycles of PCR, each cycle consisting of 30 sec at 94°C, 30 sec at 52°C and 90 sec at 72°C. There was a final elongation of 7 min at 72°C.

Cloning and sequencing

One clone library was constructed for each combination of cycle number and $MgCl_2$ concentration that yielded the expected band (30 cycles x 5 mM $MgCl_2$, 30 cycles x 7 mM $MgCl_2$, 35 cycles x 3 mM $MgCl_2$, 35 cycles x 5 mM $MgCl_2$, and 35 cycles x 7 mM $MgCl_2$). The amplicons were cloned using the TOPO TA cloning® kit for sequencing (Invitrogen, Life technologies, Grand Island, NY, United States) following the One Shot® Chemical Transformation Protocol. To maximize ligation efficiency, the 5-minute room temperature incubation step was extended to 15 minutes and 2 µl fresh PCR product was used. Clones were spread on Luria-Bertani (LB) plates containing 50 mg ml^{-1} ampicillin (Sigma-Aldrich, Saint Louis, MO, USA) and incubated for 24 hours at 37°C. Sixty colonies from each clone library were randomly picked and inoculated into 5 ml LB broth with 50 mg ml^{-1} ampicillin and grown overnight at 37°C. Positive clones were identified by growth in ampicillin based on the *amp* resistance gene on the pCR®4-TOPO® plasmid (Invitrogen, Life technologies, Grand Island, NY, United States).

Plasmid DNA extraction was performed using the QIAprep Miniprep kit (QIAGEN, Germantown, Maryland, United States) following the manufacturer's instructions. Positive clones that contained the *tet* insert were confirmed by PCR using the M13 primers (Invitrogen, Life technologies, Grand Island, NY, United States). Sanger sequencing was performed on clones using DTCS Quick Start Kit (Beckman Coulter, Brea, CA, United States) on the Beckman GeneomeLab sequencer. A 5-

minute heat treatment at 65°C was preformed prior to the cycling sequencing reaction per Beckman protocol recommendations, and 5 mM M13f primer was used for sequencing. Base calling was manually verified and poor sequences were discarded. The ends of each sequence read with poor base calling were trimmed off using BioEdit (Ibis Biosciences, Carlsbad, CA, United States). The sequences compared to GenBank sequences using Blastx to determine sequence identity to known *tet* genes in GenBank. The sequences that matched known *tet* genes were imported to Geneious version 5.6.4 (Geneious, Auckland, New Zealand) for alignment and tree construction. Trees were constructed using the Neighbor-joining method for each clone library. The sequence of ribosomal 5s binding protein L5 of *Thermus flavus* (GBIS77826.1) was used as the outgroup to root the tree. The trees were exported in Newick format for use in UniFrac analysis (Lozupone and Knight 2005). Based on weighted UniFrac analysis, the UniFrac Significance test was used to determine if the different PCR conditions (cycle numbers and $MgCl_2$ concentrations) resulted in different detection (genetic diversity and proportion) of RPP *tet* genes.

Results and Discussion

No PCR amplicons were detected on agarose gels when only 20 cycles (excluding the five touchdown cycles) of PCR were performed at any of the four $MgCl_2$ concentrations tested (data not shown). The abundance of RPP *tet* genes present in the pooled samples (containing both composted and uncomposted swine manure) might be too low to produce a visible PCR band after 25 cycles of PCR amplification. The expected PCR amplicons were not found at 30 cycles with 1.75 mM or 3 mM $MgCl_2$ and at 35 cycles with 1.75 mM $MgCl_2$ (data not shown). These results suggest that 1.75 mM $MgCl_2$ may be too low a concentration for reliable PCR detection of RPP *tet* genes in swine fecal

samples using the Ribo2_new_FW/Ribo2-RV primers. The other combinations of PCR cycle numbers and $MgCl_2$ concentrations (referred to as Cn/M combinations) yielded the expected PCR amplicons and the three new classes of RPP *tet* genes were also detected using the Ribo2_new_FW/Ribo2-RV primers (Figure 1). The new primer Ribo2_new_FW may be used in future studies to allow detection of all known classes of RPP *tet* genes.

Different numbers of high-quality *tet* sequences were obtained from the different Cn/M combinations, ranging from 41 to 55 (Figure 1). To compare the proportions of all the detected *tet* gene classes among the Cn/M combinations, the proportion of all the detected *tet* gene classes were normalized to a sum of 100% for each Cn/M combination. Differences in diversity (the classes detected) and relative abundance (proportions) were found among the different Cn/M combinations (Figure 1 and Table 2). As hypothesized, the number of RPP *tet* gene classes detected increased with increasing PCR cycles and $MgCl_2$ concentrations, with four, six, seven, eight and nine classes of RPP *tet* genes detected at 30/5, 30/7, 35/3, 35/5, and 35/7, respectively. The 35/5 combination yielded four DNA sequences that did not match *tet* genes. Interestingly, no non-specific sequences were found with the other Cn/M combinations including the least stringent 35/7 combination. This suggests that the increase in cycle number and $MgCl_2$ concentration would necessarily increase the likelihood of non-specific amplicons. UniFrac Significance test revealed significant differences in the detection of RPP *tet* genes with respect to numbers of classes detected and their relative abundance among the Cn/M combinations (Table 2).

The classes of *tet* genes detected and their proportions were affected to different degree by the different combinations of the PCR cycle numbers and the $MgCl_2$ concentrations (Figure 1). The *tet*(M), *tet*(32) and *tet*(36) genes were detected in all the PCR cycle number and $MgCl_2$ combinations with *tet*(M) being the most predominant class followed by *tet*(36) and *tet*(32) in all the Cn/M combinations.

The high *tet*(M) prevalence may be attributed to *tet*(M) being found in both Gram-positive and Gram-negative bacteria and having the broadest host range of all *tet* genes (Yu *et al.* 2005). The *tet*(S) was not detected in the pooled swine manure samples by any of the Cn/M combinations, while *tet*(O) and *tet*(Q) became detectable at 30 cycles when $MgCl_2$ concentration was increased from 5 to 7 mM. At 35 PCR cycles, the increase in $MgCl_2$ concentration from 3 to 7 mM also resulted in detection of increased *tet* gene classes. At 5 mM $MgCl_2$, simply increasing PCR cycle numbers from 30 to 35 enabled four additional classes of *tet* genes to be detected (W, O, B/P, and T). At 7 mM $MgCl_2$, the increase in PCR cycle numbers from 30 to 35 also resulted in detection of three additional classes of *tet* genes that were otherwise not detected. Variations in cycle number and $MgCl_2$ concentration also affected the proportion of the *tet* genes detected but to a different extent for different *tet* gene classes. For instance, the 2 mM increase (from 5 to 7 mM) in $MgCl_2$ concentration at 30 cycles increased the proportion of *tet*(44) and *tet*(36) at the expense of *tet*(32). At 35 cycles, increase in $MgCl_2$ concentration also affected the proportion of the *tet* gene classes detected but to different degrees for different *tet* gene classes. Such differential effect was also observed for the increase in PCR cycle number. For example, at 5 mM $MgCl_2$ concentration, the increase in PCR cycles from 30 to 35 increased the proportion of *tet*(44) and *tet*(T) while decreasing that of *tet*(M). Differences in internal sequences among the *tet* gene classes may be one factor that contributes to difference in amplification efficiency and thus detection and proportion. In addition, a degenerate primer contains a pool of different primers. The proportion of a particular primer sequence within that pool can also affect primer annealing kinetics and subsequent amplification efficiency.

The original Ribo2-FW/RV primer pair was used in PCR analysis of swine manure that used 2 mM $MgCl_2$ and 25 PCR cycles (Aminov, Garrigues-Jeanjean and Mackie 2001). In that study, only three classes of RPP *tet* genes, *tet*(M), *tet*(O), and *tet*(W), were found. In another study using the

original primer set, 1.75 mM $MgCl_2$ and 5 touchdown PCR cycles followed by 30 cycles of regular PCR were used in qPCR to quantify total RPP *tet* genes (Yu *et al.* 2005). Due to the alteration in the forward primer and the different sample types, the results from those studies cannot be directly compared to the results of the present study. Nevertheless, the current results indicate that together with touchdown cycles, increasing the cycle number to 35 and $MgCl_2$ concentration to 7 mM may help detect the RPP *tet* genes present in samples without false negative results or compromising the specificity. In addition, although the effects of PCR cycle numbers and $MgCl_2$ concentrations, among other factors, are conceivable, the results of the present study demonstrate the importance of conducting pilot studies investigating PCR assay parameters, such as cycle number and $MgCl_2$ concentration, in order to establish the optimal conditions to accurately depict the resistome. Furthermore, interpretation of research results within one study and comparison of results among different studies should be exercised with caution because the PCR conditions used can significantly affect the analysis results.

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Conflict of Interest

No conflict of interest declared.

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Figure legend

Figure 1. The classes of RPP *tet* detected in the swine manure and their proportion using different PCR cycle numbers and $MgCl_2$ concentrations. a) 30 cycles and 5 mM $MgCl_2$ (n=47), b) 30 cycles and 7 mM $MgCl_2$ (n=41), c) 35 cycles and 3 mM $MgCl_2$ (n=51), d) 35 cycles and 5 mM $MgCl_2$ (n=49), e) 35 cycles and 7 mM $MgCl_2$ (n=55). For comparison purpose, the proportions of the individual classes of RPP *tet* genes detected was normalized so that the total RRP *tet* gene summed to 100%.

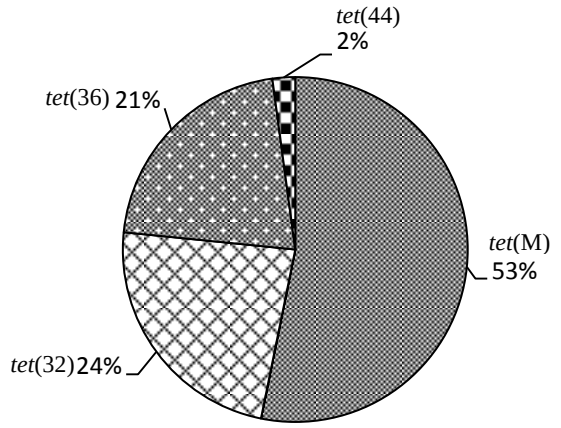
Table 1 PCR primer sequences, targets, annealing temperatures, and amplicon length

Primer	Class targeted	Primer sequence 5'→3'	Annealing temperature °C	Amplicon size (bp)	Reference
Ribo2_new_FW	M, O, W, P, Q, S, T, 32, 36, 44	IYYIAAYCCDTWYTGGGC	Touchdown[a]	233	(Aminov, Garrigues-Jeanjean and Mackie 2001)
Ribo2_RV	M, O, W, P, Q, S, T, 32, 36, 44	TCIGMIGGIGTRCTIRCIGGRC			This study

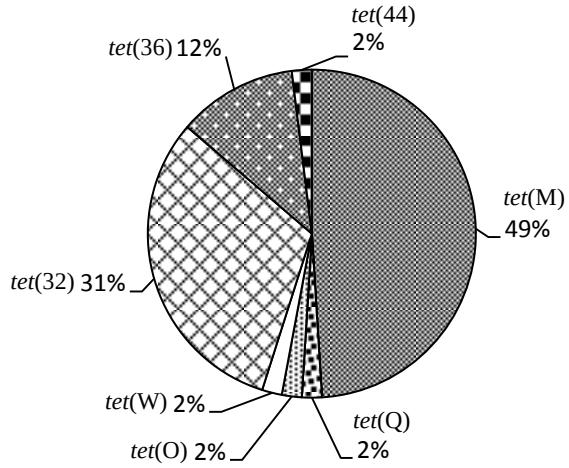
Table 2 Matrix showing of the UniFrac Significance test

Cn/M*	30/5	30/7	35/3	35/5	35/7
30/5		0.3600	0.3670	0.0600	0.0000
30/7			0.0300	0.3100	0.1000
35/3				0.0000	0.0000
35/5					0.0200
35/7					

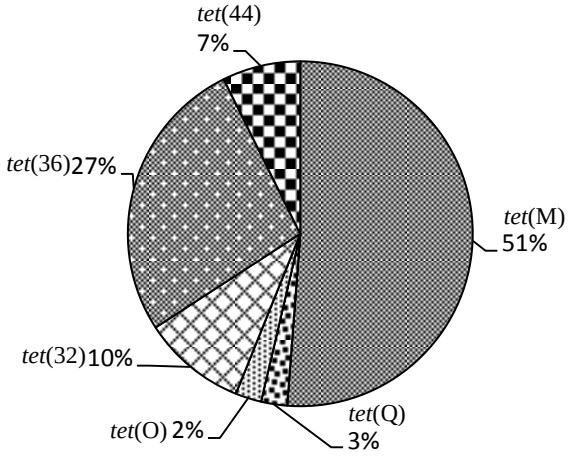
*Cn/M: PCR cycle number/$MgCl_2$ concentration (mM).


tet(44)
2%
tet(36) 21%
tet(M)
53%
tet(32)24%

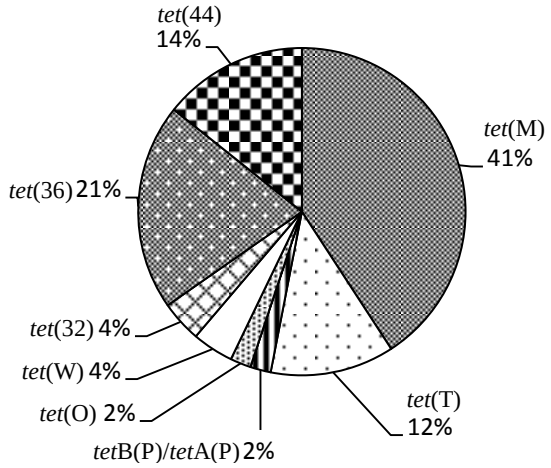
a


tet(44)
2%
tet(36) 12%
tet(M)
49%
tet(32) 31%
tet(W) 2%
tet(Q)
2%
tet(O) 2%

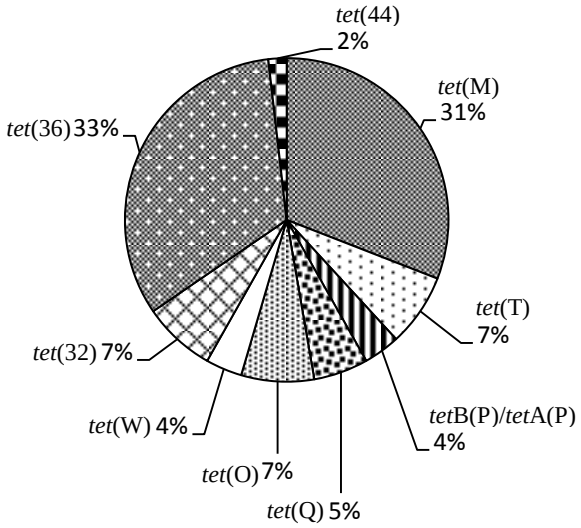
c


tet(44)
7%
tet(36)27%
tet(M)
51%
tet(32)10%
tet(O) 2%
tet(Q)
3%

b


tet(44)
14%
tet(M)
41%
tet(36) 21%
tet(32) 4%
tet(W) 4%
tet(O) 2%
tet(T)
12%
tetB(P)/tetA(P) 2%

d


tet(44)
2%
tet(M)
31%
tet(36)33%
tet(T)
7%
tet(32) 7%
tet(W) 4%
tetB(P)/tetA(P)
4%
tet(O)7%
tet(Q) 5%

e

MANUSCRIPT II

Gunilla Veslemøy Schmidt, John Elmerdahl Olsen, Lasse Engbo Christiansen, Marie Ståhl, Anders Mellerup, Øystein Angen, 2014.

Comparing Antibiotic Resistance Gene and Antibiotic Resistant Bacteria Estimates in Fecal Samples Collected Using Different Sampling and Pooling Methods in a Swine Herd.

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Quantification of Antibiotic Resistance in a Swine Herd.

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Abstract

Aims: To develop and validate 14 qPCR assays for antibiotic resistance gene quantification, and to compare estimation of antibiotic resistance levels in a swine herd using these qPCR assays and traditional quantification of resistant coliform indicator bacteria.

Methods and Results: The validated qPCR assays were used to quantify antibiotic resistance genes in total DNA from 84 swine fecal samples that were obtained using a selection of different sampling and pooling methods. Results were compared to resistance levels determination by use of coliform indicator bacteria. The results showed that the qPCR assays were capable of detecting differences in antibiotic resistance levels in individual animals that the coliform bacteria colony forming units (CFU) could not. Also, the qPCR assays accurately quantified antibiotic resistance genes when comparing individual sampling and pooling methods.

Conclusions: This study has validated qPCR assays that can be used to quantify antibiotic resistance genes in total DNA extracted from swine feces. The results indicate that regardless of the sampling and/or pooling method, there is a great deal of variation in the antibiotic resistance gene abundance within individual animals, pens, stables, and herds. This variation should be evaluated in greater detail.

Significance and impact of the Study: The use of qPCR is a promising tool in antibiotic resistance surveillance as it can capture variation between samples that is not detected by use of indicator bacteria.

Keywords: qPCR, swine, herd, feces, Genomic-DNA, sampling, pooling

Introduction

Antibiotic resistance in pathogenic bacteria is an increasing problem challenging disease treatment in humans and animals globally (Aarestrup 1999, McGowan Jr 2001, World Health Organization 2002). Resistant bacteria survive in the presence of high concentrations of antibiotics due to genetic alterations, of which the most feared are transferrable resistance genes (Roberts 1996, van den Bogaard and Stobberingh 2000, Chopra and Roberts 2001, Normark and Normark 2002, Aminov and Mackie 2007, van Hoek *et al.* 2011, Canton, Gonzalez-Alba and Galan 2012).

It is important to minimize antibiotic use in intensive agricultural practices where widespread antibiotic use is common e.g. in the pig production, as such use can have severe consequences for human health. As resistance is selected for in both commensal and pathogenic bacteria during antibiotic treatment, it is of major concern if commensal bacteria become a reservoir of antibiotic resistance genes for pathogenic bacteria (Sunde *et al.* 1998, Aarestrup 1999, van den Bogaard and Stobberingh 2000, Schwarz, Kehrenberg and Walsh 2001). Resistance genes can spread from commensal bacteria to zoonotic bacteria or to human-specific pathogens; if humans are infected with these bacteria or pathogens, this may complicate disease control and challenge treatment (van den Bogaard and Stobberingh 2000). Other significant consequences that can be ascribed to antibiotic resistance include production and economic losses and negative effects on general welfare (McGowan Jr 2001, World Health Organization 2002).

Surveillance of the presence of antibiotic resistant bacteria in individuals, populations and/or the environment facilitates risk management and may also support correct choice of drug for disease treatment. The traditional phenotypic methods for surveillance of antibiotic resistance in populations rely on cultures of indicator bacteria such as *Escherichia coli*, *Enterococcus* spp., *Salmonella* spp., and *Campylobacter* spp.. However, they neglect the remaining intestinal microflora and potentially underestimate the true antibiotic resistance levels in the bacterial community (Alali *et al.* 2009).

The amount of resources put into the development of nucleic acid-based methods utilizing total bacterial community DNA for antibiotic resistance detection has increased vastly (Fluit, Visser and Schmitz 2001, Alali *et*

al. 2009). These methods enable detection and quantification of antibiotic resistance, also in the slow-growing or non-cultivable bacteria that are neglected by the current phenotypic methods. Multiple target genes of interest can easily be targeted within the community where bacteria could be sharing resistance determinants thus reflecting the entire gene pool (Alali *et al.* 2009). Furthermore, it may also be possible to detect resistance genes earlier than is possible using phenotypic methods as cultivation of fast-growing bacteria such as *Escherichia coli* or *Salmonella* can take 1-2 days and for cultivation of slow-growing bacteria like *Mycobacterium tuberculosis* can take up to several weeks (Schmieder and Edwards 2012). In contrast, nucleic acid-based detection of antibiotic resistance can provide results within hours (Schmieder and Edwards 2012), and may also be quantitative. Real-time PCR (qPCR) enables quantification of the amount of genetic material in the sample. This principle has been used to quantify antibiotic resistance genes in fecal samples and fecal contaminated environments (Yu *et al.* 2005, Chen *et al.* 2007, Alali *et al.* 2009, Knapp *et al.* 2010).

At present, no good strategy has been published for quantification of resistance levels in animals at herd level. When designing such a strategy, it is important to consider the combination of sampling and analytical methods in order to gain representation of the true resistance level, whilst a feasible time frame and economic resources must be maintained.

The aim of the study was to develop and validate qPCR assays for antibiotic resistance quantification in swine herds that ultimately can contribute to antibiotic resistance surveillance. In the absence of a golden standard, herd level antibiotic resistance estimates by qPCR and resistance levels in coliform bacteria were compared in swine fecal samples collected at different levels (with and without pooling) and subsequently compared to each other. A total of 14 antibiotic resistance determinants were included in the qPCR and compared to the level of phenotypic resistance to sulphametizole, erythromycin, ampicillin, and tetracycline among coliform bacteria, representing the antibiotic classes that are commonly used in pig production in Denmark. Furthermore, sampling strategies were assessed including individual animal sampling, pen floor sampling, shoe cover sampling, slurry tank sampling, and laboratory pooling of the samples. We hypothesize that the qPCR assays will quantify antibiotic resistance genes in swine feces and detect differences in resistance

levels between sampling and pooling methods not detected by phenotypic resistant coliform colony forming unit (CFU) counts.

Materials and Methods

Sample collection

Pig fecal samples were collected from a single feeder pig operation in Denmark at 2 separate time points (sampling 1 and 2) that were 4 months apart. The collected samples, their sizes and corresponding pools are summarized in Table 1.

During sampling 1, fecal samples were collected from a single stable (stable 1) within the feeder pig operation. In the stable, 4 pens (pens 1 -4) were randomly chosen, and within these 4 pens all pigs (pen 1 n=22, pen 2 n=19, pen 3 n=21, pen 4 n=19; total n=84) were individually sampled by digitally extracting feces from the rectum. Furthermore, each of the 4 pens was sampled by pooling 5 separate samples of feces from the floor of each pen (pen floor samples, n=4). Four shoe cover samples were also collected in the same 4 pens. Two people each wore a pair of blue disposable polypropylene shoe covers (SEPA, Hilleroed, Denmark) and the pen area was covered by walking throughout the entire pen in a systematic snake formed pattern thus covering as much of the pen floor as possible. The same shoe covers were repeatedly used in all 4 pens and were removed when walking between pens.

During sampling 2, a total of 5 stables were sampled (stables 1–5; Table 1). Again, 4 pens were randomly chosen in each stable and sampled (n=20). The pen floor samples and shoe cover samples were collected in each stable as described above with the exception that 2 shoe cover samples were collected for each stable (pen floor samples n=20; shoe cover samples n=10) instead of 4. Furthermore, slurry tank samples were taken at 1 m, 1.5 m, and 2 m depths from 3 sampling spots that were spaced approximately 50cm apart. One ml from each of the 3 depths was mixed for each corresponding sampling spot and homogenized by vortexing. Each slurry tank sample thus consisted of a pool of the 3 depths (denoted as slurry tank 1, slurry tank 2, and slurry tank 3; total n=3).

All samples were collected in plastic containers with tight lids or sealed plastic bags and were placed in coolers immediately after sampling. They were then stored at 5ºC until analysis in the laboratory the following day. Thereafter, all samples were frozen at -80ºC.

Sample Processing

The non-sampled shoe cover weight was subtracted from the sample shoe cover weights, and defined amounts of PBS buffer were added to the sampled shoe covers. This dilution was corrected for in the statistical calculations. The shoe cover samples were then vigorously vortexed for 1 minute in order to extract as much sample from the shoe cover as possible. Thereafter, each shoe cover was strung to extract the remaining liquid. A 10^{-1} dilution was made from the resulting solutions by mixing 1 ml of the shoe cover sample solution with 9 ml PBS buffer.

Pooled samples were created in the laboratory as outlined in Table 1 and consisted of: 1) Pool of all individual animals from sampling 1 (All Animals), 2) Pools of animals within each pen (individual animal pool pen), 3) Pool of pen floor samples from pens 1-4 in each stable (pen floor pool stable), 4) Pool of the shoe cover samples in each stable (shoe cover pool stable), 5) Pool of pen floor samples from each stable (pen floor pool herd), 6) Pool of shoe cover samples from each stable (shoe cover pool herd), 7) Slurry tank samples (pool slurry). Before pooling the samples in the laboratory, a 10^{-1} dilution of the sample was made by suspending 1 g of feces in 9 ml PBS buffer. The pool samples were made by mixing 1 ml of the 10^{-1} resolution for each corresponding sample included in the pool.

CFU counts of coliform bacteria

Ten-fold dilutions from each sample were made in PBS buffer. One drop (20 µl) of each dilution was carefully placed on MacConkey plates (Oxoid) without and with antibiotics (Ampicillin (16 mg l^{-1}); Erythromycin (32 mg l^{-1}); Sulphametizole (256 mg l^{-1}); Tetracycline (16 mg l^{-1})) and incubated at 37°C for 24 hours.

DNA extraction for quantification by qPCR

DNA was extracted from the 10^{-1} dilutions. Sample preparation consisted of homogenization of the 10^{-1} dilutions by vigorous vortexing with a 5 mm stainless steel bead (Qiagen, Copenhagen, Denmark). Thereafter, 350 µl of the 10^{-1} dilutions was transferred to a new eppendorf tube and put on ice. The samples were then lyzed for 1 minute (15 Hz at room temperature) in a Tissuelyser II (Qiagen, Copenhagen, Denmark) followed by centrifugation for 90 seconds at 10000 rpm. The supernatant (300 µl) was transferred to a new eppendorf tube and 20 µl Proteinase K (Promega, Roskilde, Denmark) was added (not on ice). The samples were then immediately loaded into the QiaSymphony robot using the QiaSymphony DSP Virus/Pathogen Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions. The final elution volume was 85 µl.

Both a negative and positive DNA extraction control was run in parallel with the samples during each DNA extraction. The negative extraction control was water, and the positive extraction control was a modified pig feces sample positive for the majority of antibiotic resistance genes included in this study. The genes that were not present in this sample, defined as having a cycle number (Cq)>30 in qPCR, were spiked into the sample by adding 100 µl 10^5 amplicons μl^{-1} to a final volume of 1500 µl extraction control. This corresponded to Cq values between 20 and 30.

Primers and probes

Primers (Table 2; Table S1) for resistance genes were synthesized by TAG Copenhagen A/S (Frederiksberg, Denmark) and for 16S rDNA by DNA Technology A/S (Aarhus Denmark). Probes (Table 2) were synthesized by Applied Biosystems (Life Technologies, Naerum, Denmark).

The available antibiotic resistance determinant sequences were retrieved from GenBank (during 2011-2012), narrowed down to pig intestinal commensals, and finally aligned using ClustalX (Thompson *et al.* 1997). The conserved regions were used for primer and probe design using Primer3Plus Web Interface (Free Software Foundation, Boston, MA, USA). Previously published primers with annealing temperatures of 60-61° C and

probes with annealing temperatures of approximately 70° C were used and modified if necessary (Table 2). Potential primer and probe sequences were used to query GenBank DNA sequences using Basic Local Alignment Search Tool nucleotide (BLASTn) to determine specificity. The primer and probe sequences that matched the desired antibiotic resistant determinants were analyzed using Integrated DNA Technologies SciTools Oligoanalyzer (Integrated DNA Technologies, Inc., Coralville, IA, USA). Wherever necessary, degenerate bases were introduced into the primer/probe sequences to match all the sequences in the alignments.

Generation of amplification standards

Amplicon standards from antibiotic resistance genes included in the present study were derived from bacterial strains or pig fecal samples (Table S2). The bacterial strains positive for tetracycline and beta-lactam antibiotic resistance determinants were kindly provided by Yvonne Agersø (National Food Institute (DTU-FOOD), Lyngby, Denmark), those positive for sulphonamide resistance determinants and *ermB* by Anette M. Hammerum (Statens Serium Institut (SSI), Copenhagen, Denmark), the bacterial strain positive for *ermF* by Stefan Schwarz (Friedrich-Loeffler-Insitut (FLI) Neustadt-Mariensee, Germany), and the strain positive for *vanA* from Luca Guardabassi (Faculty of Medical and Health Sciences (SUND), University of Copenhagen). The fecal samples were provided by The Veterinary Institute, The Technical University (DTU-VET), Frederiksberg, Denmark.

Total DNA was extracted from the bacterial strains using Invitrogen-easy DNA kit (Invitrogen, Life Technologies, Naerum, Denmark) and from the fecal samples as described for the fecal samples collected for qPCR analysis. The amplicons were generated using the primers in Table S1 using a T3000 thermocycler (Biometra, Göttingen, Germany). The PCR Mastermix for *tet*(B), tet(C), *tet*(M), tet(O), *tet*(W), *sulI*, *sulII*, and *erm*B amplicon generation had the following concentrations per 25 µl reaction volume: 250 µM deoxynucleotide triphosphates (dNTPs), 1X buffer, 1.5 mM $MgCl_2$, 0.5 µM of each forward (FW) and reverse (RV) primers, 1.25 U Platinum® *Taq* DNA Polymerase (Invitrogen, Life Technologies, Naerum, Denmark) plus 2 µl DNA. Cycling conditions were: Initial denaturation for 4 min at 95º, followed by 30 cycles PCR, each cycle consisting of 15 sec at 94º, 30 sec at 58º and 60 sec at 72º. There was a final extension for 5 min at 72º.

tet(A) amplicons were generated in a 25 µl reaction volume containing 250 µM dNTPs, 1X buffer, 1.50 mM $MgCl_2$, 0.5 µM of each FW and RV primers, 1.25 U Platinum® *Taq* DNA Polymerase (Invitrogen, Life Technologies, Naerum, Denmark) plus 2 µl DNA. Cycling conditions were: Initial denaturation of 5 min at 95ºC, followed by 23 cycles of PCR, each cycle consisting of 30 sec at 95ºC, 30 sec at 62ºC, and 45 sec at 72ºC. There was a final elongation of 7 min at 72ºC (Guardabassi *et al.* 2000, Saenz *et al.* 2004, Costa *et al.* 2008).

ermF amplicons were generated in a 25 µl reaction volume containing 250 µM dNTPs, 1X buffer, 1.75 mM $MgCl_2$, 0.5 µM of each FW and RV primers, 1.25 U Platinum® *Taq* DNA Polymerase (Invitrogen, Life Technologies, Naerum, Denmark) plus 2 µl DNA. Cycling conditions were: Initial denaturation of 10 min at 95ºC, followed by 45 cycles of PCR, each cycle consisting of 15 sec at 94ºC, and 30 sec at 60ºC.

16S amplicons were generated in a 50 µl reaction volume containing 0.1 mM dNTPs, 1X buffer, 1.5 mM $MgCl_2$, 1.3 µg n^{-1} log each FW and RV primer, 0.01 U Platinum® *Taq* DNA Polymerase (Invitrogen, Life Technologies, Naerum, Denmark) plus 2 µl DNA. Cycling conditions were: Initial denaturation of 3 min at 94ºC, followed by 35 cycles of PCR, each cycle consisting of 1 min at 94ºC, 1 min at 55 ºC, and 30 sec at 72ºC. There was a final elongation for 5 min at 72 ºC.

$bla_{CTX\text{-}M\text{-}1}$ group, bla_{SHV} family, $bla_{CMY\text{-}2}$ and *vanA* amplicons were generated as previously described (Hasman *et al.* 2005, Archambault *et al.* 2006, Agersø *et al.* 2012).

Amplicon lengths were confirmed by gel electrophoresis and gene copy numbers were calculated after DNA quantification by UV spectrophotometry using a NanoDrop 3300 (Thermo Scientific, Wilmington, DE, USA). The identity of all standard amplicons were further verified by sequencing from both ends using the BigDye®Terminator v3.1 Sequencing Kit on a 3130 Genetic sequencer (Applied Biosystems, Life Technologies, Naerum, Denmark).

The standards for copy number determinations were prepared by serially diluting PCR product amplicons in nuclease-free yeast tRNA (1:100 tRNA dilutions of 10 mg mL^{-1} (Applied Biosystems, Life Technologies, Copenhagen, Denmark)). The limit of quantification (LOQ) was defined as the lowest point in the

amplicon standard serial dilution where all triplicates were positive. The limit of detection (LOD) was defined as the lowest concentration in the amplicon standard serial dilution where at least 1 of the triplicates was positive.

Internal amplification control generation for qPCR assays

Internal control amplicons (ICA) consisting of lambda (λ) phage DNA flanked by the forward (FP) and reverse (RP) primer sequences of the respective antibiotic resistance genes were included in the respective qPCR assay as an internal amplification control (IC). The primers used were the antibiotic resistance qPCR assay primers (Table 2) with a λ phage DNA sequence added to the 3´end (extra sequences 5'-3'direction: FP ATGAATATGACCAGCCAAC, RP TTCACGCAGGGGAAATATCTTTC) (Angen *et al.* 2011). The ICAs were generated on a T3000 thermocycler (Biometra, Göttingen, Germany) in a reaction volume of 50 µl with 50 µM $MgCl_2$, 1X Buffer, 10 µM dNTPs, 50 µM of each forward and reverse primers, 1.25 U Platinum® *Taq* DNA Polymerase (Invitrogen, Life Technologies, Naerum, Denmark), and 1 µL λ DNA (1 ng μl^{-1})(Invitrogen, Life Technologies, Naerum, Denmark). The cycling conditions were: 5 min incubation period at 94°C followed by 10X touchdown cycles from 58°C, each touchdown cycle consisting of 1 min at 94°C, 1 min at 58°C, and 1½ minute at 72°C.Thereafter, there were 5 cycles, each with 1 min at 94°C, 1 min at 48°C and 1½ min at 72°C followed by 8X touchdown cycling from 48 °C, each touchdown cycle consisting of 1 min at 94°C, 1 min at 48°C and 1½ min at 72°C. Next there were 12 cycles, each with 1 min at 94°C, 1 min at 40°C, and 1½ min at 72°C. Finally, there was an elongation of 10 min at 72°C.

The ICA length of 690 bp was verified by gel electrophoresis and the ICA was serially diluted to 10^{-11}. All antibiotic resistance gene qPCR assays were run on Rotorgene thermocyclers (Rotorgene Q-5plex and Rotorgene Q (Qiagen, Copenhagen, Denmark) with a λ DNA easy probe (Applied Biosystems, Life Technologies, Naerum, DK) and ICA added to the mastermix. In order to determine the concentration of ICA to use as an internal control the following was tested: 5 separate mastermixes with ICA PCR product dilutions within 10^{-3} to 10^{-11} and 1 "no internal control" were tested against the lowest 3 concentrations detectable by the respective antibiotic resistance gene qPCR assay. The ICA dilutions that did not inhibit the respective antibiotic

resistance gene qPCR assays were used as the internal controls, where the λ DNA easy probe was detected on the yellow channel (530-555 nm) and the antibiotic resistance gene qPCR probes on the green channel (470-510 nm).

Quantification of antibiotic resistance genes in pig fecal samples by qPCR

The qPCR amplicon sequences generated from pig fecal DNA were validated. Due to the short amplicon lengths sequencing attempts failed. Therefore, the amplicon products were confirmed using the High Sensitivity DNA chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Walbronn, Germany). The qPCR assays were also tested for cross reaction by running each qPCR assay against a panel of 16 different antibiotic resistant gene amplicons at a concentration of $2x10^4$ copies μl^{-1}.

Quantitative PCR amplifications for the quantification of *tet*(A),*tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W), *ermF*, *ermB*, *sulI*, *sulII*, $bla_{CTX-M-1}$ group, bla_{CMY-2}, bla_{SHV} family, *vanA* and 16S in total DNA extracted from pig fecal samples were performed with Rotorgene thermocyclers (Rotorgene Q-5plex and Rotorgene Q, 72-well rotor 1-72) (Qiagen, Copenhagen, Denmark). The mastermixes are depicted in Table 3 and cycling conditions were: 10 min incubation period at 95 ºC followed by 45 cycles of PCR, each cycle consisting of 15 sec at 94 ºC and 30 sec at 60 ºC with a single fluorescence reading at green and yellow channels at the end of the extension stage. Each sample was tested in duplicate, along with a single point from the tenfold dilution series of the specific standard in triplicate, a single negative template control (NTC) that was 23 µl mastermix and 2 µl water, and 1 positive and negative DNA extraction control.

Quantification was performed using standard curves obtained from the PCR generated positive controls. The following samples were screened where none contained low enough levels of the tested antibiotic resistance genes to be spiked for standard use (data not shown): 20 individual pigs from a single herd, 1 individual pig from 20 different herds, and feces from miniature pigs that never had received antibiotics. A final attempt was made by treating pig fecal samples with gamma rays as earlier described (Hoelzel *et al.* 2010). Unfortunately, the gamma ray-treated feces inhibited almost all the assays that were tested (data not shown). Therefore, the qPCR

standards were created by serially diluting the target gene templates in nuclease-free yeast tRNA (Applied Biosystems, Life Technologies, Copenhagen, Denmark)).

The impact of pig fecal environment on quantification

The impact of pig fecal environment DNA on the quantification of the respective antibiotic genes was tested by running nuclease-free yeast tRNA template serial dilutions in parallel to pig fecal DNA spiked with antibiotic resistance gene template serial dilutions.

Statistical analysis

All figures and statistical tests were completed using R software (Version 3.0.1). Differences in gene copy numbers gram^{-1} feces and in coliform bacteria CFU counts between pens from sampling 1 were calculated using Kruskal-Wallis rank sum test.

Results

Coliform bacteria counts in fecal samples and application to assess sampling and pooling strategies

CFU counts of coliform bacteria were chosen as the phenotypic indicator of antibiotic resistance in the collected swine fecal samples. Figure 1 (top) shows a boxplot over the CFU counts of resistant coliform bacteria in the individual animals within the 4 pens from sampling 1. Erythromycin and sulphonamide had the lowest CFU counts median values. Tetracycline's levels were also low, but a large variation was observed in pens 1 and 2. There was a significant difference between pens for the ampicillin resistant CFUs only ($P<0.05$; data not shown).

Next, the pooled samples of individual animals within pens were considered (Figure 1, top, solid circles). The individual animal pool pen samples on the plates without antibiotics were relatively close to the median in all pens, except pen 3 where the pool was above the 75th quartile. Generally, the individual animal

pool pen samples were largely dispersed compared to the median of corresponding non-pooled individual animal samples. This was most prominent in the erythromycin, sulphonamide, and tetracycline groups.

Figure 1 (bottom) depicts the pen floor, shoe cover, and individual animal coliform CFU counts for each pen from sampling 1. The respective stable pools are also included (pen floor pool stable, shoe cover pool stable, and individual animal pool stable). There was a general large variation within each sampling method with the pen floor samples having the most prominent. The pen floor CFU counts were found to be below the pen floor pool stable for all groups except erythromycin. The shoe cover and individual animal stable pools either lie among or below their corresponding pen pools.

There was no correlation between the total coliform counts and the total number of bacteria in the population represented by the 16S copy number (R^2=0.1; data not shown). Figure 2 illustrates the relative standard deviations of the fecal estimates of the coliform CFU counts and qPCR gene copy numbers. The qPCR gene copy numbers have lower relative standard deviations compared to the coliform CFU counts. For example, when regarding the coliform CFU counts, the estimated relative standard deviation was under 20% for only 50% of the cases, while the relative standard deviation of qPCR gene copy numbers was under 20% for 90% of the cases.

Accuracy of qPCR assays

Standard curves for qPCR were generated using the serial dilutions of the amplification standards. The dynamic ranges of the antibiotic resistance gene assays were all linear over a measurement range >7 orders of magnitude and 5 orders of magnitude for the 16S rDNA assay (Table S3). The amplicon standard serial dilutions were used for determining the linear dynamic range where R^2=0.99, efficiency = [0.90; 1.10] and M≈-3.2. The efficiencies of the qPCR assays, determination coefficient (R^2), dynamic range, quantification - and detection limits are all summarized in Table S3.

The qPCR primers did not give unspecific reactions when tested against a panel of 16 different antibiotic resistance gene amplicons at 2x10^4 copies μl^{-1}. The qPCR products generated from pig fecal samples were accurate when lengths were confirmed using the Agilent 2100 Bioanalyzer (data not shown).

The performance of the qPCR tests was further evaluated using extracted DNA from pig feces for spiking with antibiotic resistance genes. The efficiency remained between [0.90;1.10] and R^2 above 0.99 for all assays except for *tet*(A) which showed an efficiency of 0.85, *tet*(M) with an efficiency of 0.84 and R^2=0.87, *sulII* with an efficiency of 0.88, *vanA* with an efficiency of 0.87, and 16S rDNA with an efficiency of 0.84. Varying degrees of inhibition were observed when spiking the amplicons in pig fecal DNA extracts (1 to 4 Cq value increase), indicating a slight assay specific inhibition (data not shown). However, the dynamic ranges remained linear over a measurement range >4 orders magnitude in the spiked pig feces DNA environment (data not shown).

Application of qPCR method to assess sampling and pooling strategies

The $bla_{\text{CTX-M-1}}$ group, $bla_{\text{CMY-2}}$, bla_{SHV}, and *vanA* antibiotic resistance genes were not detected in any samples during sampling 1 and sampling 2 and were therefore excluded from further analysis and all graphs. Furthermore, the geometric mean of the qPCR replicates was used for data analysis as the replication error then became independent of the gene copy number.

Generally, the 16S rDNA levels appeared to be stable regardless of the sampling and/or pooling methods for sampling 1 and sampling 2 (Figures 3 and 4). The tendencies described for the gene copy estimates in sampling 1 and 2 did not change after normalization with 16S rDNA (Figures S1 and S2). Therefore, only the absolute quantifications by qPCR were used for further data analysis.

Figure 3 (top) illustrates the copy number distribution of each gene for all animals sampled within each pen. Genes such as *sulII*, *ermB*, and *tet*(M) were relatively constant between pens while the genes *ermF*, *tet*(A), *tet*(C), *tet*(O), and *tet*(W) varied in at least 1 of the 4 sampled pens. Generally *ermB*, *ermF*, *tet*(O), and *tet*(W)

had higher copy numbers g^{-1} feces compared to *sulI*, *sulII*, *tet*(A)-*tet*(C), and *tet*(M). *sulI* gene copy estimates were particularly low while *tet*(B) had levels below the LOQ in pens 2 and 3.

The gene copy numbers of *ermB*, *ermF*, *tet*(C), *tet*(O), and *tet*(W) were significantly different between pens ($p<0.05$ for *ermB*, *tet*(C), *tet*(W); $p<0.0001$ for *ermF* and *tet*(O)). It appears that pen 1 consistently has lower gene copy number g^{-1} feces for *ermB*, *ermF*, *tet*(O), and *tet*(W) compared to pens 2-4 with *tet*(C) also having lower gene copy number g^{-1} feces in pen 2 (Figure 3 (top)). *ermF* in particular varied between pens with a large variation within pen 2. *tet*(A) generally had large variations within pens compared to the other genes with the highest levels in pens 1 and 3.

Figure 3 (bottom) depicts the distribution of each gene within pens 1-4 for the pen floor samples, shoe cover samples, pen floor pool stable, shoe cover pool stable, individual animal pool pen, and all animals. For all genes except for *ermF* and *tet*(C), there was a tendency for lower gene copy number estimates in the laboratory pools of individual animals within each pen when comparing to the pen floor samples. The shoe cover sample estimates were highest for *sulI*, *sulII*, *tet*(B), and *tet*(M), intermediate for *ermB* and *tet*(A), and low for *ermF*, *tet*(O) and *tet*(W). The shoe cover stable pools were consistently higher than the non-pooled shoe cover samples. Furthermore, the shoe cover samples were positive for *tet*(B) in pen 2 where the individual animal samples were negative. In contrast, there were no *ermF* individual shoe cover samples above the LOQ but there were positive individual animal samples in all four pens (Figure 3 bottom).

A comparison of results from sampling 2 including pen floor samples and shoe cover samples is shown in Figure 4 (top) together with the pooled samples from each category. Each section had up to 4 pen floor samples (1 from each pen) and 2 shoe cover samples with their respective laboratory pools. For *ermB*, *ermF*, *tet*(C), *tet*(O), and *tet*(W) the shoe cover samples had lower estimates compared to the pen floor samples with the pools following the same pattern. For *sulI*, *sulII*, *tet*(A), *tet*(B), and *tet*(M), however, the shoe cover samples were higher than the pen floor samples.

Figure 4 (bottom) compares the pen floor and shoe cover pools both at stable and herd levels. The pooled and individual slurry samples from sampling 2 are also depicted in Figure 4 (bottom). The assays that had

shoe cover samples with higher levels than the pen floor samples within stables 1-5 from sampling 2 (*sulI*, *sulII*, *tet*(A), *tet*(B), and *tet*(M) (Figure 3 top)) also had higher shoe cover samples in the corresponding stable and herd level pools (Figure 4 bottom). The slurry tank samples complemented the pen floor and shoe cover herd pools, although it appeared that 1 of the 3 slurry tank samples was consistently lower than the others for all genes. Furthermore, *sulII* became negative at the pen floor herd pool despite having positive values in 2 out of 5 stable pools, and *tet*(A) had negative pen floor herd pools for and shoe cover herd pools despite their positive stable pools. *tet*(B) had a single positive shoe cover sample in stable 2 (Figure 4 top) with no positive results for stable pools, herd pools, and slurry tank samples (Figure 4 bottom). *tet*(C) had positive pen floor samples and pen floor pool stable samples in all stables except stable 4 (Figure 4 top). The *tet*(C) pen floor pool herd sample was positive (Figure 4 bottom), but the shoe cover and slurry tank samples were all negative (Figure 4 top and bottom).

Discussion

Monitoring the antibiotic resistance patterns of infectious bacteria and their distribution aids disease prevention and control. Quantification of antibiotic resistance levels facilitates antibiotic resistance surveillance, ultimately helping to contain and prevent infections caused by antibiotic resistant bacteria. In the present study, 14 qPCR assays quantifying antibiotic resistant determinants in swine fecal samples were developed, validated, and compared to antibiotic resistance estimates from coliform bacteria CFU counts. This was done by applying both methods to swine fecal samples collected using different sampling and pooling methods. The main findings of our research are that, the qPCR method detected significant differences in antibiotic resistance where the coliform CFU counts showed no significance. This implicates that the coliform bacteria were not a good representative for the general resistance level. Furthermore, qPCR gene copy estimates in swine feces had reduced relative standard deviations compared to coliform CFU counts in the same samples (Figure 2), and therefore differences in antibiotic resistance levels between samples were more readily detected.

When observing the coliform CFU counts in individual animals between pens, only a single group (ampicillin) showed a significant difference in resistant coliform bacteria. In contrast, significance was found in qPCR gene copy estimates for erythromycin and tetracycline, while no beta-lactamase genes were detected. The phenotypic ampicillin resistance could be due to other ampicillin resistance encoding genes than those included in the present study emphasizing a key limit to the qPCR method, namely not all antibiotic resistance genes are included. In contrast, the phenotypic CFU counts are limited to coliforms representing only a fraction of the intestinal bacterial population. The lacking correlation between the 16S rDNA gene copy number g^{-1} feces and the control coliform bacteria CFU counts (R^2=0.1; data not shown) demonstrates how the two methods represent each their population. When monitoring antibiotic resistance, it is favorable to quantify the genes by qPCR instead of relying on phenotypic determination. This ensures that the entire bacterial population is represented while the denoted genes of interest (and therefore resistance) are also included. This principle is illustrated when assessing the coliform bacterial CFU counts in individual animals (Figure 1 top). Here, the erythromycin group had some of the lowest CFU counts overall. This contradicts the gene copy numbers g^{-1} feces for the corresponding *ermB* and *ermF* genes which are 3rd and 4th highest after *tet*(O) and *tet*(W) (Figures 3 and 4). A tentative conclusion from this is that the *ermB* and *ermF* genes reside in bacteria found in the intestines other than coliform bacteria (Gniadkowski *et al.* 1998, Heritage *et al.* 1999, Chanawong *et al.* 2001, Navarro *et al.* 2001, Baraniak *et al.* 2002, Li *et al.* 2007, Jacoby,G. A. 2013). The coliform bacteria CFU estimates may lead to underestimates of the true antibiotic resistance levels due to the limitations of the chosen indicator bacteria.

The CFU estimates of the individual animal pool pen samples were found not to represent an average of the non-pooled individual animal samples. This means that, at pen levels, the pooled samples from individual animals were not representative for the individual animals. There were also variations when comparing different sampling methods between pens (Figure 1, bottom) making it difficult to find differences between pens using coliform CFU counts. This is likely due to the large relative standard deviation found for each coliform CFU estimate (Figure 2). Therefore, only qPCR gene copy number g^{-1} feces were used to assess the different sampling and pooling methods.

The qPCR assays in the present study allowed simultaneous quantification of major antibiotic resistance determinants in entire microbiomes of pig fecal samples. The qPCR products from pig feces were determined accurate, and there was no cross reaction when each assay was tested against a panel of 16 different antibiotic resistance gene amplicons. The design of qPCR assays containing internal, positive- and negative-extraction controls ensured a thorough PCR inhibition and DNA extraction procedure verification.

Assay inhibition in the spiked pig fecal DNA samples compared to water was seen as lower efficiencies and higher corresponding Cq values in the spiked fecal DNA samples. This discrepancy between DNA from complex environmental samples has been described and was expected (Bibbal *et al.* 2007, Koike *et al.* 2007). The dynamic range remained linear with a minimum 4-fold magnitude in the spiked pig fecal DNA samples for all assays (data not shown) and R^2 remained above 0.99 in all but 1 assay. Therefore, the slight variation seen in the spiked fecal DNA samples compared to sterile water is not considered to notably alter the assays' performance in quantifying antibiotic resistance genes in pig fecal samples. We believe that the DNA extraction protocol, primer sets, and corresponding probes possess the characteristics necessary for application to antibiotic resistance gene quantification in pig fecal samples.

The qPCR assays revealed some interesting differences when applied to assess sampling and pooling strategies within a pig stable. When looking at the samples collected from individual animals, it appeared that pen 1 consistently had lower gene copy estimates for *ermB*, *ermF*, *tet*(O), and *tet*(W) compared to pens 2-4 with *tet*(C) also having lower estimates in pen 2 (Figure 3 top). These differences in gene copy number g^{-1} feces between pens were found statistically significant, and could mean that the antibiotic resistance genes do not easily spread between pens. The apparent variation in *tet*(A) estimates seen in Figure 3 (top) in pens 1 and 3 is caused by the graph only illustrating results from positive animals. Thus, few animals had high gene copy numbers (above $1x10^6$) where for half of them, 1 of the technical replicates was below the LOQ and therefore had no effect on the graph. This was solely seen for *tet*(A).

Bibbal *et al.* (2007) monitored the bla_{TEM} excretion in pigs and found that the fecal excretion of bla_{TEM} genes showed large, individual day-to-day fluctuations (Bibbal *et al.* 2007). Similar fluctuations in gene excretion could account for variations when quantifying antibiotic resistance genes. Antibiotic resistance is dynamic as its spread and maintenance is subject to fluctuations in host organism migration and/or persistence, antibiotic gene migration, and presence of selection pressure (Koike *et al.* 2007). From the point of excretion the gene must spread horizontally within the pen's bacterial population with subsequent spread of the gene containing bacteria to a new pig. Thus, when collecting for example rectal samples at a single time point, the level found in all the individual animals within a pen may depend on the time a single animal within the pen has excreted the specific gene. The individual animal pool pen samples are more uniform than the individual animal sampling, as all of the animals and their respective antibiotic resistance levels are represented (Figure 3, top). If a single fecal sample with high levels of antibiotic resistance levels is included in a pool, it will mask the samples containing lower gene levels. On the other hand, if there also are sufficient fecal samples with low gene levels then they will dilute the high level sample (Munoz-Zanzi *et al.* 2000).

One must consider the application of detecting antibiotic resistance gene copy number estimates in individual animals. For a health hazard to occur there must be intestinal-content contamination during slaughter. The intestinal content may come from a single animal with high levels of a specific antibiotic resistance gene, or from several animals with lower gene levels. The risk involved if 1 animal has many genes versus many animals with few genes must be taken into consideration. Furthermore, if we wish to sample at herd level, then it is not optimal to implement a method where an individual animal represents the entire herd. Regardless, the results indicate that the qPCR assays are capable of detecting differences in antibiotic resistance gene copy number g^{-1} feces in individual animals between pens.

For the majority of the genes, the individual animal pen pool resulted in lower gene copy number estimates when compared to the pen floor samples for each corresponding pen (Figure 3 and Figure S1). This could be due to the dilution effect of the increased volume in the individual animal pen pools which were composed from >19 samples compared to the pen floor samples that consisted of 5 individual samples (Munoz-

Zanzi *et al.* 2006). Furthermore, pen floor samples are collected from older feces that has resided on the ground permitting liquid evaporation and run off from the feces. This may result in a higher concentration of resistance genes in the sampled portion of the pen floor fecal sample and can be an advantage when quantifying low prevalence genes.

During sampling 2, 5 stables were sampled from the same herd as sampling 1. An interesting observation was that, for some genes the shoe cover samples were lower than the pen floor samples (*ermB*, *ermF*, *tet*(O), *tet*(W)) but were higher for *sulI*, *sulII*, *tet*(A), and *tet*(M). This was observed both in sampling 1 (Figure 3 bottom) and the stable and herd pools from sampling 2 (Figure 4). The shoe cover samples varied in how much roughage they collected. Thus, if the shoe cover samples with lower gene copy number estimates had more roughage, the sample would weigh more without the entire weight being attributable to feces. Consequently, this could result in a low gene copy number g^{-1} feces. In contrast, the shoe cover with high gene copy number estimates suggest that the antibiotic resistance genes represented in the sample may depend on which sampling method is used, as different bacteria harbor antibiotic resistance genes while residing in a specific fecal fraction. The gastrointestinal tract is a complex ecosystem containing at least 400 different bacterial species residing in regional habitats (Falk *et al.* 1998). Hence, the shoe cover samples may be capable of collecting fractions of feces that pen floor or individual fecal samples cannot as the shoe cover samples were both saturated with liquid and covered with feces after sample collection. Furthermore, the entire pen floor is covered during shoe cover sampling thus increasing the likelihood of collecting a fecal sample positive for a given gene.

Several genes tested positive in at least 1 non-pooled sample type which then turned negative after pooling (sampling 1 *sulI*, *sulII*, *tet*(A) ; sampling 2 *tet*(A), *tet*(B), *tet*(C), *sulII*). Pooling may increase the risk of a sample becoming negative, especially if the gene copy number g^{-1} feces initially are relatively low. If there are sufficient numbers of negative samples included in the pool then the low prevalence gene concentration is diluted potentially resulting in levels under the assay's quantification limit (Munoz-Zanzi *et al.* 2006). This is contrary to pooling samples that are subsequently enriched, where even low numbers of bacteria can be detected.

The sensitivity of a specific assay is therefore dependent on the gene prevalence, the number of samples included in the pool, the gene concentration in samples collected from positive animals, and the quantification limit of the assay (Munoz-Zanzi *et al.* 2000, Munoz-Zanzi *et al.* 2006). A sufficient balance in the mentioned parameters could explain the apparent consistency in gene copy number g^{-1} feces between slurry tank samples, pen floor and shoe cover herd pools for the majority of the assays. Other investigations have indicated that larger pool size increased the likelihood of a pool being contaminated due to sample collection, handling or processing (Munoz-Zanzi *et al.* 2006).

When choosing a sampling method for antibiotic resistance determination at herd level by qPCR, we recommend either pen floor samples or shoe cover sampling. Both sampling types were equally sensitive in quantifying antibiotic resistance genes in swine feces. Pen floor samples were easily attainable and are representative when pooled at the stable level, but several were negative when pooled at herd level (Figure 4 bottom). In contrast, the shoe cover samples include the entire pen, thus representing more animals and increasing the likelihood of finding antibiotic resistant determinants when present. Furthermore, fecal fractions not represented in pen floor samples and individual animal samples may be represented in the shoe cover samples. However, the shoe cover samples should not be pooled as the stable pools were not representative of the individual shoe cover samples, and the herd pools were negative for several of the assays. The slurry tank samples were also promising; their gene copy levels were consistent with those quantified in the pen floor and shoe cover samples. The slurry tank contains feces from the entire herd from a time period of approximately 6 months and may therefore give a better illustration of herd antibiotic resistance levels. Further studies should be conducted where a series of slurry tank samples are taken in several pig herds over a longer time period, for example 6 months, in order to clarify the dynamics of antibiotic resistance genes in slurry tanks.

This study has validated 14 qPCR assays that can be used to quantify antibiotic resistance genes in swine feces that were compared with CFU counts of coliform bacteria in the same samples. Sampling and pooling strategies were also assessed using the qPCR assays. To our knowledge, this is the first study that tests sampling and pooling strategies for antibiotic resistance surveillance using qPCR determination of antibiotic resistance in

total DNA extracted from swine feces. Our results indicate that there is a great deal of variation in the antibiotic gene abundance within individual animals, pens, stables, and herds regardless of the sampling method. This variation could be systematically evaluated in greater detail using pen floor and/or shoe covering sampling methods supplemented with parallel slurry tank sampling.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Table S1 Primers used to generate standard amplicons. **PDF file.**

Table S2 Positive controls including bacterial isolates and fecal derived positive controls. **PDF file.**

Table S3 Efficiency, determination coefficient (R^2), dynamic range, LOQ and LOD for qPCR assays. **PDF file.**

Figure S1 Sampling 1 16S normalization of gene copy numbers g^{-1} feces. **PDF File.**

Top: Boxplot showing the distribution of gene copies normalized by 16S for individual animals within pens 1-4 from sampling 1 (pen 1, red, n=22; pen 2, green, n=20; pen 3, purple, n=22; pen 4, blue, n=20). The bottom and top of the boxes are the first and third quartiles, respectively. The black band inside the box is the median where the "dotted whiskers" represent the maximum (greatest relative gene copy values, excluding outliers) and minimum (least relative gene copy value, excluding outliers). The solid circles are the individual animal pool pen samples within each pen. Each column represents a denoted gene (the respective genes are depicted in the middle of the figure and are shared for the top and bottom section of Figure S1).

Bottom: The distribution of gene copies normalized by 16S for different sampling and pooling methods from sampling 1. The sampling methods are given under the bottom figure (Pen floor= pen floor sample; Shoe= shoe cover sample; Lab = Individual animal sample). The pens are each their colored circle (pen 1, red circle; pen 2, green circle; pen 3, purple circle; pen 4, blue circle), and the corresponding stable pools are solid black circles.

Figure S2 Sampling 2 16S normalization of gene copy numbers g^{-1} feces. **PDF file.**

Top: Copy numbers of genes normalized by 16S for pen floor samples (circles) and shoe cover samples (triangles) within stables 1-5 (stable 1, red; stable 2, green; stable 3, purple; stable 4, blue; stable 5, pink). The laboratory pooled samples are included for each stable in their respective color (pen floor pool stable, solid circle; shoe cover pool stable, solid triangles). Each column represents a denoted gene (the respective genes are depicted in the middle of the figure and are shared for the top and bottom section of Figure S2).

Bottom: Copy numbers of genes normalized by 16S for pen floor pool stable samples (solid circles); shoe cover pool stable samples (solid triangles) for stables 1-5 (stable 1, red; stable 2, green; stable 3, purple; stable 4, blue; stable 5, pink); Slurry samples 1-3 (white diamond); Pen floor pool herd samples (black solid circle); Shoe cover

pool herd sample (black solid triangle); Pool slurry (black solid diamond). The sampling methods are given under the bottom figure (Pen floor= pen floor pool herd; Shoe= shoe cover pool herd; Slurry sample).

Tables and Figures

Table 1 Overview of the samples collected during sampling 1 and sampling 2 including different sampling methods, and corresponding laboratory pools at stable and herd levels.

Pool name	Sampling 1 1 stable sampled	Sampling 2 5 stables sampled	Samples included in laboratory pool	Number of samples (n)
All animals	+	-	All individual animals from sampling 1 (digital extraction from rectum)	n=84
Individual animal pool pen	+	-	Pool of individual animals within each pen (digital extraction from rectum)	Pen 1 n=22 Pen 2 n=20 Pen 3 n=22 Pen 4 n=20
Pen floor samples	+	+	Not pooled	Sampling 1 n=4 Sampling 2 n=20
Pen floor pool stable	+	+	Pool of pen floor samples from pens 1-4 in each stable	Sampling 1 n=1 Sampling 2 n=1
Pen floor pool herd	-	+	Pool of pen floor samples from each stable (1-5)	n=1
Shoe cover samples	+	+	Not pooled	Sampling 1 n=4 Sampling 2 n=10
Shoe cover pool stable	+	+	Pool of the shoe cover samples in each stable	Sampling 1 n=1 Sampling 2 n=2
Shoe cover pool herd	-	+	Pool of shoe cover samples	n=1

			from each stable (1-5)	
Slurry tank samples	-	+	Each sample was a pool from 3 depths collected at the same spot (1m, 1.5m, and 2m)	n=3
Pool slurry	-	+	Pool of slurry tank samples 1-3	n=1

Table 2 PCR primer and probe sequences (forward primer=FP; reverse primer=RP; probe=PR), gene targets, annealing temperatures (Ann. temp.), amplicon lengths in base pairs (bp), and GenBank sequence accession number (GenBank access. no.).

Primers	Gene target	Sequence (5' → 3')	Ann. temp. (°C)	Amplicon size (bp)	GenBank access. no.*	Reference
FP_TETA_2	*tet*(A)	TTGGCATTCTGCATTCACTC	60	125	X00006	This study
RP_TETA_2		GAAGGCAAGCAGGATGTAGC	60	(840-974)		
PR_TETA_2		GATCACCGGCCCTGTAGCCG				
FP_TETB_Aminov**	*tet*(B)	TTACGTGAATTTATTGCTTCGG	60	206	NE_013365	(Aminov *et al.* 2002) and this study
RP_TETB_Aminov		ATACAGCATCCAAAGCGCAC	60	(913-1119)		
PR_TETB_Aminov_own		CGCCGACCAAATCGGTCAGA				
FP_TETC_6	*tet*(C)	GCCAGTCACTATGGCGTGCT	60	120	EU751613	This study
RP_TETC_6		CAAGTAGCGAAGCGAGCAGG	60	(124-244)		
PR_TETC_6		ACTGTCCGACCGCTTTGGCC				
FP_TETM_7	*tet*(M)	CAACGAGGACGGATAATACGC	60	191	X92947	This study
RP_TETM_7		CCATCTTTTGCAGAAATCAGTAGA	60	(119-311)		
PR_TETM_7		GGTGAACATCATAGACACGCCAGGA				
FP_TETO_Böck	*tet*(O)	AAGAAAACAGGAGATTCCAAAACG	60	75(607-682)	AY660531	(Boeckelmann *et al.* 2009)
RP_TETO_Böck		CGAGTCCCCAGATTGTTTTTAGC	60			
PR_TETO_Böck		ACGTTATTTCCCGTTATCACGGAAGCG				

FP_TETW_Smith	*tet*(W)	GCAGAGCGTGGTTCAGTCT	60	66	AJ222769	(Smith *et al*. 2004)
RP_TETW_Smith		GACACCGTCTGCTTGATGATAAT	60	(411-476)		
PR_TETW_Smith		TTCGGGATAAGCTCTCCGCCGA				
FP_SUL1_2	*sulI*	ACGAGATTGTGCGGTTCTTC	60	159	EU056266	This study
RP_SUL1_2		CCGACTTCAGCTTTTGAAGG	60	(440-598)		
PR_SUL1_2		ACCGGCTCATCCTCGATCCG				
FP_SUL2_3	*sulII*	GATATTCGCGGTTTTCCAGA	60	141	AY360321	This study
RP_SUL2_3		CGCAATGTGATCCATGATGT	60	(313-453)		
PR_SUL2_3		AAGACGGGCAGGCAGATCGG				
FP_ERMB_Böck	*ermB*	GGATTCTACAAGCGTACCTTGGA	60	86	AB563188	(Boeckelmann *et al.* 2009)
RP_ERMB_Böck**		TGGCAGCTTAAGCAATTGCT	60	(390-476)		
PR_ERMB_Böck		CACTAGGGTTGCTCTTGCACACTC AAGTC				
FP_ERMF_KNAPP	*ermF*	TCGTTTTACGGGTCAGCACTT	60	182	M14730;M17 124;	(Knapp *et al.* 2010) and this study
RP_ERMF_KNAPP		CAACCAAAGCTGTGTCGTTT	60	(24-205)	M17808;M62 487	
PR_ERMF_OWN		ATATTGGGGCAGGCAAGGGGTT				
FP_vanA_Böck	*vanA*	CTGTGAGGTCGGTTGTGCG	60	64(614-705)	AF516335	(Boeckelmann *et al.* 2009)
RP_vanA_Böck		TTTGGTCCACCTCGCCA	60			
PR_vanA_Böck		CAACTAACGCGGCACTGTTTCCCA AT				

FW3_SHV_lahey RV5_SHV_lahey Pr_SHV_Lahey2	*bla*$_{SHV}$ family	GCTGGAGCGAAAGATCCACT CGCCTCATTCAGTTCCGTTT AYGTCACCCGCCTTGACCGC	60 60	247 (258-504)	All available at http://www.lahey.org/Studies/	(Jacoby,G. A. 2013) and this study
FW3_CMY-2_Lahey RV4_CMY-2_Lahey PR_CMY-2_Lahey	*bla*$_{CMY-2}$	AGACGTTTAACGGCGTGTTG TAAGTGCAGCAGGCGGATAC TATCGCCCGCGGCGAAAT	60	127 (260-387)	All available at http://www.lahey.org/Studies/	(Jacoby,G. A. 2013)and this study
FW_CTX-M-1 RV_CTX-M-1 PR_CTX-M-1	*bla*$_{CTX-M-1}$ group	ATGTGCAGYACCAGTAARGTKATGGC ATCACKCGGRTCGCCXGGRAT CCCGACAGCTGGGAGACGAAACGT	58	335bp	X92506	(Birkett *et al.* 2007)
FW_SMI_114 R_SMI_115 PR_SMI_116	16S rDNA	CGCGAAGAACCTTACC ACTTAACCCAACATTTCAC CACGAGCTGACGACAGCC	60	126 (916-1041)	NA	The Public Health Agency of Sweden

[*] GenBank accession numbers for previously published primers and/or probes. For primers and probes designed in this study, a GenBank accession number representative of those included in the sequence alignments is given.

[**] Primer modified to fit assay in the present study.

723 **Table 3** Concentrations of reagents per reaction used in qPCR assays with a total reaction volume of 25µL including 2 µL DNA.

Gene	qPCR Mastermix					Forward Primer µM	Reverse primer µM	Probe µM	Λ Probe µM	Λ PCR product µL
	Taqman Universal (Applied Biosystems)	Buffer	$MgCl_2$ mM	Platinum® *Taq* Polymerase (Invitrogen, Life Technologies, Grand Island, NY, United States) U	dNTPs µM					
tet(A)	1X	-	-	-	-	0.5	0.5	0.2	0.2	1
tet(B)	_	1X	3.5	1.25	250	0.5	0.5	0.2	0.2	1
tet(C)	1X	-	-	-	-	0.5	0.5	0.2	0.2	1
tet(M)	-	1X	2.5	1.25	250	0.8	0.8	0.2	0.2	1
tet(O)	1X	-	-	-	-	0.6	0.6	0.2	0.2	1
tet(W)	1X	-	-	-	-	0.9	0.9	0.2	0.2	1
ermB	1X	-	-	-	-	0.5	0.5	0.2	0.2	1
ermF	-	1X	1.5	1.25	250	0.5	0.5	0.2	0.2	1
sulI	-	1X	1.5	1.25	250	0.8	0.8	0.2	0.2	1
sulII	1X	-	-	-	-	0.8	0.8	0.2	0.2	1
vanA	1X	-	-	-	-	0.6	0.6	0.2	0.2	1
$bla_{CTX-M-1}$	-	1X	1.5	1.25	250	0.5	0.5	0.2	0.2	1

group										
bla$_{CMY-2}$	-	1X	3.0	1.25	250	0.6	0.6	0.4	0.2	1
bla$_{SHV}$ family	-	1X	3.5	1.25	250	0.6	0.6	0.2	0.2	1
16S rDNA	1X	-	-	-	-	0.9	0.9	0.2	NA	NA

Figure 1

Top: Boxplot showing the distribution of the coliform bacteria CFUs from sampling 1 in individual animals within pens 1-4 (pen 1, red, n=22; pen 2, green, n=20; pen 3, purple, n=22; pen 4, blue, n=20) on MacConkey plates (No Ab. = no antibiotics, Amp= ampicillin, Erythro= erythromycin, Sulpha= sulphonamide, Tetra= tetracycline). The bottom and top of the boxes are the first and third quartiles, respectively. The black band inside the box is the median and the "dotted-whiskers" represent the maximum (greatest CFU number values, excluding outliers) and minimum (lowest CFU value, excluding outliers). The solid circles are the individual animal pool pen samples within each pen.

Bottom: The distribution of coliform bacteria CFUs from different sampling and pooling methods from sampling 1. The sampling methods are given under the bottom figure (Pen floor= pen floor sample; Shoe= shoe cover sample; Ind. animal= Individual animal sample). Each pen is represented by their colored circle (pen 1, red circle; pen 2, green circle; pen 3, purple circle; pen 4, blue circle), and the corresponding stable pools are solid black circles. The individual shoe cover samples are empty black circles, and the corresponding stable pool is a solid black circle.

Figure 2

Empirical cumulative distribution (Fn(x)) plotted against the relative standard deviations of the CFU and gene copy number estimates illustrating the relationship between uncertainties of calculated estimates and the true laboratory determined estimates for CFU counts (black) and qPCR gene copy numbers (red), respectively.

Figure 3

Top: Boxplot showing the distribution of gene copies above the limit of quantification, LOQ (grey area=below LOQ) for individual animals within pens 1-4 from sampling 1 (pen 1, red, n=22; pen 2, green, n=20; pen 3, purple, n=22; pen 4, blue, n=20). The bottom and top of the boxes are the first and third quartiles, respectively. The black band inside the box is the median and the "dotted-whiskers" represent the maximum (greatest gene copy number values, excluding outliers) and minimum (least gene copy number value, excluding outliers). The solid circles are the individual animal pool pen samples within each pen. Each column represents a denoted gene

(the respective genes are depicted in the middle of the figure and are shared for the top and bottom section of Figure 2).

Bottom: The distribution of gene copies above the LOQ (grey area=below LOQ) for different sampling and pooling methods from sampling 1. The sampling methods are given under the bottom figure (Pen floor= pen floor sample; Shoe= shoe cover sample; Ind. animal= Individual animal sample). Each pen is represented by their colored circle for the pen floor and Ind. animal samples (pen 1, red circle; pen 2, green circle; pen 3, purple circle; pen 4, blue circle). The shoe cover samples are the same 4 shoe covers that were used in all 4 pens (individual shoe cover samples are empty black circles). The stable pools of the respective sampling method, pen floor, shoe cover, Ind. animal, are solid black circles.

Figure 4

Top: Copy numbers of genes gram^{-1} feces above the limit of quantification, LOQ (grey area=below LOQ) for pen floor samples (circles) and shoe cover samples (triangles) within sections 1-5 in sampling 2 (section 1, red; section 2, green; section 3, purple; section 4, blue; section 5, pink). The laboratory pooled samples are included for each section in their respective color (pen floor pool stable, solid circle; shoe cover pool stable, solid triangles). Each column represents a denoted gene (the respective genes are depicted in the middle of the figure and are shared for the top and bottom section of Figure 3).

Bottom: Copy numbers of genes gram^{-1} feces above the LOQ (LOQ=grey area) for pen floor pool stable samples (solid circles); shoe cover pool stable samples (solid triangles) for stables 1-5 (stable 1, red; stable 2, green; stable 3, purple; stable 4, blue; stable 5, pink); Slurry samples 1-3 (white diamond); Pen floor pool herd samples (black solid circle); Shoe cover pool herd sample (black solid triangle); Pool slurry (black solid diamond). The sampling methods are given under the bottom figure (Pen floor= pen floor pool herd; Shoe= shoe cover pool herd; Slurry sample).

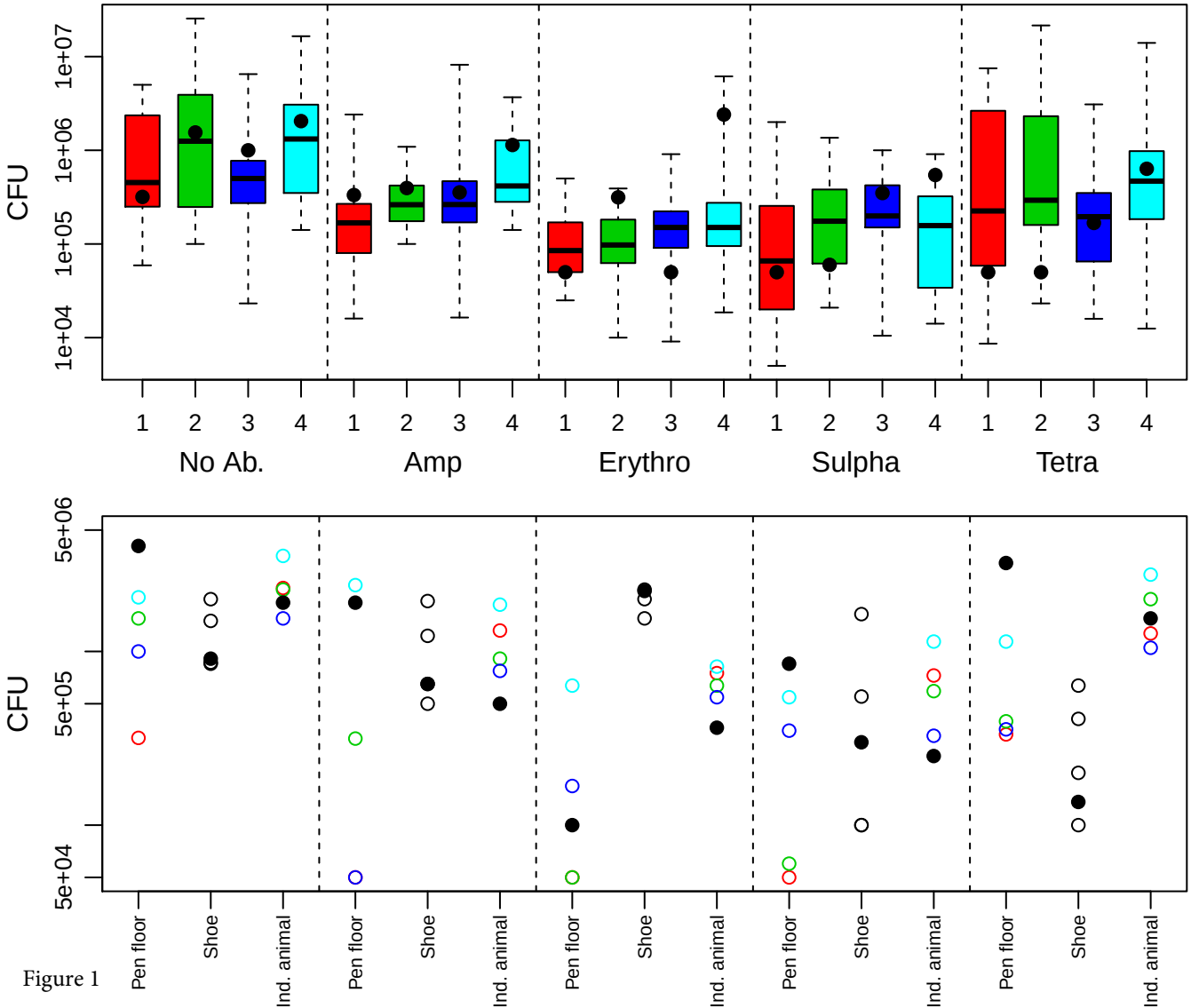
CFU
1e+04
1e+05
1e+06
1e+07
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
No Ab.
Amp
Erythro
Sulpha
Tetra
CFU
5e+04
5e+05
5e+06
Pen floor
Shoe
Ind. animal

Figure 1

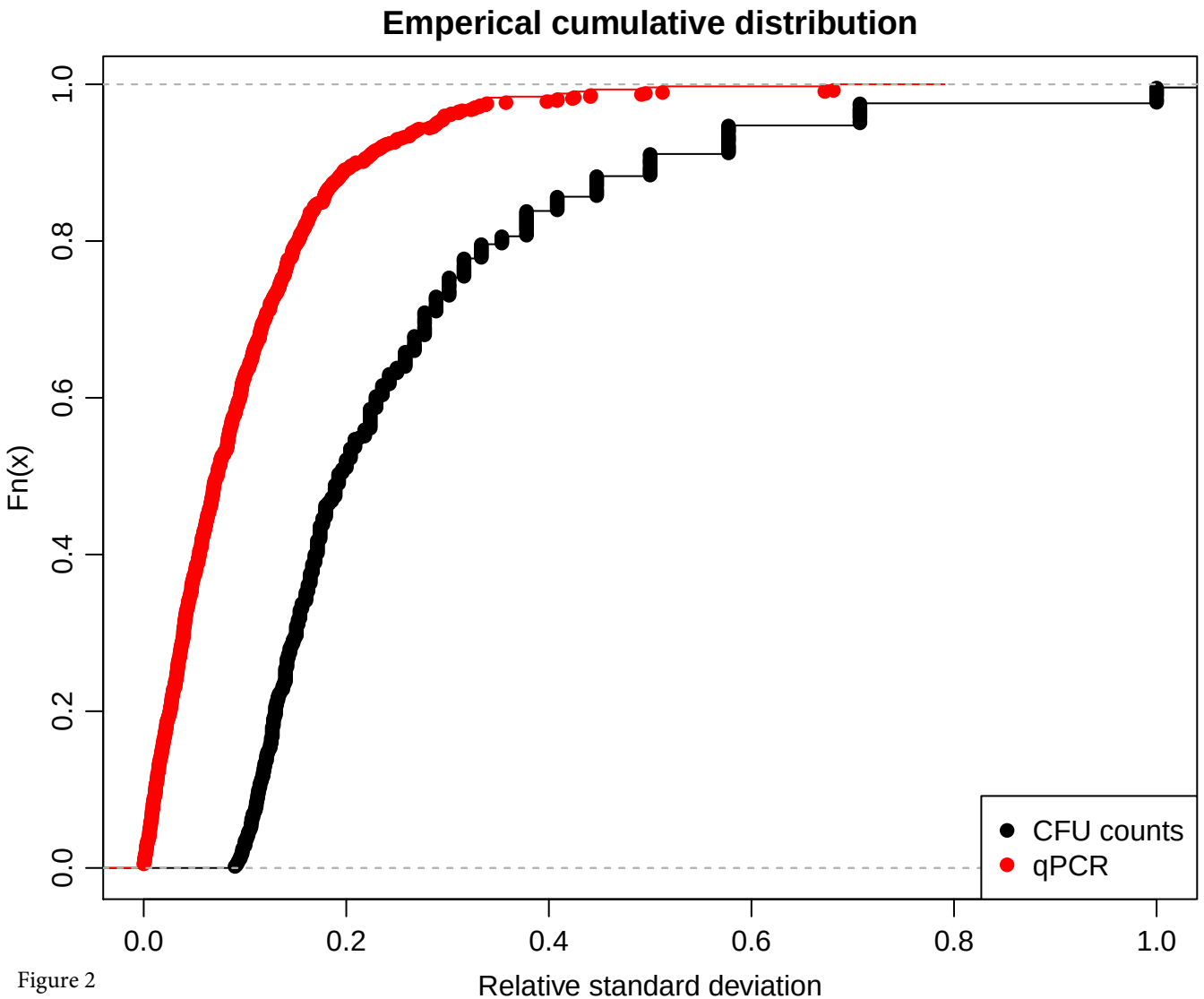
Emperical cumulative distribution
Fn(x)
0.0
0.2
0.4
0.6
0.8
1.0
0.0
0.2
0.4
0.6
0.8
1.0
Relative standard deviation
CFU counts
qPCR

Figure 2

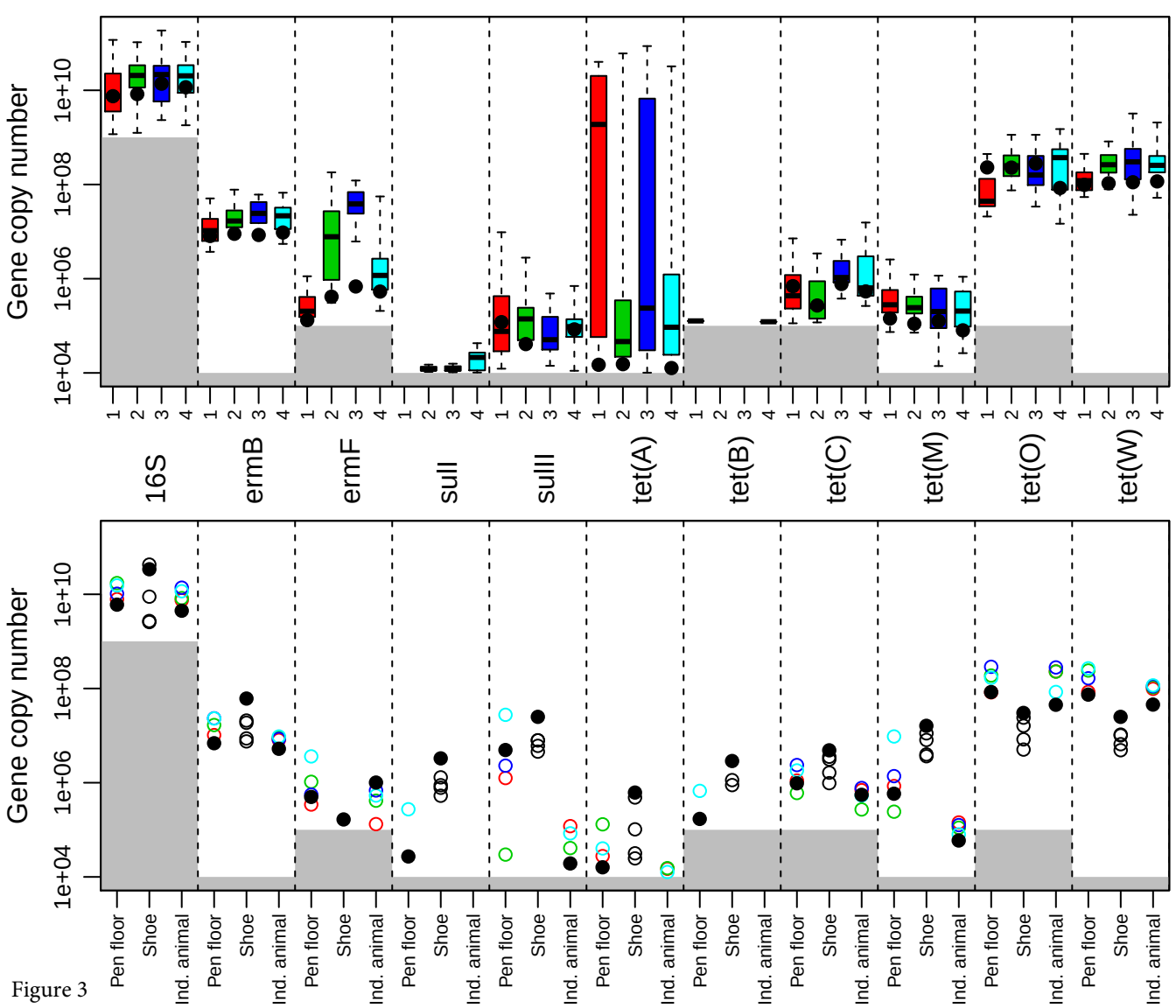
Gene copy number
1e+04
1e+06
1e+08
1e+10
16S
ermB
ermF
sulI
sulII
tet(A)
tet(B)
tet(C)
tet(M)
tet(O)
tet(W)
1
2
3
4
Pen floor
Shoe
Ind. animal

Figure 3

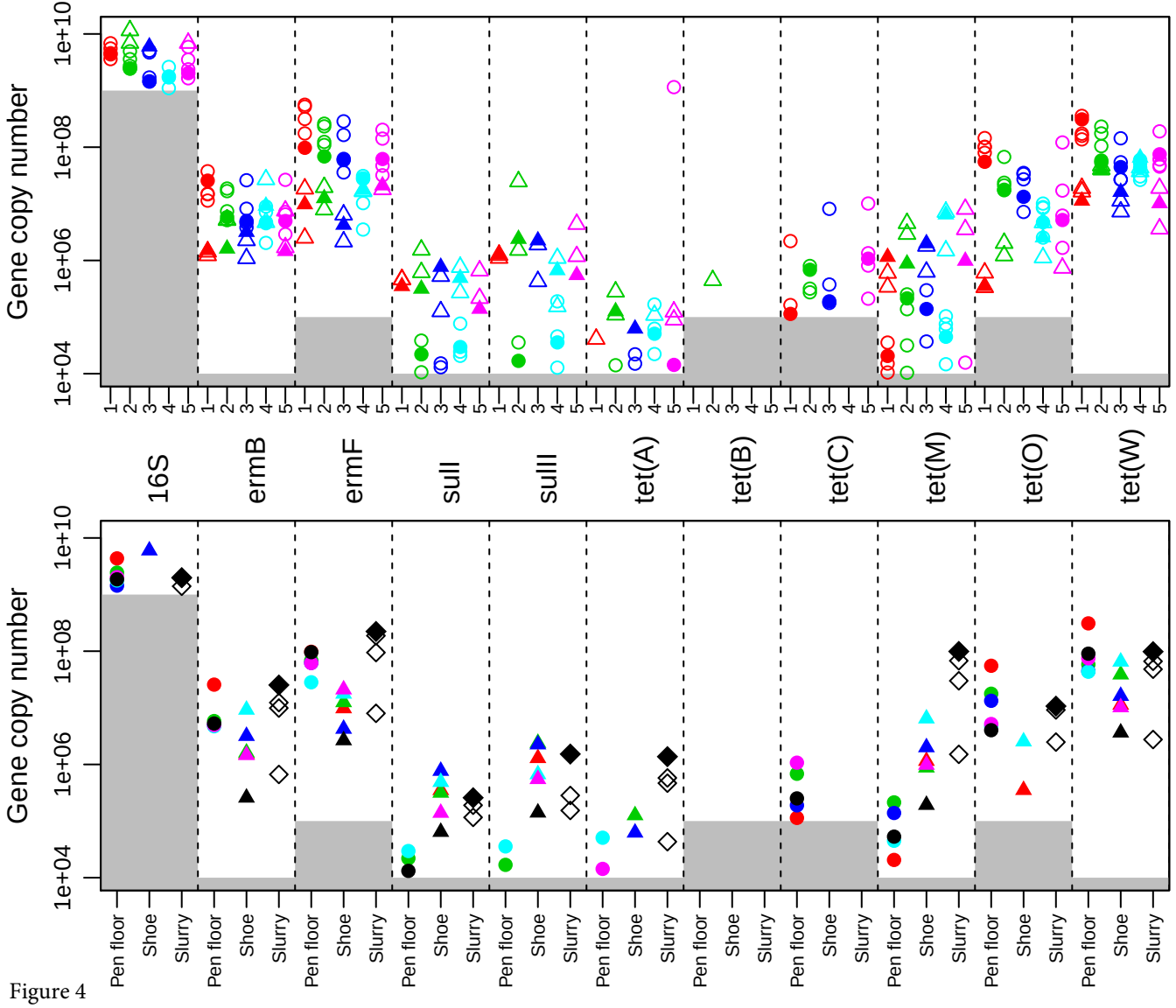
Gene copy number
1e+04
1e+06
1e+08
1e+10
16S
ermB
ermF
sulI
sulII
tet(A)
tet(B)
tet(C)
tet(M)
tet(O)
tet(W)
Pen floor
Shoe
Slurry
1
2
3
4
5

Figure 4

Table S1 Primers used to generate standard amplicons (forward primer=FP, reverse primer=RV). The amplicon size is in number of base pairs (bp)

Primer	Target gene	Base sequence 5'-3'	Amplicon size (bp)	Reference
tet(A)_std_FW *tet*(A)_std_RV	*tet*(A)	GTAATTCTGAGCACTGTCGC CTGTCCTGGACAACATTGCTT	937	(Guardabassi *et al.* 2000, Saenz *et al.* 2004, Costa *et al.* 2008)
tet(B)_std_FW *tet*(B)_std_RV	*tet*(B)	ACTTGTCTCCTGTTTACTCCCCTGAGC GCCTTATCATGCCAGTCTTGCCAACG	1142	This study
tet(C)_std_FW *tet*(C)_std_RV	*tet*(C)	CGCTCATCGTCATCCTCGGCAC CAACCCGTTCCATGTGCTCGC	1091	This study
tet(M)_std_FW *tet*(M)_std_RV	*tet*(M)	GAAGCGTGGACAAAGGTACAACGAGG CGACGGGGCTGGCAAACAGG	1768	This study
tet(O)_std_FW *tet*(O)_std_RV	*tet*(O)	CTGGCTCACGTTGACGCAGGAAAG TATTCGGGCGGCGGGGTTG	1852	This study
tet(W)_std_FW *tet*(W)_std_RV	*tet*(W)	AAAGACGACCTTGACGGAGAGCC TCTCGCCAGTAAAGACAACTTCATCC	1697	This study
*sulI*_std_FW *sulI*_std_RV	*sulI*	ATGGTGACGGTGTTCGGCATTCTG GATCTAACCCTCGGTCTCTGGCGT	832	This study
*sulII*_std_FW *sulII*_std_RV	*sulII*	CGGCATCGTCAACATAACCTCGGAC CGGCTCGTGTGTGCGGATGAAG	730	This study
*ermB*_std_FW *ermB*_std_RV	*ermB*	GGGCATTTAACGACGAAACTGGCT ACTTTGGCGTGTTTCATTGCTTGATG	539	This study
*ermF*_std_FW *ermF*_std_RV	*ermF*	TCGTTTTACGGGTCAGCACTT CAACCAAAGCTGTGTCGTTT	182	(Knapp *et al.* 2010)
*vanA*_std_FW *vanA*_std_RV	*vanA*	GAAATCAACCATGTTGATGTAGCA TTTGCCGTTTCCTGTATCCGT	572	(Boeckelmann *et al.* 2009)
CMY-2_std_FW CMY-2_std_RV	bla_{CMY-2}	ATGATGAAAAAATCGTTATGC GCTTTTCAAGAATGCGCCAGG	758	(Hasman *et al.* 2005, Archambault *et al.* 2006, Agerso *et al.* 2012)

FW_SHV_OS5 FW_SHV_OS6	*bla*$_{SHV}$ family	TTATCTCCCTGTTAGCCACC GATTTGCTGATTTCGCTCGG	854	(Hasman *et al*. 2005, Archambault *et al*. 2006, Agerso *et al.* 2012)
CTX-M-U1_FW CTXXM-U2nds_RV	*bla*$_{CTX-M-1}$ group	ATGTGCAGYACCAGTAARGTKATGGC GGGTRAARTARGTSACCAGAAYSAGCGG	593	(Hasman *et al*. 2005, Agerso *et al*. 2012)
8FX 1407RX	16S rDNA	AGAGTTTGATCCTGGCTNAG TGACGGGCGGTGTGTACAA	1392	(Angen, Ahrens and Tegtmeier 1998)

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Table S2 Positive controls including bacterial isolates and fecal derived positive controls.

Species	Name	Genes	Relevant resistance	Provider
NA	NA	*tet*(A)	Tetracycline	PCR amplification from swine fecal sample
E.coli	tetB, CSH50:Tn10	*tet*(B)	Tetracycline	Yvonne Agersø (DTU-Food)
E.coli	tetC, D07 pBR 322, Tet	*tet*(C)	Tetracycline	Yvonne Agersø (DTU-Food)
E.coli	tetM HB 101	*tet*(M)	Tetracycline	Yvonne Agersø (DTU-Food)
E.coli	tetO HB 101	*tet*(O)	Tetracycline	Yvonne Agersø (DTU-Food)
E.coli	tetW HB 101 pTnB1230, pUTetWup3	*tet*(W)	Tetracycline	Yvonne Agersø (DTU-Food)
E.coli	sul1, NCTC 50001	*sulI*	Sulphonamide	Anette M. Hammerum Statens Serum Institute(SSI)
E.coli	sul2, NCTC 50020	*sulII*	Sulphonamide	Anette M. Hammerum (SSI)
E.faecalis	ermB, D516C1 pAD2	*ermB*	MLS	Anette M. Hammerum (SSI)
E.coli	ermF, Tn4551, original plasmid pFD292 cloned into pUC19 (is ampicillin resistant)	*ermF* (0,7KB)	MLS	Stefan Schwarz (FLI)
E.coli	CTX-M-1,O149 77-30108-11	$bla_{\text{CTX-M-1}}$ group	ESBL	Yvonne Agersø (DTU-Food)
E.coli	CMY-2,F1 from ESC 1009 data	$bla_{\text{CMY-2}}$	AmpC Cephalosporinase	Yvonne Agersø (DTU-Food)
E.coli	SHV-12,F21 from ESC2009 data	bla_{SHV} family	ESBL	Yvonne Agersø (DTU-Food)
E. faecium	vanA, BM4147	*vanA*	Vancomycin	Luca Guardabassi (SUND)
NA	NA	16S rDNA	NA	PCR amplification from swine fecal sample

Table S3 Efficiency, determination coefficient (R^2), dynamic range, LOQ and LOD for qPCR assays.

Assay	Efficiency	R^2	Dynamic range copies reaction^{-1}	LOQ copies g^{-1} feces	LOD copies g^{-1} feces
tet(A)	0.92	0.99781	$1x10^9$-$1x10^1$	$1x10^4$	$1x10^3$
tet(B)	0.93	0.99942	$1x10^8$-$1x10^2$	$1x10^5$	$1x10^5$
tet(C)	0.96	0.99938	$1x10^8$-$1x10^1$	$1x10^5$	$1x10^3$
tet(M)	1.02	0.99690	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^1$
tet(O)	1.13	0.99875	$1x10^8$-$1x10^2$	$1x10^5$	$1x10^1$
tet(W)	0.89	0.99908	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^1$
ermB	0.91	0.99909	$1x10^9$-$1x10^1$	$1x10^4$	$1x10^2$
ermF	0.94	0.99587	$1x10^8$-$1x10^2$	$1x10^5$	$1x10^4$
sulI	1.03	0.99745	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^3$
sulII	0.92	0.99845	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^3$
vanA	0.78	0.99891	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^3$
$bla_{CTX-M-1}$ group	0.98	0.99673	$1x10^9$-$1x10^1$	$1x10^4$	$1x10^2$
bla_{CMY-2}	0.96	0.99697	$1x10^8$-$1x10^2$	$1x10^5$	$1x10^4$
bla_{SHV} family	0.96	0.99791	$1x10^8$-$1x10^1$	$1x10^4$	$1x10^3$
16S rDNA	0.64	0.99045	$1x10^9$-$1x10^5$	$1x10^8$	NA

Figure S1

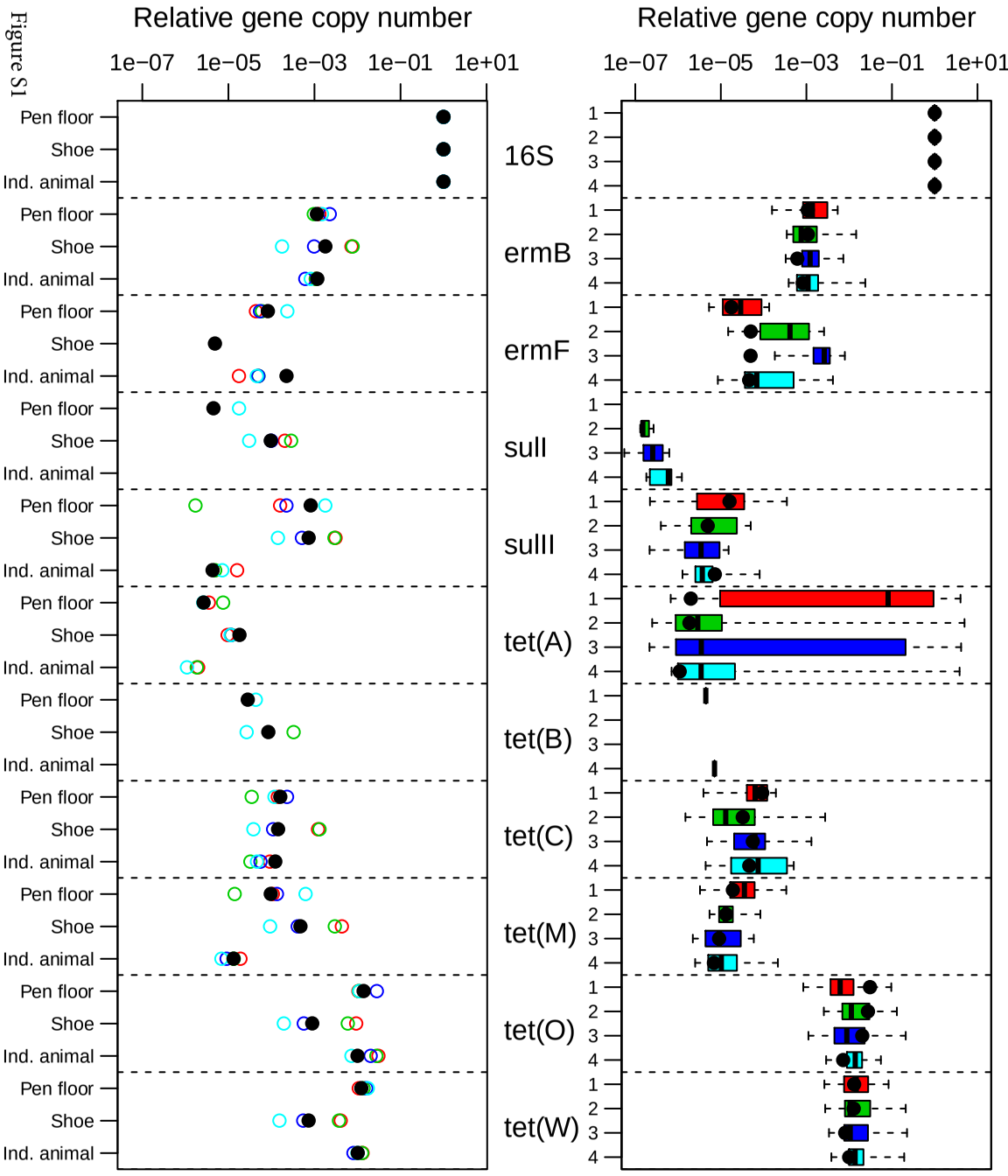
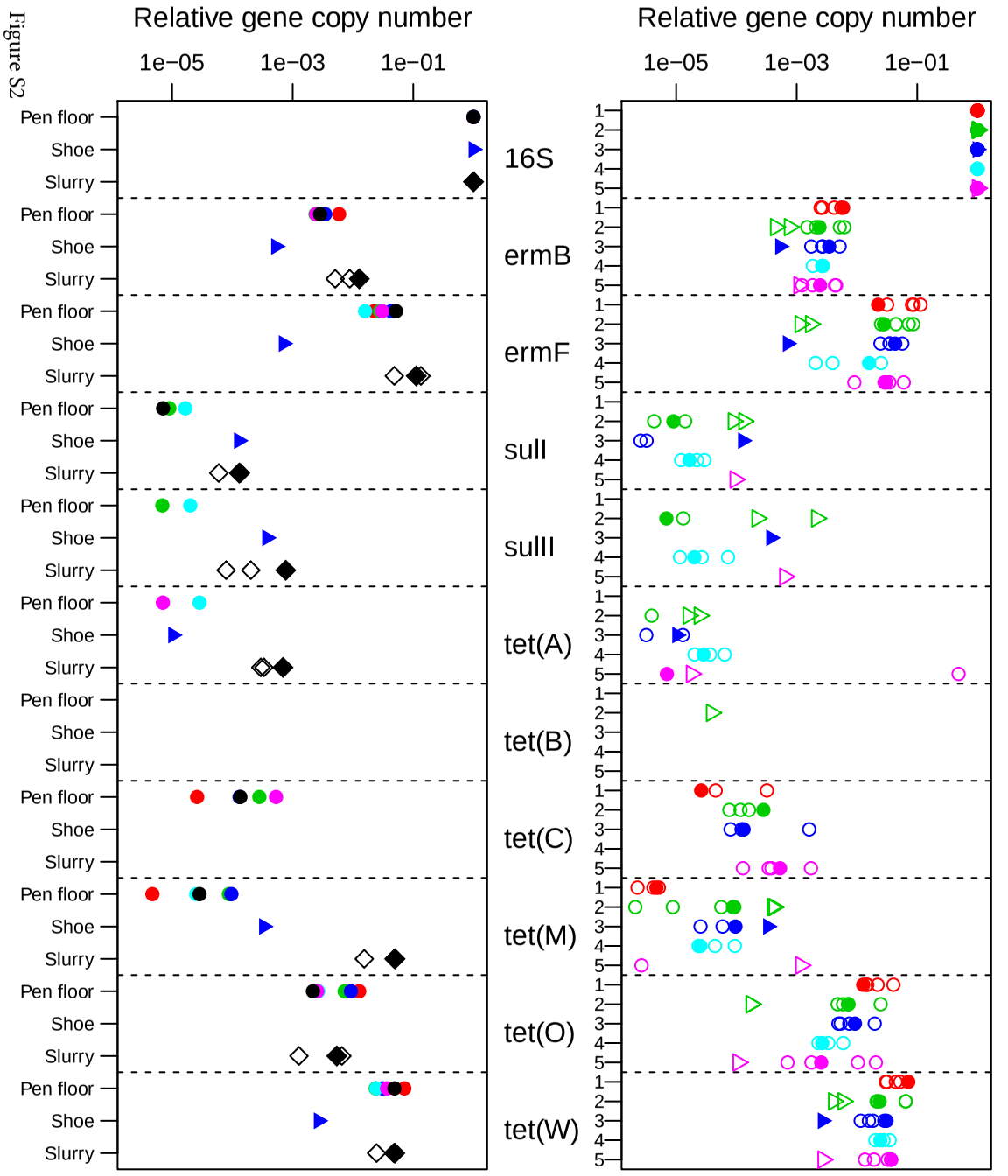

Relative gene copy number
1e−07
1e−05
1e−03
1e−01
1e+01
Pen floor
Shoe
Ind. animal
16S
ermB
ermF
sulI
sulII
tet(A)
tet(B)
tet(C)
tet(M)
tet(O)
tet(W)
1
2
3
4

Figure S2


Relative gene copy number
1e−05
1e−03
1e−01
Pen floor
Shoe
Slurry
16S
ermB
ermF
sulI
sulII
tet(A)
tet(B)
tet(C)
tet(M)
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1
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MANUSCRIPT III

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Quantitative measurement of antibiotic resistance in swine feces using qPCR, colony hybridization, and coliform CFU estimates.

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Title: Quantitative measurement of antibiotic resistance in swine feces using qPCR, colony hybridization, and coliform CFU estimates.

Running title: Quantifying antibiotic resistance.

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Introduction

Genetic alterations enable antibiotic resistant bacteria to survive in the presence of high concentrations of antibiotics. Resistant commensal bacteria in animals may transfer antibiotic resistance genes to zoonotic bacteria or, upon transmission to the human gut, human specific pathogens. When these pathogens cause infections of humans, there can be severe consequences for human health (van den Bogaard and Stobberingh, E. E. 2000). There is increased focus on the spread of resistant bacteria, emphasizing the significance of minimizing antibiotic use, monitoring antibiotic consumption, and performing surveillance of antibiotic resistance (van den Bogaard and Stobberingh, E. E. 2000).

The present methods used for estimating the level of antibiotic resistance at population and herd levels rely on culture of indicator bacteria such as *Escherichia coli*, *Enterococcus* spp., *Salmonella* spp., and *Campylobacter* spp. possibly underestimating the antibiotic resistance levels within the entire community (Alali *et al.* 2009). Furthermore, interpretation of phenotypic tests such as minimum inhibitory concentration (MIC) and disk diffusion may be compromised if bacteria are slow growing in a specific media and/or in the presence of a specific antibiotic (Jorgensen and Ferraro, M. J. 1998, Phillips 1998). It is therefore of interest to evaluate the risk of antibiotic resistance spread within food producing animal herds, and identify methods that rapidly give an estimate of the amount of antibiotic resistance genes at herd level.

To our knowledge, there are currently no validated direct and simple methods for quantifying the amount of antibiotic resistance at herd level within feeder pig operations. Real-time PCR (qPCR) quantifies the amount of specific target DNA in a sample, and has previously been used to quantify antibiotic resistance genes in fecal samples and diverse environments (Fluit, Visser, M. R. and Schmitz, F. J. 2001, Yu *et al.* 2005, Birkett *et al.* 2007, Chen *et al.* 2007, Manuzon *et al.* 2007, Alali *et al.* 2009, Boeckelmann *et al.* 2009, Knapp *et al.* 2010). Being a nucleic acid-based method, qPCR can target genes within the DNA of total bacterial communities, reflecting the entire gene pool (Alali *et al.* 2009).

We have recently developed a method for quantifying 14 antibiotic resistance genes commonly found in swine feces (Schmidt, G., Mellerup, A., Christiansen, L. E. *et al*. 2014). The method was found suitable for quantifying antibiotic resistance at a herd level and to be more stable than a CFU-based method. In order to compare the principle of resistance gene quantification by qPCR, the current study initially quantified selected resistance genes in fecal samples collected from pigs by qPCR. The determined number of resistance genes was subsequently compared to the corresponding colony forming units (CFUs) of resistant bacteria from selected indicator groups, which is a method commonly used to phenotypically estimate levels of resistance. Colony hybridization (CH) was used for further validation by quantifying the antibiotic resistant bacteria using probes corresponding to the fragments amplified with the qPCR primers.

Materials and methods

Sample collection

Swine fecal samples were collected from a feeder pig operation in Denmark. Fecal samples were collected from 4 randomly allocated pens within a single stable. All individual animals within these 4 pens were sampled by digitally extracting feces from the rectum (total 84 samples). The samples were collected in plastic containers with tight lids and placed in coolers immediately after sampling. After analysis, samples were stored at -80ºC. Twenty fecal samples were randomly selected from these 84 samples and were analyzed using the different methods.

Quantification of resistant bacteria by coliform CFU counts

Ten-fold dilutions of each sample were made in PBS buffer. One drop (20 µl) of each dilution was placed on MacConkey plates (Oxoid A/S, Roskilde, Denmark) with and without antibiotics. The antibiotics used were ampicillin (16 mg l^{-1}), erythromycin (32 mg l^{-1}), sulphametizole (256 mg l^{-1}), and tetracycline (16 mg l^{-1}). Once dry, the plates were incubated at 37° for 24 hours.

Colony hybridization

The 20 random fecal samples were analyzed for the presence of antibiotic resistance genes by colony hybridization, essentially as described by Olsen et al. (1995). Briefly, dilutions of samples were spread on Hybond-N colony hybridization filters (Amersham) placed on the surface of one MacConkey agar plate (Oxoid A/S, Roskilde, Denmark) and one blood-agar (BA) plate (Oxoid blood agar base I with 5% calf blood). Plates were incubated 18 hours at 37 °C. The BA plate was incubated anaerobically in a chamber using commercially available gas pack (Oxoid A/S, Roskilde, Denmark). Filters were pre-treated for hybridization as previously described (Olsen *et al.* 1995), air dried, and sealed in plastic bags at ambient temperature until hybridization was performed.

Probes consisted of P^{32}-labeled PCR fragments, corresponding to the amplicons of the qPCR method (Table S1). Labeling was performed using either Ready-to-go DNA labeling beads (GE Healthcare, Brøndby, Denmark) or Rediprime II DNA labelling system (GE Healthcare, Brøndby, Denmark) according to the instructions given by the supplier.

Hybridization was performed at 65°C in 0.5 M NaPi buffer + 7 % sodium dodecyl sulfate (SDS). After post-hybridization washes, as described (Olsen *et al.* 1995), signals were developed using Perkin Elmer Cyclone Plus system (Perkin Elmer, Skovlunde, Denmark). Colony-filters with proper controls, consisting of bacteria with known resistance genes (Schmidt, G., Mellerup, A., Christiansen, L. E. *et al*. 2014) were processed in parallel.

DNA extraction for antibiotic gene quantification by qPCR

DNA was extracted from the fecal samples using 10% dilutions in phosphate buffered saline (PBS) as described previously (Schmidt, G., Mellerup, A., Christiansen, L. E. *et al*. 2014). Briefly, the 10% dilutions were homogenized and 350 µl of the 10% dilution was lyzed for 1 minute 15 Hz^{-1} at room temperature using a Tissuelyser II (Qiagen, Copenhagen, Denmark). After centrifugation for 90 seconds at 10000 rpm the supernatant was transferred to a new eppendorf tube and 20 µl proteinase K (Promega,

Roskilde, Denmark) was added. The samples were loaded into the QiaSymphony robot using the Pathogen complex 200-V5-DSP default IC kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions. The final elution volume was 85 µl. Both a negative and positive DNA extraction control was run in parallel with the samples during each DNA extraction.

Assays for antibiotic resistance

The following antibiotic-resistance genes were investigated by qPCR: tetracycline resistance *tet*(A),*tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W); sulphonamide resistance *sulI*, *sulII*. The primers, internal controls, PCR conditions, PCR mastermixes, and standard curves used are described elsewhere (Schmidt, G., Mellerup, A., Christiansen, L. E. *et al*. 2014). Each sample was tested in duplicate along with a positive control template in triplicate, and 1 positive and negative DNA extraction control. Furthermore, a negative template control (NTC) (23 µl mastermix and 2 µl water) was also included.

Statistical analysis

All figures and statistical tests (paired t-test) were performed on the log transformed values using GraphPad Prism version 5 (La Jolla, CA, USA). The samples that were negative were set to 0.1 for statistical analysis.

Results

The 20 fecal samples were analyzed for tetracycline and sulphonamide resistance using coliform CFU counts, colony hybridization, and qPCR (resistance estimates gram^{-1} feces, Table 1). The left part of Figure 1a shows the number of coliform bacteria growing on MacConkey agar plates with tetracycline or no antibiotics. The right part of Figure 1a shows the number of colonies containing one or more of the tetracycline resistance genes among bacteria growing on MacConkey agar and on blood agar incubated anaerobically. The number of tetracycline resistance genes and 16S rDNA found per gram feces are

shown in Figure 1b. Similar results for the sulphonamide resistance in the fecal samples are shown in Figure 2(a,b).

The tetracycline and sulphonamide resistant estimates attained using the different methods were compared, and the p-values are summarized in Table 2 and Table 3, respectively. There was a significant difference between the numerical estimates of tetracycline resistance by all three methods (Table 2). The qPCR *tet* gene copy number estimate were significantly higher than all colony hybridization estimates and coliform bacteria estimates both resistant and without antibiotics ($P<0.0001$). There was no significant difference between the CH values for bacteria grown on MacConkey agar and anaerobic growth on blood agar for both sulphonamide and tetracycline.

There were also significant differences between the sulphonamide resistance estimates using the different methods (Table 3). The number of coliform bacteria on MacConkey agar without antibiotics was significantly higher than the number of coliform bacteria growing on MacConkey agar added sulphonamide ($P<0.001$), and significantly different from the qPCR sulphonamide gene copy number.

Figure 3(a,b) depicts the relationship between the genotypic antibiotic resistance estimates given as their ratios (MacConkey CH:CFU (red columns); blood agar (BA) CH:qPCR (blue columns); MacConkey CFU:qPCR (green columns) for tetracycline resistance (a) and sulphonamide resistance (b). Eight out of the 20 tetracycline MacConkey CH:CFU ratios were close to 100%, meaning that 100% of the tetracycline resistant coliforms were detected by the colony hybridization. Another 8 ratios were close to 10% of the tetracycline resistant coliform bacteria being detected by the colony hybridization, and the remaining 2 close to 1%. In contrast, 4 of sulphonamide resistance CH:CFU ratios were over 100%, meaning that over 100% sulphonamide resistant coliforms were detected using colony hybridization (Figure 3b, red columns). Nine sulphonamide CH:CFU ratios were between 10%-100%, with the majority being close to 10%, 5 were under 10%.

The ratio between the colony hybridization estimates on BA and the qPCR gene copy number estimates (CH:qPCR) was very low for tetracycline resistance (Figure 3a, blue columns) ranging from below 0.01% to 0,1%. The sulphonamide CH:qPCR ratio was larger, with 4 over 100%, 8 above 10%, and the remaining 6 at 1% or above (Figure 3b, blue columns). For tetracycline, the CH:CFU ratio was significantly higher than the CH:qPCR ratio ($P < 0.0001$), while there was no difference between these values for sulphonamide ($P = 0,4826$).

The tetracycline CFU:qPCR ratios (green columns) varied between animals with the 3 highest between 10% and 100%. The tetracycline CFU:qPCR ratios were significantly different from the CH:CFU ratios ($P<0.0001$), and the CH:qPCR ratios ($P=0.0101$), respectively. There was also a significant difference between the sulphonamide CH:CFU ratio and the CFU:qPCR ratio ($P=0.0291$), but not the CFU:qPCR ratio ($P=0.1020$).

Discussion

In the present study, antibiotic resistance gene quantification by qPCR was compared to phenotypic antibiotic resistant coliform CFU counts and antibiotic resistant bacteria by colony hybridization with probes corresponding to the qPCR primer amplicons. This investigation shows that the method chosen for quantification has a high influence on the results obtained.

There were highly significant differences between all of the different estimates of tetracycline resistance obtained using tetracycline resistance coliform counts, colony hybridization on MacConkey and BA (anaerobic), and the qPCR gene copy number estimates ($P<0.0001$). This reflects that the methods measure the antibiotic resistance in different populations and that there might be a difference between the detection of a gene and a corresponding phenotype. qPCR differs from both colony hybridization and CFU counts by being a culture independent method. However, the culture dependent estimates also differed significantly, and can be due to the different bacterial populations that are represented in each method. The CFU estimates represent the tetracycline resistance in coliform

bacteria, the MacConkey colony hybridization estimates represent the enterobacteria, while the BA (anaerobic growth) estimates represent those bacteria that grow on a non-selective agar under anaerobic incubation.

There was a higher level of tetracycline resistance observed in the coliform bacteria (CFU counts) compared to the colony hybridization despite there being a smaller bacterial population represented in the CFU estimates. A tentative conclusion is that the majority of the tetracycline resistant bacteria are not detected by colony hybridization because they do not carry the specific genes that were used as probes (Mendez, Tachibana, C. and Levy, S. B. 1980, Khan and Novick, R. P. 1983, Waters *et al.* 1983, Guillaume *et al.* 2000, Chopra and Roberts, M. 2001, Roberts,M. C. 2013, Roberts, Schwarz, S. and Aarts, H. J. M. 2012, van Hoek *et al.* 2011). This is also supported by the differences between the CH:CFU ratios (Figure 3a). For the majority of the samples, close to 10% of the tetracycline resistant coliform bacteria were also detected using colony hybridization on MacConkey plates, a couple had 100%. In contrast, the CH:qPCR ratios were very low (0,01%-0,1%) illustrating how the *tet* genes detected by qPCR were not detected using colony hybridization on BA (anerobic growth). This could reflect that bacteria containing the genes detected using qPCR are not present on BA plates (anaerobic growth), or a single bacterium might harbor more than one resistance gene (Chopra and Roberts, M. 2001, van Hoek *et al.* 2011, Roberts,M. C. 2013, Roberts, Schwarz, S. and Aarts, H. J. M. 2012). The tetracycline CH:qPCR ratios were significantly lower than the CFU:qPCR ratios meaning that the tetracycline resistant coliforms were more readily detected by the qPCR than the colony hybridization estimates. This may be due to the fact that there was a selection pressure in favor of tetracycline resistant coliforms containing *tet* genes compared to the BA plates that did not contain antibiotics. Therefore, there may be more genes per bacteria in the coliforms compared to those on the BA plates (anaerobic growth).

There were also significant differences in sulphonamide resistance estimates determined using the different methods (Table 3), however, regarding the qPCR *sul* gene copy numbers, they only differed significantly from the CFU estimates from plates without antibiotics (P=0.0061). The qPCR *sul*

estimates that are not significantly different from the culture dependent methods could be due to the fact that there are few genes that code for sulphonamide resistance compared to for example tetracycline (Sköld 2000, Roberts, Schwarz, S. and Aarts, H. J. M. 2012). This indicates that when fewer genes cause an antibiotic resistance phenotype, the chance of detecting the antibiotic resistance is increased and there will be less discrepancy between the genotype and phenotype. This is supported by the sulphonamide CH:qPCR and CFU:qPCR ratios that did not significantly differ (P=0.1020). Hence, the *sul* qPCR results indicate that the *sulI* and *sulII* genes detected are represented in the culture dependent method regardless of the method used and bacterial population that is represented. Furthermore, if *sulI* and *sulII* are present, even in a complex bacterial population, the qPCR assays are sensitive enough to detect them. This is also supported by the ratios in Figure 3b where the CH:CFU ratios and the CH:qPCR ratios are very similar. Furthermore, 14 of the sulphonamide CH:qPCR ratios were close to 10% emphasizing the fact that these methods represent different populations. The estimates were not significantly different, but only a fraction of the genes detected using qPCR were detected with colony hybridization on BA (anaerobic growth).

This study emphasizes the importance of defining which bacterial population is relevant in the specific goal of antibiotic resistance quantification. When the aim is to monitor and quantify antibiotic resistance at herd level, using a method where a few chosen indicator bacteria represent the resistance in the intestinal bacterial population let alone the herds' is not optimal due to the large portion of neglected bacteria. However, if the aim is to quantify resistance in a known pathogen, it can be an advantage to cultivate the bacteria and phenotypically test the antibiotic resistance to avoid false negatives if the resistance is due to another gene than those tested.

There can also be variation between methods that cannot solely be ascribed the bacterial population, but other confounding influences. For example, the number of resistant coliforms detected depends on the concentration of antibiotic that is added to the MacConkey agar. Certain antibiotic resistance determinants may not be sufficient to enable growth at the set resistance breakpoint. Furthermore, sampling from individual animals also gives rise to variation due to the fluctuations in gene

excretion that can show large, individual day-to-day fluctuations (Bibbal *et al.* 2007). Antibiotic resistance is subject to fluctuations in the migration of its host organism and/or persistence in a given environment, antibiotic gene migration, and the presence of selection pressure (Koike *et al.* 2007). Therefore, when collecting a fecal sample from an individual animal at a given time point, the level found may depend on the time other animals within the pen excreted the specific gene.

The present study compared antibiotic resistance determination using coliform CFU, colony hybridization, and qPCR estimates. The results show how the method chosen for quantification has an influence on the results obtained. Each method represents a separate bacterial population as indicated by the significant differences observed between methods. Whether a phenotypic antibiotic resistance estimate is signified by the genotype or not may depend on the number of corresponding resistance determinants. Future studies must be conducted to investigate this further.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Table S1 PCR primer and probe sequences for qPCR, gene targets, annealing temperatures, amplicon lengths, and GenBank accession number for the assays used in this study.

Figures and tables

Figure 1 Boxplot showing the distribution of the tetracycline antibiotic resistance estimates in the tested animals (n=20) using different methods a) left, colony forming units on MacConkey plates without antibiotics (CFU No antibiotics), colony forming units on MacConkey plates with antibiotics (CFU tet Mac); right, colony hybridization on MacConkey plates (Colony tet Mac), colony hybridization on blood agar plates anaerobically incubated (Colony tet BA) b) qPCR gene copy estimates (cc) for *tet* genes (qPCR tet sum), qPCR estimates of 16S rDNA in total DNA (qPCR 16S). The top of the box is the upper quartile and the bottom the lower quartile. The black band inside the box is the median and the "whiskers" represent the maximum (greatest number values, excluding outliers) and minimum (lowest CFU value, excluding outliers). The dotted line indicates that the CFU estimates should be read off the left Y-axis, and the colony hybridization estimates the right Y-axis.

Figure 2 Boxplot showing the distribution of the sulphonamide antibiotic resistance estimates in the tested animals (n=20) using different methods a) left, colony forming units on MacConkey plates without antibiotics (CFU No antibiotics), colony forming units on MacConkey plates with antibiotics (CFU sul Mac) ; right: colony hybridization on MacConkey plates (Colony sul Mac), colony hybridization on blood agar plates anaerobically incubated (Colony tet BA) b) qPCR gene copy estimates (cc) for *sul* genes (qPCR tet sum), qPCR estimates of 16S rDNA in total DNA (qPCR 16S). The top of the box is the upper quartile and the bottom the lower quartile. The black band inside the box is the median and the "whiskers" represent the maximum (greatest number values, excluding outliers) and minimum (lowest CFU value, excluding outliers). The dotted line indicates that the CFU estimates should be read off the left Y-axis, and the colony hybridization estimates the right Y-axis.

Figure 3 Histogram showing the ratios between antibiotic resistance estimates from the colony hybridization from MacConkey agar (CH) and the coliform colony forming units (CFU) (CH:CFU, red); colony hybridization on Blood Agar anaerobically incubated (CH) and the qPCR (CH:qPCR, blue); CFU on MacConkey agar and the qPCR (CFU:qPCR, green) for tetracycline (a) and sulphonamide (b) for the tested animals (n=20).

Table 1 The results from the coliform colony forming unit (CFU) counts on MacConkey with antibiotics (tetracycline, sulphonamide), and without antibiotics (No antibiotics); colony hybridization estimates of *sul* or *tet* positive bacteria on MacConkey agar and blood agar (anaerobic growth); qPCR gene copy number estimates given as the sum of the gene copy numbers for *tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W) for tetracycline, and *sulI* and *sulII* for sulphonamide. 16s rDNA gene copy number estimates are also included. All results are given gram^{-1} feces. The sample numbers denote the randomly selected pig number.

	CFU gram^{-1} feces on MacConkey			Colony Hybridization number of positive bacteria gram^{-1} feces				qPCR gene copy numbers gram^{-1} feces		
Sample number	No antibiotics	Tetracycline	Sulphonamide	Tetracycline MacConkey	Tetracycline Blood agar anaerobic	Sulphonamide MacConkey	Sulphonamide Blood Agar anaerobic	16S	Tetracycline	Sulphonamide
4	4,00E+06	7,50E+06	4,50E+05	4,20E+05	2,40E+05	4,83E+05	4,70E+05	4,28E+09	1,88E+08	9,68E+06
5	2,68E+05	5,00E+04	6,82E+04	5,00E+03	1,93E+05	1,33E+05	1,75E+04	7,55E+09	1,47E+08	2,64E+06
7	5,00E+06	7,50E+06	4,55E+04	9,68E+05	5,50E+04	2,75E+04	3,25E+04	5,46E+10	5,71E+08	8,19E+05
10	4,50E+05	2,50E+05	3,18E+05	3,20E+05	1,73E+05	5,80E+05	4,13E+05	3,55E+09	2,02E+08	1,25E+05
14	6,76E+04	5,86E+04	5,45E+03	1,23E+05	5,00E+04	5,00E+03	1,50E+04	9,14E+09	1,61E+08	5,10E+05
18	5,00E+06	5,32E+06	5,00E+03	1,17E+06	1,33E+05	1,25E+04	1,25E+04	5,74E+09	1,53E+08	9,72E+04
22	4,55E+05	1,73E+05	1,41E+04	5,90E+05	7,23E+05	2,50E+03	7,50E+03	2,88E+08	1,03E+08	4,11E+04
28	2,18E+05	2,32E+04	6,00E+04	2,50E+04	1,50E+04	1,25E+04	1,50E+04	1,22E+10	8,78E+08	4,29E+05
32	2,55E+07	2,15E+07	2,52E+05	0,00E+00	0,00E+00	2,50E+04	3,25E+04	2,65E+10	3,43E+08	5,23E+04
35	4,09E+06	2,32E+06	5,00E+04	2,73E+05	2,08E+05	4,00E+04	2,75E+04	7,00E+10	1,41E+09	1,61E+05
46	5,91E+05	2,00E+05	4,82E+05	1,25E+05	2,43E+05	1,00E+05	5,50E+04	3,13E+10	8,11E+08	1,55E+05
49	2,32E+04	1,59E+04	1,05E+04	2,50E+03	5,00E+03	2,50E+03	5,00E+03	8,74E+09	1,45E+08	0,00E+00
55	4,55E+05	1,86E+05	5,00E+05	1,40E+05	7,25E+04	3,93E+05	1,75E+05	4,11E+09	2,26E+08	1,42E+04
59	1,50E+05	4,50E+04	1,85E+05	1,25E+04	2,25E+04	3,25E+04	3,50E+04	4,03E+10	6,17E+08	6,84E+04
64	9,50E+05	7,27E+05	1,00E+05	4,25E+04	2,75E+05	1,68E+05	1,15E+05	1,96E+10	5,09E+08	3,12E+04
67	2,70E+05	8,18E+04	1,41E+05	8,25E+04	1,03E+05	5,00E+04	2,55E+05	8,20E+09	2,20E+08	0,00E+00

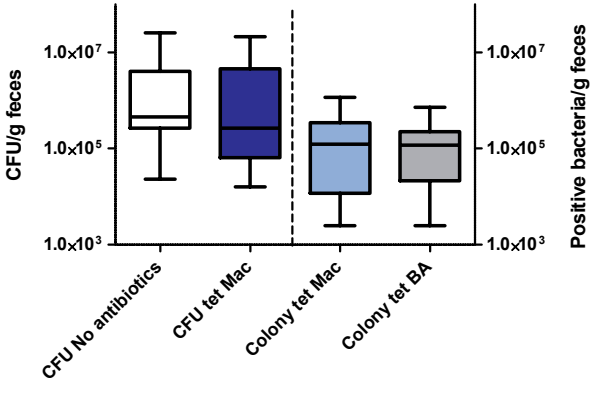
70	3,09E+06	2,82E+05	2,30E+05	2,05E+05	2,20E+05	5,00E+04	7,00E+04	5,66E+09	1,78E+08	1,60E+05
78	6,50E+06	1,40E+07	3,64E+04	0,00E+00	0,00E+00	3,00E+04	1,75E+04	4,60E+10	1,38E+09	1,51E+05
79	3,00E+05	6,82E+05	2,00E+04	1,00E+04	2,50E+03	6,75E+04	4,00E+04	8,97E+09	6,30E+08	8,68E+04
82	3,50E+05	3,27E+05	2,50E+05	7,50E+03	1,75E+04	2,35E+05	2,95E+05	3,41E+10	4,70E+08	1,07E+05

Table 2 Matrix showing the paired t-test P-values for the different methods for tetracycline antibiotic resistance estimation. Colony forming unit=CFU; No antibiotics=No Ab; colony hybridization on MacConkey= CH MacConkey; colony hybridization blood agar anaerobe=CH BA anaerobic growth; qPCR for tetracycline resistance gene *tet*(A), *tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W) estimates' sums.

	CFU No Ab	CFU tet MacConkey	CH MacConkey	CH BA anaerobic	qPCR *tet* genes
CFU No Ab		0,0178	0,0002	0,0002	< 0.0001
CFU tet MacConkey			0,0018	0,0043	< 0.0001
CH MacConkey				0,7939	< 0.0001
CH BA anaerobic					< 0.0001
qPCR *tet* genes					

Table 3 Matrix showing the paired t-test P-values for the different methods for sulphonamide antibiotic resistance estimation. Colony forming unit=CFU; No antibiotics=No Ab; colony hybridization on MacConkey= CH MacConkey; colony hybridization blood agar anaerobe=CH BA anaerobic growth; qPCR for sulphonamide resistance gene *sulI* and *sulII* estimates' sums.

	CFU No Ab	CFU sul MacConkey	CH MacConkey sul	CH BA anaerobic sul	qPCR *sul* genes
CFU No Ab		< 0.0001	< 0.0001	< 0.0001	0,0061
CFU sul MacConkey			0,0393	0,0304	0,5718
CH MacConkey sul				0,9664	0,9271
CH BA anaerobic sul					0,9265
qPCR *sul* genes					


CFU/g feces
1.0x10^7
1.0x10^5
1.0x10^3
Positive bacteria/g feces
1.0x10^7
1.0x10^5
1.0x10^3
CFU No antibiotics
CFU tet Mac
Colony tet Mac
Colony tet BA

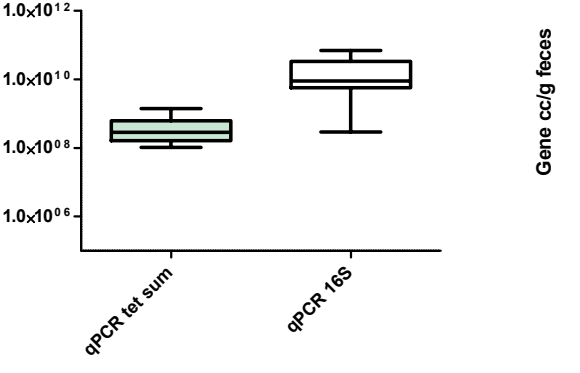
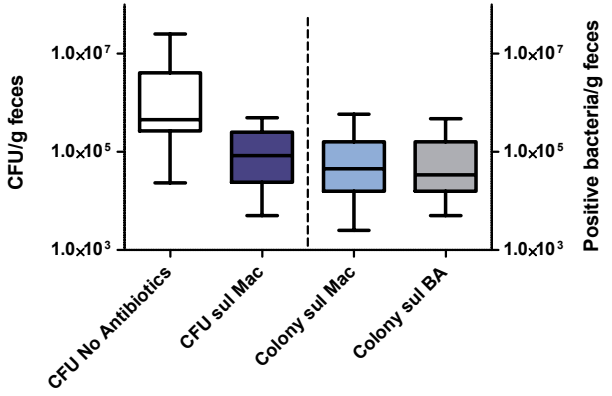

1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
Gene cc/g feces
qPCR tet sum
qPCR 16S

Figure 1 a b


CFU/g feces
1.0x10^7
1.0x10^5
1.0x10^3
Positive bacteria/g feces
1.0x10^7
1.0x10^5
1.0x10^3
CFU No Antibiotics
CFU sul Mac
Colony sul Mac
Colony sul BA

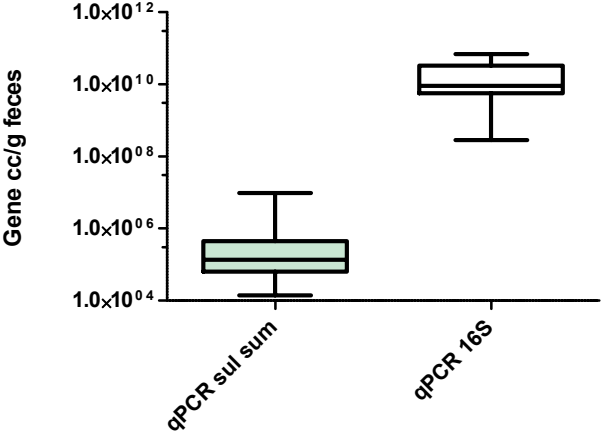
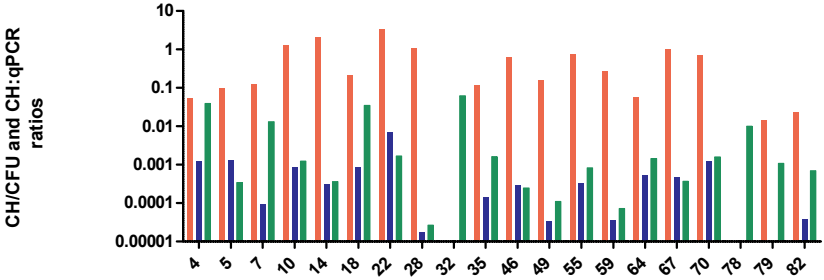
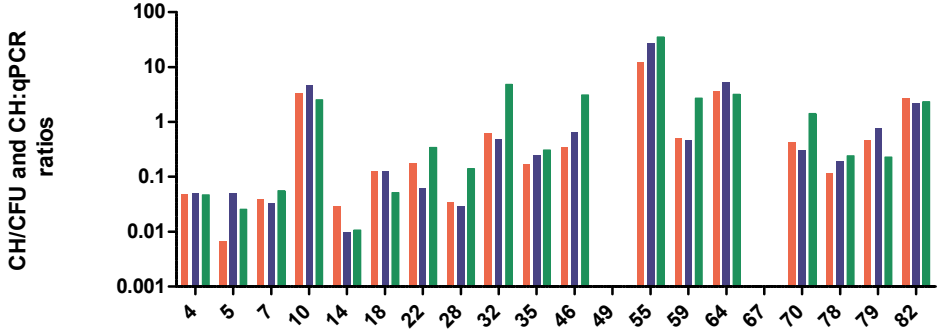

Gene cc/g feces
1.0x10^12
1.0x10^10
1.0x10^08
1.0x10^06
1.0x10^04
qPCR sul sum
qPCR 16S

Figure 2 a b


CH/CFU and CH:qPCR ratios
10
1
0.1
0.01
0.001
0.0001
0.00001
4
5
7
10
14
18
22
28
32
35
46
49
55
59
64
67
70
78
79
82

a


CH/CFU and CH:qPCR ratios
100
10
1
0.1
0.01
0.001
4
5
7
10
14
18
22
28
32
35
46
49
55
59
64
67
70
78
79
82

b

Figure 3

Table S1 qPCR primer and probe sequences (forward primer=FP; reverse primer=RP; probe=PR), gene targets, annealing temperatures (Ann. temp.), amplicon lengths in base pairs (bp), and GenBank sequence accession number (GenBank access. no.). The probes used in the colony hybridization correspond to the DNA fragments amplified by the qPCR primer pairs.

Primers	Gene target	Sequence (5' → 3')	Ann. temp. (°C)	Amplicon size (bp)	GenBank access. no.*	Reference
FP_TETA_2	*tet*(A)	TTGGCATTCTGCATTCACTC	60	125	X00006	This study
RP_TETA_2		GAAGGCAAGCAGGATGTAGC	60	(840-974)		
PR_TETA_2		GATCACCGGCCCTGTAGCCG				
FP_TETB_Aminov**	*tet*(B)	TTACGTGAATTTATTGCTTCGG	60	206	NE_013365	(Aminov *et*
RP_TETB_Aminov		ATACAGCATCCAAAGCGCAC	60	(913-		*al.* 2002) and
PR_TETB_Aminov_own		CGCCGACCAAATCGGTCAGA		1119)		this study
FP_TETC_6	*tet*(C)	GCCAGTCACTATGGCGTGCT	60	120	EU751613	This study
RP_TETC_6		CAAGTAGCGAAGCGAGCAGG	60	(124-244)		
PR_TETC_6		ACTGTCCGACCGCTTTGGCC				
FP_TETM_7	*tet*(M)	CAACGAGGACGGATAATACGC	60	191	X92947	This study
RP_TETM_7		CCATCTTTTGCAGAAATCAGTAGA	60	(119-311)		
PR_TETM_7		GGTGAACATCATAGACACGCCAGGA				
FP_TETO_Böck	*tet*(O)	AAGAAAACAGGAGATTCCAAAACG	60	75(607-	AY660531	(Boeckelmann
RP_TETO_Böck		CGAGTCCCCAGATTGTTTTTAGC	60	682)		*et al.* 2009)
PR_TETO_Böck		ACGTTATTTCCCGTTATCACGGAAGCG				
FP_TETW_Smith	*tet*(W)	GCAGAGCGTGGTTCAGTCT	60	66	AJ222769	(Smith *et al.*
RP_TETW_Smith		GACACCGTCTGCTTGATGATAAT	60	(411-476)		2004)
PR_TETW_Smith		TTCGGGATAAGCTCTCCGCCGA				

FP_SUL1_2	*sulI*	ACGAGATTGTGCGGTTCTTC	60	159	EU056266	This study
RP_SUL1_2		CCGACTTCAGCTTTTGAAGG	60	(440-598)		
PR_SUL1_2		ACCGGCTCATCCTCGATCCG				
FP_SUL2_3	*sulII*	GATATTCGCGGTTTTCCAGA	60	141	AY360321	This study
RP_SUL2_3		CGCAATGTGATCCATGATGT	60	(313-453)		
PR_SUL2_3		AAGACGGGCAGGCAGATCGG				
FW_SMI_114	16S rDNA	CGCGAAGAACCTTACC	60	126 (916-1041)	NA	The Public health agency of Sweden Solna, Sweden
R_SMI_115		ACTTAACCCAACATTTCAC				
PR_SMI_116		CACGAGCTGACGACAGCC				

[*] GenBank accession numbers for previously published primers and/or probes. For primers and probes designed in this study, a GenBank accession number representative of those included in the sequence alignments is given.

[**] Primer modified to fit assay in the present study.

MANUSCRIPT IV

Gunilla Veslemøy Schmidt, Abdul Ahmed Selemani Katakweba, John Elmerdahl Olsen, Øystein Angen, 2014. Eight antibiotic resistance genes including the *bla*$_{\text{CMY-2}}$ gene detected in wildlife grazing with and without cattle in Tanzania

Target Journal *Letters in Applied Microbiology*

Title: Eight antibiotic resistance genes including the *bla*$_{CMY-2}$ gene detected in wildlife grazing with and without cattle in Tanzania.

Running title: Antibiotic resistance genes in Wildlife

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Keywords: Cattle, Wildlife, antibiotic resistance, qPCR, CMY-2

Abstract

In the present study a total of 8 samples collected from interacting wildlife and cattle (NCA=Ngorongoro Conservation Area) and 4 samples from wildlife not interacting with cattle (MNP= Mikumi National Park) were screened for the presence of 14 antibiotic resistance genes. All of the wildlife samples contained antibiotic resistance genes that also were found in the cattle, regardless of the sampling site. Eight antibiotic resistance genes were detected in the samples, the most prevalent being *tet*(W) and $bla_{\text{CMY-2}}$ with the latter being of concern due to the nature of the antibiotic resistance spectrum. Based on these results, it appears that wildlife, irrespective of contact with domesticated cattle, constitutes an unexpected reservoir for important antibiotic resistance determinants, and further studies are indicated to determine the antibiotic resistance gene-pool among the wildlife.

Introduction

Antibiotic resistance is a global manifestation and has been described in developed and undeveloped countries and also in a variety of environments both in nature and agricultural surroundings (Koike *et al.* 2007, Costa *et al.* 2008, CheeSanford *et al.* 2009, Poeta *et al.* 2009, Poeta *et al.* 2010, Koike *et al.* 2010, Goncalves *et al.* 2013). Bacteria residing in the intestines of humans and animals, such as *Escherichia coli*, pose a potential threat to the human population if they carry antibiotic resistance genes as the bacteria are readily distributed among different environments through water, soil, and food (Skurnik *et al.* 2006, Costa *et al.* 2008). It has been demonstrated that antibiotic-resistant bacteria are released into the environment from food-producing animals via feces, urine, animal food products and that further spread can be facilitated by flies feeding on feces and urine (Aarestrup *et al.* 2000). Infectious diseases in humans and animals caused by antibiotic resistant pathogenic bacteria are often associated with treatment failure when the use of antibiotics is essential (Costa *et al.* 2008).

Human activity in natural environments is increasing as populations grow in size, ultimately narrowing the human and wildlife proximity (Skurnik *et al.* 2006, Pesapane, Ponder and Alexander 2013). As a consequence of this close proximity, infectious diseases are exchanged between humans and wildlife, where the most threatening are caused by antibiotic resistant bacteria (Benavides *et al.* 2012, Pesapane, Ponder and Alexander 2013). Skurnik *et al.* (2006) found a correlation between the level of exposure to humans and/or human activities and the prevalence of resistance in wildlife from environments that had minimal exposure to humans, healthy pets, and farm animals (Skurnik *et al.* 2006). Furthermore, the tested antibiotic resistant *E.coli* strain patterns were extremely heterogeneous, indicating that the presence of resistant strains was not due to clonal spread but to the spread of antibiotic resistance genes. The antibiotic resistance genes can spread indirectly by transmission of either the resistant bacteria themselves or horizontal gene-transfer (Skurnik *et al.* 2006). It has also been

implied that wild animals can act as reservoirs for resistant bacteria consequently facilitating antibiotic resistance gene transfer throughout the environment (Dolejska *et al.* 2007).

In the Ngorongoro Conservation Area (NCA), Tanzania, Massai shepherds migrate with their short horned zebu cattle interacting with wildlife species such as wildebeest (*Connochaetes taurinus)*, zebra (*Equus burchelli)* and buffalo (*Syncerus caffer)* through grazing and gathering at water holes (Voeten and Prins 1999, Charnley 2005). South from NCA is the Mikumi National Park (MNP) where the Massai are prohibited to migrate with their cattle in the park. It is therefore assumed that the wildlife in MNP have limited contact with cattle, if any. In the present study, fecal samples were collected from wildlife (wildebeest, zebra and buffalo) and cattle residing in the NCA, while in MNP fecal samples were only collected from wildlife. The DNA extracted from fecal samples was screened for the presence of 14 antibiotic resistance encoding genes using qPCR. The level of antibiotic resistant coliform bacteria was also determined. A study by Katakweba et al. (2013) found *tet*(W) and *sulII* genes in the same animal species. We hypothesize that, if the samples were positive for two antibiotic resistance genes it is likely that there are more genes, and identical genes will be present in both the wildlife and cattle samples.

Materials and Methods

Study Area and samples

The samples used in this study (Table 1) were collected in the MNP and NCA areas from Tanzania (Selemani Katakweba 2013).

Colony forming unit (CFU) counts of coliform bacteria

Ten-fold dilutions from each sample were made in phostphate buffer saline (PBS) and 100 µl of each dilution was spread on MacConkey plates without and with antibiotics (Ampicillin (16 mg l^{-1});

Cefotaxime (2 mg l^{-1}); Sulphametizole (256 mg l^{-1}); Tetracycline (16 mg l^{-1})) and incubated at 37°C for 24 hours.

DNA extraction

Ten-fold dilutions from each sample were made in phosphate buffer saline (PBS). After thorough vortexing, 200 µl of the 10^{-1} dilution was used for DNA extraction using the QIA amp DNA Stool Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's instructions. The DNA was eluted in 200 µl elution buffer and stored at -20°C until analysis (Selemani Katakweba 2013).

Assays for antibiotic resistance

The following antibiotic-resistance genes were studied by qPCR: tetracycline resistance *tet*(A),*tet*(B), *tet*(C), *tet*(M), *tet*(O), *tet*(W); macrolide, lincosamide, streptogramin B (MLS_B) resistance *ermB*, *ermF*; sulphonamide resistance *sulI*, *sulII*; beta-lactam resistance $bla_{CTX\text{-}M\text{-}1}$ group, $bla_{CMY\text{-}2}$, bla_{SHV} family; glycopeptide resistance *vanA*. The primers, conditions, and standard curves used are described elsewhere (Schmidt *et al.* 2014). Each sample was tested in duplicate along with a positive control template in triplicate. Furthermore, a negative template control (NTC) (23 µl mastermix and 2 µl water) was also included.

Results and discussion

One or more fecal samples collected from the NCA wildlife and cattle were positive for 8 out of the 14 antibiotic resistance genes tested, as were several samples collected from the MNP wildlife that had not interacted with cattle. Table 2 summarizes the gene copy numbers g^{-1} feces for the different antibiotic resistance determinants and for 16S rDNA that is used as a proxy for the overall bacterial population size. In addition, the isolation of antibiotic resistant coliform bacteria are indicated when present. The results from this screen give rise to concern of the antibiotic resistance genes residing within the wildlife

population in the NCA and MNP areas of Tanzania. Wild animals have previously been implicated as potential reservoirs of resistant bacteria, and might act as vehicles for the dissemination of resistant bacteria throughout the environment (Jardine *et al.* 2012).

The cattle were all positive for *tet*(W), *ermF*, *sulI*, and $bla_{\text{CMY-2}}$. *tet*(A), *tet*(M), *tet*(O), and *sulII* were also detected in minimum 1 of the 4 cattle samples (Table 2). No wildlife samples from NCA or MNP were positive for antibiotic resistance genes not also detected in the cattle. The finding of similar antibiotic resistance gene profiles in the NCA wildlife samples and the cattle was expected as samples from wildlife were deliberately obtained from animals that were observed grazing in close proximity of cattle. Three MNP samples from wildlife not interacting with cattle, Buffalo M3, Buffalo M13, and Buffalo M15, were positive for *tet*(W) and $bla_{\text{CMY-2}}$, Buffalo M3 was also positive for *sulI*. It has been postulated that buffalo and zebras may exhibit a behavior where they are frequently found in close proximity to staff housing, offices, and lodges. Therefore, the buffalo and zebra may come in contact with human refuse or even human excretes (Rolland *et al.* 1985, Selemani Katakweba 2013) accounting for the antibiotic resistance gene detection in these samples.

With a few exceptions, all samples displaying phenotypic resistance also contained the corresponding antibiotic resistance gene encoding the resistant phenotype. The Buffalo M13 sample was the only sample that was negative for all the tested antibiotic resistance gene determinants. However, this sample had a relatively low amount of 16S rDNA implying that there may not have been sufficient DNA for qPCR gene detection. In contrast, the Buffalo M15 sample had phenotypic sulphametizole resistance, but neither *sulI* nor *sulII* were detected. This could be due to the presence of another sulphonamide resistance encoding gene causing the resistant phenotype, for example *sulIII* or a mutated DHPS encoding *folP* gene (Huovinen *et al.* 1995, Bean, Livermore and Hall 2009).

The *bla*$_{CMY-2}$ gene is reported to be found on plasmids and encodes extended spectrum cephalosporinases (ESC) that confer resistance to penicillins, beta-lactam-beta-lactamase inhibitor combinations, and cephalosporins (Li *et al.* 2007, Jorgensen *et al.* 2010). The plasmids are often associated with other multiple resistance genes and transposons, and it has been suggested that the *bla*$_{CMY-2}$ spread is both clonal and horizontal (Li *et al.* 2007, Agersø *et al.*2014). This emphasizes the *bla*$_{CMY-2}$ significance in the spread of antibiotic resistance. The location of the *bla*$_{CMY-2}$ in positive strains in the current investigation was not determined, but the amount of samples that tested positive for the *bla*$_{CMY-2}$ gene (10 out of 12) was alarming. A study in Denmark demonstrated that the *bla*$_{CMY-2}$ gene was present in samples obtained from broiler flocks where cephalosporin administration has been banned for 10 years (Agersø *et al.* 2014). This shows how the *bla*$_{CMY-2}$ gene can persist without the selection pressure induced by cephalosporin antibiotics. Our data suggests that the wildlife might have a role as s reservoir for antibiotic resistance genes. If these genes spread from nonpathogenic bacterial species in the wildlife reservoir to a human or zoonotic pathogen, the consequences can be severe. Therefore, the finding of *bla*$_{CMY-2}$ among 10 of the 12 screened animals gives reason for further investigation of the antibiotic resistance gene prevalence among the wildlife in the NCA and MNP areas.

The present study investigated a total of 12 samples collected from wildlife and cattle (NCA) and wildlife samples not having interaction with cattle (MNP) for the presence of 14 antibiotic resistance genes. Despite the wildlife at MNP not interacting with cattle, resistance was observed in these samples. This resistance could arise through birds that migrate between national parks or ingestion of plants with antimicrobial properties (Adesiyun and Downes 1999, Ushimaru *et al.* 2007, Radhouani *et al.* 2013, Selemani Katakweba 2013). Based on these results, further studies should be conducted to study the antibiotic resistance gene-pool among the wildlife in northern Tanzania in depth. It is

important to minimize excessive antibiotic use in order to reduce selection of antibiotic resistant bacteria both among humans, livestock, and wildlife.

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Conflict of Interest

No conflict of interest declared.

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Table 1 Sampling site in Tanzania (Ngorongoro Conservation Area (CA), Mikumi National Park (NP)), animal species, laboratory number, and cattle interaction status (+= interaction; -=no interaction) for the fecal samples included in the present study.

Sampling site	Animal species	Laboratory number	± Cattle interaction
Ngorongoro CA	Cattle	N22	+
Ngorongoro CA	Cattle	N23	+
Ngorongoro CA	Cattle	N43	+
Ngorongoro CA	Cattle	N54	+
Ngorongoro CA	Buffalo	N21	+
Ngorongoro CA	Buffalo	N58	+
Ngorongoro CA	Zebra	N1	+
Ngorongoro CA	Zebra	N5	+
Mikumi NP	Buffalo	M3	-
Mikumi NP	Buffalo	M13	-
Mikumi NP	Buffalo	M15	-
Mikumi NP	Wildebeest	M16	-

Table 2 Resistance genes g^{-1} feces quantified in total DNA extracted from the following samples: Massai cattle and wildlife from Ngorongoro Conservational Area (N22, N23, N43, N54, N21, N58, N1, N5) or wildlife from Mikumi National Park (M3, M13, M15, M16) in Tanzania. Dark grey sections=antibiotic resistance genes levels were over the assays' limit of quantification. Unshaded numbers= resistance genes levels were over the assays' limit of detection. 0 = no resistance genes detected. The samples with phenotypically resistant coliform bacteria are denoted with a letter, each representing their antibiotic group (A=Ampicillin, C=Cefotaxime, S=Sulphametizole,T=Tetracycline).

Genes / Sample	Gene copies g^{-1} feces															
	tet(A)	*tet*(B)	*tet*(C)	*tet*(M)	*tet*(O)	*tet*(W)	*ermB*	*ermF*	*sulI*	*sulII*	bla_{SHV} family	$bla_{CTX-M-1}$ group	bla_{CMY-2}	*vanA*	16S rDNA	Phenotypic resistance
Cattle N22	0	0	0	2.57E+03	0	7.03E+04	0	1.27E+05	2.38E+03	0	0	0	2.25E+04	0	3.98E+09	A
Cattle N23	0	0	0	0	0	2.66E+05	0	1.27E+04	5.49E+02	0	0	0	3.23E+03	0	6.47E+09	T
Cattle N43	1.03E+04	0	0	0	6.29E+04	3.43E+05	0	1.09E+07	7.90E+04	2.42E+06	0	0	8.54E+03	0	2.01E+09	A
Cattle N54	0	0	0	0	1.05E+04	1.58E+05	0	2.45E+04	2.46E+02	0	0	0	8.04E+03	0	1.91E+09	
Buffalo N21	0	0	0	0	0	3.63E+03	0	2.31E+04	1.36E+02	0	0	0	6.44E+03	0	1.09E+09	T, A, C
Buffalo N58	0	0	0	0	0	1.76E+03	0	2.15E+04	4.23E+03	0	0	0	9.73E+03	0	2.02E+09	T, A
Zebra N1	0	0	0	0	0	5.55E+03	0	0	1.51E+03	0	0	0	6.27E+03	0	8.34E+08	A
Zebra N5	0	0	0	0	0	2.82E+03	0	8.17E+03	8.01E+02	0	0	0	7.69E+03	0	9.95E+08	T, A, C
Buffalo M3	0	0	0	0	0	4.72E+04	0	0	8.05E+03	0	0	0	3.83E+03	0	1.38E+09	S, C
Buffalo M13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6.72E+07	T, S, A
Buffalo M15	0	0	0	0	0	3.87E+04	0	0	0	0	0	0	4.62E+03	0	5.01E+08	T, S, A
WildebeestM16	0	0	0	0	0	5.93E+03	0	0	0	0	0	0	0	0	1.87E+08	T, S, A