ANTIMICROBIAL USE AND RESISTANCE

IN BELGIAN PIG PRODUCTION

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List of abbreviations

ADD_{pig}	animal daily dose pig
AMCRA	Antimicrobial Consumption and Resistance in Animals
APP	*Actinobacillus pleuropneumoniae*
AR	atrophic rhinitis
CBP	clinical breakpoint
CIA	critically important antimicrobials
DCDA	defined course dose animal
DDDA	defined daily dose animal
ECV	epidemiological cut-off value
ESBL	extended-spectrum beta-lactamase producing *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
FAO	Food and Agriculture Organization
GAP	good agriculture practices
GVP	good veterinary principles
HGT	horizontal gene transfer
I	intermediate
IM	intramuscular
LA	long acting
LA-MRSA	livestock-associated methicillin-resistant *Staphylococcus aureus*
LS	lincomycine-spectinomycine

non-WT	non-wild type
MIC	minimum inhibitory concentrations
MPC	mutant prevention concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MSW	mutant selection window
NRI	normalized resistance interpretation
OIE	Office Internationale des Epizooties
OR	odds ratio
PCT	percentile
PDDA	prescribed daily dose for animal
PR	proportion of resistance
R	resistant
S	susceptible
SD	standard deviation
SPC	summary of product characteristics
STEC	Shiga toxin-producing *Escherichia coli*
TI	treatment incidence
TI_{ADDpig}	treatment incidence based on the ADD_{pig}
TI_{UDDpig}	treatment incidence based on the UDD_{pig}
TMS	trimethoprim/sulfadiazine
UDD_{pig}	used daily dose pig
UDDA	used daily dose for animal

VCIA	veterinary critically important antimicrobial agents
WHO	World Health Organization
WT	wild type
ZnO	zinc oxide

Chapter 1

General introduction

ANTIMICROBIAL USE IN FOOD-PRODUCING ANIMALS

1. DEFINING ANTIMICROBIALS *IN LATU* OR *STRICTU SENSU*

Antimicrobials in broad sense are defined as all compounds with a direct action on micro-organisms. They include antibacterial, antiviral, antifungal and antiprotozoal compounds (EPRUMA, 2013). Antibacterial compounds or antibiotics are then determined as compounds with a direct action on bacteria, both naturally occurring and (semi)synthetic chemical compounds, such as β-lactams and quinolones, respectively. Next to antibacterial activity, certain antibiotics such as sulfonamides and pyrimidines show activity against coccidia (Giguère, 2013). Throughout this review, we will speak both of antimicrobials and antibiotics or antibacterial agents/compounds when referring to substances with antibacterial activity.

2. DIFFERENT AIMS FOR THE USE OF ANTIBIOTICS IN FOOD-PRODUCING ANIMALS

The use of antibacterial compounds in veterinary practice started soon after they became available for the treatment of human diseases in mid 1940s (Aryal, 2000). Sulfonamide was the first antibiotic to be formally introduced to food animal medicine in the 1940s, but penicillin was most likely already used before as the first antibacterial compound to treat bovine mastitis (Gustafson and Bowen, 1997; Aryal, 2000). Currently, in veterinary medicine, antibiotics are used to prevent and to treat bacterial infections of an individual animal or a group of animals (Table 1). **Preventive treatment or prophylaxis** is defined as the treatment before clinical signs of disease, in order to prevent the onset of disease or infection (EPRUMA, 2013), which might result in decreased production results. Prevention is based on a high probability of disease to occur, at key time points where animals are generally recognized as more susceptible to infections (Schwarz and Chaslus-Dancla, 2001). Current livestock production systems rely on the application of antibiotics to groups of animals. Examples of when these strategic treatments are performed are prevention of diarrhoea in weaned piglets or veal calves through medicated feed or medicated milk replacer respectively, necrotic enteritis in broilers, caesarean section or other surgery, transport and mixing of animals, or intra-mammary dry cow treatment at the end of lactation in dairy cattle (Schwarz and Chaslus-Dancla, 2001). The treatment of an ill animal or group of animals preceded by a diagnosis of a disease or infection is called **curative or therapeutic** (EPRUMA, 2013). In veterinary medicine, curative treatment is historically linked to the individual animal, as large groups of animals are often already treated before infection is spread to all animals. A third common practice is **metaphylaxis or control treatment**. This can be defined as the treatment of a group of animals after the diagnosis of clinical disease in part of the group, with the aim of treating the clinically sick animals and controlling the spread of disease to animals in close contact and at risk which may already be (subclinically) infected (EPRUMA, 2013). Metaphylaxis may not only reduce the number of sick or dead

animals. It could also decrease the total amount of antibacterial agents needed to treat a large number of symptomatically ill animals, consequently reducing treatment costs (Schwarz et al., 2001).

The introduction of antibiotics to cure or prevent diseases and infections came along with the discovery of advantageous production effects in animals of small amounts of antibiotics in feed, after experimental observations of feeding fermentation waste from tetracycline production to chickens (Gustafson and Bowen, 1997). The mechanisms behind the benefits of using antibacterial growth promoters (AGP) are only partially revealed (Edqvist and Pedersen, 2000; Reti et al., 2013). Though, the use of antibacterial compounds at a low, subtherapeutic dose over long periods of time, showed to promote growth rates and feed conversion, to improve egg production, to increase litter size in sows and to prevent a reduced milk yield associated with ketosis in dairy cows (Edqvist and Pedersen, 2000; Page et al., 2005). Furthermore, they provided protection against certain diseases promoted by intensification of animal production. As a result, AGP became soon an integrated part of several industrialised animal husbandry production systems.

Table 1. Reasons for antibiotic use in food-producing animals individually or at group level and the most frequently applied administration routes.

	Antibiotic administration:	Individual or group treatment in food-producing animals?	Administration route
Preventive treatment or prophylaxis	Before clinical signs of disease	Group of animals	Oral, via feed or water Topical
Curative or therapeutic	Preceded by a diagnosis of a disease or infection	Mostly individual	Parenteral
Metaphylaxis or control treatment	After the diagnosis of clinical disease in part of the group	Subgroup of animals	Oral, via feed or water
Growth promotion*	To promote growth rates and feed conversion, to improve production	Group of animals	Oral, via feed

* The use of the last 4 antibacterial growth promoters have been banned in the European Union in 2006 (European Commission, 2005).

3. ANTIBIOTIC CLASSES IN VETERINARY MEDICINE

The number and variety of antibacterial compounds available for veterinary use have increased rapidly since penicillin was used as the first antibiotic in veterinary medicine. In total around 26 different antibiotic classes are currently licensed in the world. An antibiotic class can be defined as a group of agents with a similar mechanism of action, regardless of chemical structure (Collignon et al., 2009). Within classes, divergent subgroups or generations might be present with activity against different spectrums of bacteria. For instance, 3th generation cephalosporins have decreased Gram-positive but increased Gram-negative antibacterial activity compared to the 2nd generation (Prescott, 2013). Yet, in general, newer generations have been developed to improve pharmacodynamic and pharmacokinetic traits and to overcome resistance problems (Chopra and Roberts, 2001).

Besides working mechanism and spectrum, classes of antibiotics are characterized by several features and these might differ or match to various degrees between and within classes. These characteristics include oral bioavailability, tissue penetration, elimination routes, toxicity and interaction with heavy metals or other antibacterial compounds. The latter can result in either synergism or antagonism. As the characteristics of antibiotics are not a part of this thesis, they will not be further discussed.

4. ROUTES OF ADMINISTRATION

Antibacterial drugs, as all veterinary drugs, can be administered via various routes. Local or topical use in food-producing animals involves cutaneous (through the skin), nasal, intra-articular, intra-ocular, intra-auriculair, and finally intra-mammary and intra-uterine as the main routes. Systemic use is defined as each administration via oral or parenteral routes. The latter includes then intravenous, intramuscular, intraperitoneal, transdermal and subcutaneous injection (EPRUMA, 2014).

Oral administration in food-producing animals is performed either by medicated feed or water. Medicated premixes or water soluble drugs are then mixed in feed and water under controlled conditions. Also, oral powders are available and can be mixed into the feed on the farm.

As mentioned above, in animals for food production, the number of animals treated is often consistent with what wants to be achieved by giving antibiotics, prevention, metaphylaxis or treatment. This can however be expanded to the routes of administration (Table 1). The preventive and metaphylactic treatment of groups of animals by oral administration through feed or water has been reported for veal calves (Pardon et al., 2012) and poultry (Persoons et al., 2012). Nevertheless, in dairy cattle, at the end of the lactation period, dry-cow therapy is applied via intra-mammary injection and thus topical therapy is here used to a large number of animals (Schwarz et al., 2001). In intensive livestock farming, parenteral therapy might be the route of choice in order to cure sick individual animals (Schwarz et al., 2001).

Furthermore, concerning dosing and administration routes, in veal calves, it has been seen that orally administered antibiotics are often underdosed compared with the recommendations on the prescription leaflet (Pardon et al., 2012). In poultry, orally administered doses were generally more respected (Persoons et al., 2012). Parenteral treatment was more often overdosed (Pardon et al., 2012). Uncorrect dosing might have specific implications regarding development and selection of resistant mutants depending on the antibiotic used and the bacteria on which selection pressure is exerted. Potential effects on the prevalence of resistance related to dosing are more explained into detail in ***Chapter 1.3.*** of this thesis.

5. IMPACT OF ANTIMICROBIAL RESISTANCE EMERGENCE ON THE ANTIMICROBIAL USE POLICY

The potential risks associated with the extended use of antibiotics, and more in particular of penicillin were already expressed by Alexander Fleming in 1945. Indeed, for many antibiotics the time coincidence between the discovery and production of antimicrobial agents, their introduction into clinical use as well as the emergence of resistant bacteria is unmistakable (Schwarz and Chaslus-Dancla, 2001). For decades, scientists, public health services as well as organizations dealing with animal health have warned for the threats associated with resistance, namely a decreased animal welfare, endangered animal and human health as well as a continued food production under pressure (Vose et al., 2001; EFSA-ECDC, 2013).

5.1. THE SWANN REPORT

In 1969, a joint committee of scientists expressed their concerns on a potential higher risk of the prevalence of resistant pathogenic bacteria in man associated by giving antibiotics to animals (Swann Report, 1969). Clear recommendations on the use of antimicrobial compounds in animal husbandry were formulated to inform policymakers. They urged for a control of antibiotics in feed, as well as therapeutically used. Global surveillance of animal and human related resistant bacteria and research on possible other ways to reduce the burden of infectious diseases was proposed (Swann report, 1969).

5.2. THE BAN ON ANTIMICROBIAL GROWTH PROMOTERS (AGPs)

Following the Swann Report, a broader debate on the potential hazards of antibiotic resistance linked to antibiotic use was initiated in both animals and humans. The prospective farm study by Levy in 1975 (Levy et al., 1976) and many other studies in the following decades clearly demonstrated the selective nature of low-dose, nontherapeutic AGP on both the pathogenic and commensal flora of food animals such as poultry, swine and cattle (Marshall and Levy, 2011). In Sweden, findings on the risks associated with AGP, led to the use of antibacterial compounds solely allowed for therapy and on veterinary prescription from 1986 onwards (Wierup, 2001). The European Union followed with the ban of the growth promoter avoparcin in 1997 and bacitracin, spiramycin, tylosin and virginiamycin in 1999 (Casewell et al., 2003). Still, the last 4 antibiotics

permitted as feed additives to help fatten livestock have only been banned in the EU in 2006, which was the final step of phasing out all antibiotics used for non-medical purposes (European Commission, 2005).

Outside Europe, the use of antibiotics as growth promoters and without veterinarian prescription is still accepted for a large range of compounds, such as tetracyclines, macrolides, bambermycine and streptogramins (Chopra and Roberts, 2001; Reti et al., 2013). Yet, their use is under increasing regulatory and political scrutiny in both the United States (US) and China. The use of antibiotics for growth promotion, increased performance, and improved feed efficiency would no longer be permitted in the US (Allen et al., 2013). Recently, the Center for Veterinary Medicine of the American Food and Drug Administration (FDA) announced the withdrawal of 16 antimicrobial drug applications in 2014 in order to phase out antibiotic use for production purposes, necessary for assuring health in food-producing animals (FDA, 2014). Additionally, certain antibiotics of critical importance, such as 3th generation cephalosporins, are likely to be restricted to human use in the near future even if they are important for animal disease treatment (FDA, 2013). Furthermore, they urge on the requirement for veterinarian involvement in the decision to use antibiotics (FDA, 2012).

5.3. CLASSIFICATION OF ANTIBIOTICS ACCORDING TO ANIMAL AND PUBLIC HEALTH

Many antibacterial classes and/or subgroups within classes are used both in human and veterinary medicine (Moulin et al., 2008; Collignon et al., 2009). Currently, last line agents like carbapenems, lipopeptides and oxazolidinones have no veterinary equivalent. Colistin and even some of the carbapenems, although used as last resort in the treatment of extended-spectrum β-lactamase producing *Escherichia coli* (ESBL) infected patients in human medicine, are in a more or lesser extent used in veterinary medicine, in the framework of the cascade legislation (Gibson et al., 2008). As a result , the use of certain antibiotics give particular rise to debate regarding the impact of increased resistance in human and/or veterinary medicine and the potential transmission of resistant determinants between animals and humans. In 2005, the World Health Organization (WHO) developed criteria to rank antibiotics according to their importance in human medicine (‘critically important’ – ‘highly important’ or ‘important’) to ensure that critically important antimicrobials (CIA) are used prudently both in human and veterinary medicine (2011). These lists aim at helping regulators and stakeholders to determine which types of antibacterial agents could be used in food animal production and to determine how these agents might be managed (e.g., single animal therapy or mass treatment via water, prohibiting extra-label use, etc.). Meanwhile, a second and third revision of the list have been made in 2007 and 2011 respectively to re-evaluate the classification of antimicrobials and update the list on the basis of recent developments (WHO, 2011). Similarly as in human medicine, in 2007, the World Organisation for Animal Health (OIE) established a criteria based list of all antibacterial agents used in food-producing animals with the objective to safeguard the efficacy and availability of veterinary antimicrobial agents for animal diseases where they are few or no antimicrobial alternatives (OIE, 2007). The list furthermore intended to help

veterinarians in their therapeutic choice. A revision of the list occurred in 2012 and within the One Health concept, a joint expert group of the OIE, the Food and Agriculture Organisation (FAO) and the WHO assigned specific recommendations to the fluoroquinolones and the 3rd and 4th generation of cephalosporins (OIE, 2014). Among the Veterinary Critically Important Antimicrobial Agents (VCIA) in the OIE List, these two classes are considered to be critically important with respect to transmission of resistance.

Only very recently, a French group of experts established a methodology in order to evaluate and recognize antibiotic use practices at risk in order to decrease human and animal health threats associated with resistance determinants selection (ANSES, 2014). The method does not solely take into account the importance of a certain class to treat infections in animal and human health, according to OIE and WHO respectively, but equally the routes (local, parenteral, oral and other routes) and reasons for administration (curative, metaphylactic or preventive). Furthermore, additional factors specific per animal species and production stage to which antibiotics are administered and the potential for alternatives are taken into account. This methodology resulted into a categorization per animal production sector of antibiotic (sub)classes in 4 groups : 'Les pratiques à risque à abandonner sans délai, 'Mesures visant à abandonner la pratique à terme', 'Les pratiques à encadrer' and 'Les pratiques sans encadrement supplémentaire'.

Furthermore, in Belgium, AMCRA, the Belgian "centre of knowledge on antimicrobial use and resistance in animals", launched in 2012, has developed colour codes assigned to active substances licensed in veterinary medicine. The aim of the colour codes 'yellow', 'orange' and 'red' is to inform veterinarians, farmers and policy makers on their importance for human and animal health. Also, the colour codes represent conditions, presented in table 2, which have to be fulfilled before the active substance can be used. The assignment of the colour codes is based on the WHO and OIE lists ranking antibiotics with importance for human and animal health (WHO, 2011; OIE, 2014). Also the advice of the Health Council of the Netherlands has been taken into account. When producing the lists priority was given to human health. Within the red-coloured substances, the fluoroquinolones and 3rd and 4th generation cephalosporins, can be found, emphasizing their relevance in human and veterinary medicine (AMCRA, 2014a).

Table 2. Conditions for use of active substances with colour code 'yellow', 'orange' or 'red', proposed by AMCRA (2014a).

Can the active substance be used for preventive reasons?	**Can the active substance be present in the stock of 2 months at the herd?**	**Should an additional diagnostic laboratory test be done?**	**Should an antimicrobial susceptibility test be performed?**
No	Yes	It is preferred	It is preferred
No	Yes	Condition for use	It is preferred
No	No	Condition for use	Condition for use

5.4. MEASURE TO MANAGE

Antibiotic consumption data are a key element in the establishment of strategies for containing resistance. At national and/or regional levels such data are vital for detecting trends in use, assessing the effect of responsible use campaigns, risk management measures such as legislative restrictions of use, and implementation of animal health prevention measures.

As a result, surveillance of antimicrobial use has been widely recommended internationally in recent decades and data collection promoted in all sectors of use (human medicine, veterinary medicine and agriculture) (FAO/OIE/WHO, 2003).

6. QUANTITIES OF ANTIBIOTICS USED

6.1. HOW TO MEASURE?

Sweden and Denmark, followed by Norway and the Netherlands were the first to start collecting national sales data from the nineties onwards (Ungemach et al., 2006; NORM-VET, 2012; SWEDRES-SVARM, 2012; Bos et al., 2013; DANMAP, 2013; MARAN, 2013). Different indicators and technical units of measurement for antibiotic use in food-producing animals can be used with advantages and disadvantages for each of them (Table 3). In the above reports, the total use is expressed in kg or tons of active substance and the actual biomass of the animal population at risk is used as denominator data (Ungemach et al., 2006; NORM-VET,

2012; SWEDRES-SVARM, 2012; Bos et al., 2013; DANMAP, 2013; MARAN, 2013). This method allows a crude estimation of how usage evolves in time and between countries or geographical areas. More detailed denominator data equally make it possible to compare animal species or production stages within one species, farmers or food animal producers, and veterinarians (Grave et al., 2010). Yet, many of the veterinary antimicrobial products are licensed for several species, and an approximation is required to reallocate the amounts sold to the different species. The relative use does still not take into account differences in potency of an active substance (Jensen et al., 2004). For instance, 3th and 4th generation cephalosporins have a higher potency compared to old substances as tetracyclines and trimethoprim, resulting in different dosages per kilogram of body weight between and within antibiotic classes (LEI, 2011; Silley et al., 2012). Therefore, an alternative way to quantify consumption data is by expressing the use in terms of number of dosages applied in an animal species. Yet, differences in daily dose between antibiotic agents, pharmaceutical forms, animal species and countries give rise to the need of standardized doses or defined daily dose animals (DDDA), as proposed by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). The ESVAC consortium has defined the Defined Daily Dose Animal (DDDA) as "The assumed average maintenance dose per day per kg body weight for the main indication in a specified species". The DDDA is not defined at product level but for each antimicrobial agent, administration route and animal species and when appropriate, also age group, and is not related to one country. As these standardised DDDAs are currently missing, a group of experts recently attempted to assign standardised DDDAs for antibiotics authorized in pig production in Belgium, Germany, France and Sweden (Postma et al., 2015). Ideally, to allow on comparison of countries, the use of DDDAs is combined with reliable usage data per animal species, because a country overall average is influenced by animal demographics and therefore an inaccurate indication of true differences of exposure, per species (Bondt et al., 2012).

In order to take into account simultaneously dose and duration of treatment, ESVAC equally defined the Defined Course Dose Animal (DCDA) as a second technical unit. Whereas the DDDA and the DCDA have the potency of being harmonised between countries, the Prescribed Daily Dose for Animal (PDDA) and Used Daily Dose for Animal (UDDA) are units expressing the prescribed and real dose administered under field conditions. The UDDA reflects valuable information regarding the precise amounts of antibiotics exerting selection pressure and allows to estimate the correctness of administered doses relative to the DDDA. As a result, it would be in conflict with their nature to standardise them between countries. For European comparisons, numbers of DDDA or DCDA used by animal species or specific weight group divided by the number of animals produced or livestock by country and year, has been proposed by ESVAC as the indicator for reporting consumption. This presents then the number of standardized daily doses or course doses respectively administered for an animal species of a specific weight group in year by country (EMA, 2013a).

Table 3. Indicators and corresponding technical units of measurements of antibiotic use in food-producing animals. For each indicator, nominator and denominator data are shown. Pro's and con's are listed for the technical units of measurement.

Indicator for antibiotic use	Technical unit of measurement	Nominator	Denominator	Pro's	Con's
Amount of active substance /biomass of animal population at risk	Weight of active substance (kg)	Total amount of active substance sold (kg)	Number of live and slaughtered animals x standard weight at treatment	- Direct accessible - Allows combining different animal species	- Requires reallocation of the amounts of antibiotics sold to the different species - Differences in potency are not taken into account
DDDA/1000 animals/year	DDDA	Number of DDDA used by animal species or weight group	Number of animals produced or livestock per time span (year)	- No bias due to differences in potency	- Requires harmonization for comparisons
UDDA/1000 animals/year	UDDA	Number of UDDA used by animal species or weight group	Number of animals produced or livestock per time span (year)	- Estimates correctness of the administered dose relative to the DDDA	- Requires detailed data at the herd level
DCDA/1000 animals/year	DCDA	Number of DCDA used by animal species or weight group	Number of animals produced or livestock per time span (year)	- Takes into account both dose and number of days of treatment	- Requires harmonization for comparisons
PDDA/1000 animals/year	PDDA	Number of PDDA used by animal	Number of animals produced or livestock per	- Estimates correctness of the prescribed	- Requires detailed data at the level of the individual

		species or weight group	time span (year)	dose relative to the DDDA	veterinarian

Besides which technical units of measurement and indicator might be used, ESVAC also reported on which data sources on consumption are appropriate (EMA, 2013a). Different data sources fulfil to a more or lesser extent the objectives set when collecting data (FAO/OIE/WHO, 2003). Ideally, data are continuously collected at the level of the farm or veterinarian. As mentioned above, data collection systems aim at targeted and accurate action, namely where high usage patterns are observed. Detailed information on both quantitative and qualitative aspects of use are then required. Animal species or production stage specific information on used quantities, on which active substances, for which indications, and through which routes administered, are preferable (Silley et al., 2012). Thereto, collection at the level of the farm is essential (EMA, 2013a). Surveillance systems at the national level, unless provided by marketing authorisation holders (MAHs), do not provide the species specific information. Yet, despite these shortcomings, they might serve as validation of other data sources (FAO/OIE/WHO, 2003).

6.2. TRENDS IN OVERALL USAGE IN ANIMAL HUSBANDRY

IN EUROPE

Only few countries started collecting data from the nineties onwards and trends of their antibiotic consumption during time can be studied. Only qualitative between country comparisons will be made, in order not to conflict with the abovementioned assumptions of comparisons. Data from the United Kingdom, the Netherlands and Denmark showed a substantial increase in the use of antibiotics for food animals from 1999 onwards (Casewell et al., 2003; DANMAP, 2013; MARAN, 2013). In Finland, the overall consumption increased between 2001 and 2008 (Finnish Food Safety Authority, 2011). The overall increase coincides with the ban of growth promoters, whereas this ban can be seen as a first attempt to decrease veterinary use of antimicrobials. Also the Fédération Européenne de la Santé Animale (Fedesa) reported an increase in antimicrobial use in veterinary medicine in the European Union and Switzerland by 408 tons for the year 1999, while in the meantime the non-therapeutic use of antibiotics as growth promoters in farm animals declined by 51% to 786 tons (Ungemach et al., 2006). A ban on AGP was predicted by sceptics to increase the number of both subclinical and clinical infections (Castanon et al., 2007). Indeed, after the ban, infections associated with *Lawsonia intracellularis* in pigs and with *Clostridium perfringens* in poultry were amongst the increased reported diseases in Denmark (WHO, 2002). The above mentioned data confirmed that the ban had most likely led to, at least partially, an increase in use for therapeutic reasons (Casewell et al., 2003). Remarkably, Norway reported no increase at all, but even succeeded in a continued decrease in antibiotic

use in food producing animals from 1995 to 2012 by approximately 36%, while the total animal population remained more or less stable (Norm-Vet, 2012). This has been explained by a reduction target of 25% from 1995 onwards, set by Norwegian husbandry organisations and simultaneously prudent use campaigns.

In 2010, The Netherlands, at that time among the top of high users of antimicrobials in European Union (EU)/European Economic Area (EEA) countries (EMA, 2012), declared to respond to its antibiotic resistance crisis with a mandate to reduce their use in food animals by 50% by 2013 and 70% by 2015 and furthermore to establish a registration process for veterinary prescriptions of antibiotics. Indeed, the MARAN report showed for 2012 a decrease with the pre-established objective of 50% (MARAN, 2013). Furthermore, a decrease in the sales of antimicrobial compounds between 2010 and 2011 for both food producing (including horses) and companion animals has been reported for 19 out of the 20 EU/EEA countries that provided data to the European Medicines Agency (EMA, 2013b). Suggested explanations provided by the countries for the decline in sales are, among others, implementation of responsible-use campaigns, restrictions of use, increased awareness of the threat of antimicrobial resistance, and/or the setting of targets (EMA, 2013b).

IN BELGIUM

In Belgium, national sales data are annually reported from 2007 onwards and consist of all veterinary antibiotics sold to a veterinarian or pharmacist in Belgium and of antibacterial premixes incorporated in medicated feed intended to be used in Belgium (BelVet-SAC, 2015). Data include thus consumption data for farm animals as well as companion animals. The denominator for animal production is the biomass (in kg) calculated as the sum of the amount of beef, pork and poultry meat produced in 2010, plus the number of dairy cattle present in Belgium times 500 kg of metabolic weight per individual head. Since the onset of the data collection in 2007, the highest usage was also observed for that year (168.66 mg active substance per kg biomass). Figure 1 shows the evolution of national sales data for antibacterial pharmaceuticals and premixes (BelVet-SAC, 2015). When looking at the data from 2007 onwards, a decrease of 14,3% in total consumption can be observed by 2011. A substantial part of this decrease was realized between 2007 and 2008 (11.4%), but the level of use remained more or less stable between 2008 and 2011.

After an overall reduction (in terms of mg per kg of biomass produced) of 12.7% between 2011 and 2013, in 2014, a small increase of 1.1% was recorded. When using 2011 as a reference, still a reduction of 11.8% is achieved, distributed over a reduction of 12.2% in antibacterial pharmaceuticals and 10.0% in antibacterial premixes (BelVet-SAC, 2015). Yet, additional efforts will be needed to be in line with the recently set objective of a 50% reduction in antimicrobial use in animals by 2020 (2011 is the reference year), proposed by the partners of AMCRA (AMCRA, 2014b). Additionally, a reduction of 75 % in CIA 3rd and 4th generation cephalosporins and fluoroquinolones has been proposed by AMCRA by 2020. Compared with 2011, the use of quinolones dropped with 18.9% in 2013, but increased again by 5.3% in 2014 compared to 2013. The use

of 3th and 4th generation cephalosporins can be considered decreasing with 6.7% between 2011 and 2014 (AMCRA, 2015).

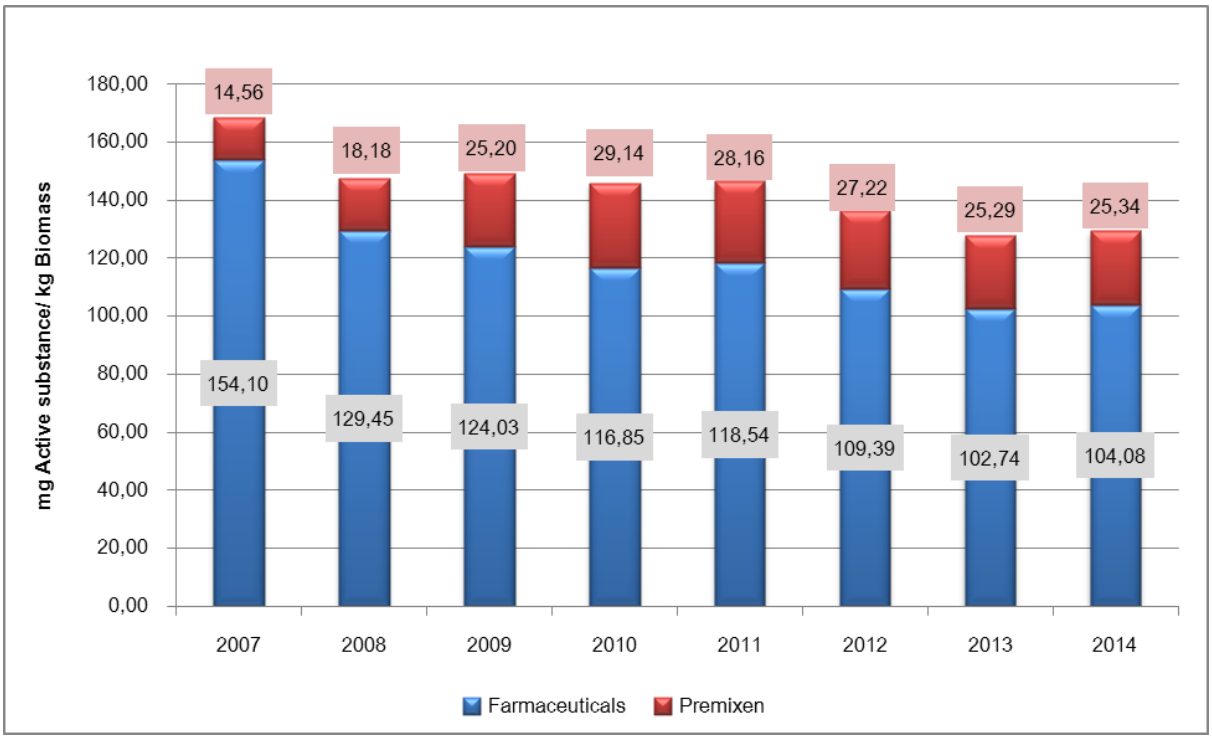

Figure 1. Evolution of national sales data for antibacterial pharmaceuticals and premixes expressed in mg active substance per kg biomass for all animals in Belgium (BelVet-SAC, 2015).

7. ANTIMICROBIAL USE IN PIG PRODUCTION

Pig production is characterized by 3 major production stages, based on the function, weight and age of the animals. Average animal weights for a certain production category and at the end of fattening period, before slaughter can differ between countries. This might result in different weights used when calculating incidences of antimicrobial treatment. Sows are breeding animals and used for the production of piglets. Sows and piglets are housed in the farrowing unit until piglets are weaned, generally between the age of 21 and 28 days. After weaning, piglets are placed in the so called 'nursery unit'. From 10 weeks onwards, the growing pigs are raised until slaughter. In general, pig production management consists of open, closed or semi-closed herds. Closed herds breed and rear fattening pigs (farrow to finish). Semi-closed farms may have animal supply, for instance, the purchase of gilts. Ideally, the number of sources should be limited to minimize the odds for infectious agents to enter, compared to closed farms. Open herds solely breed or raise pigs. Sow herds raise the piglets and these are moved at weaning or at the onset of fattening to fattening pig herds that only receive piglets from these sow herds.

7.1. IN EUROPE

The pig production sector is believed to be amongst the highest users of antimicrobials in intensive animal husbandry production (Bondt et al., 2012; Bos et al., 2013; DANMAP, 2013). In pig production, as well as in broiler and veal calf production, animals have a short lifetime and younger animals demand relatively more treatment with antibiotics, as they are more susceptible to diseases (Bondt et al., 2012). This might partially result into a relatively higher use per time period in pigs compared to sectors with longer production cycles, for instance in beef cattle. Denmark used to compare antibiotic usage in pigs with other species based on kilograms of meat produced (DANMAP, 2013). However, this measure overestimates the selection pressure in species with long lives (e.g. cattle), while underestimating the selection pressure in species slaughtered at an early age (e.g. broilers). Also, comparing different animal categories or production stages have their pitfalls, as these categories have inherent differences in farm management, including antibiotic administration. Suckling and weaned piglets, for instance, are more susceptible to enteric diseases than other age groups. Consequently, results on antimicrobial use data in pigs of different ages should be interpreted with a certain background knowledge.

As mentioned above, data on a specific animal species are needed to indicate the true levels of exposure (Bondt et al., 2012), and these data can be collected at different levels. Detailed information on pigs, animal categories (sows, piglets, fattening pigs) and individual herds can be obtained by means of single surveillance studies, eventually repeated in time. Both Canada and France gathered information on quantitative as well as qualitative aspects of antibiotic use in pigs, through a survey of Canadian swine producers and French pig veterinarians respectively (Dunlop et al., 1998a; Dunlop et al., 1998b; Chauvin et al., 2002).

Secondly, well-established surveillance systems on a continued basis can be used and this demands sources such as feed mills, pharmacies and veterinarians to deliver this data. Sweden has antibiotic sales data for pigs expressed in mg active substance per kg slaughtered pig (SWEDRES-SVARM, 2012). A decrease of 13% was seen between 2008 and 2012. In 2012, the sales of CIA/VCIA fluoroquinolones for pigs were 32% lower than in 2008. Sales of CIA/VCIA 3th generation cephalosporins were insignificant (SWEDRES-SVARM, 2012).

So far, only two countries register and report data on pig sector consumption of antimicrobials based on veterinarian prescriptions (Jensen et al., 2004; Bos et al., 2013). Denmark collects prescription based data since 2001 and reports annually per animal species, including pigs (DANMAP, 2013). The Danish national reports used to express the ‘on prescription based consumption data’ as the ADD related to total biomass per year at risk, kg meat produced or number of animals produced (DANMAP, 2013). Yet, from 2012 onwards, the Defined Animal Daily Dose (DADD) is used in order to cope with different ADDs between products with the same active compound, route of administration and formulation. The urge for such country-independent standardized doses has been expressed by the ESVAC (standardized DDDA as defined above) (EMA,

2013a). Yet, DADD has been specifically defined for use in DANMAP and is currently not standardized with other countries. DADDs are expressed per 1000 animals per day (DAPD). The DAPD is the proportion (in thousands) of animals treated daily with an average maintenance dose of a particular antimicrobial agent.
During the last decade, the DAPD in pig production increased by 49% from 2003 to 2009. However, in 2010 and 2011, a decrease in DAPD by 23% compared with 2009 was observed, probably as a response to the Danish Veterinary and Food Administration (DFVA) implementation of the "yellow card initiative" – a special provision for reduction of antibiotic consumption in pig production. Yet, continued efforts on awareness are needed as in 2012, again an increase by 8-9% in antibiotic use was seen for the production of a slaughter pig (total DAPD equalled 30). Highest DAPDs are observed in the weaned pigs (100), whereas the DAPDs in sows and finishers are more similar in magnitude (20). The DAPD increased the most in weaners (15%) and finishers (10%) and less in sow herds (2.9%), and this was almost entirely (97%) associated with an increasing use of primarily tetracyclines and macrolides in all age groups. The DAPD of tetracycline increased by 15%, while the use of macrolides increased by 19%. No use of CIA's and VCIAs cephalosporins and quinolones has been reported in one of the pig categories in 2012.

The Netherlands reported data on an annual basis from 1999 onwards for all animal species. Data on pigs are available from 2004 onwards, for a selection of pig herds, based on stratified sampling (Bondt et al., 2012). Meanwhile, Dutch large animal production sectors recently implemented centralised registration systems, monitoring the use on all farms (Bos et al., 2013). From the data on all farms, it was seen that the outcomes based on the sample of farms may give a biased estimate of antimicrobial consumption (Bos et al., 2013). Furthermore, information on all farms allow to get insight in the shape and width of the distribution of individual herd use and large variations in use between herds were observed (Bondt et al., 2012; Bos et al., 2013). In the Netherlands, data on antimicrobial consumption in pigs are expressed as ADD per animal year (dd/ay) for the stratified sample (Bondt et al., 2012), and more recently for all pig herds, the Animal Daily Defined Dose per year (ADDD/Y) is used (Bos et al., 2013).

As mentioned above, the Dutch government set a policy objective for 2013 and 2015, namely a 50% and 70% reduction in antibiotic use in animals compared to 2009. The 50% objective was already achieved in 2012, partially due to a decrease in use in pig sector. Between 2004 and 2012, annual variation in the dd/ay in sows and piglets was seen, with a strong decrease as from 2009, which seemed to level off slightly in 2012. In 2012, the average use in sows/piglets is estimated to be 10 dd/ay and most antimicrobials are likely used for the treatment of the piglets, and only incidentally for the sows. In fattening pigs, an increase in dd/ay was seen until 2008, and a strong decrease from 2008 to 2012 (6 dd/ay) (Bondt et al., 2012). Regarding the use of CIA's and VCIA's, since 2009, the use of macrolides decreased substantially. In 2012, the use of 3th

and 4th generation cephalosporins and the use of fluoroquinolones in sows, piglets and fattening pigs dropped to zero.

In order to achieve the 50% and 70% goals, thresholds for veterinary antimicrobial use on individual livestock herds were determined in 2011 by the Veterinary Medicines Authority (SDa). These thresholds were based on the 50th and 75th percentiles of the distribution of DDDA over all pig herds in The Netherlands and were translated into signal and action categories, respectively (Bos et al., 2014). These levels are now used to benchmark each pig herd in the Netherlands and to identify herds that need to take action to reduce their use. Additionally, the SDa has recently started with describing the veterinary prescription patterns. Based on this, again thresholds were determined based on the percentage of herds exceeding the farm action benchmark threshold for all farms for which the veterinarian is the contracted veterinarian (Bos et al., 2015). By setting benchmarks, it is expected that veterinarians will mirror their use with colleagues and that this will trigger discussions between veterinarians. Also, by making prescription patterns transparent, farmers can make informed decisions regarding the veterinarian who they will contract.

Comparing antibiotic consumption in pigs between the Netherlands and Denmark requires equal indicators for reporting. Bondt et al. (2012) used sales data and animal census data, combined with average dosages, to estimate the average number of treatment days per average animal per year (NADD/y), for several animal species, including pigs, in order to make comparisons between both countries. They concluded that the overall use in pigs was 35% higher in the Netherlands than in Denmark.

7.2. IN BELGIUM

During the last decade, Belgium has increased efforts to gather more information on animal specific antimicrobial consumption. For pig production, a first attempt was made in 2003 by Timmerman et al. (2006). Data on antibiotic group treatments in fattening pigs were retrospectively collected for 50 randomly selected herds with a closed or semi-closed production system (Timmerman et al., 2006). In this first report, treatment incidences (TI) based on the animal daily dose pig (ADD_{pig}) and the used daily dose pig (UDD_{pig}) were introduced to report herd specific data for pigs. Additionally, the ratio UDD_{pig}/ADD_{pig} was calculated in order to get an estimation of the correctness of dosing. Qualitative aspects of use, namely which antibiotic classes and administration routes were used, and for which indications, were equally collected. Based on the obtained information in 2003, in Belgium, for the first time, clear recommendations on correct dosing were asked, concern on the high number of prophylactic group treatments in pig production were expressed, and the replacement by other disease-preventive measures was proposed (Timmerman et al., 2006).

Surveillance studies, as performed by Timmerman et al. (2006), have to be repeated, as different factors may influence use in time. Although restricted in both number of times and cooperating herds they might partially compensate for the absence of well-established continuous surveillance programmes.

From January 1st 2014, antibiotic use data on the level of the individual pig herd are collected by the private quality label Belpork. The input of data is delivered by the veterinarian, who is responsible for prescribing veterinary antimicrobials and antimicrobial premixes. AMCRA is operational in the analysis of the data through an independent scientific unit. Their tasks consist of safeguarding the quality of the data collection (input), managing and analysing the data and to set thresholds in order to benchmark between herds and veterinarians. Finally, communication and feedback to the animal sector is crucial in order to achieve a sustainable reduction in use.

Recently, a review study of Filippitzi et al. (2014) has attempted to extrapolate the results of several in depth studies on antibacterial use in pigs, poultry and veal calves in Belgium towards the whole population in order to make a rough estimate of the proportion of use in the different species. Findings of this study are discussed in relation with antimicrobial use data from Belgian pig herds in the discussion of this thesis (***Chapter 7***).

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MONITORING ANTIMICRIOBIAL RESISTANCE IN COMMENSAL, PATHOGENIC AND ZOONOTIC *ESCHERICHIA COLI* AND *STREPTOCOCCUS SUIS* FROM PIGS

In addition to the collection of antimicrobial consumption data, the monitoring of antimicrobial resistance in bacteria in food-producing animals is unmistakable an important first step in strategies aiming to contain resistance.

Monitoring of resistance in bacterial pathogens aims at effective interventions to counter the problem of resistance for animal health or welfare threatening infections and at developing guidelines on the appropriate choice of therapy in a clinical setting. Resistant bacteria with a zoonotic aspect are of interest as they might compromise animal and/or human health, but equally might transfer their acquired resistance genes to human commensal or pathogenic bacteria through direct contact between animals and humans or indirect via the food chain or the environment. Finally, resistance in commensal bacteria is thought to reflect the magnitude of the selection pressure enforced by the use of antimicrobials. These commensal bacteria are therefore referred to as 'indicator bacteria'.

1. COMMENSAL, PATHOGENIC AND ZOONOTIC *ESCHERICHIA COLI* FROM PIGS

The rationale of monitoring antimicrobial resistance in commensal *Escherichia coli* (*E. coli*) is that these form a reservoir of mobile resistance genes that can be transferred to other bacteria, thereby playing a key role in the dissemination and persistence of resistance (Marshall and Levy, 2011). Several studies have indicated the capability of *E. coli* to acquire and spread antimicrobial resistance associated with antimicrobial drug administration. *E. coli* is one of the bacterial species with an exceptional capacity of exchanging resistance genes (Smith et al., 2007). Moreover, *E. coli*, commonly present in the intestinal tract of pigs, other animals and humans (Sörum and Sunde, 2001), are exposed to antimicrobials, directly after oral treatment, and potentially indirectly via the enterohepatic circulation after parenteral treatment (Guggenbichler et al., 1985). Finally, *E. coli* is easy to isolate and identify. These aspects make *E. coli* an internationally used indicator for resistance of (intestinal) Gram-negative bacteria (Wray and Gnanou, 2000).

Some *E. coli* strains are also major pathogens in several animal species, including pigs. In pigs, enterotoxigenic *E. coli* (ETEC) are associated with neonatal diarrhea, diarrhea in 2 to 4-week old pigs as well as with post-weaning diarrhea, whereas Shiga toxin-producing *E. coli* (STEC) are responsible for oedema disease (Martins et al., 2011).

Some strains of *E. coli* are examples of zoonotic bacteria which can infect people by the food-borne route. From a zoonotic point of view, Shiga toxin-producing *E. coli* (STEC) is the only group of intestinal pathogenic *E. coli* of major interest, as the Shiga toxin-producing strains are able to cause bloody diarrhea, potentially evolving into a hemolytic uremic syndrome in humans, when being transmitted through the food chain from

their animal reservoirs (Wasteson, 2001). A restricted range of serotypes (i.e. O157, followed by O26, O103, O91, O145 and O111) are associated with public health risks and O157 is the serotype most frequently associated with severe human infections in the European Union (ECDC and EFSA, 2011). Pigs have not been identified as a major source of STEC for human infection in Europe, whereas ruminants, and more particularly cattle, are recognised as the main natural reservoir of STEC, in particular for STEC O157 (Wasteson, 2001; ECDC and EFSA, 2011). Within the different serotypes associated with public health risks, isolates are not necessary pathogenic when recovered from food-producing animals. Therefore, serotyping alone when applied to STEC isolates from food and animals is not the optimal method of identifying public health risks (EFSA, 2007). The role of additional virulence factors, next to the main virulence factors known, is currently being included for the assessment of the human pathogenic potential of different STEC serotypes (ECDC and EFSA, 2011).

Recently, it has been suggested that extra-intestinal pathogenic *E. coli* (ExPEC) of food-producing animals, particularly chicken and to a lower extent pigs and cattle, are involved in urinary tract infections in humans following direct contact or contamination of meat (Jakobsen et al., 2010; Bélanger et al., 2011; Tan et al., 2011). Extra-intestinal pathogenic *E. coli* is by far the most common agent infecting the human urinary tract and is equally involved as a major pathogen in meningitis and bloodstream infections in humans (Stenutz et al., 2006; Bélanger et al., 2011). The sharing of apparently clonal ExPEC between humans and animals suggests a possible role as a reservoir for these animals, especially avian species. Yet, the demonstration of these clonal relationships should be more robust through the use of sensitive methods, such as genome sequencing (EFSA, 2014). As a result, a clear zoonotic nature of ExPEC human infections can not yet been hypothesized.

2. COMMENSAL, PATHOGENIC AND ZOONOTIC S*TREPTOCOCCUS SUIS* FROM PIGS

Streptococcus suis (*S. suis*) is part of the normal microbiota of the pig. It can be found in the upper respiratory, alimentary, and urogenital tract of healthy pigs (Staats et al., 1997). Primarily, *S. suis* is known as an important swine pathogen causing meningitis, arthritis, septicemia, endocarditis, polyserositis, bronchopneumonia, and abortion (Higgins and Gottschalk, 1990; Amass et al., 1996; Staats et al., 1997). Within the bacterial species of *S. suis* 35 serotypes based on antigenic differences in its capsule polysaccharides are described. All serotypes, except 17-19 and 21, have been isolated from clinical cases (Gottschalk et al., 1989). Serotype 2 is the most prevalent type isolated from diseased pigs, followed by serotypes 1 and 9. *S. suis* colonizes piglets at birth and the pathogen is known to affect pigs of different ages. Yet, disease incidence decreases with age after weaning (Amass et al., 1996; Staats et al., 1997). Management, husbandry and preexisting injury may predispose to infection (Neumann et al., 2009).

Furhermore, *S. suis* has been reported as an emerging zoonotic pathogen evidenced by a few large-scale outbreaks of severe *S. suis* epidemics in Asia (Ye et al., 2006; Yu et al., 2006; Mai et al., 2008).

Due to its economic and zoonotic importance, reports include results on the monitoring of antimicrobial susceptibility mainly of clinical isolates of *S. suis* (Kataoka, 2000; Martel et al., 2001; Marie et al., 2002;

Wisselink et al., 2006), whereas *S. suis* from clinically healthy animals are only seldom included (Marie et al., 2002).

Monitoring resistance identifies trends in the emergence, spread and persistence of resistance to antimicrobials and is therefore a prerequisite for understanding the epidemiology of resistance. In the next chapter, the key aspects and drivers of the stages in the epidemiology of antimicrobial resistance in animal production are reviewed (***Chapter 1.3.***).

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EPIDEMIOLOGY OF ACQUIRED ANTIMICROBIAL RESISTANCE IN BACTERIA FROM FOOD-PRODUCING ANIMALS

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SUMMARY

In the epidemiology of antimicrobial resistance a succession of 4 stages can be distinguished: development, selection and spread, persistence and reversion or reduction. Resistance, a self-defense of bacteria against antibiotic-producing organisms in their surroundings, or against antibiotics produced by bacteria themselves, is inherent to the bacterial nature. Yet, anthropogenic antimicrobials equally play a role in the emergence of resistance, as they might induce mutations and facilitate gene transfer. In the next stage, resistance determinants are selected and spread through a bacterial population due to the exertion of a selection pressure. Antimicrobial use at large scale in animal production is believed to contribute substantially to the rapid spread and persistence of resistance determinants. However, selectors other than antimicrobials are also involved, as well as evolutionary adaptations in bacteria with reduced fitness, rendering the persistence of resistance determinants without antimicrobial selection pressure possible. A reversion to susceptibility involves a decreased growth rate of bacteria harboring resistance determinants or the loss of determinants. This entails every selection pressure to be discontinued and mechanisms interfering with the spread of resistance determinants through a bacterial population. This paper reviews key aspects and drivers of the stages in the epidemiology of antimicrobial resistance in animal production.

INTRODUCTION

Since their discovery, antimicrobials have become indispensable tools in countering bacterial diseases in both humans and animals, and to a lesser extent in horticulture. Yet, their use has become overshadowed by a phenomenon that has become increasingly more threatening over the last decades, namely antimicrobial resistance. Antimicrobial resistance in pathogenic and zoonotic bacteria results in decreased efficiency and/or therapeutic failure, and commensal bacteria may play a key role in the dissemination and persistence of this resistance. Antimicrobial resistance is a complex issue, both in origin as well as spread. A plethora of resistance mechanisms and transfer systems have been described (Levy and Marshall, 2004; van Hoek et al., 2011). Understanding these mechanisms is crucial for explaining phenomena in the past and to predict future tendencies. In addition, the development of appropriate strategies to stop further selection of resistance against the current available antimicrobial agents, the development of new agents as well as the refinement of infection control measures should be based on thorough knowledge of both basic mechanisms of resistance development and transmission and epidemiologic aspects of antimicrobial resistance selection and spread.
Many in-depth studies on resistance mechanisms have been conducted in the past. Recently, the number of studies approaching antimicrobial resistance from an epidemiological perspective is increasing.

In the epidemiology of antimicrobial resistance a succession of 4 different stages can be distinguished: development, selection and spread, persistence and finally reversion or reduction of antimicrobial resistance (Figure 1). This review aims to focus on the key aspects and drivers of each of these stages. Firstly, the emergence of antimicrobial resistance determinants in a certain pathogenic or commensal bacterium is essential, which we refer to as the development of antimicrobial resistance. Secondly, antimicrobial resistance is selected for and may spread within a bacterial species, but also across species. Once antimicrobial resistance is selected for, it may persist in a population. Therefore, a continuous selection pressure might be needed, even though in some cases resistance persists in the absence of any antimicrobial selection pressure. Finally, in the absence of a selection pressure, reduction of antimicrobial resistance may occur, due to the loss or silencing of resistance genes or due to replacement of resistant bacteria by susceptible ones.

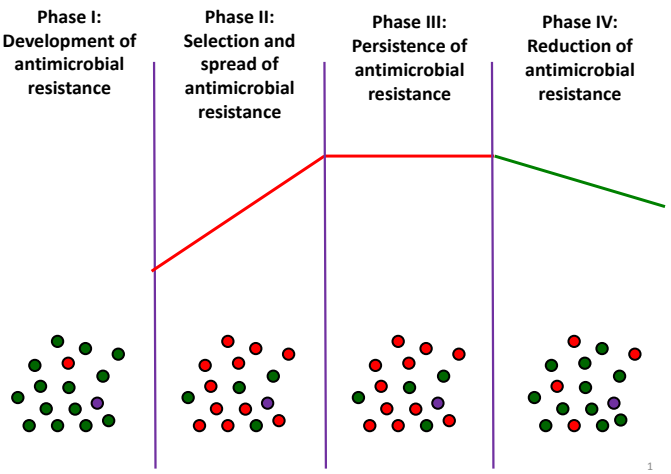

Figure 1. Stepwise process of the epidemiology of antimicrobial resistance. Green, blue and red dots: susceptible, intermediate and resistant isolates.

Before describing these 4 stages, antimicrobial resistance must be well defined. Resistance to an antimicrobial is either intrinsic or acquired. Intrinsic or natural resistance is caused by a structural or functional feature inherently associated with a certain bacterial taxonomic group, resulting in the inability of the antimicrobial to exert its effect. In addition, bacteria can acquire antimicrobial resistance (extrinsic or acquired resistance). This is the phenomenon that bacteria, previously susceptible to a specific antimicrobial, become resistant due to genomic alterations in a part of the population of a bacterial genus or species. Bacteria have developed various resistance mechanisms to neutralize the action of the antimicrobial (Schwarz and Chaslus-Dancla, 2001; Cloete, 2003). They can be divided into three major categories: enzymatic inactivation, a reduced intracellular drug accumulation, and target site alteration (Schwarz and Chaslus-Dancla, 2001). One resistance mechanism can mediate resistance to different antimicrobial classes because of the alteration of a common target site within the bacterial cell. For instance, methylation of adenine residues in the 23S rRNA of the 50S ribosomal subunit prevents macrolides, lincosamides and group B streptogramins to bind to their target site (Schwarz and Chaslus-Dancla, 2001). Different mechanisms conferring resistance to the same antimicrobial agent have been described. The main tetracycline resistance mechanism is the extrusion of the drug from the cytoplasm via efflux (Chopra and Roberts, 2001). Also, resistance genes coding for protection of the target site via ribosomal protection proteins, for enzymatic inactivation and for another, still unknown mechanism have been detected in various Gram-positive and Gram-negative bacteria (Taylor and Chau, 1996). In addition to active efflux, a diminished intracellular drug accumulation can be achieved by a reduced drug uptake (Kumar and Schweizer, 2005). Impaired uptake can be due to changes in the charge of the lipopolysaccharides of the outer membrane of Gram-negative bacteria. Also, outer membrane proteins, an

entry of antimicrobials to the bacterial cell, can be lost or down-regulated, resulting in reduced influx (Kumar and Schweizer, 2005). The abovementioned examples report only briefly the existence of resistance mechanisms. A detailed overview of resistance mechanisms towards antimicrobial classes related to a specific taxonomic group can be consulted from a variety of reviews on this topic (Chopra and Roberts, 2001; Schwarz and Chaslus-Dancla, 2001; Butaye et al., 2003; Cloete, 2003; Kumar and Schweizer, 2005).

To classify bacterial isolates as resistant, 4 different criteria, each with its own threshold value, are available. First, the clinical criterion for resistance evaluates the outcome of treatment of an infection with a specific pathogen compared to its Minimum Inhibitory Concentration (MIC). This is the lowest concentration of an antimicrobial necessary to prevent growth of the bacterial strain considered under specific *in vitro* conditions. An isolate is considered resistant to a specific antimicrobial agent, when symptoms of disease are not resolved, while the antimicrobial is used according to the standard therapeutic protocol. As a consequence of the definition, this criterion is uniquely used to describe resistance in pathogenic bacteria in a specific animal species for a specific diagnosis. Second, the microbiological or epidemiological criterion evaluates the *in vitro* susceptibility of a specific isolate, compared to the wild type population (Kahlmeter et al., 2003). The latter does not exhibit any mechanism of resistance that increases the MIC. When an isolate shows a considerably higher MIC to an antimicrobial than the wild type population, it is considered to have acquired resistance and these bacteria are then defined as “non-wild type”. Third, the genetic criterion for resistance is based on the detection of resistance genes (Cai et al., 2003). The phenotypically detectable antimicrobial resistance may be encoded by acquired genes or gene mutations. Yet, identical phenotypic bacteria (identical MIC) can reflect different genotypes, because different mutations in different genes or mobile elements can reflect similar antimicrobial resistance phenotypes (Martinez and Baquero, 2000). Finally the pharmacological criterion evaluates the relationship between antimicrobial concentration in blood or tissue and the MIC of the respective bacterial species or isolate (Guardabassi and Courvalin, 2006). Thus, bacterial antimicrobial susceptibility and the antimicrobial’s pharmacodynamic and pharmacokinetic properties are taken into account. A bacterium is considered resistant in the pharmacological criterion if the *in vitro* MIC of the antimicrobial tested is higher than the concentration of the antimicrobial in the plasma or tissue.

For interpretation of results of minimum inhibitory concentration (MIC) determinations of antimicrobials, either epidemiological cut-off values (ECV) or clinical breakpoints (CBPs) can be used (Schwarz et al., 2010). CBPs aim to predict how a patient will respond to a treatment and they classify bacteria as susceptible, intermediate or resistant (Schwarz et al., 2010). These breakpoints are mainly based on pharmacological and clinical criteria. They try to correlate the *in vitro* susceptibility of a bacterium towards a certain antimicrobial agent (the MIC value) with the chance to successfully treat an animal with the normal, recommended dose of this antimicrobial agent (Turnidge and Patterson, 2007; Schwarz et al., 2010). ECVs are mainly based on the microbiological criterion, sometimes in combination with the genetic criterion and allow to distinguish wild type

populations of bacteria from those with acquired resistance (non-wild type). They do not necessarily predict how a patient will respond to antimicrobial therapy (Schwarz et al., 2010). In the absence of a clear bi- or multimodal distribution of MICs for different isolates within a bacterial species, the epidemiological cut-off value can be difficult to establish (Kronvall et al., 2011). For practical use in monitoring of antimicrobial resistance both in humans and different animal species, this cut-off value should be fixed for one antimicrobial agent within a bacterial species, independent of time, country, animal or organ (Aarestrup et al., 2007). Although determined by a different approach, CBP and ECVs may be very similar or even identical for some bacteria/drug combinations. Yet, they can also diverge, resulting in misleading conclusions when comparing resistance results based on discordant ECVs and CBP (MARAN, 2005). ECVs are often used in monitoring and surveillance programs for describing evolutions in resistance prevalence or detecting emerging resistance in commensal bacteria. They are also very valuable in detecting small changes in the population distribution indicating the acquisition of new resistance mechanisms, of which the clinical implications are not yet clear. Isolates are said to have “a decreased susceptibility” when they have MICs that are above ECV, but less than or equal to the susceptible clinical breakpoint (Simjee et al., 2008). Although infections caused by these isolates may be treatable, they can be of concern as they represent an introductory step to full resistance, for example due to consecutive stepwise mutations.

Throughout this review resistance refers to the epidemiological criterion, unless otherwise mentioned, as this is of particular interest with regard to studying the epidemiology of antimicrobial resistance.

1. DEVELOPMENT OF ANTIMICROBIAL RESISTANCE IN A BACTERIAL SPECIES

The origins of resistance genes are often found in bacteria or fungi producing antimicrobial compounds in order to protect the organisms themselves from the compounds produced (Benveniste and Davies, 1973). These natural products are defined ‘antibiotics *sensu stricto*’, and are ubiquitously present (Waksman and Woodruff, 1940; D'Costa et al., 2011). Bacteria in the direct environment of antibiotic producing organisms will only survive and multiply in the presence of antibiotic concentrations if they can circumvent the potential effect of the antibiotic. Multidrug resistance efflux pumps are commonly observed in large numbers in antibiotic producing bacteria of natural environments (Martinez, 2008). These are likely the source of drug efflux pump genes found in pathogenic bacteria (Vecchione et al., 2009). For instance, *Pseudomonas aeruginosa* (*P. aeruginosa*) is known as multidrug-resistant by nature, insensitive to a large number of antimicrobial classes, such as various β-lactams, tetracyclines, potentiated sulfonamides and some aminoglycosides (Ferrara, 2006).

The first resistance mechanisms might have evolved from pathways involved in other physiological processes, such as detoxification of metabolic intermediates, virulence, and other functions (Piddock, 2006). It might explain the ancient nature of antimicrobial resistance, existing in nature long before the presence of

anthropogenic antimicrobials, defined as antimicrobials produced and used by humans (Allen et al., 2009; D'Costa et al., 2011). The "antibiotic resistome" can be defined as the collection of all resistance determinants in bacterial strains that might confer phenotypic resistance (Wright, 2007). The resistome encompasses diverse genes having original functions that are not exclusively for avoiding the effects of antibiotics (Gillings, 2013).

Unlike intrinsic resistance, acquired resistance is associated with specific isolates of a particular bacterial genus or species. Bacterial isolates can obtain resistance by genetic changes in the bacterial genome. It results from *mutations* (endogenous resistance), from *horizontal acquisition* of foreign genetic information (horizontal gene transfer, exogenous resistance) or a combination of both mechanisms (Martinez, 2000; Normark and Normark, 2002).

1.1. MUTATIONAL RESISTANCE

Antimicrobial resistance genes can originate from mutations in the bacterial genome. Mutation of genes has for a long time been believed to be a random and spontaneous process only. Yet, bacteria exposed to sublethal stress factors show increased mutation rates (stress-induced mutagenesis) (Shapiro, 1997), which might be a well-regulated phenomenon in order to generate heterogeneous populations with clones able to adapt and to survive adverse conditions. These stress factors can be diverse: depletion of nutrients, new environments, inhibitory effects of host defense mechanisms, temperature, pH, osmotic pressure as well as antimicrobial treatment. Bacteriostatic (sublethal) food preservation systems with increased salt or reduced pH conditions has been shown to increase antimicrobial resistance in *Escherichia coli* (*E. coli*), *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) and *Staphylococcus aureus* (*S. aureus*), with some of the strains showing a continued expression of higher resistance levels after removal of the stress factors (McMahon et al., 2007). Low concentrations of certain antimicrobials such as fluoroquinolones and β-lactams, have been reported to stimulate mutagenesis (increased mutation rate) (Couce and Blázquez, 2009), whereas high concentrations of fluoroquinolones might reduce the mutation rate (Martinez and Baquero, 2007). Furthermore, antimicrobials may also select cells with increased frequency of mutation and recombination (so called hypermutators) (Gustafsson et al., 2003). Antimicrobials are thus not only acting as mere selectors of resistant clones, but they also affect development of mutational resistance.

In veterinary medicine, antimicrobials for which resistance by mutations have been described are numerous. It has limited the clinical use of streptomycin, rifampin, erythromycin and it is increasingly limiting the use of fluoroquinolones (Gershwin, 2013). Mutations in the multiple antimicrobial resistance (Mar) locus are involved in an efflux system responsible for the resistance towards a variety of drugs in *E. coli* and other *Enterobacteriaceae*, including tetracycline, chloramphenicol, ampicillin, nalidixic acid, and rifampin (Alekshun and Levy, 1999).

Although the widespread presence of resistance genes is mainly due to the horizontal transfer of resistance genes, mutation processes are central in the diversification of the acquired genes and thus in the evolution of antimicrobial resistance. An example can be found in extended-spectrum-beta-lactamases (ESBL) producing organisms. Mutations have led to a diversification of enzymes responsible for resistance to different generations of cephalosporins, after the initial penicillin resistance (Woodford and Wellington, 2007; Smet et al., 2009). AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the *Enterobacteriaceae* and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation (Jacoby, 2009). For instance, mutational inactivation of *AmpD, which expresses an enzyme* preventing the overexpression of *AmpC*, is the main mechanism found to lead to the constitutive hyperproduction of chromosomal AmpC, and consequently to clinically relevant β-lactam resistance, in *Enterobacteriaceae* (Juan et al., 2006). Furthermore, specific spontaneous mutations in the promoter or attenuator region of the chromosomal *AmpC* gene have recently been reported in *E. coli* from cattle, which may be an emerging mechanism contributing to resistance towards extended spectrum cephalosporins in the animal population (Haenni et al., 2014).

The frequency of mutations required (single or multiple-step resistance) for clinical resistance to occur is dependent on the antimicrobial agent, the bacterial species and mechanism of resistance. For streptomycin, a 1000-fold increase in the MIC can be the result of a single mutation (Springer et al., 2001). In *Enterobacteriaceae*, mutational resistance to fluoroquinolones is a stepwise process where an increasing number of mutations generally is linked with increasing MICs (Woodford and Ellington, 2007) and thereby, clinical resistance occurs less readily. However, for *C. jejuni* and *P. aeruginosa*, a single nucleotide change at a particular site in the *gyrA* DNA gyrase gene can lead to clinical resistance by altering the target site for the fluoroquinolone (Gershwin, 2013).

1.2. HORIZONTAL GENE TRANSFER OR ACQUISITION OF RESISTANCE DETERMINANTS

Horizontal gene transfer (HGT) is the most common way for bacteria to acquire antimicrobial resistance genes (Ochman et al., 2000). Horizontal acquisition can be described as the acquisition of DNA from another organism by any of 3 mechanisms.

(1) Bacteria can exchange mobile genetic elements through physical contact (conjugation). It is thought to be the most important mechanism for spread of resistance (Schwarz and Chaslus-Dancla, 2001). Different mobile genetic elements can be transferred. Plasmids are circular or linear extrachromosomal DNA molecules of variable size, capable of autonomous replication (Stokes and Gillings, 2011). Transposons are linear DNA elements of variable size that mediate their excision and random incorporation into a recipients chromosomal or plasmid DNA (Iyer et al., 2013). Integrons contain collections of incorporated genes (gene cassettes) in

their attachment site, a promoter that drives expression of incorporated genes and integrase (Partridge et al., 2009). Unlike transposons, integration of integrons, mediated by integrase, is site specific and highly stable (Demarre et al., 2007). Class 1 integrons in particular, are associated with transfer of resistance (Partridge et al., 2009).

The horizontal transfer of a conjugative plasmid carrying multiple antimicrobial resistance genes has been described between different bacterial genera among the enteric microbial flora. This has for example been demonstrated in calves with resistant *E. coli* and susceptible *Salmonella* Typhimurium prior to apramycin treatment and *E. coli* and *Salmonella* Typhimurium carrying the same resistance genes after treatment (Hunter et al., 1992). This has equally been shown in the avian gastrointestinal tract between *E. coli* and *Salmonella* Newport, without anthropogenic selection pressure (Poppe et al., 2005). Also, transfer of resistance determinants from a (transiently) colonizing animal strain to human commensal microbiota has been observed, even in the absence of selective pressure, emphasizing the importance of this mechanism of resistance transfer *in vivo* (Dahl et al., 2007; Smet et al., 2011).

(2) Under specific conditions, bacteria are capable of incorporating exogenous DNA from the environment into the their genome (transformation of a competent receptor bacterium). This may require proteins involved in the assembly of type 4 fimbriae and type 2 secretion systems, as well as a DNA translocase complex at the plasma membrane (Chen and Dubnau, 2004). Some bacteria, such as *Helicobacter pylori* and probably also zoonotic, animal-associated gastric *Helicobacter* species use a type 4 secretion system for natural competence (Alverez-Martinez and Christie, 2009; Haesebrouck et al., 2009; Vermoote et al., 2011; Smet et al., 2013). Compared to mobile genetic elements the efficacy of transformation may be rather low, especially for bacterial species that are not naturally competent. However, antimicrobials may enhance transformation as has been shown for ciprofloxacin that induces expression of competence genes in *Helicobacter pylori* (Dorer et al., 2010).

(3) Bacterial viruses, called bacteriophages, can accidentally package host DNA in their capsid and subsequently infect another bacterium, introducing bacterial DNA into the recipient (transduction) (Ochman et al., 2000). Transduction is mainly observed between bacteria of the same species, but can equally occur between bacteria of different species and genera (Ammann et al., 2008). Transduction has been considered as a rare event, occurring around once every 10^7–10^9 phage infections. Yet, recent studies showed transduction to occur at frequencies of several orders of magnitudes greater (Kenzaka et al., 2010). Moreover, a potential major role of bacteriophages in the transfer of resistance genes from natural environments, such as water and soil, to human and animal microbiomes has been suggested (Muniesa et al., 2013).

The rate of horizontal transmission of resistance genes is dependent on the properties of the genetic element it is encoded on. Resistance genes on a (conjugative) plasmid have a higher rate of horizontal transfer

compared to resistance genes incorporated in the chromosome. Yet, the segregational stability of the chromosome is higher than that of plasmids, meaning that resistance genes encoded on the chromosome are less likely to be lost in the absence of selective pressure compared to those encoded on a plasmid (Wassenaar et al., 2005).

In vivo rates of HGT are probably higher than *in vitro* rates for both chromosomal and plasmid located resistance genes (Dahl et al., 2007). At a high rate of HGT, resistance genes may disseminate quickly between bacteria, slowing down a potential reversion to susceptibility (Levin et al., 1997; Johnsen et al., 2009).

2. SELECTION AND SPREAD OF ANTIMICROBIAL RESISTANCE

Once antimicrobial resistance has emerged, the number of isolates with resistance determinants might increase under a certain selection pressure. Resistance genes can be multiplied within the population by vertical dissemination and/or isolates can pass resistance determinants to other members of the population and to different populations (spread).

Selection and spread are therefore the second step in the epidemiology of resistance. This selection occurs under the driving force of a selection pressure which can be multiple.

Three conditions are required to allow spread of antimicrobial resistance in a bacterial population: at first, the presence of resistance genes; secondly, the viability of the resistant clone in case of mutations, or the mobility of resistance genes (transferability of genes) allowing vertical or horizontal spread of the resistance genes; and thirdly a selection pressure (Schwarz et al., 2001). Selection pressure alters populations by both selecting as well as affecting the rate of spread within and between the exposed animals (Olofsson et al., 2007). As a result, selection and spread are not independent and are therefore described here as one event.

2.1. ANTIMICROBIAL RESISTANCE SELECTION PRESSURE AND SPREAD THROUGH THE USE OF ANTIMICROBIALS

The rate at which existing resistant strains increase in prevalence has been described as a function of the level of antimicrobial exposure in bacterial populations. The pharmacodynamic and pharmacokinetic aspects of antimicrobial administration, possibly together with the presence or absence of other selecting factors affect whether the present strains will be cleared or selected. Describing the relationship between antimicrobial use and antimicrobial resistance selection is often challenging, because the association can be influenced by a large number of factors.

A first approach involves the effect of specific antimicrobial use on the resistance development against that particular antimicrobial. A direct relationship between the use of a specific antimicrobial and the emergence of resistance determinants to the antimicrobial concerned in bacterial populations has been described at

several levels (Stamey et al., 1976; Chaslus-Dancla and Lafont, 1985; Wray et al.; 1986; Cloeckaert and Chaslus-Dancla, 2001; Asai et al., 2005; Akwar et al., 2007; Dewulf et al., 2007; Harada et al., 2008; Chantziaras et al., 2013).

Describing and quantifying the association between use and resistance is more complicated when accounting for antimicrobial use factors, involving volumes used, number and category of animals treated, dose and duration of administration and qualitative factors such as administration route. Furthermore, mechanisms such as cross-resistance and co-selection may participate in the emergence and maintenance of antimicrobial resistance (Harada et al., 2008) and render the relationship between use and resistance more difficult to interpret.

2.1.1. TOTAL AMOUNT OF ANTIMICROBIALS USED

Levy et al. (1994) introduced the threshold theory, suggesting that a certain level of antimicrobial drug consumption is required to trigger the emergence of resistance in a particular environment. This theory is based on the equilibrium between a number of susceptible and resistant bacteria and the potential of the population of susceptible bacteria to return to their original number after an antimicrobial treatment. Austin et al. (1999) supported this theory by describing the sigmoidal rise in resistance over time in the presence of a constant rate of antimicrobial consumption. Again, a critical level of drug consumption is required to trigger the increase of resistance to certain levels. When this occurs, a crucial variable is time for the resistance to change from its low starting prevalence to a certain prevalence of resistance. This highlights the importance of reacting on emerging resistance at the onset of the resistance development. Moreover, the strength of the association between use and resistance and the time required to observe certain levels of resistance is most likely antimicrobial specific and related to the underlying resistance mechanism.

The concept of Austin et al. (1999) also suggests that at a certain level the resistance prevalence arrives at a maximum level where further use will no longer result in a substantial increase in the resistance prevalence. This is in agreement with the conclusions of Handel et al. (2006) who reported that small changes in the volumes of antimicrobials, used in a population with a low level of antimicrobial resistance, lead to much larger changes in resistance when compared with changes in antimicrobial volumes used at a high level of resistance. *In vivo* studies in cattle (O'Connor et al., 2002) and pigs (Dunlop et al., 1998b) aiming to evaluate the effect of different administration routes on the selection and spread of resistance observed that in animals already receiving an in-feed antimicrobial, no further increase of the resistance prevalence was observed after an additional subcutaneous administration of the same antimicrobial. Though, resistance prevalence to antimicrobials not present in the feed increased after administering them via subcutaneous injection. Berge et al. (2006) equally observed no resistance increase in high level resistant *E. coli* from pre-weaned calves after individual antimicrobial treatment concurrently with antimicrobials administered in the milk replacer. The calves

not receiving in-milk antimicrobials but treated with individual antimicrobial therapy transiently shed a more resistant *E. coli* population than the untreated calves (Berge et al., 2006). This again suggests that the in-feed antimicrobials might have increased the level of resistance to a saturation level, and additional treatments did not result in a further increase of the prevalence of resistance (Dunlop et al., 1998b; O'Connor et al., 2002). This non-linearity of the association between use and resistance may partially explain the often observed weak, or even apparent absence of a link between antimicrobial use and resistance selection (Checkley et al., 2008).

2.1.2. ANTIMICROBIAL DOSE AND DURATION OF TREATMENT

One way to apply treatment strategies to manage selection and spread is to control the dose and duration of therapy, ideally at the moment a compound is introduced on the market for a certain indication. The influence of dosage regimens on the emergence of certain resistance mechanisms has only recently been recognized and emphasized (Drusano, 2003; Olofsson and Cars, 2007). Appropriate dosage regimens should aim for the highest microbiological and clinical efficacy of a treatment as well as minimize the selection of resistance (Roberts et al., 2008) both in the targeted pathogens as well as commensal bacteria. This requires a good understanding of resistance mechanisms involved as well as the antimicrobial pharmacodynamics and kinetics (Roberts et al., 2008). However, diverging results between *in vitro* and *in vivo* studies on the impact of different dosage regimens on resistance selection and spread complicate the understanding of the relationship (Smith et al., 2003; Roberts et al., 2008). The appropriate dosage regimens are diverse for different antimicrobial classes and bacterial species. In the current literature, the relationship between the dosage on the one hand and selection and spread of antimicrobial resistance on the other hand has only been well described for mutational stepwise resistance (Smith et al., 2003). This is well illustrated for many fluoroquinolones, where many of the evaluated resistance mechanisms were the same *in vitro* as in the clinical setting (Smith et al., 2003). In bacteria where the majority of known resistance mechanisms occur via single or multi-stepwise mutations, such as in *Brachyspira spp.* (Pringle et al., 2012; Hillen et al., 2014), the correlation between MIC's and clinical efficacy is high, as has been seen for *B. hyodysenteriae* in swine (Vyt and Hommez, 2006). This allows determining the dosage that limits the selection of resistant mutants, also referred to as the "mutation prevention concentration" (MPC). Tam et al. (2005) demonstrated the possibility of combining optimal treatment dosages and the suppression of resistance emergence for garenoxalin, a quinolone, in *P. aeruginosa* in an *in vitro* infection (Tam et al., 2005). The inverted "U" shape relationship between exposure and resistance selection indicated that a range of antimicrobial concentrations might favor isolates with higher MIC's and cause a considerable amplification of the resistant subpopulation (Baquero et al., 1997; Tam et al., 2007). When a maximal value of resistance selection was attained, a further increase in concentration of quinolones caused a decline in the number of resistant colonies towards baseline resistance (Tam et al., 2007). A critical minimal concentration is also required to select resistant strains *in vitro* and is

called the "minimal selective concentration" (MSC) (Drusano et al., 2010). The MPC concept has encouraged the use of high dose regimens to reduce the likelihood of selection of resistant mutants. Lubbers et al. (2011) demonstrated that doses above the MIC were equally effective in suppressing the transfer of antimicrobial resistance plasmids between donor (*Salmonella* Typhimurium) and recipient (*E. coli*) bacteria *in vitro*.

Insight into the concept of the "mutation selection window" (MSW), defined at the top by the MPC and at the bottom by the MSC, for mutational stepwise resistance, gave rise to the establishment of single and high treatment dose regimens (above the MPC) instead of lower and repeated doses, in order to reach a maximum time of the antimicrobial concentration above the MPC. In grass carp *(Ctenopharyngodon idella)*, 0.010µg/g, 0.020µg/g and 0.030µg/g doses of enrofloxacin were compared in order to choose the most appropriate dosage to control infection and prevent mutant selection in *Aeromonas hydrophila*. The time above the MPC, needed to prevent selection of resistant mutants, appeared to be prolonged after a single day of 0.030µg/g enrofloxacin administration compared to twice daily of 0.020µg/g (Xu et al., 2013). Similar findings were seen for ciprofloxacin, enrofloxacin and marbofloxacin treatment of canine pyoderma caused by *Staphylococcus pseudintermedius* isolates, where only the highest doses within the clinically recommended dose ranges could achieve sufficient high concentrations to cross the MPC based on a 24 h interval (Awji et al., 2012).

Smith et al. (2003) commented on overstretching the MPC measurements beyond its limits and suggested that the measurement of MPC only applies to fluoroquinolones and not to aminoglycosides, β-lactams and macrolides. This has in turn been questioned by Blondeau et al. (2012), by defining the measurement of the MPC as a concentration that does not further allow for selection and amplification of resistant sub-populations, regardless of the mechanisms of reduced susceptibility and antimicrobial drug class. In this respect, the MPC and MSW against *Rhodococcus equi* have been determined for 10 antimicrobial agents from different classes (Berghaus et al., 2013). The authors concluded that the combination of a macrolide with rifampin considerably decreases the emergence of resistant mutants, based on the achievement of much lower MPCs of the antimicrobial combination *in vitro* and antimicrobial concentrations in the lungs above the MPC. However, at present, no results have been reported on the *in vivo* validation of the MSW for preventing the selection of resistant strains to other antimicrobials than fluoroquinolones (Cui et al., 2006; Almeida et al., 2007).

The use of antimicrobial growth promoters in livestock is clearly in contrast with the concept of the establishment of single and high treatment dose regimens, as growth promoters can be regarded as under-dosed antimicrobial compounds. For commensal bacteria, not only mutational resistance is of concern, but especially the organization of multi-drug resistance clusters as demonstrated, e.g. in *Enterobacteriaceae* (Leverstein-van Hall et al., 2002). This type of resistance is frequently encoded by genes located on mobile genetic elements, such as plasmids or transposons. The acquisition of such resistance genes may lead to a

100 to 1000-fold increase of the MIC and thus underdosing is less likely to speed up the selection of isolates with such increased MICs. Nevertheless, numerous studies have linked the use of antimicrobial growth promoters to the occurrence of antimicrobial resistance among Gram-positive and Gram-negative commensal bacteria (Sunde et al., 1998; van den Bogaard et al., 2000; Swartz, 2002; Alexander et al., 2008). In contrast, several studies in swine *E. coli* (Langlois et al., 1984; Wagner et al., 2008), *Salmonella enterica* (*S. enterica*) (Wagner et al., 2008) and anaerobes (Holman and Chénier, 2012) after administrating different dosages of tetracycline did not confirm the statement that underdosing would lead to a higher degree of resistance in comparison to correct dosing. Moreover, the study of Langlois et al. (1984) found a significantly higher resistance level in the group receiving the higher dosage compared to the group receiving the lower dosage during first weeks after administration. However, this turned to the opposite from 30 days after first administration to the last sampling at 84 days, suggesting the time period after administration also needs to be taken into consideration. Contrary, feeding sub-therapeutic concentrations of tylosin to pigs on a continuous basis resulted in a significant and rapid increase in the proportion of tylosin resistant anaerobes (Holman and Chénier, 2012). Alexander et al. (2008) described the effect of the sub-therapeutic administration of chlortetracycline in combination with sulfamethazine to cattle feedlot. The number of animals shedding tetracycline and ampicillin resistant *E. coli* increased as well as the number of resistant *E. coli* from one animal (Alexander et al., 2008). The authors noted that their findings on changed resistance prevalence may have been related to additional environmental factors such as diet.

These contrasting results for *Enterobacteriaceae*, anaerobes and different antimicrobials tested emphasize that different outcomes on the relation between dose and resistance selection can be expected, depending on the antimicrobial used, the resistance mechanism and bacteria involved.

Several authors have questioned the MPC concept and the derived dosage regimes for preventing the emergence of antimicrobial resistance, as this concept suggests that there is no selection for mutants at concentrations less than the MSC (Courvalin, 2008; Canton and Morosini, 2011; Macia et al., 2011). This is contradictory to observations that concentrations below MSC may facilitate hypermutation and HGT (Canton and Morosini, 2011; Macia et al., 2011). Furthermore, resistant mutants may have benefits compared to the susceptible strains at sub-MSC levels, as long as their fitness cost is lower than the growth reduction of the susceptible isolates (Gullberg et al., 2011). Gullberg et al. (2011) used *in vitro* models demonstrating the evolution of low to high level resistance for streptomycin resistant *Salmonella* Typhimurium mutants and for ciprofloxacin resistant *E. coli* after 500-600 generations in sub-therapeutic doses, up to several hundred-fold below the MIC of susceptible strains.

More objections towards dosage regimens have been raised. As mentioned above, dosage regimens are generally determined in function of good clinical treatment and preferably also minimal antimicrobial resistance

selection for specific pathogens. Yet, even if the correct dosing regimen - to treat specific pathogens while avoiding resistance selection as much as possible - is known and applied in the field, at the same moment the commensal microbiota of the animal, for which the treatment dose is not specifically adapted, is also exposed to these treatment doses. Therefore, what is a correct dosing for a specific pathogen in a specific organ may be an over- or underdosing for a commensal present in the same or another organ.

Since no general applicable conclusions can be drawn on the link between dosage and resistance selection, the use of specific dosing regimens as a potential way of reducing resistance emergence might be restricted to specific antimicrobial-bacterial relations. A dosing regimen adapted to control mutant selection of one strain, might undesirably encourage development of resistance in other strains by mutation or horizontal gene transfer.

2.1.3. CHOICE OF ANTIMICROBIAL AGENT

Antimicrobial agents are characterized by several features that may play a role in the selection and spread of resistance. In addition to their working mechanism (and thus activity against spectrum of bacteria) and whether they have bacteriostatic or bactericidal effects, they might have diverging pharmacodynamic and kinetic parameters, such as plasma half-life and tissue persistence, which is important for their time- or concentration-dependent activity.

The use of broad-spectrum antimicrobial agents rather than narrow spectrum antimicrobials affects a higher number of different bacterial taxa and thereby increases the risk for selection of bacteria carrying resistance genes and for suppressing or eliminating broadly the susceptible commensal microbial flora, which generally out-competes resistant strains (Levy and Marshall, 2004). Thus, these broad-spectrum agents might encourage the survival of more resistant strains.

The type of antimicrobial agent also influences the antimicrobial selection and spread. A bacteriostatic agent only inhibits growth and thus gives more chance for selecting resistant sub-populations (Dagan et al., 2011) compared to bactericidal agents killing the bacteria. Yet, the latter may eradicate fully susceptible populations giving the opportunity for resistant strains to colonize certain ecological niches (Catry et al., 2008). The distinction between bactericidal and bacteriostatic effect is far from being absolute and depends on both the drug concentration at the site of infection and the bacterial species involved (Giguère, 2013). Sub-lethal antimicrobial concentrations might induce stress in the targeted bacteria. As previously mentioned, stress might result in a transient decrease in antimicrobial susceptibility due to increased copy numbers of resistance genes (McMahon et al., 2007).

Resistance to macrolides, lincosamides and streptogramin B (MLS_B resistance) encoded by *erm* genes can be either constitutive (permanently expressed) or inducible (expressed after antimicrobial exposure). This inducible resistance can have clinical implications as *in vivo* exposure to macrolides may result in resistance

higher than predicted by *in vitro* determined MIC's in the absence of the inducer (Chancey et al., 2011). In staphylococci, within the macrolides class, only the 14- and 15-member rings are good inducers for resistance expression (Chancey et al., 2012). Thus, isolates harboring inducible MLS_B resistance and exposed to 16-membered antimicrobials can remain susceptible, whereas constitutive MLS_B resistance refers to all macrolide members (Chancey et al., 2012).

For time-dependent antimicrobials, such as β-lactams, tetracyclines, macrolides, sulfonamides and lincosamides, the antibacterial effect is highest when the concentration is maintained above the MIC throughout the dosing interval. Long-acting formulations, based on long half-lives, result in prolonged plasma concentrations, and offer a solution for the required repeated administrations inherent to treatment regimens of time-dependent antimicrobials. Such long-acting formulations have been developed for certain third-generation cephalosporins (for example cefovecin) or macrolides (azithromycin, tulathromycin). They are characterized by a long half-life and a slow release after tissue binding, resulting in a pronounced post-treatment effect (Van Bambeke and Tulkens, 2001). For azithromycin, this effect has been shown to significantly select more for macrolide-resistant streptococci until about 4 weeks after the end of therapy than clarithromycin, characterized by a shorter plasma half-life and tissue persistence (Malhotra-Kumar et al., 2007). Moreover, concentrations of macrolides below the MIC and long-term presence due to a long half-life, can induce mutational resistance at concentrations below the MIC, as has been shown *in vitro* for *S. pneumoniae* (Pankuch et al., 1998; Nagai et al., 2000).

Along with different pharmacodynamic and kinetic parameters, differentiated underlying resistance mechanisms as well as cross- and co-resistance mechanisms might play a role in different resistance outcomes observed after the use of antimicrobials belonging to the same class. Clarithromycin, but not azithromycin, perturbs the distribution of macrolide resistance genes *erm*(B) and *mef* in streptococci (Malhotra-Kumar et al., 2007). This is the result of clarithromycin's greater efficacy against *mef* carrying streptococci up to 8 µg/ml (Noreddin et al., 2002), whereas azithromycin is far less potent and produces only a bacteriostatic effect against *mef* isolates with MIC's up to 2 µg/ml (Zhanel et al., 2003). *In vitro* pharmacodynamic studies have shown that both macrolides failed to eradicate *erm*(B) strains, which generally have MICs of 32 µg/ml or higher (Noreddin et al., 2002; Zhanel et al., 2003). The higher efficacy of clarithromycin against *mef* isolates results in a steeper decrease of *mef* carrying macrolide-resistant streptococci, which in turn allows for an expansion of *erm*(B) isolates (Malhotra-Kumar et al., 2007). Furthermore, due to equal resistance mechanisms, the *erm*(B) gene confers equally resistance against lincosamides and streptogramin B antimicrobials (co-resistance), whereas *mef* genes only encode resistance to 14- and 15-member macrolides. Moreover, the *erm*(B) gene is often located on the same mobile genetic element as the tetracycline resistance determinant *tet*(M). As a result, clarithromycin use might restrict the use not only of all macrolides, but also of lincosamides, streptogramins B, and even of tetracyclines (Malhotra-

Kumar et al., 2007). The phenomena of co- and cross-resistance are explained more in detail in following text.

In conclusion, the choice of a specific antimicrobial should not be based exclusively on the elimination of the target pathogen, but should equally take into account all aspects aiming at a minimal selection of resistance determinants. For example, the different risks for selection and spread are not restricted to different classes, but are also present within one class. One specific antimicrobial agent might play a role in the shift towards resistant strains with higher MIC's and might even select for resistance to other classes due to genetic linkage, whereas other similar antibiotics do not have the same selective effects.

2.1.4. ADMINISTRATION ROUTE

Different factors might play a role in the effect of different administration routes on resistance selection and spread. At first, the route of administration will affect tissue/intestinal content concentrations (Baggot, 2006) and thus also the degree of the selection pressure exerted on both pathogens and commensal bacteria. Oral administration of antimicrobials exerts a selection pressure on the intestinal microbiota that is, most likely, higher than seen for parenteral injections, except for parenterally administered antimicrobials which undergo enterohepatic circulation to a high extent, such as tetracyclines (del Castillo, 2013). Nevertheless, the extent and pattern of antibiotic tissue distribution might equally be involved in the exertion of a selection pressure. Distribution into the intestinal lumen varies between antimicrobial agents of different classes due to differences in their chemical nature (Baggot and Giguère, 2013). In this respect, parenterally administered antimicrobials, not undergoing enterohepatic circulation might possibly achieve selective concentrations in the intestinal lumen.

The intestinal microbiota is often described as a commensal reservoir of resistance genes (Levy and Marshall, 2004) and the oral administration of antimicrobials might be an increased risk (compared to parenteral administration) for selection of resistant commensal bacteria and spread of resistance genes to other commensal and pathogenic bacteria.

Yet, only limited specific research data on the effect of different administration routes on resistance selection and spread are available. Zhang et al. (2013) described the development of resistance in intestinal bacteria of mice as significantly less or delayed when the same doses of antimicrobials were administered via intravenous injection rather than oral administration. Moreover, the difference in intravenous or oral therapy was more significant for ampicillin, eliminated via the kidney, than for tetracycline, excreted via both kidneys and the gastrointestinal tract. Wiuff et al. (2003) included a parenteral and an oral treatment group in their study and found no difference in the speed of selection for resistance in *S. enterica* infected pigs between intramuscular administration of enrofloxacin and oral administration of the same dose. Yet, a selection pressure might also have been present in the intestines following parenteral administration, as enrofloxacin

and its major metabolite, ciprofloxacin, is passing through the intestinal tract after excretion in the bile (Koningstein et al., 2010). From these studies, it appears that the effect of different administration routes on the resistance selection again depends on the antimicrobial used, as this is linked to a specific excretion route.

In food producing animals, individual and group treatments often coincide with parenteral and oral administration routes, respectively (Pardon et al., 2012). Treatment of only one or a few animals (individual treatment) or an entire group of animals can effect observed resistance levels, since resistance selection, as the result of antimicrobial treatment of a single animal, may be partially diluted at the population level due to the presence of a susceptible microbiota excreted by the contact animals. In chickens, previously fed tetracycline feed, a decrease in the excretion of resistant *E. coli* was seen after housing them with larger numbers of cage mates that excreted susceptible microflora (Levy, 1978). However, when the entire population is treated, the odds of dilution to occur by susceptible bacteria will be lower and a commensal reservoir of resistance genes can be formed (Levy and Marschall, 2004). Moreover, the transfer of resistant bacteria might occur more rapidly to animals being treated, due to the inhibition of the commensal microbiota, which exert a protective effect against colonization and infection by exogenous organisms (Barza and Travers, 2002). Dunlop et al. (1998b) compared the effect of individual and group treatment on resistance in *E. coli* from swine using aminoglycosides and tetracycline and found lower resistance levels in the group receiving individual parenteral treatment compared to the group receiving oral administration. Feedlot bulls showed a higher proportion of resistant *E. coli* after the oral administration of tetracyclines compared to a subcutaneous treatment (Checkley et al., 2010), yet the prominent difference disappeared after a few weeks, showing that other factors were involved. The authors suggested an interaction between the groups, as all bulls were kept together. On the one hand, a dilution effect could have occurred in the animals treated with feed antimicrobials after the antimicrobial selection pressure dropped. On the other hand, resistance might have spread horizontally between the different groups of animals, explaining the rise in resistance after cessation of antimicrobial therapy in both the control and the parenterally treated group.

2.2. ANTIMICROBIAL RESISTANCE SELECTION PRESSURE AND SPREAD THROUGH FACTORS OTHER THAN ANTIMICROBIAL USE

Besides the use of antimicrobials, other factors can also be involved in the selection and spread of resistance determinants. These factors can either be selection pressure originating from biocides or heavy metals or can be due to entirely different drivers as discussed below.

2.2.1. BIOCIDES

It is known that biocides and preservatives, such as quaternary ammonium derivates (QAD), triclosan and chlorhexidine, might have working mechanisms in common with antimicrobials (McMurry, 1998) through which similar mechanisms of bacterial insusceptibility may occur. Various studies in *E. coli* and *S. enterica* have demonstrated that efflux pumps play an important role in resistance to antimicrobials or disinfectants, including QAD and triclosan (Levy et al., 2002). This results in induced cross-resistance following increasing concentrations of a biocide or antimicrobial under laboratory conditions (Braoudaki and Hilton, 2004). Sub-inhibitory concentrations of QAD and triclosan, due to poor disinfection procedures, can lead to the selection of *Salmonella* Typhimurium strains with reduced susceptibility or resistance to ampicillin, ciprofloxacin and tetracycline (Karatzas et al., 2007).

Besides similar resistance mechanisms, the possibility of genetic linkage on plasmids between *qac* genes for QAD resistance and β-lactamase genes for β-lactam resistance has been described for *S. aureus* (Fraise, 2002). This might also be the case for Gram-negative bacteria, such as *S. enterica* and *E. coli* from farm animal origin where *qac* genes are often together with *sul1*genes, encoding sulfonamide resistance, located on mobile genetic elements that can harbor various other resistance genes (Sidhu et al., 2001; Sidhu et al., 2002; Chuanchuen et al., 2007; Cocchi et al., 2007).

According to Russell (2003) the translation of biocide resistance laboratory studies to *in vivo* situations might lead to premature or even wrong conclusions, as the presence of resistant bacteria is not necessarily higher in settings with a higher biocide use. Karatzas et al. (2007) suggest a lower virulence of strains due to QAD and triclosan selection as a possible explanation. Nevertheless, QAD resistant staphylococci isolated from human patients with bacteremia showed a significantly higher prevalence of resistance to several antimicrobials than QAD sensitive staphylococci, indicating an association between biocide and antimicrobial resistance (Sidhu et al., 2002).

A third factor, along with cross-resistance and co-selection, might be the selective stress exerted by biocides. The expression of the broad-specificity efflux AcrAB pump is up regulated by the *mar* operon responding to toxic substances, such as biocides and antimicrobials (Levy, 2002). Furthermore, stress induced by biocides, favors not only the expression of resistance mechanisms, but also their dissemination by horizontal transmission of integrons (Beaber et al., 2004) and plasmids (Feld et al., 2008), and thereby accelerating the spread of resistance.

Despite *in vitro* evidence for associations between biocide use and selection and spread of antimicrobial resistance through the abovementioned mechanisms, data related to the occurrence of bacterial resistance following exposure to biocides in the veterinary field are scarce. The correct use of biocides for biosecurity measures in animal husbandry as a part of disease prevention to avoid the need of antimicrobials is strongly arguing in favor of biocide use.

These conflicting arguments in combination with the limited field data available indicate that there is a need for further studies to elucidate the potential interaction between the use of biocides in animal facilities and the emergence of antimicrobial resistance.

2.2.2. HEAVY METALS

Metal-containing compounds are widely used as feed supplements, both to address metabolic needs and for the prevention of gastro-intestinal diseases in food animals (Cavaco et al., 2011).

Multidrug efflux systems have been shown to be important mechanisms of resistance against antimicrobials and other structurally unrelated compounds, such as heavy metals, in several bacterial genera from different animal species (Delmar et al., 2014).

A correlation between copper resistance on the one hand and glycopeptide and macrolide resistance on the other hand in *Enterococcus faecium* isolates has been observed in pigs, but not in broilers, calves and sheep in Denmark (Hasman and Aarestrup, 2005). This might be partly due to higher copper exposure in pigs through feed additives compared to other livestock. Most likely, this has resulted in the co-selection of the *tcrB*, Tn*1546* and the *erm*(B) gene, responsible for copper, glycopeptide and macrolide resistance respectively, as they are closely located to each other on a conjugative plasmid (Hasman and Aarestrup, 2005). Cross-resistance has also been seen in *Listeria monocytogenes* by means of a multiple-drug resistance pump exporting metals in addition to antimicrobials (Mata et al., 2000). The *czrC* gene, conferring resistance to zinc and cadmium in *S. aureus*, was found to be located within the clonal complex SCC*mec* type V, prevalent in MRSA from pigs and veal calves (Cavaco et al., 2010).

In fecal multidrug resistant *Salmonella* serotypes from swine, statistical associations were found between ampicillin, streptomycin, tetracycline and kanamycin resistance and the *pcoA* gene, conferring resistance to copper, and between ampicillin, chloramphenicol, streptomycin, sulfisoxazole, tetracycline and the *czcD* gene, conferring resistance to zinc (Medardus et al., 2014).

2.2.3. HOST FACTORS

Animals experiencing stress can show increased and prolonged shedding of bacteria, whereby the spread of resistance into the environment can be promoted (Sorum and Sunde, 2001; Verbrugghe et al., 2012). This has been observed for fecal *E. coli* from slaughter pigs, exposed to heat stress and without previous antimicrobial use (Moro et al., 2000).

Alongside an increased shedding of bacteria, a higher prevalence of resistance has been observed in animals exposed to stress. For instance, in pigs exposed to cold and overcrowding an increased resistance prevalence to apramycin in *E. coli* was observed (Mathew et al., 2003). Another example of a possible stress-associated resistance effect was seen in a study on the effect of florfenicol injection in steers on multiresistance in fecal *E. coli* where cattle were rounded up from two pastures and transferred to a research institute. Higher levels of multi-resistance and prolonged resistance following a single injection of florfenicol was seen in calves that were immediately weaned prior to transfer to the research institute compared to calves from a source where they had been weaned one month prior to shipment (Berge et al, 2005b).

Several studies support that increasing age is linked with a decreased prevalence of resistant *E. coli* in dairy cattle (Berge et. al, 2005a; Sato et al., 2005; Berge et al., 2010; Khachatryan et al., 2004) and in coliforms from pigs (Langlois et al., 1988; Dewulf et al., 2007; Akwar et al., 2008). In poultry, the prevalence of resistance for multiple agents in enterococci was significantly higher for the maximum 42 days old broilers compared to older laying hens (van den Bogaard et al., 2002). The higher antimicrobial use in the more susceptible younger animals is often suggested as the main reason. However, higher levels of resistance in young preweaned calves that had not had previous exposure to antimicrobials compared to adult animals has been noted, and the age-related resistance prevalence cannot completely be explained by increasing antimicrobial exposure (Berge et al, 2010). Also, for poultry, broiler production coincides with higher infection pressure and thus antimicrobial use compared to laying hens production systems (van den Bogaard et al., 2002). Another possible explanation has been given by Walk et al. (2007). The authors suggest that the fitness cost of resistant bacteria becomes too large as the host gastrointestinal tract matures and competition with other microbes increases.

2.2.4. HOUSING CONDITIONS

Farm types or housing conditions have been identified as a significant factor in the prevalence of resistance in different animal sectors. These differences have often been assigned to divergent antimicrobial use however. Antimicrobials in the milk replacers throughout the pre-weaning period might provide a selective advantage to resistant enteric *E. coli* in calves. This could explain phenotypic resistance to more antimicrobials in *E. coli* isolated from calves from calf ranches than from dairy farms (Berge et. al, 2005a; Berge et al., 2010). Similarly, the fecal coliforms (Berge et al., 2001) and respiratory tract *Pasteurellaceae* (Mevius & Hartman, 2000; Catry et al., 2005) from calves for fattening show a higher degree of resistance than isolates from dairy or beef calves. Most likely, the routinely administered in-feed medication exerting a selection pressure in the nasopharynx, through systemic distribution or through direct contact (nasopharynx or tonsils) with the microbiota of the upper respiratory tract, is responsible for the higher resistance levels (Catry et al., 2005). Other factors, different from antibiotic use, playing a role in the selection and spread of resistance in pigs and inherent to farm type were reported by Langlois et al. (1988). These authors may have been the first to report

the potential beneficial effect of moving animals towards outdoor production on the resistance prevalence. A greater proportion of *E. coli* isolated from pigs on pasture were sensitive to 13 antimicrobial agents tested than were isolates from pigs housed in a farrowing house or concrete-floored finishing unit (Langlois et al., 1988). This phenomenon may be referred to as 'environmental dilution' and results in a microbial population with an equilibrium between susceptible and resistant subpopulations or even a predominance in susceptible bacterial populations. A comparable effect has been seen in broiler chickens and fattening pig farms where a lower hygiene standard in farms was associated with lower resistance in intestinal *Enterobacteriaceae* (Dewulf et al., 2007; Persoons et al., 2010). The authors suggested the possibility of a dilution effect by susceptible bacteria due to a soiled environment, resulting in a more diverse intestinal microbiota and thus less resistant strains. Apparently, hygienic measures play an ambiguous role in the prevalence of resistance. In disease control, hygiene and sanitation are very important and modifiable assets to prevent disease introduction and spread in a herd or flock and thus also to prevent antimicrobial intervention. Furthermore, good hygiene standards in a farm assume the prevention of the development of bacterial reservoirs. Yet, in contrast they might result in less dilution and thus exposure to a more homogeneous (resistant) bacterial population.

2.2.5. DIET

The possible impact of diet on the prevalence of resistant enteric bacteria in the feces has been suggested by several studies. As a result of a change in the composition of diets, environmental stressors, such as pH, can vary within the intestinal tract (Alexander et al., 2008). Cattle on a grain diet, previously treated with antimicrobials, showed a higher prevalence of tetracycline resistant *E. coli* in fecal samples, compared to control cattle on a silage diet (Alexander et al., 2008). A pH decrease in the rumen, after feeding the grain diet, might act as trigger for the expression of membrane-bound transporters, a common mechanism of tetracycline resistance in *E. coli* (Roberts et al., 1994). Alexander et al. (2008) suggested the presence of other environmental stressors, such as bacteriocins and osmolarity in the intestines, which might be able to induce genes linked to antimicrobial resistance genes. Khachatryan et al. (2006) assumed a multifactor selective system for streptomycin-sulfadiazine-tetracycline (SSuT) resistant *E. coli* strains from dairy cattle. Animals receiving a dietary vitamin D supplement showed a nearly twofold increase in the prevalence of SSuT resistant strains compared to animals that did not receive any supplement. The authors performed *in vitro* experiments, showing that SSuT resistant strains attained a higher density of cells at stationary phase than non-SSuT resistant strains in the presence of the vitamin D additive. They concluded that the relationship between the prevalence of SSuT resistant strains and the vitamin D additive may be related to genetic linkage of the SSuT determinants to other genes, so called 'beneficial genes', that confer a selective advantage in the presence of the vitamin D additive (Khachatryan et al., 2006).

Further investigations are needed in order to determine how changes in diet composition may impact the prevalence of antimicrobial resistant enteric bacteria and thus the spread of resistance into the environment.

2.2.6. BACTERIAL FACTORS

In certain bacterial strains there is substantial evidence for a common mechanism for virulence and resistance or a linked presence of particular virulence and resistance genes. The hazard of these common mechanisms or linked occurrence of virulence and resistance genes can be defined as the possibility of co-selection of virulence genes by use of antimicrobials and consequentially maintenance of resistance in populations of pathogenic bacteria (Boerlin et al., 2005). Yet, the mechanisms involved affect whether positive or negative associations between resistance and virulence are seen (Martinez and Baquero, 2002; Beceiro et al., 2013). The AcrAB-TolC efflux pump, widely distributed in Gram-negative bacteria, expels antimicrobial agents, but also host-derived compounds with bactericidal activity such as fatty acids and bile salts (Perez et al., 2012). Several studies have demonstrated that the efflux pump is required for bacteria to be pathogenic and to show resistance towards several classes, such as β-lactams, aminoglycosides, fluoroquinolones, tetracyclines and macrolides (Martinez et al., 2009). For *Klebsiella pneumoniae* it has been shown that porin deficiency can increase antimicrobial resistance, but decrease virulence at the same time (Tsai et al., 2011). Resistance to colistin in Gram-negative bacteria is caused by either the loss or by changes in their lipopolysaccharide (LPS), thereby preventing or reducing the affinity of polymyxins (Landman et al., 2008; Beceiro et al., 2014). A loss or change in LPS has been associated with a noticeable cost in terms of overall fitness and virulence in colistin resistant *Acetinobacter baumanni* (Lopez-Rojas, 2011; Beceiro et al., 2014).

Besides common mechanisms of resistance and virulence, mobile genetic elements such as plasmids and integrative conjugative elements (ICE's) may carry both virulence and resistance genes, which can be concurrently transmitted between and within bacterial species. For porcine enterotoxigenic *E. coli* (ETEC), diverse resistance and virulence genes profiles have been seen (Smith et al., 2010), which might explain the diverged outcomes on clustering of resistance and virulence genes. Only few or no associations between resistance and virulence genes were reported in porcine multidrug resistant ETEC by Smith et al. (2010). On the other hand, field data obtained by Boerlin et al. (2005) showed statistical associations between these genes for ETEC isolated from pigs. This has been supported by the clustered prevalence of the tetracycline resistance gene *tetA* and several virulence factors on a common plasmid in porcine ETEC (Goswami et al., 2008). The latter confirmed the hypothesis that antimicrobial resistance is more common in ETEC than in other porcine *E. coli* (Boerlin et al., 2005). Yet, where positive associations were found for *tetA*, this was not the case for certain virulence factors and *tetB*. Other studies report the presence of a pTC plasmid, linking resistance and enterotoxin virulence genes in porcine ETEC (Fekete et al., 2012), in F18-positive strains (Olsz et al., 2005) and in an *E. coli* O149:H10 strain shown to have enhanced virulence (Goswami et al., 2008). Studies on enterohemorrhagic *E. coli* (EHEC) in cattle (Valat et al., 2012) also aiming at investigating the possible link between resistance and virulence genes reported only few or no associations. Avian *E. coli*

strains have been shown to carry a conjugative R plasmid containing both tetracycline and ampicillin resistance genes and virulence genes (Johnson et al., 2002).

Field studies have shown more phenotypic resistance in bacteria from diseased than in bacteria from healthy animals, such as *in E. coli* from Swedish dairy calves with diarrhea (de Verdier et al., 2012), in *E. coli* from dairy cows with mastitis (Suojala et al., 2010) and in *Streptococcus suis* from pigs with diverse clinical conditions (Li et al., 2012). Yet, findings on the statistical relationship between virulence factors and resistance phenotypes in the field should be interpreted with caution. Though phenotypic resistance to one or more antimicrobials was associated with the presence of virulence genes in *E. coli*, none of these virulence factors were associated with the respective disease (Suojala et al., 2010; de Verdier et al., 2012), suggesting that detection of virulence factors might not always predict virulence in field conditions (Li et al., 2012) and that other factors may explain higher resistance prevalences in diseased animals. Higher prevalence of resistance in pathogenic isolates from diseased animals as a consequence of antimicrobial treatment has been suggested by Harada and Asai (2010).

Recently, another connection between virulence and resistance to antimicrobials has been described (Arnoldini et al., 2014; Diard et al., 2014). In *Salmonella* Typhimurium, the expression of a type three secretion system, encoded by genes on the *Salmonella* pathogenicity island (SPI) 1, triggers gut tissue invasion followed by intracellular growth retardation and antibiotic tolerance. This results in the so called ‘persister cells’ with increased tolerance against antimicrobial agents and thus promoting persistence in an antimicrobial environment (Arnoldini et al., 2014; Diard et al., 2014). Upon cessation of antibiotic treatment, former persister cells reseed the gut lumen and thereby facilitate disease transmissibility to new hosts (Diard et al., 2014).

Another bacterial factor is the fitness cost after having acquired a resistance determinant, either by mutation or HGT. This will be further discussed below.

3. PERSISTENCE OF ANTIMICROBIAL RESISTANCE

3.1. PERSISTENCE OF ANTIMICROBIAL RESISTANCE IN THE PRESENCE OF ANTIMICROBIAL SELECTION PRESSURE

When evaluating resistance monitoring results, often a relative steady state of the levels of resistance in a population can be observed. Rates of resistance in commensal *E. coli* from cattle, pigs and broiler chickens in Japan, remained stable at intermediate level for ampicillin and at high levels for streptomycin and tetracycline from 2000 to 2007, commonly used antimicrobials in Japan (Harada and Asai, 2010). Also, in pathogenic porcine and bovine *E. coli* in Belgium, tetracycline resistance remained stable between 2005 and 2011(VAR, 2012). The MARAN data report relatively stable resistance levels in *E. coli* from different animal species over time, with a tendency to increase until 2011 (MARAN, 2014). From 2012 onwards, a limited decrease of resistance rates was recorded for most antimicrobials included, which is most likely the result of the substantial decrease in antimicrobial use in the Netherlands since 2009 (MARAN, 2014).

Administering antimicrobials to an individual animal generally results in an increase in resistance level in the individual animal and by doing so maintains a resistance pool that can influence the entire population (Levy, 1998). In calves, prior to treatment with apramycin, resistant *E. coli* were detected but all *Salmonella* Typhimurium strains were susceptible. Following treatment, apramycin-resistant *Salmonella* Typhimurium strains, carrying the same resistance genes as resistant *E. coli*, were isolated (Hunter et al., 1992). Levin et al. (1997) suggested a model where treatment of individual animals exerts sufficient selective pressure to maintain a commensal reservoir of resistance genes, which contributes to the spread and persistence of these genes at a population level. This, together with earlier described factors including vitamin D selection by milk intake, could explain why resistant *E. coli* are found in neonatal calves and piglets not previously exposed to antimicrobials. These young animals are exposed to a pool of resistant commensal bacteria in the dam, which thereafter is maintained by the treatment of other individuals (Berge et al., 2005a).

The persistence of resistance can also be promoted by cross-selection, referred to as the selection of resistance to antimicrobial agents by any other antimicrobial of the same antimicrobial class, and by co-selection, defined as resistance selection through the use of unrelated antimicrobials as a result of linkage of multiple resistance genes on the same genetic element. The latter will result in the collective positive selection of all genes in the presence of a selective pressure for one trait. Co- and cross-selection can explain the persistence of resistance, even though the actual antimicrobial has not been used for a certain period. This phenomenon is well known in veterinary medicine. For example, swine *E. coli* have been reported resistant to chloramphenicol in spite of the absence of a direct selection pressure exerted by chloramphenicol use for 25 years, as this product was withdrawn from the market for food producing animals in 1989 in Europe (Berendsen et al., 2010). Cross-resistance to florfenicol and co-selection by the use of aminoglycosides,

tetracycline and sulfonamides may explain this persistent chloramphenicol resistance (Bischoff et al., 2005). Cefazolin-resistant *E. coli* strains, harboring extended spectrum class A or class C β-lactamases on plasmids, have been isolated from broiler chickens in Japan (Kojima et al., 2005). Since no cephalosporins are approved for use in poultry in Japan, the selection of these strains is most likely enhanced by the presence of other resistance genes on the same plasmid (Harada and Asai, 2010). Co-selection of *strA* and *sul2* genes, conferring resistance to streptomycin and sulfonamides respectively, during the treatment of chickens with streptomycin has been reported by Faldynova et al. (2013).

3.2. PERSISTENCE OF ANTIMICROBIAL RESISTANCE IN THE ABSENCE OF ANTIMICROBIAL SELECTION PRESSURE

In the absence of a selection pressure exerted by antimicrobials, one could assume no evolutionary advantage anymore for the presence of antimicrobial resistance determinants, especially when a certain cost is associated with the resistance trait. It has been described that resistance achieved by both mutation of chromosomal genes (Wichelhaus et al., 2002; Giraud et al., 2003) and through acquired resistance genes (Johnsen et al., 2002) may impose a fitness cost both *in vitro* and *in vivo*. A loss in fitness can be reflected in a reduced growth rate (Andersson, 2006; Majcherczyk et al., 2008), a reduced transmission rate (Randall et al., 2008), a higher clearance rate (Gustafsson et al., 2003) and a decreased invasiveness (Fernebro et al., 2008), which can make the resistant strains less competitive than the susceptible ones in the absence of antimicrobials. This should result in a gradual reduction of resistance prevalence if no selection pressure is present. However, it is often observed that a reduction or discontinuation of antimicrobial use in farm environments does not necessarily result in a decreased prevalence of antimicrobial resistant isolates, at least not in the short term (Enne et al., 2001; Khachatryan et al., 2004; Thakur and Gebreyes, 2005; Bunner et al., 2007). The horizontal transfer of resistance genes in the absence of an antimicrobial selection pressure has for instance been described for the tetracycline resistance gene *tetO* between *Campylobacter jejuni* strains in the intestinal tract of chickens (Avrain et al., 2004) and for MLS_B resistance in the Gram-positive nasal and tonsillar microflora of pigs (Martel et al., 2003). Recently, an *in vivo* chicken model showed the spread of genetic determinants of MLS_B resistance independent of any antimicrobial pressure (Marosevic et al., 2014).

Several mechanisms might be responsible for the persistence of resistance mechanisms in bacterial strains in the absence of antimicrobial use. These are described below.

3.2.1. COMPENSATORY MUTATIONS AND OTHER MECHANISMS RESULTING IN PERSISTENCE OF ANTIMICROBIAL RESISTENCE

Some resistance-conferring mutations bear low or no fitness cost or may even enhance the bacteria's fitness (Luo et al., 2005). This was observed for a porcine *E. coli* isolate where the fitness impact imposed by the

carriage of antimicrobial resistance elements was generally low or non-existent (Enne et al., 2005). Moreover, carriage of transposon Tn1 A improved fitness *in vitro* and in pig infection models (Enne et al., 2005). Most likely, the insertion of Tn1 A disrupted a gene that imposed fitness cost or the transposon itself conferred fitness advantage (Enne et al., 2005). Enhanced fitness has been seen in fluoroquinolone resistant *Campylobacter jejuni*, directly linked to the single point mutation in *gyrA*, conferring high-level resistance to fluoroquinolones (Luo et al., 2005).

Additionally, a mechanism of compensatory mutations has been described (Andersson and Levin, 1999) enabling a resistant strain to compensate for fitness loss and successfully compete with, or even prevail over susceptible strains (Handel et al., 2006; Johnsen et al., 2009). The level to which compensation is attained and the number of compensatory mutations depends on the bacterial strain, the resistance mechanism and the environmental conditions (Andersson and Hughes, 2010; Beceiro et al., 2013). *In vitro* experiments and animal models have revealed compensatory mutations for amelioration of fitness costs caused by chromosomal mutations, such as in *Salmonella* Typhimurium (Bjorkman et al., 1998), *E. coli* (Marcusson et al., 2009) and *S. aureus* (Nagaev et al., 2001).

In *E. coli*, it has been observed that the addition of a fourth fluoroquinolone resistance mutation in low-fitness mutant strains caused a further reduction in susceptibility to fluoroquinolones and an increase in relative fitness in *in vitro* models as well as *in vivo* (Marcusson et al., 2009).

Besides chromosomal mutations, plasmid encoded resistance genes might equally acquire compensatory mechanisms for fitness costs (Poole et al., 2011). Plasmids such as pCT, carrying the extended-spectrum-β-lactamase (ESBL) resistance gene *bla*CTX-M-14 have evolved to impose little impact on host strains *in vitro* (Cottell et al., 2012). In clinical *Salmonella* Typhimurium strains, the biological cost of plasmid-encoded high level AmpC production is compensated by plasmid-encoded factors, other than the repression of AmpC expression (Hossain et al., 2004). The emergence of *Salmonella* populations resistant to extended-spectrum cephalosporins in animal reservoirs (Chen et al., 2004) supports the presence of such evolved mechanisms to compensate the loss of fitness associated with resistance to beta-lactams (Zhang et al., 2006).

As a result of no- and low cost mutations, and compensatory evolution, antimicrobial resistance genes and their vectors are able to show a rapid emergence, stabilization and persistence in environments in the absence of antimicrobial use once established in bacterial populations (Enne et al., 2005; Cottell et al., 2012).

3.2.2. REGULATION OF RESISTANCE MECHANISMS FOR PERSISTANCE OF RESISTANCE

Antimicrobial resistance is in general only transiently advantageous to bacteria, namely in the presence of the antimicrobial. Therefore, bacteria can regulate the expression of resistance, following chromosomal mutations or acquisition of mobile genetic elements (Depardieu et al., 2007). Induction is the process where the phenotypic resistance is brought to expression after having acquired an antimicrobial resistance determinant (Chancey et al., 2013). The antimicrobial to which the resistance gene is targeted can be involved in the

induction of the expression of the gene (Butaye et al., 2003) and furthermore, can even induce the dissemination of the resistance determinant (Deparieu et al., 2007). This phenomenon has for instance been described for inducible MLS_B resistance (Chancey et al., 2013) and for vanA- and vanB-type resistance (Arthur et al., 1992; Foucault et al., 2010) in Gram-positive bacteria, for AmpC β-lactam resistance (Jacoby, 2009) in Gram-negative bacteria and for several resistance mechanisms, such as specific efflux systems (Butaye et al., 2003) and ribosome methylation (Chancey et al., 2013).

Regulation of expression of resistance mechanisms in bacterial strains may explain why isolates are more resistant *in vivo* than *in vitro* (Chancey et al., 2012). It drastically reduces the biological cost associated with resistance (Andersson and Hughes, 2010) and therefore it can account for the widespread dissemination of such strains (Foucault et al., 2010).

3.2.3. GENETIC LINKAGES AS A MECHANISM FOR PERSISTANCE OF RESISTANCE

Co-selection does not solely occur by the use of antimicrobials of diverse classes. As previously described, resistance genes can be linked to a much broader spectrum of genes due to common mobile genetic elements, such as plasmids, transposons or integrons. These genes can attribute an advantage to bacteria in certain conditions, such as in the presence of heavy metals (Cavaco et al., 2011), biocides (Levy et al., 2002), nutritional components in a diet (Khachatryan et al., 2006) and immune defense mechanisms (Goswami et al., 2008). As a result, selection of resistance genes can occur by other selectors, in the absence of an antimicrobial selection pressure. Specific efflux and multidrug efflux systems for antimicrobials can for instance be involved in additional physiological functions related to a wide range of potentially toxic substances occasionally including also antimicrobial agents (Wang et al., 2000; Butaye et al., 2003). This may confer advantages to bacteria even when antimicrobials are not present (Wang et al., 2000; Butaye et al., 2003), resulting in the persistence of such systems.

3.2.4. POST SEGREGATIONAL KILLING AS A MECHANISM FOR PERSISTANCE OF RESISTANCE

Post segregational killing (PSK) systems imply the killing of bacterial cells after the loss of a plasmid, due to the imbalance between a toxic and an anti-toxic substance that are co expressed. The linked presence of a PSK system to antimicrobial resistance determinants on one plasmid has been reported for glycopeptides in *Enterococcus faecium* from poultry and poultry farmers (Sorum et al., 2006). The system also prevents the development of plasmid-free daughter cells (Johnsen et al., 2009) and thus contributes to persistence of resistance. In some cases the resistance genes themselves increase fitness (Groh et al., 2007) thus losing the genetic element they are located on presents a fitness cost, effectively selecting for resistance.

3.2.5. ENVIRONMENTAL RESERVOIRS OF RESISTANCE GENES

Bacteria in soil, containing resistance elements, are phylogenetically diverse and many are closely related to pathogenic species, suggesting that they can play an important role as reservoir of resistance genes through horizontal gene transfer (Dantas et al., 2008).

Resistance in *Campylobacter* spp. and *S. enterica* strains has been reported to persist in farm animals in antimicrobial-free and organic production systems (Rollo et al., 2010; Keelara et al., 2013). Identical resistance profiles were observed in *S. enterica* and *Campylobacter* spp. from pigs and their environment on farms and at slaughter (Quintana-Hayashi and Thakur, 2012a; Keelara et al., 2013). Moreover, results from multilocus sequence typing, genotypically characterizing bacterial strains, revealed a clear overlap between swine and environmental farm- and slaughterhouse-associated *Campylobacter coli* (*C. coli*) sequence types (STs). This demonstrates the swine environment to play a key role in the persistence of multidrug resistant *C. coli* strains on pig herds, even in the absence of antimicrobial selection pressure (Quintana-Hayashi and Thakur, 2012b).

4. REVERSION TO SUSCEPTIBILITY

A final possible phase in the epidemiology of antimicrobial resistance is the disappearance of resistance. From the above, it is clear that, despite the absence of an antimicrobial selection pressure, selection and spread of resistance through other mechanisms decrease the likeliness for a rapid reduction in resistance prevalence. However, some studies showed a decline in the resistance level of bacterial populations when antimicrobial selection pressure was removed. In a multi-resistance-plasmid-carrying *E. coli* population, a tetracycline-sensitive subpopulation emerged in the absence of antimicrobials (De Gelder et al., 2004). This was observed in an *in vitro* experiment aiming at constructing mathematical models for predicting the reversibility to a susceptible population in the absence of an antimicrobial selection pressure. The model incorporated the mutation rate (number of mutations per generation), and the selection coefficient (reduction in growth rate due to the fitness costs associated with harboring the multi-resistance plasmid). As expected, the time required to reduce the number of resistant bacteria appeared to be inversely related to the fitness cost. Consequently, relatively less time is needed for strains with a high fitness cost which can be seen e.g. for a constitutively expressed tetracycline operon on a high-copy number plasmid (Modi et al., 1991). Yet, in order to cope with this high cost, bacteria may adapt to the environmental condition in which they reside, allowing them to remain competitive with susceptible isolates in the absence of antimicrobials. This has been illustrated for amplification of the *bla*A gene in *Yersinia enterocolitica*, where the copy number of the plasmid carrying *bla*A is adjustable according to changes in the environment and as a result, only a basal resistance level remained once the selective pressure was removed (Seoane et al., 2003).

In the abovementioned example, absence of resistance is the result of genetic alterations, yet exclusively in the absence of antimicrobials. The final step in the reversion to susceptibility leading to a continued reduction

in resistance prevalence, should be a decreased growth rate of bacteria carrying resistance determinants or even the disappearance of these determinants. Bambermycin or flavophospholipol, primarily used as a feed additive in poultry, swine and cattle (Butaye et al., 2003) because of its suppressive effect on Gram-positive bacteria, has been shown to be effective in inhibiting growth of Gram-negative intestinal bacteria carrying antimicrobial resistance plasmids both *in vitro* and *in vivo* (Pfaller, 2006). In addition, it can decrease the frequency of transfer of resistance plasmids via conjugation in *E. coli*, *S. enterica*, *S. aureus* and *E. faecium in vitro* and might thus be effective in the prevention of horizontal dissemination of resistance genes (Pfaller et al., 2006). Yet, the latter has not yet been confirmed for all mentioned bacterial species *in vivo* (Poole et al., 2006). Nevertheless, flavophospholipol might play a role in decreasing the transferable antimicrobial resistance gene pool within the intestinal bacterial population.

An additional role may be attributed to vaccination. In human medicine, the introduction of a vaccine against invasive pneumococcal disease has been shown to decrease the incidence of antibiotic-resistant invasive disease. By reducing the risk of carriage and transmission of vaccine-type resistant *Streptococcus pneumoniae* strains the number of resistant *S. pneumoniae* decreases in the population (Kyaw et al., 2006).

Results of antimicrobial resistance monitoring programs in countries where during several years a substantial decrease of antimicrobial use in veterinary medicine was achieved, demonstrate that a reduction in resistance prevalence is possible after a few years (MARAN, 2013). It remains to be seen how long it will take before a substantial and stable decrease is reached.

5. CONCLUSIONS

Antimicrobial resistance is an old phenomenon with a widespread prevalence in animal production, mainly attributed to the use of antimicrobial agents for growth promotion, prophylaxis, metaphylaxis and therapy. As a result, one would expect that reducing the main selection pressure, namely the antimicrobial use, could result in a decrease in resistance prevalence. Yet, several non-antimicrobial factors may contribute to the selection, spread and persistence of resistance. Co-selection between a resistance mechanism and another selected determinant is believed to play a considerable role in the spread and persistence of resistance. Furthermore, bacteria have evolved mechanisms to cope with the potential fitness cost accompanying the acquisition of resistance determinants. Since these fitness costs are believed to be the main driving forces for reduction of the frequency of resistant bacteria in the absence of antimicrobials, it makes a decrease in resistance after cessation of antimicrobial administration uncertain. The role of antimicrobials in the treatment of bacterial diseases cannot be seen as anything but essential. Therefore, good insights in the epidemiology of resistance at the animal and population level is crucial as it can help to develop appropriate strategies to stop further selection of resistance.

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Chapter 2

Scientific aims

Intensive pig production has been identified as subject to high antimicrobial exposure. The emergence of antimicrobial resistance in commensal, zoonotic, and pathogenic bacteria from pigs, mainly as a result of antimicrobial consumption, and potentially threatening treatment options in both human and veterinary medicine, is worrisome.

Closely monitoring antimicrobial use and antimicrobial resistance levels are both important first steps in strategies aiming to contain antimicrobial resistance. Commensal bacteria, commonly present in animals, are exposed to any antimicrobial administration and therefore good indicators for antimicrobial resistance.

The overall objective of this thesis was to gain insight in the extent to which antimicrobial agents are used in Belgian pig production, in the presence of resistance in commensal bacteria from pigs and in the relationship between the occurrence of resistance and the use of antimicrobials in sows and piglets.

The specific objectives of this thesis were:

(1) to collect and quantify herd level-data on the group use of antimicrobial agents in Belgian pig herds in 2010 and to compare the results to a similar study conducted in 2003 (Chapter 3)

(2) to report the level of antimicrobial resistance in the Gram-positive indicator bacterium *Streptococcus suis,* isolated from clinically healthy fattening pigs at slaughter age (Chapter 4)

(3) to report the level of antimicrobial resistance in the Gram-negative indicator bacterium *Escherichia coli* isolated from clinically healthy pigs at slaughter age (Chapter 5)

(4) to investigate whether the presence of antimicrobial resistant *Escherichia coli* in sows and the administration of antimicrobials to sows and piglets during farrowing influenced the antimicrobial resistance in fecal commensal *E. coli* in sows and their offspring (Chapter 6)

Chapter 3

Antimicrobial use in Belgian fattening pig herds

PROPHYLACTIC AND METAPHYLACTIC ANTIMICROBIAL USE IN BELGIAN FATTENING PIG HERDS

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ABSTRACT

The monitoring of antimicrobial use is an essential step to control the selection and spread of antimicrobial resistance. Between January and October 2010 data on prophylactic and metaphylactic antimicrobial use were collected retrospectively on 50 closed or semi-closed pig herds. Ninety-three percent of the group treatments were prophylactic whereas only 7% were metaphylactic. The most frequently used antimicrobials orally applied at group level were colistin (30.7%), amoxicillin (30.0%), trimethoprim-sulfonamides (13.1%), doxycycline (9.9%) and tylosin (8.1%). The most frequently applied injectable antimicrobials were tulathromycin (45.0%), long acting ceftiofur (40.1%) and long acting amoxicillin (8.4%). The treatment incidences (TI) based on the used daily dose pig (UDD_{pig}[1] or the actually administered dose per day per kg pig of a drug) for all oral and injectable antimicrobial drugs was on average 200.7 per 1000 pigs at risk per day (min = 0, max = 699.0), while the TI based on the animal daily dose pig (ADD_{pig}[2] or the national defined average maintenance dose per day per kg pig of a drug used for its main indication) was slightly higher (average = 235.8, min = 0, max = 1322.1). This indicates that in reality fewer pigs were treated with the same amount of antimicrobials than theoretically possible. Injectable products were generally overdosed (79.5%), whereas oral treatments were often underdosed (47.3%). In conclusion, this study shows that prophylactic group treatment was applied in 98% of the visited herds and often includes the use of critically important and broad-spectrum antimicrobials. In Belgium, the guidelines for prudent use of antimicrobials are not yet implemented.

[1] UDD_{pig} or Used Daily Dose is equal to UDDD, as proposed by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (EMA, 2013)

[2] ADD_{pig} or Animal Daily Dose is equal to ADDD, as proposed by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (EMA, 2013)

INTRODUCTION

The use of antimicrobials in modern pig production is of essential importance in maintaining animal health (McEwen and Fedorka-Cray, 2002). Yet, under some circumstances, the risks associated with their use could negate their benefits (Collignon et al., 2009). The potential risks, consisting of the exposure to antimicrobial residues in food or environment (WHO, 2002; McEwen and Singer, 2006; Wei et al., 2011), and in particular the selection of antimicrobial resistance in both animal and human related bacteria, might compromise animal and human health (Bywater, 2004; Ungemach et al., 2006). The demonstrated contribution of antimicrobial use in livestock in the emergence of both methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum beta-lactamase producing *Escherichia coli* (ESBL) in production animals has increased the public health concern over the consumption of antimicrobials in livestock (van Duijkeren et al., 2008a; Graveland et al., 2010; Horton et al., 2011).

Over the years, several measures have been taken to safeguard the efficacy of antimicrobial agents and to prevent the emergence of antimicrobial resistance. In 2005, the World Health Organization (WHO) developed criteria to rank antimicrobials according to their importance in human medicine, to help preserve the effectiveness of currently available antimicrobials (Collignon et al., 2009). Guidelines for the responsible and prudent use of antimicrobials in food-producing animals have been suggested by several institutions (Stöhr et al., 2000; BTK and ArgeVet, 2000; CODEX, 2005), intended to prevent or reduce the selection pressure that contributes to the spread of antimicrobial resistant bacteria in humans and animals. German guidelines recommend the prescription of antimicrobials to animals only for therapeutic or metaphylactic reasons and only after the identification and antimicrobial sensitivity testing of the causal pathogen (BTK and ArgeVet, 2000). According to the WHO guidelines (Stöhr et al., 2000) the prophylactic use of antimicrobials in control programs has to be regularly assessed for effectiveness and whether use can be reduced or stopped.

The most far reaching change up to now, in order to decrease the antimicrobial use was the total ban of antimicrobial growth promoters in the European Union (Bengtsson and Wierup, 2006; Aarestrup et al., 2010). Sweden was the first to discontinue growth promoter use in 1986 (Phillips, 2007). The use of the four final growth promoting antibiotics had ceased in the entire European Union by 2006.

The appropriate assessment of the selection pressure exerted by the use of antimicrobial agents is a crucial first step in the control of emergence of antimicrobial resistance (Chauvin et al., 2002; McEwen and Fedorka-Cray, 2002; Aarestrup, 2005). This requires detailed knowledge on the reasons for antimicrobial use, the treatment duration and administered dose as well as the accuracy of dosing (Catry et al., 2003; Regula et al., 2009). Furthermore, monitoring antimicrobial use allows to evaluate the appropriateness of antimicrobial drug application according to the prudent use guidelines as established by several institutions (Stöhr et al., 2000;

Ungemach et al., 2006; Regula et al., 2009). Moreover, data on antimicrobial use also provide an insight into disease burden. Finally, interventions cannot be evaluated properly unless a standardized monitoring system can measure the relationship between exposure and outcome.

In 2003–2004 a detailed study of the antimicrobial use in pig production in Belgium was performed (Timmerman et al., 2006). A relatively high level of group treatments was noted and a considerable amount of broad spectrum antibiotics used for prevention was highlighted. Since 2003, little new information was collected and it was therefore not known whether the appropriateness of use has improved or not in the last seven years.

The purpose of this study was to collect and quantify herd-level data on the use of antimicrobial agents in Belgian pig herds and to assess the changes in consumption of antimicrobial drugs in 2010 and to compare the results to a similar study conducted in 2003.

MATERIALS AND METHODS

Selection of herds and data collection

A list of 140 pig herds that fulfilled the selection criteria were randomly selected from the Belgian farm-animal identification and registration database (SANITEL, 2010). The sampling frame consisted of all farrow to finish herds that used a closed or semi-closed production system and held at least 150 sows and 600 fattening pigs. Only farrow to finish herds were selected since these allow to collect data on the antimicrobial use of the fattening pigs during their entire lifespan. The sample was stratified by province (n = 5), proportional to the number of pig herds per province. Random selection was performed using a computer-generated list (Toolbox, Cameron, 1999).

All selected herds were contacted by telephone and the first 50 herds that were willing to cooperate in the study were visited between January and October 2010. The herds were visited when the oldest fattening pigs were less than 2 weeks before slaughter (average body weight at slaughter varied between 105 and 110 kg at the average age of 206 days). In this way, we aimed to assess the antimicrobial use during the entire lifespan of the fattening pigs. One hundred thirty-two herds were contacted by telephone to obtain 50 cooperative herds (response rate of 38%). Of the non-responders, 30% (25/82) had stopped their activities, 28% (23/82) were not interested and 28% (23/82) were unable to participate due to lack of time. Thirteen percent (11/82) of the non-responders claimed other reasons. The number of sows and fattening pigs present in the non-responding herds (on average 181 and 1046 respectively) was significantly ($p < 0.05$) lower than in the responding herds (on average 289 and 1420 respectively).

Despite the high number of non-responders, a sufficient number of responders was available for the different provinces in order to fulfill the number of herds pro-posed after stratification. The herds were located in the 5 different provinces of Flanders. 94% (47 of 50 herds) of the selected herds lay in the most dense pig areas of Belgium (West-, East-Flanders and Antwerp with 0.9 herds/km^2, 0.3 herds/km^2 and 0.2 herds/km^2 respectively). Three herds were located in the less dense regions of Limburg and Flemish-Brabant (0.1 herds/km^2 and 0.1 herds/km^2 respectively). No herds from the southern part of Belgium were included since 90% of the Belgian pig production is located in Flanders and the remaining herds (10%) located in Wallonia are mainly fattening sites that were not within the selection criteria for this study.

Quantitative and qualitative data on group-level antimicrobial use in the sampled herds were collected by means of a questionnaire during a face-to-face on site interview with the farmer. All the interviews were taken by the same interviewer (first author) in order to avoid different interpretation of the answers provided by the farmer. The questions aimed to collect retrospective data on the antimicrobial group treatments applied between birth and time of the herd visit for the oldest group of fattening pigs present (within two weeks of slaughter). A group treatment was defined as each prophylactic or metaphylactic administration of antimicrobials to all the pigs of the same production group. Prophylactic use of antimicrobials was defined as treatment of healthy pigs to prevent disease from occurring, whereas metaphylactic use was defined as treatment of clinically healthy pigs belonging to the same group as animals that showed clinical symptoms of disease (Aarestrup, 2005). For each group treatment, following data were gathered: product name, indication, duration of therapy (in days), dose, administration route (feed, water or by injection (intramuscular)), age of the treated animals (in days) and body weight at time of treatment. To check for completeness, prescription documents or order forms were consulted if available. This was only possible for 10% of the herds.
Indications for treatment were categorized by the interviewer based on the symptoms described by the farmer prior to the administration of antimicrobials.

Quantification of drug consumption

Antimicrobial drug consumption was quantified as treatment incidences (TI) based on the animal daily dose pig (ADD_{pig}) and the used daily dose pig (UDD_{pig}). The ADD_{pig} is the national defined average maintenance dose per day per kg pig of a drug used for its main indication (Jensen et al., 2004). Values of the ADD_{pig} were based on the dose recommendations in the Belgian Compendium for Veterinary Medicines and on the drug's instruction leaflet. The UDD_{pig} is defined as the actually administered dose per day per kg pig of a drug. In order to calculate the UDD_{pig}, an estimate of the body weight at time of treatment was made. The average body weight (bw) between birth and the end of the nursery period (at 10 weeks) was standardized over the different herds using a standard growth table for a given age of the pigs (1.5 kg bw at birth and 6.1

kg bw at weaning age of 4 weeks). In order to estimate the body weight between the start of the fattening period and slaughter time, the average daily weight gain was consulted for the individual herd.

The treatment incidence is defined as the number of pigs per 1000 that is treated daily with one ADD_{pig} or UDD_{pig}. The TI_{ADDpig} and TI_{UDDpig} were calculated based on the acquired data, according to the method described by Timmerman et al. (2006). The following formula was applied:

$$\frac{Total\ amount\ of\ antimicrobial\ administered\ (mg)}{UDD\ or\ ADD\ \left(\frac{mg}{kg}\right) * number\ of\ days\ at\ risk * kg\ pig}$$

The number of days at risk was set as the total lifetime of slaughter pigs.

A distribution (minimum, percentiles, maximum) of the treatment incidences was used because the data were not fully normally distributed (Table 1). The proportional TI_{ADDpig} and TI_{UDDpig} for each individual antimicrobial drug was calculated by dividing the TI_{ADDpig} and TI_{UDDpig} of each individual antimicrobial by the total TI_{ADDpig} and TI_{UDDpig} for injectable and oral administrations, respectively (Timmerman et al., 2006). The UDD_{pig}/ADD_{pig} ratio of each antimicrobial drug gives an idea of the correctness of dosing. A variation of 0.2 under or above 1 (=theoretically correctly dosed) was considered as within an acceptable range (0.8–1.2) of correct dosing (Timmerman et al., 2006).

Data analysis

Comparison of herd characteristics of responding and non-responding was performed by means of Student's t-test.

RESULTS

Prophylactic antimicrobial group treatments were responsible for 93% of all group treatments. Metaphylactic treatments constituted only 7% of all group treatments. In only one herd, no antimicrobials at group level were used. The forty-nine other herds applied at least one group-level treatment between birth and the time of the herd visit. Fig. 1 represents the distribution per herd of average treatment incidences based on either the ADD_{pig} or the UDD_{pig} for all group treatments.

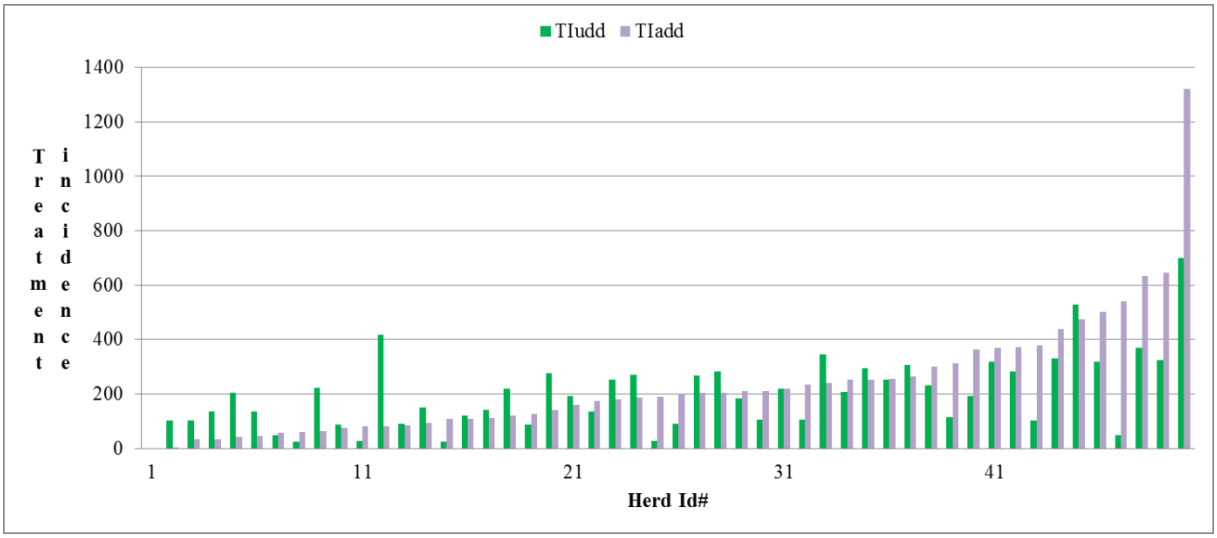

Figure 1. Distribution per herd of average treatment incidences based on ADD_{pig} (TI_{ADDpig}) and UDD_{pig} (TI_{UDDpig}) for all group treatments in Belgian closed and semi-closed pig herds (between birth and two weeks within slaughter), for 2010.

The distribution (minimum, percentiles, maximum) of the TI_{ADDpig} and TI_{UDDpig} for the different oral and injectable antimicrobial agents as well as their relative importance, expressed as the proportional TI_{ADDpig} and TI_{UDDpig}, are presented in Table 1. Penicillins were the most frequently used antimicrobial class (proportional TI_{UDDpig} equals 27.6%), mainly due to the frequent use of amoxicillin both as injectable and oral administration, together with the less frequently used injectable penicillin and ampicillin. Polymyxins follow very closely with a frequency of 27.0%. Antimicrobial classes with a moderate rela-tive importance are the macrolides/lincosamides (17.7%), the trimethoprim-sulfonamides (11.5%) and tetracyclines (10.0%). Cephalosporins represent 5.3% of the total use and aminoglycosides (0.7%), phenicols (<0.1%) and quinolones (<0.1%) are less frequently used.

During 47.7% of the time, animals were administered antimicrobials belonging to the WHO critically important list. The 3rd and 4th generation cephalosporins ceftiofur and cefquinome were used in 48% of the visited herds. Tulathromycin, a long acting 15-ring macrolide, was administered in 29% of the visited herds, mostly in

combination with iron mineral supplements that are given to prevent iron deficiency and anemia in newborn piglets.

The average TI_{ADDpig} for all oral and injectable antimicrobial drugs was 235.8. In reality fewer animals were treated, as the TI_{UDDpig} was 200.7. Animals were more often exposed to oral antimicrobial therapy (TI_{ADDpig}, oral = 183.5 and TI_{UDDpig}, oral = 176.5) than injectable administrations (TI_{ADDpig}, injectable = 52.3 and TI_{UDDpig}, injectable = 24.2). Discrepancies between TI_{UDD} and TI_{ADD} are the result of incorrect dosing. On average, antimicrobials were mostly overdosed, since with the same amount of antimicrobials fewer animals were treated than based on the theoretically TI_{ADDpig} ($TI_{UDDpig} < TI_{ADDpig}$).

Injectable antimicrobials were mostly overdosed (79.5% overdosed, 8.2% correctly dosed, 12.3% underdosed) whereas oral administrations mostly were underdosed (47.3% underdosed, 23.3% correctly dosed, 29.4% over-dosed). The two most often oral administered antimicrobials, colistin and amoxicillin were underdosed in 53% and 43% of the cases, respectively, whereas injectable amoxicillin was always overdosed. Tulathromycin was under-, correctly and overdosed in an equal number of treatment occasions. Ceftiofur was overdosed in 88% of the administrations and cefquinome was always overdosed.

Table 1. Distribution of the oral and injectable drugs and the proportional TI_{ADDpig} or TI_{UDDpig} administered as group treatments to fattening pigs between birth and slaughter age, in 50 Belgian closed or semi-closed pig herds expressed as treatment incidence per 1000 pigs at risk per day and based on animal daily doses or used daily doses (ADD_{pig} or UDD_{pig}/1000 pigs at risk/day). Antimicrobials are classified according to their importance in human medicine (WHO, 2007). Class I, critically important antimicrobials; Class II, highly important antimicrobials; Class III, important antimicrobials.

Class according to importance in human medicine	Active substance		TI_{ADDpig} [a]				TI_{UDDpig} [a]					Proportional TI_{ADDpig} %	Proportional TI_{UDDpig} %
	Oral	Min	25th PCT[b]	50th PCT	75th PCT	Max	Min	25th PCT	50th PCT	75th PCT	Max		
I	Amoxicillin	0	0	0	57.7	185.2	0	0	0	72.8	203.9	24.9	30.0
	Tylosin	0	0	0	0	197.1	0	0	0	0	305.8	8.5	8.1
	Oxytetracycline	0	0	0	0	4.9	0	0	0	0	135.9	0.1	1.5
	Tilmicosin	0	0	0	0	45.4	0	0	0	0	53.4	0.7	1.0
II	Colistin	0	0	14.4	52.5	532.2	0	0	41.3	101.9	203.9	32.3	30.7
	Trimethoprim-sulfadiazine	0	0	0	0	444.1	0	0	0	0	184.5	17.0	13.1
	Doxycycline	0	0	0	0	523.4	0	0	0	0	194.2	12.6	9.9
	Spectinomycin	0	0	0	0	55.4	0	0	0	0	68.0	0.6	0.8

	Lincomycin-spectinomycin	0	0	0	0	46.1	0	0	0	0	126.2	0.6	3.3
III	Lincomycin	0	0	0	0	143.6	0	0	0	0	68.0	2.7	1.6
	Injectable												
I	Tulathromycin	0	0	0	12.9	76.1	0	0	0	34	68.0	22.4	45.0
	Ceftiofur LA[c]	0	0	0	58.5	135.1	0	0	0	24.3	48.5	57.2	40.1
	Amoxicillin LA[c]	0	0	0	9.5	51	0	0	0	4.9	14.6	11.1	8.4
	Ceftiofur	0	0	0	0	27	0	0	0	0	4.9	2.9	2.5
	Cefquinome	0	0	0	0	18.7	0	0	0	0	9.7	1.0	1.2
	Procaïne-benzylpenicillin	0	0	0	0	46.2	0	0	0	0	4.9	4.0	1.2
	Ampicillin LA[c]	0	0	0	0	12.9	0	0	0	0	4.9	0.8	0.8
	Enrofloxacin	0	0	0	0	6.4	0	0	0	0	4.9	0.2	0.4
II	Florfenicol	0	0.2			8.7	0	0			4.9	0.4	0.4

a TI_{ADDpig}, treatment incidence based on ADD_{pig}; TI_{UDDpig}, treatment incidence based on UDD_{pig}

b PCT, percentile

c LA, long acting

Fig. 2 shows the distribution of the oral and injectable group treatments for the different antimicrobial classes used during the four production stages. These can be defined as the farrowing period (from birth until weaning between 21 and 28 days of age), the nursery period (from weaning age until 70 days of age), the grower period (from 70 until 126 days of age) and the finisher period (from 126 days of age until slaughter). Of all 206 group treatments, 90% (n = 186) was administered between birth and 10 weeks of age (farrowing and nursery period). Only 20% of all injectable and oral group treatments were administered during the fattening period (grower and finisher period).

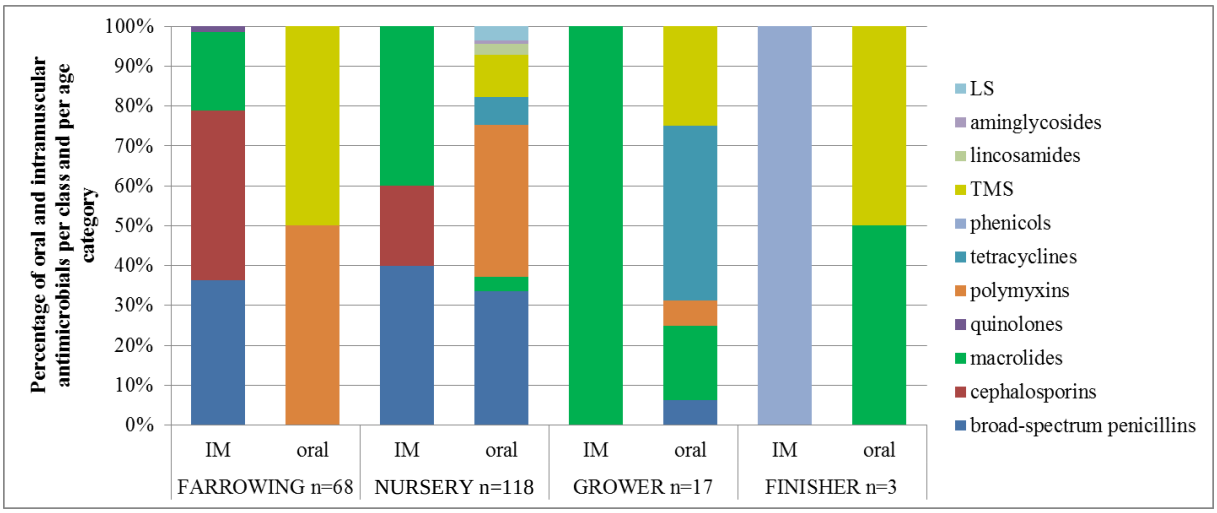

Figure 2. Distribution of oral and injectable group treatments for the different antimicrobial classes administered to fattening pigs for the different stages of production in 50 Belgian pig herds in 2010. IM, intramuscular (injectable group treatments); oral, water and feed antimicrobial medication; farrowing, from birth until weaning between 21 and 28 days of age; nursery, from weaning age until 70 days of age; grower, from 70 until 126 days of age; finisher, from 126 days of age until slaughter; n, total number of injectable and oral group treatments for all used antimicrobial classes per unit on 50 pig herds (n_{total} = 206); LS, lincomycine-spectinomycine; TMS, trimethoprim/sulfadiazine.

Results of the indications of treatment and the administered antimicrobial classes are shown in Table 2. Prophylactic group treatments were mainly applied shortly after birth, around castration and in prevention of piglet diarrhea during the farrowing period. Cephalosporins and broad-spectrum penicillins were the most frequently administered antimicrobials for these indications with 44% and 9.3% of the injectable administered treatments, respectively. Tulathromycin was administered in 22% of the injectable administrations to prevent suckling piglets from coughing and sneezing as main indication. Group injection with enrofloxacin and florfenicol after birth was recorded in one herd. In this study, the main indication for oral treatment with colistin was post-weaning E. coli infections, whereas amoxicillin was mainly administered as a preventive measure against streptococcal infections. Respiratory disease was mainly prevented with doxycycline and trimethoprim-sulfadiazine followed by tylosin and tilmicosin.

Table 2. Antimicrobial classes orally and injectable administered as group treatments to fattening pigs between birth and slaughter age, in 50 Belgian closed or semi-closed pig herds per age category for different indications.

FARROWING[a] *(n= 68)*			**NURSERY[b] *(n= 118)***		
Indication	Antimicrobial class *Number of treatments n*	% of used class per age group	**Indication**	Antimicrobial class *Number of treatments n*	% of used class per age group
Birth	Cephalosporins *13*	19.1	**Coughing**	TMS[e] *8*	6.8
	Broad-spectrum penicillins *11*	16.2		Macrolides *6*	5.1
	Fluoroquinolones *1*	1.5		Tetracyclines *5*	4.3
Castration	Cephalosporins *13*	19.1	**Diarrheae**	Polymyxins *38*	32.5
	Broad-spectrum penicillins *9*	13.2		Broad-spectrum penicillins *5*	4.3
	Macrolides *5*	7.4		Lincosamides *3*	2.6
Coughing	Macrolides *7*	10.3		TMS[e] *2*	1.7
				LS[f] *2*	1.7

	TMS[e] *1*	1.5		Macrolides *1*	0.9
Diarrheae	Cephalosporins *1*	1.5		Cephalosporins *1*	0.9
				Lincosamides *2*	1.7
	Polymyxins *1*	1.5		Aminoglycosides *1*	0.9
Streptococcal infections	Broad-spectrum penicillins *4*	5.9	**Oedema disease (*Escherichia coli*)**	Polymyxins *2*	1.7
	Macrolides *1*	1.5		Broad-spectrum penicillins *1*	0.9
Tooth cutting	Cephalosporins *1*	1.5	**AR[g]**	Tetracyclines *3*	2.6
				Broad-spectrum penicillins *1*	0.9
			Streptococcal infections	Broad-spectrum penicillins *32*	27.4
				Polymyxins *3*	2.6
				TMS[a] *2*	1.7
GROWER[c] *(n= 17)*			**FINISHER[d] *(n= 3)***		
Indication	Antimicrobial class *Number of treatments n*	% of used class per age group	**Indication**	Antimicrobial class *Number of treatments n*	% of used class per age group
Coughing	Tetracyclines *6*	35.3	**APP[h]**	TMS[e] *1*	33.3
	TMS[e] *4*	23.5		Phenicols *1*	33.3
	Macrolides *4*	23.5			
Diarrheae	Polymyxins	5.9	**Coughing**	Macrolides	33.3

	1			*1*	
APP[h]	Tetracyclines *1*	5.9			
Streptococcal infections	Broad-spectrum penicillins *1*	5.9			

[a] Farrowing, from birth until weaning between 21 and 28 days of age.

[b] Nursery, from weaning age until 70 days of age.

[c] Grower, from 70 until 126 days of age.

[d] Finisher, from 126 days of age until slaughter.

[e] TMS, trimethoprim-sulfadiazine.

[f] LS, lincomycin-spectinomycin.

[g] AR, Atrophic Rhinitis.

[h] APP, *Actinobacillus pleuropneumoniae*.

DISCUSSION

Methodology

In intensive livestock production such as pig, veal and poultry production, antimicrobials are often administered on a regular basis by the farmer himself upon advice and receipt of the prescription documents by the herd veterinarian (Dunlop et al., 1998). Therefore, pig farmers play a crucial role in the administration of antimicrobials to pigs. As a result, valid data on the actual dose and treatment duration of antimicrobial group treatment can be obtained directly from the farmer (Chauvin et al., 2008).

Yet, the collection of retrospective data based on an interview with the farmer may be subject to recall and intervention bias. Recall errors may occur when a farmer's answers were affected by a lack of memory (Vrijheid et al., 2006). In this study, systematic external validation, such as prescription documents or order forms, were not readily available. However, in intensive pig production, group-level use of antimicrobials is the most important way of antimicrobial administration (Schwarz et al., 2001; Regula et al., 2009). Group level-treatments are mostly standardized treatments, and therefore well known by the farmer and so less subject to recall bias. This methodology would not work for the collection of data on incidental therapeutic use of antimicrobials since these are likely very prone to recall biases. This is the primary reason why in this study data collection was restricted to (standardized) group level-treatments. If accidentally recall bias occurred, this most likely led to an underestimation of the group level-antimicrobial use. Intervention bias could have occurred if a farmer had elected to deliberately misstate treatment data. This was avoided as much as possible by guaranteeing confidentiality of the individual herd data. Besides, observed differences with the results from 2003 are likely real as the authors from 2003 had similar challenges to confront.

A different response rate was found between 2003 and 2010 (60% and 38% respondents in 2003 and 2010 respectively) (Timmerman et al., 2006). The higher rate of non-responders can mainly be attributed to the higher number of farmers which had stopped their activities since the latest update of the SANITEL database (25 farmers had stopped their activities in 2010 whereas only 3 in 2003). This is in agreement with a decreasing number of pig herds found in the national agricultural census in Belgium between 2009 and 2010 (Landbouwtelling, 2009). A current disadvantageous economic situation could be a reason for the higher number of farmers which have stopped their activities. Thus this higher none response rate is not seen as an increased resistance of the farmers to cooperate but rather as a result of the not fully updated data in the I&R database. Therefore, this lower response rate is believed not to influence an adequate comparison of the results between 2003 and 2010. Moreover, since data collection was performed in exactly the same way, it can be assumed that the biases are similar. Therefore, observed differences are likely to be real.

In interpreting antimicrobial use data, the unit of measurement is crucial. The use of treatment incidences (TI) based on the Defined Daily Dose (DDD) and the animal daily dose (ADD) as described earlier for human (WHO, 2003) and veterinarian antimicrobial use (Jensen et al., 2004; Timmerman et al., 2006; Persoons et al., 2012) is an appropriate way to express the selection pressure exerted by the administered antimicrobial drugs for time at risk and average weight of the pigs at time of treatment.

The repeated performance of surveillance studies are restricted in both number of times and cooperating herds. As a result, well established surveillance programs using sources such as feed mills, pharmacies and veterinarians are imperative. Yet, only few countries have well established surveillance programs (DANMAP, MARAN). Recently, in Belgium, the first national antimicrobial consumption report in food animals has been published (BelVet-Sac, 2011). The reported data consist of all veterinary antimicrobials sold to a veterinarian or pharmacist in Belgium for the years 2007, 2008 and 2009. Yet, the obtained results in the BelVet-Sac report are still crude and do not give any details on animal species, indications, correctness of dosages, individual herd usages, number of treatments attributed to an animal during its life span etc. In this context the collection of more detailed and accurate animal species-level data implies the collection of data directly on the end user level. Countries such as Denmark, have organized structures which facilitates the monitoring of antimicrobial use patterns at the individual herd level (Vieira et al., 2010). In Belgium, plans to develop a comparable system are currently studied, however it will probably take some more years before this system will be fully operational. In this understanding the current study delivered very valuable information on species and herd specific data.

Antimicrobial drug consumption

In 2003, six out of the 50 herds (12%) did not administer antimicrobials in group (Timmerman et al., 2006). In the present study, antimicrobials were not administered in group in only one herd (2%) (Fig. 1). As in 2003 (Timmerman et al., 2006) large between herd variation exists which fattening pigs were treated (Fig. 1). These differences may be related to herd differences in disease incidence, management, husbandry, biosecurity as well as differences in farmer and veterinarian attitudes (Hybschmann et al., 2011). More thorough studies are needed to identify the influencing factors.

The average TI_{ADDpig} and TI_{UDDpig} in 2010 (235.8 and 200.7 respectively) were higher than those in 2003 (178.1 and 170.3 respectively). The higher group-level use is reflected in an increased number of prophylactic group treatments whereas a drastic decrease of the portion of metaphylactic group treatments (7%) to the total number of group treatments (prophylactic and metaphylactic) is observed since 2003 (44% metaphylactic and 56% prophylactic) (Timmerman et al., 2006). The high number of prophylactic group treatments is not in agreement with the “Good Agriculture Practices” (GAP) formulated by the Food and Agriculture Organization

of the United Nations (FAO, 2003). GAP refer to a minimization of the non-therapeutic use of antimicrobials and highlight a reduction of infection and disease in terms of prevention. Yet, they refer to prevention as in vaccination programs, proper management and housing, good hygiene standards in housing by proper cleaning and disinfection, etc. Moreover, appropriate veterinary advice in order to avoid disease and health problems is set as an example in both the GAP principles and the Good Veterinary Principles (GVP) (FVE, 2002).

Taking into account the suggested guidelines by several institutions on restricted therapeutic or metaphylactic treatments, identifying the causal pathogen and appropriate dosing, it can be stated based on the present study that the guidelines for prudent use are currently not implemented in pig production in Belgium. In this study 93% of the group treatments were for preventive reasons and antimicrobials administered for these reasons often lack a precise diagnosis. Although there is no well-founded justification for the repeated use of prophylactic group treatments. Farmers often consider the prophylactic use of antimicrobials, in spite of the associated high cost, as a necessity to achieve less disease, lower mortality and better production results. Moreover pig production is rapidly evolving into a highly organized production system where standardized management procedures are used in order to prevent production losses. In this type of highly organized production, standard prophylactic therapies are easier and less labor intensive to implement than treatment of clinically diseased animals and after losses have occurred. Selected herds were on average larger than those in 2003 (216 and 289 sows in 2003 and 2010 respectively, 1250 and 1420 fattening pigs in 2003 and 2010 respectively). A yearly increase in the number of pigs per herd is confirmed by the national agricultural census in Belgium evaluating changes in herd size (Landbouwtelling, 2009). It could be assumed that a larger herd size includes a greater risk of transmission of pathogens within herds resulting into a higher antimicrobial use. Yet, Danish and Dutch studies reported higher TI rates being associated with smaller herds (Vieira et al., 2010; Poortwachter, 2010). On the other hand, compared with small herds, large herds might more frequently adopt management and housing practices decreasing this risk (Gardner et al., 2002). Therefore in Belgium, a non-adapted herd management for an increased number of pigs per herd could be a possible reason for an increased antimicrobial group-level use. Pharmaceutical companies serve more and more as advisers in disease management, linked to the provision of antimicrobial agents and vaccines. A recent study showed that in Belgium, on average, 43% of the income of pig veterinarians results from the selling of medicines, including vaccines, antimicrobials and other drugs (Maes et al., 2010). New active and more potent antimicrobial substances, like long acting critically important compounds (tulathromycin, crystallic ceftiofur), offer advantages to the farmer and as a result are easily introduced. These new substances could be another reason for an increased antimicrobial group-level use in food producing animals. Although most of the antimicrobials used in 2003, are currently still in use, a substantial shift in the relative importance between the commonly used antimicrobials and route of administration was seen. In particular the oral group treatments

with doxycycline and potentiated sulfonamides (trimethoprim-sulfonamides) appeared to have been replaced by long acting injectable group treatments. The introduction of ceftiofur in a long acting formulation since 2003 could explain the current higher use, as farmers see practical advantage in a single administration instead of repeated administration of short acting formulations. The contribution of 3rd and 4th generation cephalosporins to the total group medication increased from 0.1% in 2003 to 5.3% in 2010. The same is seen for injectable amoxicillin and ampicillin. In 2003, 1.8% of all injectable amoxicillin and ampicillin was administered as a short acting formulation in contrast to 2010, where all use of these compounds was in long acting form. Similarly, the overall use of macrolides (tulathromycin, tylosin and tilmicosin) has increased (proportional TI_{UDDpig} equals 5.5% and 13.4% respectively in 2003 and 2010), in particular due to the newly introduced long acting tulathromycin in 2004. A slight decrease in use was seen for tilmicosin whereas the use of tylosin, having the same spectrum as tilmicosin (Prescott, 2000), has increased. Iron supplementation shortly after birth is often performed in combination with tulathromycin in a single injection, mainly because of reduced labor. The use of fluoroquinolones was lower compared to 2003 (proportional TI_{UDDpig} to all injectable antimicrobials equals 6.5% and 0.4% respectively in 2003 and 2010). The high cost of both enrofloxacin and marbofloxacine could interfere with the choice for this large spectrum antimicrobial.

An indication of the appropriateness of dosing of individual antimicrobials was obtained by evaluating the distribution of the UDD/ADD ratios (Timmerman et al., 2006). In accordance with results from 2003, injectable antimicrobials were generally overdosed ($UDD_{pig}/ADD_{pig} > 1.2$), whereas orally administered group treatments were generally underdosed ($UDD_{pig}/ADD_{pig} < 0.8$). Sub-therapeutic doses can lead to a lack of efficiency and in some cases, may increase antimicrobial resistance (Regula et al., 2009). The relation between dosing and the selection and spread of antimicrobial resistance has been studied very often (Drusano, 2003; Olofsson and Cars, 2007). No general conclusion can be drawn on the impact of different dosage regimens on resistance selection and spread as this is complicated by different resistance mechanisms and differences in results between *in vitro* and *in vivo* studies (Smith et al., 2003; Roberts et al., 2008). Yet, for mutational stepwise resistance, seen e.g. for the fluoroquinolones, the use of antibiotic concentrations within a certain range has been shown to favor isolates with higher MIC's and to cause a considerable amplification of the resistant subpopulation (Tam et al., 2007). The finding that many of the administered doses differ from the recommended dose is consistent with a study in Switzerland on prescription patterns in veterinary medicine (Regula et al., 2009) and a study on antimicrobials described in pig feed in Germany (Ungemach et al., 2006). Reasons for non-compliance of prescription doses could be: misevaluation of the bodyweight at moment of administration (Timmerman et al., 2006), intentional overdosing to aim at less disease or lack of precision of dosing.

Another guideline suggests the selection of an appropriate antimicrobial, based on defined lists of antimicrobials of first, second or third choice (van Duijkeren et al., 2008b) or based on Good Veterinary Practices (FVE, 2002). Both refer to the choice of an antimicrobial with a spectrum as narrow as possible and the use of critically important antimicrobials only in single animals for a limited number of strict indications when other antimicrobials would fail based on susceptibility testing. Largely used broad-spectrum antimicrobials recorded in this study were aminopenicillins, 3rd and 4th generation cephalosporins, tulathromycin, trimethoprim-sulfadiazine and doxycycline. Also critically important antimicrobials were extensively used (β-lactam antimicrobials, 3rd and 4th generation cephalosporins and macrolides). The WHO classification of critically important antimicrobials serves as a factor in guiding decisions regarding risk management strategies for antimicrobial use in food animals and agriculture (Collignon, 2009). The high number of antimicrobials classified as either critically or highly important for human proves that from now on, the reduction of use should be emphasized and be set as the major objective, prior to a well-considered choice of antimicrobial agent when use is required.

The higher use of group treatments in suckling pigs and weaners compared to growers and finishers (Fig. 2) has also been reported in the other studies. The MARAN report (2009) published data assuming that 83% of antimicrobial treatments are administered to pigs younger than 74 days of age (age at beginning of the fattening unit). This higher use can be explained by the application of group treatment at critical time points and key intervals, such as castration and weaning. At these time points, it is often expected by the farmer that pigs will become diseased shortly after (Schwarz et al., 2001). Besides, most of the vaccines are administered to prevent pigs from getting ill during fattening period and less during the stressful farrowing and nursery periods.

Although the use of antimicrobial growth promoting antibiotics is banned in Europe since 2006, the use of antimicrobials in food producing animals is continued under the pretext of treatment, control or prevention of infectious diseases but may still be driven by the hope of better production results. In order to ensure the efficiency of antimicrobials, any prophylactic use other than in very limited, clearly defined situations, should be phased out. Some European countries like Denmark, Sweden and the Netherlands (Nielsen et al., 2007; Cogliani et al., 2011) are pioneer in the prudent use of antimicrobials as they prohibited the prophylactic use of antimicrobials.

These results clearly show that the need for clear information about correct dosing and a reduction of group-level prophylactic antimicrobial use, stated as a conclusion on the results obtained in 2003, has not been answered ever since. Herd veterinarians, pharmaceutical companies, farmers and other stakeholders have a responsibility in the prudent use of antimicrobials in pig and other animal production and should be trained on the implementation of the guidelines for prudent antimicrobial use.

CONCLUSIONS

The guidelines for prudent use of antimicrobials are not yet implemented in Belgium. An overall higher use of prophylactic antimicrobial group-level therapy was recorded in 2010 compared to 2003. This shift was marked by a partial yet substantial replacement of older, orally administered compounds by new injectable long acting products. This evolution warrants an assessment of antimicrobial resistance trends in commensal and pathogenic bacteria. Critically important antimicrobials to human and veterinarian medicine were used on a regular basis and 82% of the administered doses were incorrect, with large between herd variations.

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CHAPTER 4

CLINICAL RESISTANCE AND DECREASED SUSCEPTIBILITY IN *STREPTOCOCCUS SUIS*

CLINICAL RESISTANCE AND DECREASED SUSCEPTIBILITY IN *STREPTOCOCCUS SUIS* ISOLATES FROM CLINICALLY HEALTHY FATTENING PIGS

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ABSTRACT

Streptococcus suis (*S. suis*) has often been reported as an important swine pathogen and is considered as a new emerging zoonotic agent. Consequently, it is important to be informed on its susceptibility to antimicrobial agents. In the current study, the Minimum Inhibitory Concentration (MIC) population distribution of nine antimicrobial agents has been determined for nasal *S. suis* strains, isolated from healthy pigs at the end of the fattening period from 50 closed or semiclosed pig herds. The aim of the study was to report resistance based on both clinical breakpoints (clinical resistance percentage) and epidemiological cut-off values (non-wild type percentage). Non-wild type percentages were high for tetracycline (98%), lincomycin (92%), tilmicosin (72%), erythromycin (70%), tylosin (66%), and low for florfenicol (0%) and enrofloxacin (0.3%). Clinical resistance percentages were high for tetracycline (95%), erythromycin (66%), tylosin (66%), and low for florfenicol (0.3%) and enrofloxacin (0.3%). For tiamulin, for which no clinical breakpoint is available, 57% of the isolates did not belong to the wild type population. Clinical resistance and non-wild type percentages differed substantially for penicillin. Only 1% of the tested *S. suis* strains was considered as clinically resistant, whereas 47% of the strains showed acquired resistance when epidemiological cut-off values were used. In conclusion, MIC values for penicillin are gradually increasing, compared to previous reports, although pigs infected with strains showing higher MICs may still respond to treatment with penicillin. The high rate of acquired resistance against tiamulin has not been reported before. Results from this study clearly demonstrate that the use of different interpretive

criteria contributes to the extent of differences in reported antimicrobial resistance results. The early detection of small changes in the MIC population distribution of isolates, while clinical failure may not yet be observed, provides the opportunity to implement appropriate risk management steps.

INTRODUCTION

Streptococcus suis (*S. suis*) is an important swine pathogen affecting pigs of different ages, although susceptibility to the disease decreases with age after weaning (Amass et al., 1996; Staats et al., 1997). It is known to cause meningitis, arthritis, septicemia, endocarditis, polyserositis, bronchopneumonia, and abortion, (Higgins and Gottschalk, 1990; Amass et al., 1996; Staats et al., 1997) but can also be found in the upper respiratory, alimentary, and urogenital tract of healthy pigs (Amass et al., 1996; Han et al., 2001). *S. suis* has also been implicated in disease in humans, especially among people in close contact with swine and pork (Ma et al., 2008; Gottschalk et al., 2010). Moreover, *S. suis* has recently been reported as an emerging zoonotic pathogen evidenced by a few large-scale outbreaks of severe *S. suis* epidemics in Asia (Ye et al., 2006; Yu et al., 2006; Mai et al., 2008). The most frequently applied treatment for pigs with clinical signs of *S. suis* infection is feed medication with antimicrobials, particularly, broad-spectrum penicillins (Gottschalk et al., 1991; Timmerman et al., 2006; Callens et al., 2012). Currently, no effective commercial vaccine is available. Prevention is based on the optimization of management, autogenous vaccines, and primarily the strategic administration of antimicrobial agents at periods with the highest risk, for example, weaning (Haesebrouck et al., 2004; Wisselink et al., 2006). High levels of resistance to tetracyclines (Kataoka et al., 2000; Martel et al., 2001), macrolides, and lincosamides (Martel et al., 2001) have been reported.

Different methods are often applied for interpreting the results of antimicrobial susceptibility testing. In most studies, clinical breakpoints have been used resulting in the categorization of the tested isolates in susceptible, intermediate, or resistant against the tested antimicrobials (clinical resistance) (CLSI, 2011). The use of clinical interpretive criteria may be sufficient from the point of view of the clinician as it predicts the antimicrobial effect of the drug in the patient at the prescribed dose (Dudley and Ambrose, 2000; Simjee et al., 2008; Turnidge and Paterson, 2008). However, these breakpoints can vary over time and between countries (Kahlmeter et al., 2003), making comparisons between different studies and evolution of antimicrobial resistance patterns in *S. suis* over time hard. Moreover, this categorization precludes the detection of small changes in the population distribution that may indicate the acquisition of new resistance mechanisms of which the clinical implications are not yet clear, as has been noted for fluoroquinolones and Gram-negative bacteria (de Jong et al., 2012). For such changes to be noticed, epidemiological cut-off values are very valuable. These cut-off values are based on the differentiation between the wild type and the non-wild type population (Kahlmeter et al., 2003; EUCAST, 2015; CLSI, 2011). They enable to detect strains with a decreased susceptibility, which are isolates with Minimum Inhibitory Concentrations (MICs) that are non-wild type, but less than or equal to the susceptible clinical breakpoint (CLSI, 2011; Simjee et al, 2008). However, only few studies report resistance results as MIC population distributions, necessary for setting the epidemiological cut-off values. Finally, *S. suis* from diseased animals have been tested more often (Salmon et al., 1995; Marie et al., 2002; Wisselink et al., 2006) than *S. suis* from clinically healthy animals. This could

lead to biased results, since isolates from diseased animals may represent a different population (Allgaier et al., 2001) and since they have often been exposed to an antimicrobial selection pressure shortly before sampling (Silley et al., 2011).

This study aimed to report the level of resistance in *S. suis* isolates from clinically healthy fattening pigs at slaughter age. Resistance percentages were calculated based on both clinical breakpoints and epidemiological cut-off values.

MATERIALS AND METHODS

Study design, sample, and data collection

For the isolation of *S. suis*, nasal swabs were taken from clinically healthy fattening pigs from 50 different pig herds in Belgium. A list of 140 pig herds that fulfilled the selection criteria were randomly selected from the Belgian farm-animal identification and registration database (Sanitel-Pigs, 2005). The sampling frame consisted of all farrow-to-finish herds that used a closed or semi-closed production system and held at least 150 sows and 600 fattening pigs. The sample was stratified by province (n = 5), proportional to the number of pig herds per province. A random selection was performed using a computer-generated list (Cameron, 1999). All selected herds were contacted by telephone and the first 50 herds that were willing to cooperate in the study were visited between January and October 2010.

The pigs were sampled ~ 2 weeks before the slaughter age. The average age of the pigs was 182 days (minimum 156 days; maximum 220 days). In each herd, 20 fattening pigs were randomly sampled.

Bacterial isolation

Swabs were plated on Columbia agar plates with 5% defibrinated sheep blood, supplemented with colistin and nalidixic acid (CNA; Oxoid, Basingstoke, United Kingdom) within 24 hr after collection and cultured at 35°C in a 5% CO_2-enriched atmosphere for 24 hr. Colonies showing alpha-hemolysis were purified for further identification (Aarestrup et al., 1998; Lun et al., 2007). Isolates showing a positive amylase reaction, a negative catalase reaction, and a negative Vogues-Proskauer test were considered to be *S. suis* (Higgins and Gottschalk, 1990; Aarestrup et al., 1998; Han et al., 2001). The identity of 28 randomly chosen *S. suis* isolates was confirmed by sequencing the 16s rRNA gene as described before (Baele et al., 2003). *S. suis* isolates were stored at – 80°C until antimicrobial susceptibility testing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on all isolates using the agar dilution method according to the standardized methods described by the Clinical and Laboratory Standards Institute (CLSI, 2013). Inocula were prepared suspending colonies in sterile 0.9% NaCl to a turbidity equivalent of 0.5 Mac Farland and

diluted 1/10. Using a Steers inoculum applicator, the suspensions were inoculated on the Muller-Hinton II agar (BBL; Cockeysville, MD) supplemented with 5% sheep blood and containing doubling concentrations, ranging from 0.03 µg/ml to 128 µg/ml of the following antimicrobial agents: enrofloxacin, erythromycin, florfenicol, lincomycin, penicillin, tetracycline, tiamulin, tilmicosin, and tylosin. The plates were incubated at 35°C in 5% CO_2-enriched atmosphere for 24 hr. The MIC was defined as the lowest concentration producing no visible growth. *Staphylococcus aureus* ATCC_29213, *Enterococcus faecalis* ATCC_29212, and *Streptococcus pneumoniae* ATCC_49619 were included as quality control (QC) strains. Interpretation of the MIC values was done using both clinical breakpoints (CLSI, 2013) and epidemiological interpretative criteria (Turnidge and Paterson, 2007).

For lincomycin, tiamulin, tilmicosin, and tylosin, no clinical breakpoint for *S. suis* is available (CLSI, 2013). For florfenicol and tetracycline, the clinical breakpoint for swine respiratory disease caused by *S. suis* was used (CLSI, 2013). For erythromycin and penicillin, clinical breakpoints were used, as described by the Clinical and Laboratory Standards Institute (CLSI) for veterinary pathogens, but which were based on CLSI breakpoints for human Streptococci (CLSI, 2013). Since no epidemiological cut-off values are available for *S. suis* from the European Committee on Antimicrobial Susceptibility (EUCAST, 2015), acquired resistance was assumed when MIC values showed a bimodal or multimodal distribution or tailing (Butaye et al., 2003; Dung et al., 2008). Isolates in the higher range of MICs were considered not to belong to the wild type population. For antimicrobials for which no clear bimodal distribution was present, ECV were used available from a previous study carried out in the same laboratory using identical test conditions. This was done for the following antimicrobials: penicillin, tilmicosin, erythromycin, lincomycin, tiamulin, and tetracycline (Martel et al., 2001). The MIC50 and MIC90 were calculated and presented the lowest MIC at which at least 50% and 90% of the isolates in a test population are inhibited, respectively.

RESULTS

In the current study, *S. suis* was recovered in 33.2% of all nasal samples (332/1000). The number of isolates obtained per herd was normally distributed, with on average, 6.6 isolates recovered from one herd (minimum number of isolates per herd equaled 5 isolates; maximum equaled 8 isolates; median equaled 7 isolates). The MIC values of 10 antimicrobial agents were determined for 332 *S. suis* isolates. Yet, a number of *S. suis* isolates showed poor growth under the prescribed conditions, as has been observed before (Wisselink et al., 2006) and their MIC could not be determined. Therefore, in this report, MIC data have been reported for a variable number of *S. suis* isolates (Table 1). The MIC values for QC strains were within the acceptable QC ranges when available (CLSI, 2013). For lincomycin, QC strains *S. aureus* ATCC_29213 and *E. faecalis* ATCC_29212 had similar MIC values as described earlier (Salmon et al., 1995; Marie et al., 2002).

In table 1, the MIC distribution for all tested *S. suis* isolates is shown. A bimodal distribution was seen for enrofloxacin. A monomodal distribution was seen for florfenicol. For penicillin, a distribution with tailing toward higher MIC values was noted. No clear bimodal distribution was seen for erythromycin, lincomycin, tylosin, tilmicosin, tiamulin, and tetracycline.

In table 2, the clinical breakpoints (CLSI, 2013) and the ECV for the different antimicrobials tested are shown. Based upon clinical breakpoints, percentage of susceptible, intermediate, and resistant *S. suis* strains are shown. Equally, based upon ECV, % of wild type and non-wild type strains are presented.

No or very low percentages of clinical resistance were found against enrofloxacin (0.3%), florfenicol (0.3%), and penicillin (1%). High to very high-resistance percentages were observed against erythromycin (66%) and tetracycline (95%).

Using the ECV, low percentages of non-wild type isolates were seen to enrofloxacin and florfenicol (0.3% and 0%, respectively). Acquired resistance was observed for penicillin (percentage of non-wild type isolates equals 47%), tiamulin (57%), erythromycin (70%), tylosin (66–67%), tetracycline (98%), tilmicosin (72%), and lincomycin (92%).

Table 1. Minimum Inhibitory Concentration (MIC) distribution for *Streptococcus suis* isolates obtained from clinically healthy fattening pigs on 50 closed or semi-closed pig herds.

Antimicrobial agent	**Number of strains with MIC (µg/ml)**														**Number of isolates tested**
	≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	>128	
Erythromycin	24	40	33	8	6	8	7	16	22	9	7	2	8	136	326
Lincomycin	2	0	3	6	3	9	24	11	10	3	7	7	7	214	306
Tylosin	3	6	1	1	27	63	8	2	3	1	1	4	13	197	330
Tilmicosin	6	0	0	1	2	13	15	20	9	15	9	14	10	174	288
Tiamulin	3	1	5	9	12	25	57	30	10	16	33	34	43	54	332
Tetracycline	0	0	4	2	4	5	9	6	19	40	115	112	14	0	330
Penicillin	22	32	48	71	86	54	13	1	2	0	0	0	0	0	329
Florfenicol	0	0	2	0	11	97	218	2	1	0	0	0	0	0	331
Enrofloxacin	1	9	22	129	122	17	0	0	1	0	0	0	0	0	301

Table 2. Minimum Inhibitory Concentrations (MIC) clinical breakpoints (CBP) and epidemiological cut-off values (ECV) of 10 antimicrobial agents for *Streptococcus suis* isolates obtained from clinically healthy fattening pigs on 50 closed or semi-closed pig herds. The percentage of resistant (%R), intermediate (I%) and susceptible (%S) strains is provided based on clinical breakpoints.

The percentage of wild type (WT) and non-wild type (non-WT) is provided based on epidemiological cut-off values.

Antimicrobial agent	**Epidemiological Interpretive Criteria**			**Clinical Interpretive Criteria**							
	ECV	Wild type[a]	Non-wild type[b]	CBP[c]			%R	%I	%S	MIC_{50}[d]	MIC_{90}[e]
		%	**%**	**S**	**I**	**R**					
Erythromycin[g]	0.12	30	70	0.25	0.5	1	66	2	32	16	>128
Lincomycin[f, g]	1	8	92	-	-	-	-	-	-	>128	>128
Tylosin[f]	2-4	33-34	66-67	-	-	-	-	-	-	>128	>128
Tilmicosin[f, g]	16	23	72	-		-	-	-	-	>128	>128
Tiamulin[f, g]	4	43	57	-	-	-	-	-	-	32	>128
Tetracycline[g]	0.25	2	98	0.5	1	2	95	2	3	64	128
Penicillin[g]	0.25	53	47	0.12	0.25-2	4	1	68	31	0.5	2
Florfenicol	8	100	0	2	4	8	0.3	0.6	99.1	4	4
Enrofloxacin	1	99.7	0.3	0.5	1	2	0.3	5.6	94	0.5	1

[a] Wild type describes isolates with minimal inhibitory concentrations below the epidemiological cut-off value (WT ≤ X µg/ml)

[b] Non-wild type describes isolates with minimal inhibitory concentrations above the epidemiological cut-off value (non-WT > X µg/ml)

[c] Clinical breakpoints were obtained from Clinical and Laboratory Standards Institute standards (CLSI, 2011). S: Susceptible, I: Intermediate, R: Resistant (R ≥ Y µg/ml; Z µg/ml < I < Y µg/ml; S ≤ Z µg/ml)

[d, e] MIC_{50} and MIC_{90} are the lowest MIC at which at least 50% and 90% of the isolates in a test population are inhibited in their growth

[f] For lincomycin, tiamulin, tilmicosin and tylosin, no clinical breakpoint is available (CLSI, 2012)

[g] Epidemiological cut-off value was based on study from Martel et. al (2011)

DISCUSSION

The choice of the epidemiological cut-off value, based on the distinction between the wild type and the non-wild type population within a bacterial population, should be fixed for one antimicrobial agent within a bacterial species, independent of time. Moreover, given that wild type MIC distributions of bacteria of human and animal origin coincide, the same epidemiological cut-off value can be used for monitoring resistance in humans and in different animals (Aarestrup et al., 2007). Yet, discrepancies between antimicrobial susceptibility test protocols may result in the establishment of a different epidemiological cut-off value between studies within one bacterial species for one antimicrobial agent (Butaye et al., 1998, Silley et al., 2001). Nevertheless, the preferred method for reporting MIC results is to present all data in a distribution table, containing the quantitative data (Watts and Lindeman, 2006) to allow the reader to interpret the data with changing interpretive criteria over time (clinically or epidemiologically).

The high percentages of non-wild type *S. suis* isolates for erythromycin, lincomycin, tilmicosin, tylosin, and tetracycline are in accordance with other studies reporting percentages of non-wild type *S. suis* isolates for macrolides, lincosamides, and tetracyclines (Martel et al., 2001; Wisselink et al., 2006). Despite differences in interpretive criteria (clinical breakpoints or epidemiological cut-off values), susceptibility testing methods (disk diffusion, microdilution, and agar dilution), sampled animals (clinically healthy or diseased pigs, sows or fattening pigs), and geographical location, there seems to be a similarity concerning results on clinical resistance percentages, when available, and percentages of non-wild type *S. suis* isolates for those antimicrobials, which in some studies have been supported by the identification of genotypic resistance mechanisms (Martel et al., 2001; Princivalli et al., 2009). In the farms included in the current study, macrolides were frequently used during the farrowing and nursery period (Callens et al., 2012). Genes encoding cross resistance to macrolides, lincosamides, and streptogramin B are widespread among *S. suis* isolates (Martel et al., 2001). As a result, the administration of macrolides may select for resistance against these antimicrobials. Similarities between the current study results and others have equally been found for the low-resistance percentages against florfenicol (Wisselink et al., 2006) and enrofloxacin (Martel et al., 2001; Wisselink et al., 2006).

For tiamulin, a high percentage of *S. suis* isolates did not belong to the wild type population, defined as having a MIC of ≤ 4 mg/ml, demonstrating acquired resistance in these isolates against this antibiotic. For tiamulin, no clinical breakpoints are available for *S. suis* and epidemiological cut-off values do not necessarily predict how a patient will respond to therapy. However, for 49% of the isolates, the MIC of tiamulin varied between 32 and > 128 mg/ml, being at least 8 to more than 32 times higher than for isolates belonging to the wild type population. Although it has not yet been tested, the likelihood that pigs infected with isolates demonstrating the higher MIC values of tiamulin will respond well to treatment with this antibiotic should be

considered to be low (Silley et al., 2011). For evaluation of tiamulin resistance in *S. suis* isolates, Zhang et al. (2008) used the clinical breakpoint reported by CLSI (2013) for *Actinobacillus* spp. causing respiratory tract disease in pigs (32 mg/ml) and reported that 34.4% of their isolates were resistant. Although this clinical breakpoint cannot be extrapolated as such to other bacterial species or disease conditions (Schwarz et al., 2010), the percentage of isolates with a MIC of ≥ 32 mg/ml was clearly higher in this study. Also based on MIC determinations from *S. suis* isolates recovered between 1999 and 2000 from clinically diseased pigs, carried out in the same laboratory using identical test conditions (Martel et al., 2001), a clear shift towards higher MIC values was observed in this study. The sampled pigs from this study did not receive tiamulin for prophylactic or metaphylactic reasons (Callens et al., 2012). Yet, the use of tiamulin as a therapeutic antimicrobial agent against *Brachyspira* spp. and *Mycoplasma hyopneumoniae* infections is common and cannot be ruled out for this study.

Broad-spectrum penicillins were the most frequently used antimicrobial class in pigs from this study, as described in a former study conducted in the same pig herds (Callens et al., 2012). Based on the clinical breakpoint for penicillin (CLSI, 2013) in this study, only three isolates could be categorized as resistant. Yet, when considering isolates with MICs beyond the wild type cut-off value, a high number of isolates showed a decreased susceptibility. Penicillin resistance in streptococci is the result of the acquisition of stepwise mutations in genes encoding penicillin binding proteins (Aarestrup et al., 2007). A single-point mutation results in isolates with a modest increase in MIC, and infections due to these isolates may still be treatable with penicillins, but they are of great concern as they represent an introductory step to full resistance (Amyes, 2007). Isolates showing higher values of MICs are associated with additional mutations and most likely lead to therapy failure (Chambers, 1999). Additionally, these mutations are selected by the use of β-lactam antimicrobials (Chambers, 1999). As a result, reporting a decreased susceptibility based on epidemiological cut-off values is important as it can act as an early warning for an emerging clinical problem (Aarestrup et al., 2007; Silley et al., 2011).

CONCLUSIONS

The current study on *S. suis* isolates from healthy carrier pigs confirms the high level of acquired resistance to macrolides, lincosamides, and tetracycline. MIC values for penicillin are gradually increasing, compared to previous reports (Martel et al., 2001), as has been seen for *S. pneumoniae* in humans, although pigs infected with strains showing higher MICs may still respond to treatment with this antibiotic. The high rate of acquired resistance against tiamulin has not been reported before.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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CHAPTER 5

CLINICAL RESISTANCE AND DECREASED SUSCEPTIBILITY IN *ESCHERICHIA COLI*

ANTIMICROBIAL RESISTANCE SURVEILLANCE IN *ESCHERICHIA COLI* BY USING NORMALIZED RESISTANCE INTERPRETATION

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ABSTRACT

Objectives: To improve antimicrobial surveillance accuracy for results obtained by the disk diffusion technique for porcine *Escherichia coli,* by comparing traditional clinical breakpoint interpretation with the Normalized Resistance Interpretation (NRI) method.

Methods: The susceptibilities of 921 *E. coli* isolates from clinically healthy pigs at slaughter age was determined for 15 antimicrobials by the Kirby Bauer disk diffusion technique. NRI with previously established optimal controlled parameters for *E. coli* ATCC25922 was used to reconstruct the fully susceptible population of the tested *E. coli* isolates. Based on a lower limit for susceptibility, set at 2.5 standard deviations below the mean of the reconstructed susceptible population, the percentage of wild type (WT) and non-wild type isolates was compared with the percentage of clinical resistance as determined by traditional breakpoints to categorize strains as susceptible, intermediate or resistant.

Results: The NRI method was applicable for 11 out of the 15 antimicrobials tested. Antimicrobials for which no normal distribution of inhibition zones for the population of susceptible isolates was seen, could not be used to reconstruct the susceptible population and had to be discarded from the calculations. Clinical breakpoints much lower than the epidemiological cut-off values (ECV) resulted into presumptively identifying isolates as clinically susceptible, but likely carrying acquired resistance determinants. On the other hand, clinical breakpoints did cut through the WT population for several antibiotics tested, categorizing isolates from the WT population as not susceptible.

Conclusion: NRI was shown to be a valid method to define the WT population for disk diffusion outcomes provided a normal distribution of the susceptible bacterial species population is present. Until international harmonization of breakpoints is achieved, it might give rise to a wide application in monitoring antimicrobial resistance in veterinary medicine.

INTRODUCTION

Escherichia coli (*E. coli*) is internationally used as Gram-negative indicator organism for resistance surveillance (Wray and Gnanou, 2000) and included in large scale monitoring studies at the national and supranational level (Hendriksen et al., 2008; MARAN, 2014; EFSA and ECDC, 2015). In addition, local microbiological laboratories often have access to a historical large databank of *E. coli* antimicrobial susceptibility test results. From the mid-2000s onwards, resistance in *E. coli* is increasingly reported using minimum inhibitory concentrations (MIC) distributions, obtained from dilution methods, to which clinical breakpoints and/or epidemiological cut-off values (ECV) are applied (Finnish Food Safety Authority, 2011; DANMAP, 2013; Chantziaras et al., 2014; CODA-CERVA, 2014; MARAN, 2014). However, the previously more commonly used disk diffusion method is still generally applied as a method of antimicrobial susceptibility testing in routine microbiology testing (Matuschek et al., 2014). It is a fairly reproducible and accurate method, technically easy to perform with a relatively low cost which allows large number of isolates to be tested (Matuschek et al., 2014). Yet, differences in the standardization of the methodology over time and between laboratories, as well as differences between recommended clinical breakpoints result in a lack of comparability between the test results of disk diffusion assays. Potentially valuable data collected in laboratories worldwide is hence precluded from resistance surveillance studies (Silley et al., 2011). The variations in disk content potency and in clinical breakpoints over time, between countries, animals and organs can be circumvented by analysing the population distribution by the ECV if available. These are based on the differentiation between the wild type (WT) and the non-wild type (non-WT) population, are fixed for a certain bacterium- antimicrobial agent combination, and make the detection of even small susceptibility changes in the bacterial species population possible (Kahlmeter et al., 2003; EUCAST, 2015; CLSI, 2011). ECVs are established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for a primary public health relevant selection of bacterium-antibiotic combinations (EUCAST, 2015). In the absence of an ECV, one might define the WT and non-WT populations based on the examination of the observed outcome distributions in the available study population. The emergence of resistance may evoke an unclear transition between the WT and non-WT populations, which makes the determination of the ECV sometimes difficult for results obtained by disk diffusion techniques as well as by the 'golden standard' dilution techniques (Turnidge and Paterson, 2007). Whereas disk diffusion techniques rely on a concentration gradient of an antimicrobial agent throughout an agar medium, dilution techniques rely on defined two-fold dilutions of standard antimicrobial concentrations. The latter is therefore more discriminatory and reproducible.

For disk diffusion results the normalized resistance interpretation (NRI) method has been described in human medicine as an objective method to define the WT population in a bacterial collection, and thereby identifies a subpopulation of isolates with decreased susceptibility (= subset of strains with decreased inhibition zone diameters) (Joneberg et al., 2003; Kronvall, 2010). The method uses the high zone side of the susceptible

peak in a zone diameter histogram as an internal calibrator to construct the real standard distribution of susceptible isolates (Kronvall et al., 2003a). Therefore, it has made comparisons possible of zone diameter population distributions despite discrepancies in methodology over time and between individual laboratories (Kronvall, 2010).

For the first time, the NRI method was used on a dataset of animal related bacteria, i.e. *E. coli* isolated from clinically healthy pigs at slaughter age to define the WT and non-WT population and to compare the obtained results with the percentage of *E. coli* categorized as susceptible, intermediate or resistant according to described clinical breakpoints.

MATERIALS AND METHODS

Study design, sample, and data collection

For the isolation of *E. coli*, fresh fecal samples were collected after rectal stimulation from clinically healthy fattening pigs from 50 Belgian herds. A list of 140 swine herds that fulfilled the selection criteria, mentioned below, were randomly selected from the Belgian farm-animal identification and registration database (Sanitel-Pigs, 2005). The sampling frame (selection criteria) consisted of all farrow-to-finish herds that used a closed or semi-closed production system and held at least 150 sows and 600 fattening pigs. The sample was stratified by province, proportional to the number of pig herds per province, only including the 5 provinces (out of the 10) with a substantial pig breeding activity (covering 95% of pig breeding in Belgium). A random selection was performed using a computer-generated list (Cameron, 1999). All selected herds were contacted by telephone and the first 50 herds that were willing to cooperate in the study were visited between January and October 2010.

The pigs were sampled approximately 2 weeks before the slaughter age. The average age of the pigs was 182 days (minimum 156 days; maximum 220 days). In each herd, 20 fattening pigs were randomly sampled.

Isolation and identification

For the isolation of *E. coli*, faecal samples were inoculated on MacConkey agar plates (MacConkey Agar No. 3; Oxoid Ltd.). Plates were incubated aerobically for 24h at 35°C ± 2°C. From each culture, one suspected *E. coli* colony was identified by means of positive glucose and lactose fermentation, gas production and absence of H_2S production using Kligler Iron Agar (Oxoid Ltd.), indole production (Indole spot on; Becton Dickinson), and the absence of aesculin hydrolysis (Bile Aesculin Azide Agar; Oxoid Ltd.) (Callens et al., 2015).

Antimicrobial susceptibility testing

For antimicrobial resistance profiling of *E. coli*, the Kirby-Bauer disk diffusion method with Mueller-Hinton II agar was used for susceptibility testing of fifteen different antimicrobial agents. The Clinical Laboratory

Standards Institute (CLSI) standards were followed for inoculum standardization, incubation conditions, and internal quality control organisms (CLSI, 2013). Antimicrobial tablets used, including potency in µg, are presented in tables 1 and 2. After 18h of aerobic incubation at 35°C ± 2°C, inhibition zones were read and interpreted according to the manufacturer's guidelines, based on CLSI standards (Rosco, 2007). Using these clinical breakpoints, shown in Table 2, the *E. coli* isolates were categorized as susceptible, intermediate or resistant (Rosco, 2007).

Normalized Resistance Interpretation (NRI) method

Based upon the obtained inhibition zone diameters, a population distribution was plotted for each antimicrobial. Visual examination of the distribution did not allow for clear distinction between isolates with acquired resistance (non-WT) and the WT population. Therefore, the NRI method was used to construct the standard distribution of susceptible isolates in the possible presence of isolates carrying resistance determinants as previously described in detail by Kronvall et al. (2003a).

The method uses the upper high-zone region of the susceptible peak in an inhibition zone histogram to reconstruct the distribution of the fully susceptible population (Kronvall et al., 2003a). The upper high-zone region groups the observations with the largest (=most susceptible) inhibition zone values. Since it is assumed that this part of the distribution is not influenced by the presence of any acquired resistance, it is used to reconstruct the full susceptible population. For this, first the high-zone side of a zone histogram distribution is analyzed by calculating the moving averages of the number of observations per zone value based on a certain window of observations going from the highest zone diameter values downwards (Fig. 1). By applying the moving average, the ruggedness of the histogram is evened out and therefore the determination of the peak position is facilitated (Kronvall, 2003a). The optimal window for determination of the moving average has previously been investigated for (external quality) control strain *E. coli* ATCC25922, i.e. a four-zone value average (e.g. 32 mm; 31mm; 30 mm; 29 mm), and was consequently applied to *E. coli* in this study (Joneberg et al., 2003). When the moving average is starting to decrease the peak position of the susceptible population is reached or even passed. Subsequently, the peak position is determined by adjusting the observed peak (peak adjustment). For control *E. coli* ATCC25922 the optimal peak adjustment has been set at 2.5 for a four-zone value average (Joneberg et al., 2003) and was therefore used in this study (Fig. 1). The next step in the NRI method is to calculate the accumulated percentages of observations of the different zone values, from the highest zone values down to the peak position. The total number of isolates included in the susceptible population is defined to be twice the number of isolates for the upper half of the susceptible peak (Kronvall, 2003a) (Fig. 1). Given the total number, the accumulated percentage of isolates can easily be calculated. Assuming a normal distribution for the susceptible population in a histogram according to earlier studies (Kronvall et al., 1991) the probit values of the accumulated percentages will form a straight line

against the zone diameter values (Fig. 2). The accumulated percentages were, therefore, converted to probit values by using an Excel function and plotted. Subsequently the best fitting line, determined by means of the least-squared differences, is plotted. The slope and intercept of this line are equal to and therefore provide the mean and standard deviation (SD) of the ideal normal distribution of the susceptible population. The mean and SD have been shown earlier to describe histogram populations accurately (Kronvall et al., 1991). The susceptible population is thereby defined and a lower limit for susceptibility can then be set to any value of the SD below the mean, e.g. 2.5 SD below the mean. All isolates with zone values lower than the calculated lower limit can be considered different from the normally distributed susceptible isolates, i.e. non-WT. A 2.5 SD limit will theoretically include 97.725% of the susceptible isolates. Finally, SD of the normalized NRI-calculated distributions were used as estimates for the appropriateness of the NRI method for the observed disk diffusion results. Limits for SD to accept results of the NRI calculations have not yet been established (Smith and Kronvall, 2014). In this study, highly deviating SD within all SD calculated were taken as indicative for the NRI method not being applicable to the disk diffusion data.

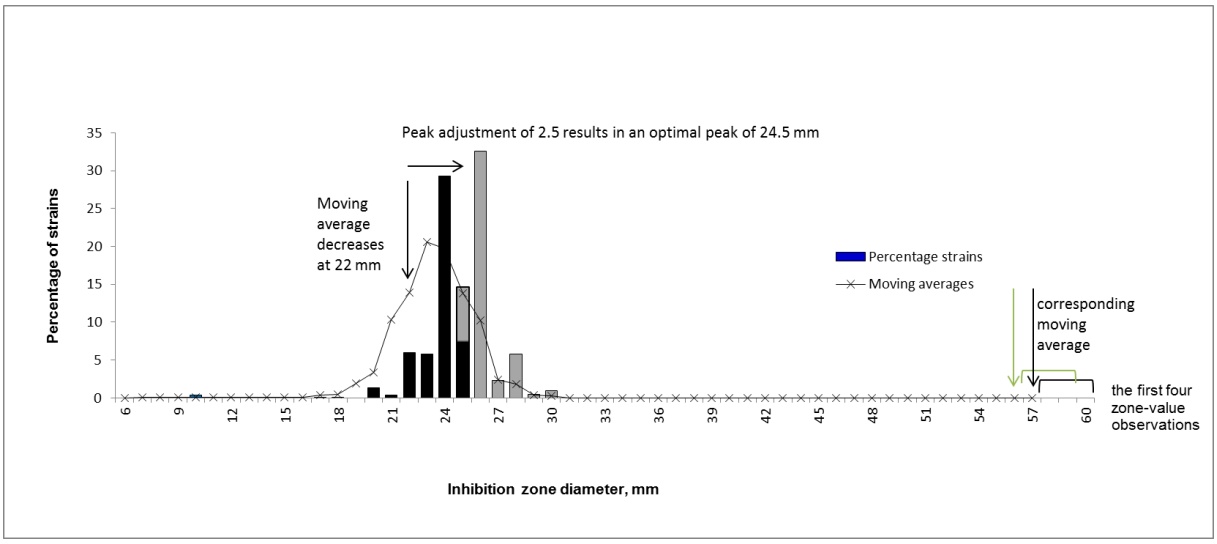


Figure 1. Histogram of zone diameter values of apramycin (40 µg) disk diffusion results for 921 *Escherichia coli* isolates from healthy pigs. The moving average of the four-zone value average is shown as a line plot. The switch to the lower position is noticed at position 22 mm (down arrow). The strains of half the ideal peak are marked as grey bars. Twice these isolates will give the total number of the ideal susceptible peak, the denominator when calculating accumulated percent values.

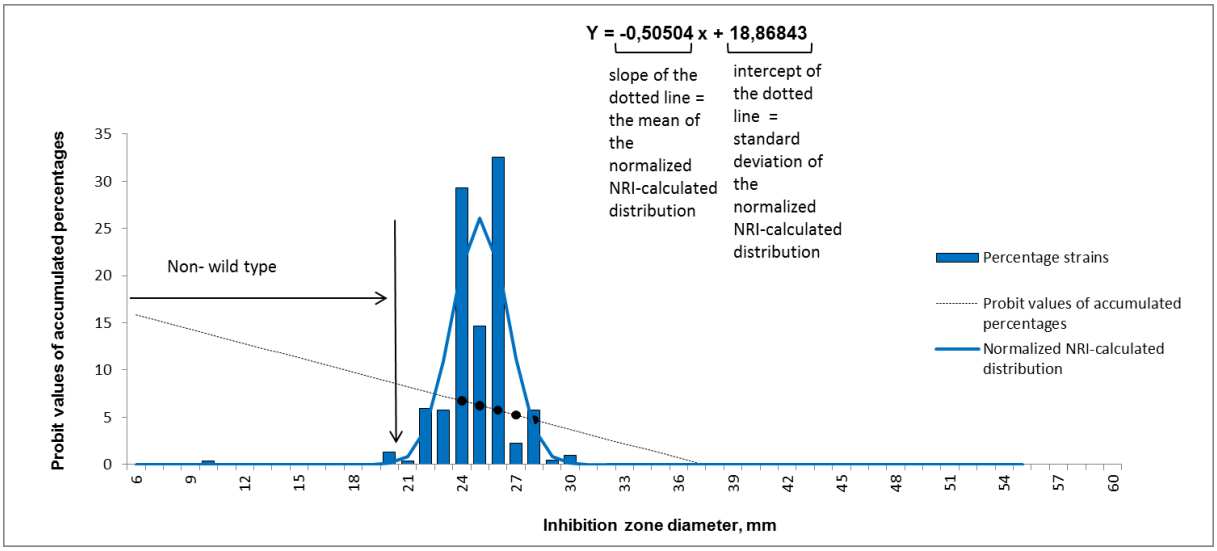


Figure 2. Histogram of zone diameter values of apramycin (40 µg) disk diffusion results for 921 *Escherichia coli* isolates from healthy pigs. The straight dotted line represents the correlation between probit values of the accumulated percentages and zone diameter values (mm). The normalized resistance interpretation (NRI)-calculated distribution is shown as a line graph and the 2.5 SD (standard deviation) lower limit is indicated by the vertical arrow (21 mm).

RESULTS

NRI

E. coli isolates were obtained from 921 of the 1000 faecal samples that were collected, showing a high rate of isolation success (92.1%) for *E. coli* in this study.

Figure 3 (a-o) represents the inhibition zone diameter distribution for 15 antimicrobials tested on these *E. coli* isolates. The normalized NRI-calculated distribution was based on an optimal peak adjustment set at 2.5, for a moving average based on the four-zone value, and is shown as a line curve.

For chloramphenicol, enrofloxacin and trimethoprim, the calculations resulted in erroneously wide normalized distributions. SD of the normalized distributions were deviant for these antibiotics (14.1, 44.9 and 11.9 for chloramphenicol, enrofloxacin and trimethoprim, respectively) compared with the SD of the other antibiotics used in the calculations (range between 1.0 and 3.2) (Table 1). An attempt to reconstruct better fitting normalized distributions was done by shifting the 2.5 peak adjustment to 2. This resulted in better fitting distributions for chloramphenicol and trimethoprim, but provided no optimized distribution for enrofloxacin. Moreover, a 2.5 to 2 shift decreased the appropriateness of NRI distribution of nalidixic acid (Fig. 3i). Chloramphenicol, enrofloxacin, nalidixic acid and trimethoprim were consistent with each other in their absence of a normal distribution of the population of susceptible isolates with a steep slope on the high zone value side (Fig. 3e, f, j and o) and highly varying SD when switching between the 2 and the 2.5 peak adjustment, whereas this was not the case for the other antibiotics tested (Table 1).

The use of the same optimal parameters through all the distributions has been emphasized, as they take into account the laboratory-specific methodology (Kronvall et al., 2003b). A peak adjustment of 2.5 was therefore chosen and chloramphenicol, enrofloxacin, nalidixic acid and trimethoprim were discarded from the NRI calculations.

Table 1. Standard deviations (SD) of the normalized NRI-calculated distribution applied to a bacterial collection of 921 porcine commensal *E. coli* for a peak adjustment of 2 and 2.5 zone diameters.

Antibiotic	AMC	AM	APR	CE	CHLO	ENR	FLO	GENT	KAN	NA	NE	STRE	SU	TETR	TRI
Disk	30+1	P	A	F	R	O	R	A	A	L	O	P	L	A	M
content µg	5	30	40	30	60	10	30	40	100	130	120	100	240	80	5.2
Peak adjustment = 2	2,6	2.4	1.7	1.9	1.8	17.8	2.7	1.9	2.1	0.8	1.4	2.8	3.4	2.7	2.4
Peak adjustment = 2.5	2.8	2.4	1.5	1.9	14.1	44.9	2.6	1.8	2.4	3.2	1.0	2.9	3.6	2.7	11.9

AMC: amoxicillin/clavulanic acid; AMP: ampicillin; APRA: apramycin; CEF: ceftiofur; CHLOR: chloramphenicol; ENRO: enrofloxacin;

FLOR: florfenicol; GENTA: gentamicin; KANA: kanamycine; NAL: nalidixic acid; NEO: neomycin; STREP: streptomycin;

SUL: sulfadiazine; TETRA: (oxy)tetracycline; TRIM: trimethoprim

E. coli *susceptibility based on clinical interpretive criteria and normalized interpretation*

Highest percentages of clinical resistance (R+I) were seen for streptomycin (58.5), tetracycline (57.4%), sulfadiazine (56.9%), and trimethoprim (49.5%). Moderate resistance was present against ampicillin (38.5%) and chloramphenicol (23.5%). Low levels of clinical resistance were seen for florfenicol (9.6%), nalidixic acid (4.9%), ceftiofur (3.1%), amoxicillin/clavulanic acid (3%), gentamicin (3%), neomycin (2.2%), apramycin (2%), enrofloxacin (1.7%), and kanamycin (1,2%).

For amoxicillin/clavulanic acid, ampicillin, ceftiofur and neomycin, a gap was present between the clinical breakpoint for susceptibility and the epidemiological cut-off value of the reconstructed distribution of the susceptible population (Fig 3a, b, d, k). For neomycin, this resulted into 97.8% susceptible isolates according to clinical interpretive criteria and 90.7% WT using normalized interpretation (Table 2). However, for amoxicillin/clavulanic acid, ampicillin and ceftiofur, due to the low number of isolates within the gap, the difference between the percentage of susceptible isolates according to clinical interpretive criteria and WT using normalized interpretation was very small (between 0.3% and 0.6%; Table 2).

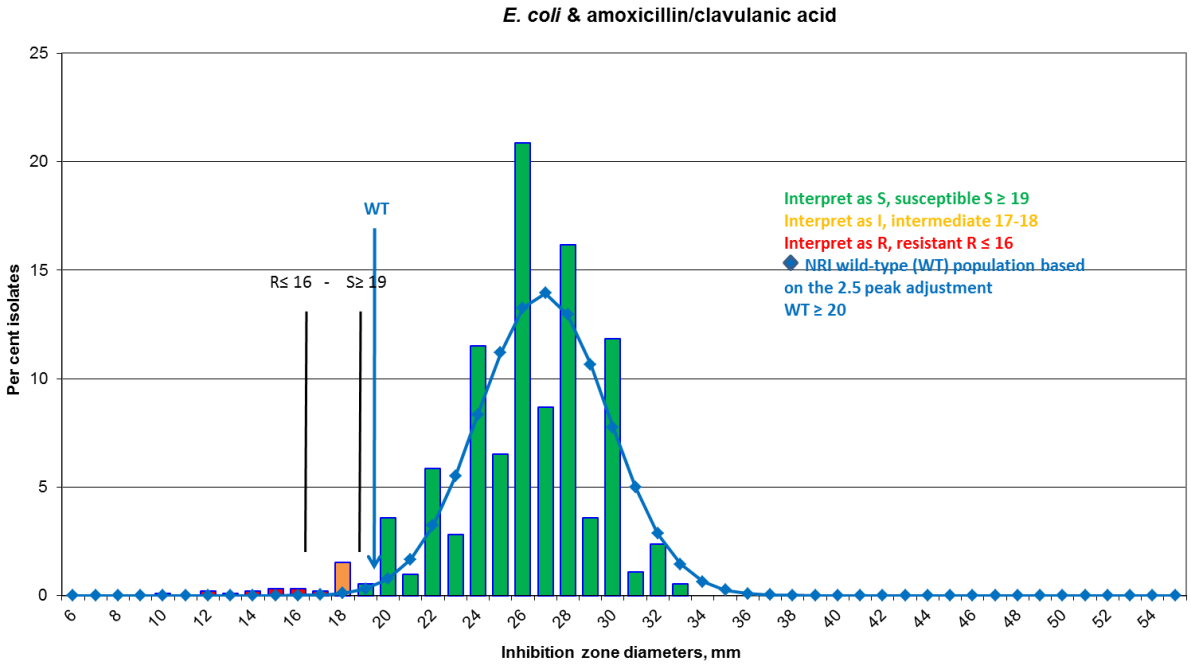
For florfenicol, clinical breakpoints divided the NRI calculated WT population in a way that 7.1% and 2.5% of WT isolates were classified as intermediate and resistant, respectively (Fig 3g). The percentage of susceptible isolates was then 90.4%, whereas according to NRI and a 2.5SD limit below the mean, susceptibility of strains equaled 98.4% (Table 1). A similar result was seen for sulfadiazine, with 1.2% of the isolates interpreted as intermediate and 0.8% as resistant, whereas the NRI distribution includes these isolates as belonging to the WT population. Also for gentamicin, 2% of the isolates were divided as ‘intermediate’, where the normalized distribution indicates them as member of the WT population (Fig 3h, i). For streptomycin, overlapping populations were seen (Fig. 3l). Mid zone values were shared by the low zone side and the high zone side of both parts of the distribution.

Table 2. Percentages of wild type (WT) and non-wild type (non-WT) for 921 *Escherichia coli* isolates, from healthy pigs at slaughter age, based on a lower limit of 2.5 standard deviations (SD) below the mean. The percentage of susceptible (S), intermediate (I) and resistant (R) strains are provided based on clinical breakpoints (Rosco, 2007). The Clinical Laboratory Standards Institute (CLSI) standards were followed for inoculum standardization, incubation conditions, and internal quality control organisms (CLSI, 2013).

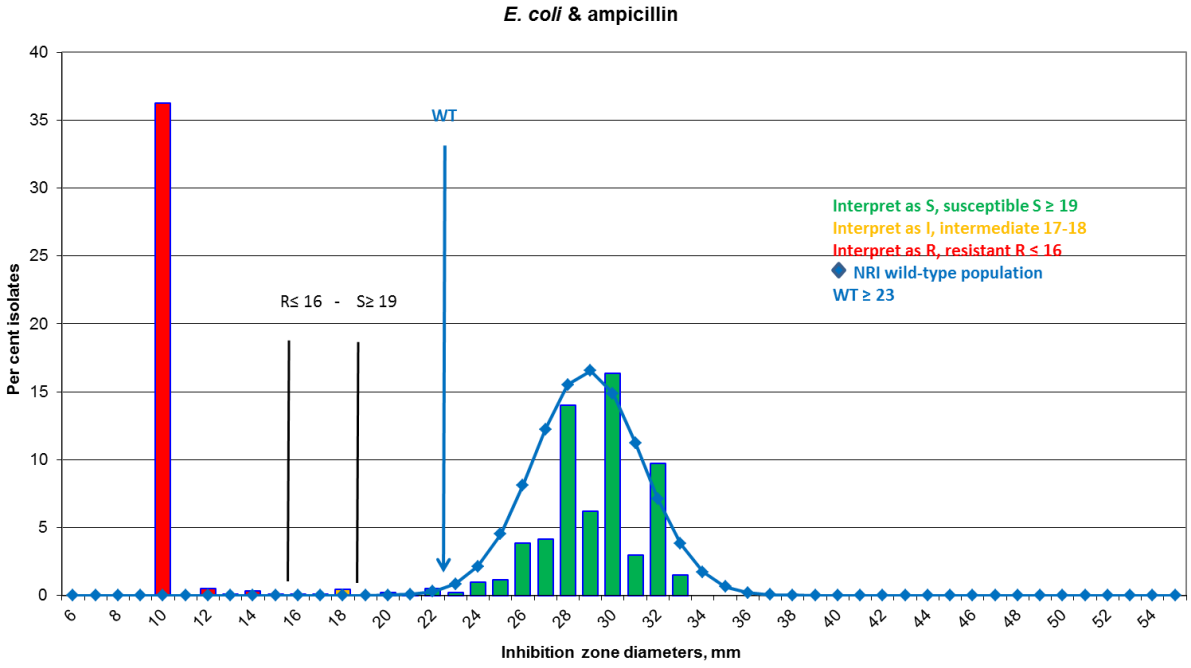
Antibiotic	AMC	AMP	APRA	CEF	CHLOR	ENRO	FLOR	GENTA	KANA	NAL	NEO	STREP	SUL	TETRA	TRIM
Disk content µg	30+15	30	40	30	60	10	30	40	100	130	120	100	240	80	5.2
Clinical breakpoints	R ≤ 16 S ≥ 19	R ≤ 16 S ≥ 19	R ≤ 19 S ≥ 22	R ≤ 19 S ≥ 22	R ≤ 20 S ≥ 24	R ≤ 16 S ≥ 22	R ≤ 18 S ≥ 21	R ≤ 20 S ≥ 24	R ≤ 19 S ≥ 22	R ≤20 S ≥ 24	R ≤ 19 S ≥ 22	R ≤ 22 S ≥ 25	R ≤ 19 S ≥ 25	R ≤ 19 S ≥ 22	R ≤ 16 S ≥ 19
Non-WT	3.6	38.8	2,.0	3.6	-	-	1.6	1.1	1.2	-	9.3	28.4	54.9	57.4	-
WT	96.4	61.2	98.0	96.4	-	-	98.4	98.9	98.8	-	90.7	71.6	45.1	42.6	-
S	97.0	61.5	98.0	96.9	76.4	98.3	90.4	97.0	98.8	95.1	97.8	41.5	43.1	42.6	50.5
I	1.7	0.5	1.3	0.4	1.8	0.3	7.1	2.0	0.2	0.8	1.4	8.5	1.2	0.3	0.1
R	1.3	38.0	0.7	2.7	21.7	1.4	2.5	1.0	1.0	4.1	0.8	50.0	55.7	57.1	49.4
R+I	3.0	38.5	2.0	3.1	23.5	1.7	9.6	3.0	1.2	4.9	2.2	58.5	56.9	57.4	49.5

AMC: amoxicillin/clavulanic acid; AMP: ampicillin; APRA: apramycin; CEF: ceftiofur; CHLOR: chloramphenicol; ENRO: enrofloxacin; FLOR: florfenicol; GENTA: gentamicin; KANA: kanamycine; NAL: nalidixic acid; NEO: neomycin; STREP: streptomycin; SUL: sulfadiazine; TETRA: (oxy)tetracycline; TRIM: trimethoprim

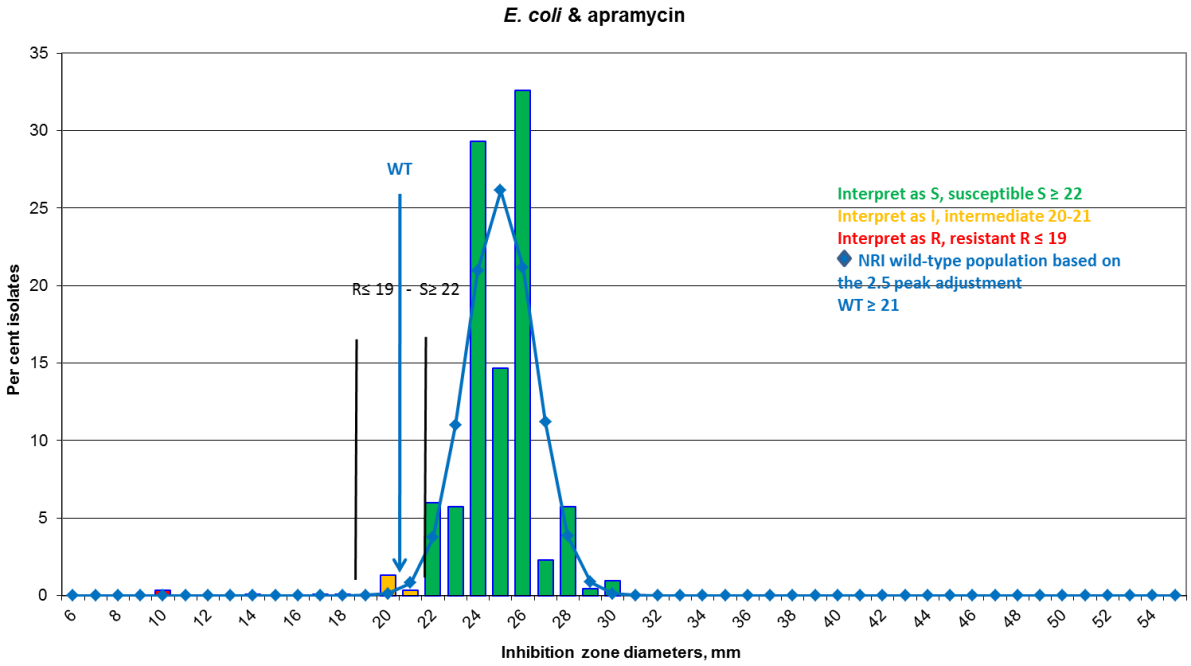
-: results were discarded


E. coli & amoxicillin/clavulanic acid
Per cent isolates
Inhibition zone diameters, mm
WT
R≤ 16 - S≥ 19
Interpret as S, susceptible S ≥ 19
Interpret as I, intermediate 17-18
Interpret as R, resistant R ≤ 16
NRI wild-type (WT) population based on the 2.5 peak adjustment
WT ≥ 20

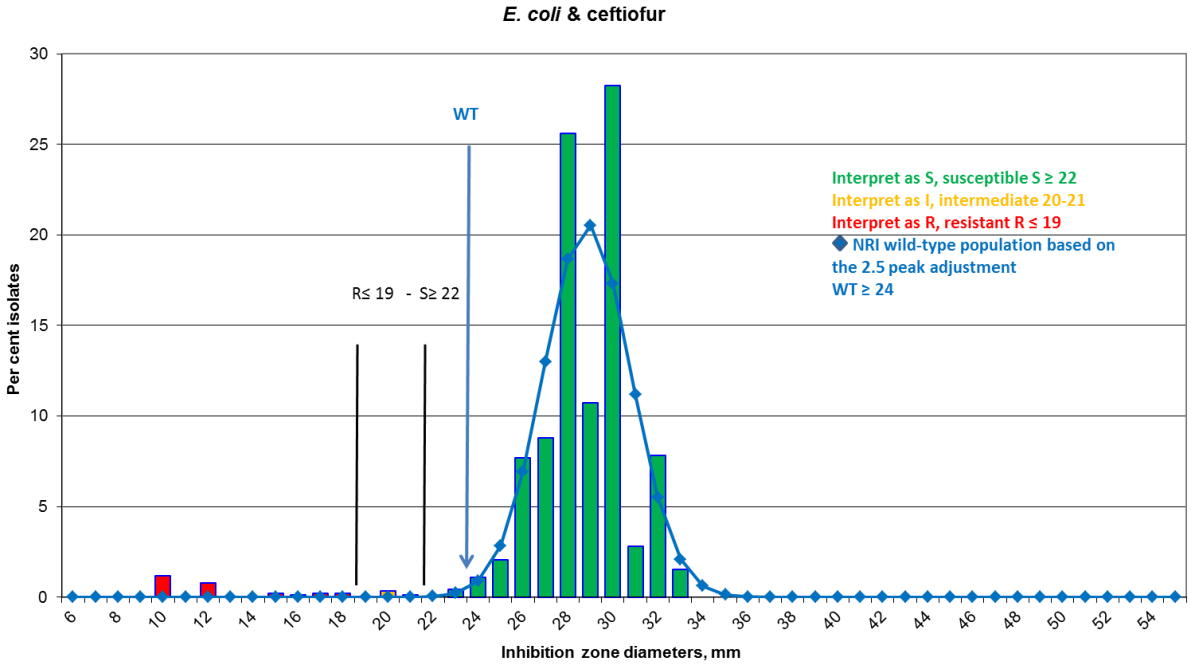
(a)


E. coli & ampicillin
Per cent isolates
Inhibition zone diameters, mm
WT
R≤ 16 - S≥ 19
Interpret as S, susceptible S ≥ 19
Interpret as I, intermediate 17-18
Interpret as R, resistant R ≤ 16
NRI wild-type population
WT ≥ 23

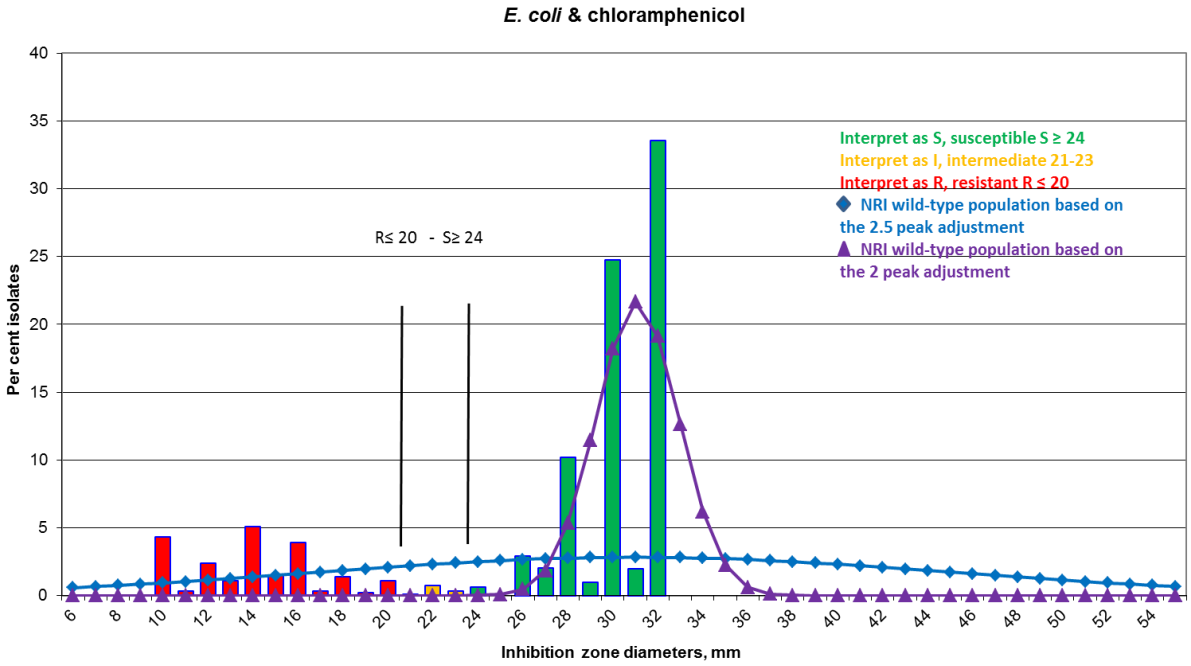
(b)


E. coli & apramycin
Per cent isolates
Inhibition zone diameters, mm
WT
R≤ 19 - S≥ 22
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 21

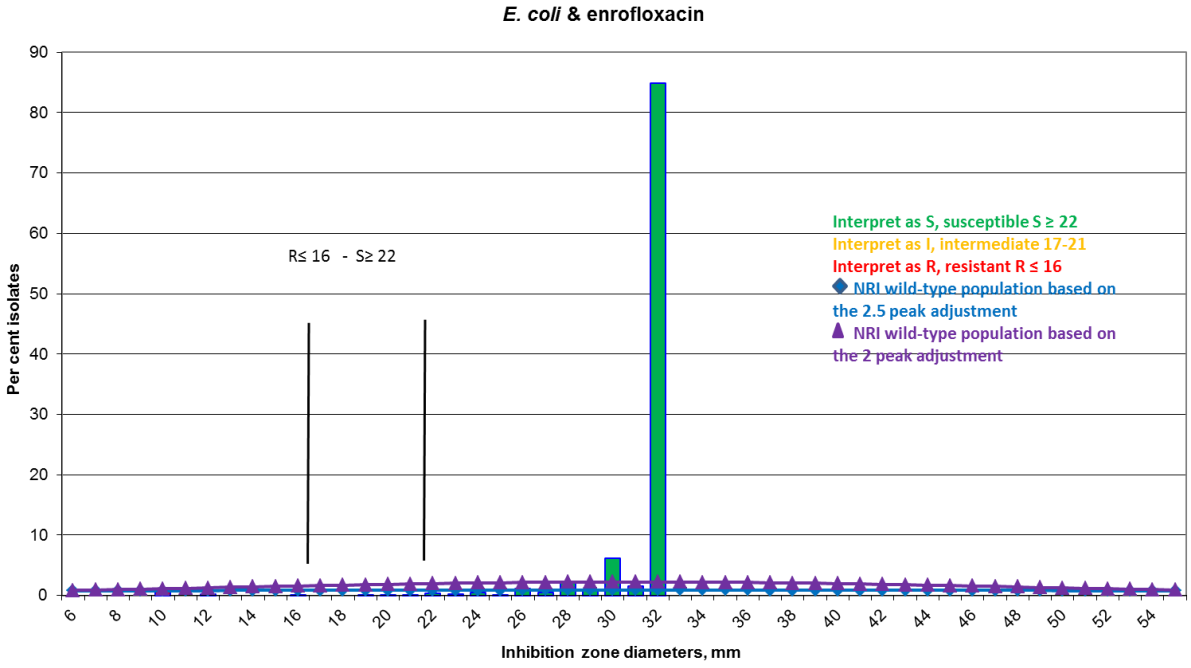
(c)


E. coli & ceftiofur
Per cent isolates
Inhibition zone diameters, mm
WT
R≤ 19 - S≥ 22
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 24

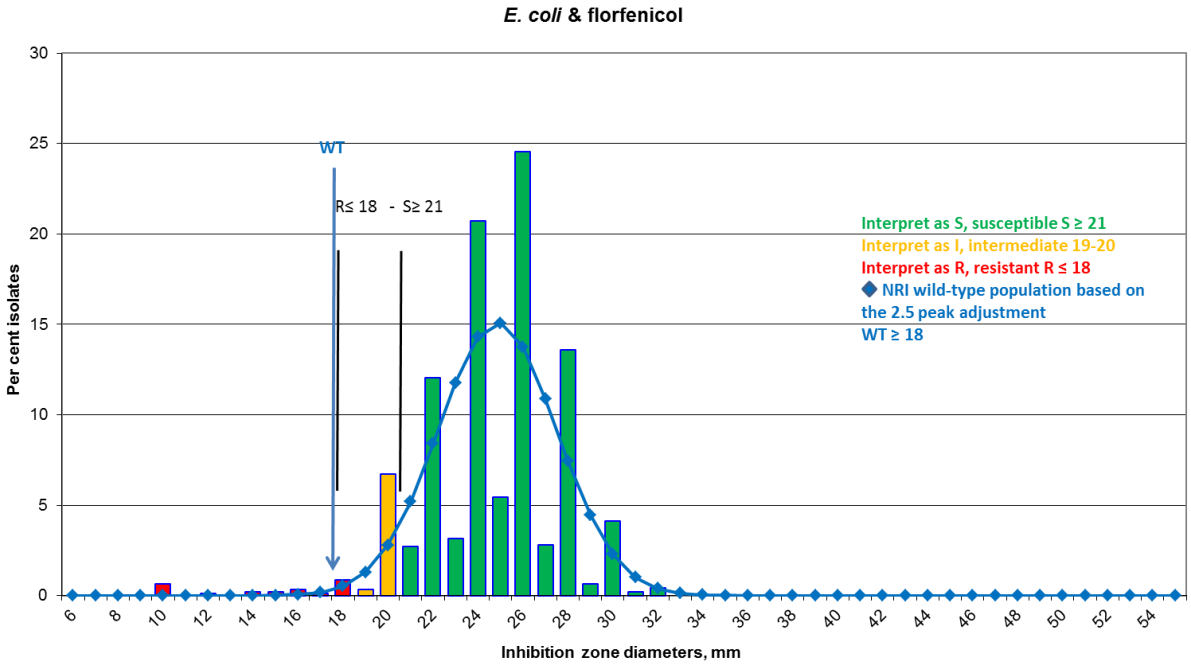
(d)


E. coli & chloramphenicol
Per cent isolates
Inhibition zone diameters, mm
R≤ 20 - S≥ 24
Interpret as S, susceptible S ≥ 24
Interpret as I, intermediate 21-23
Interpret as R, resistant R ≤ 20
NRI wild-type population based on the 2.5 peak adjustment
NRI wild-type population based on the 2 peak adjustment

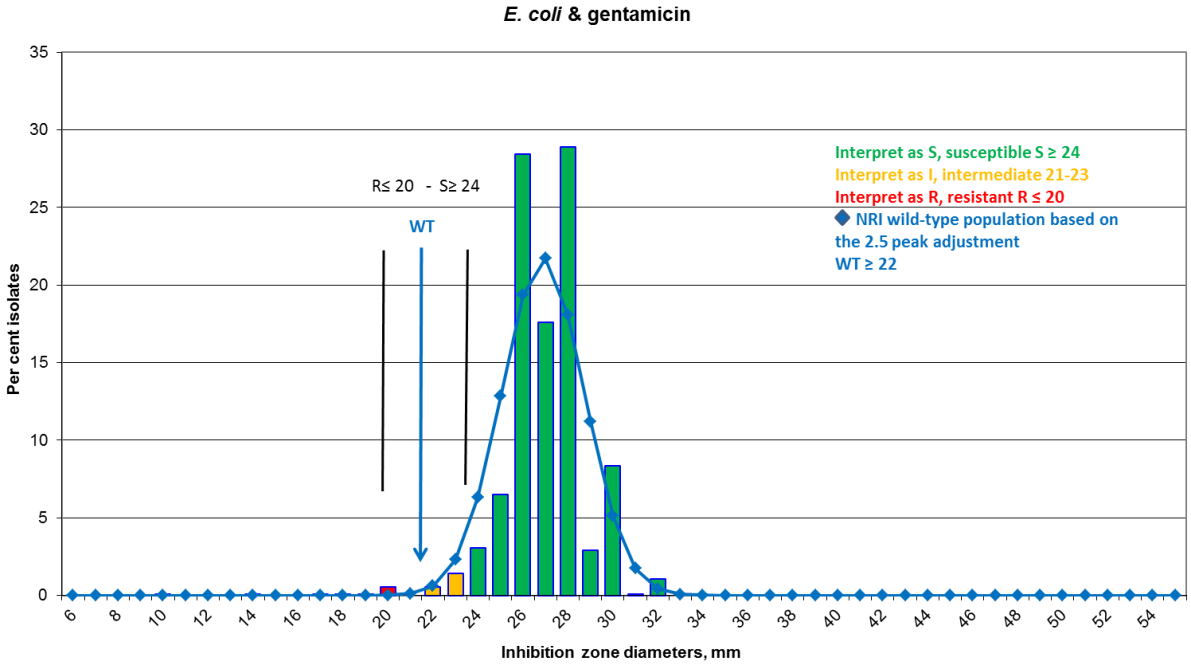
(e)


E. coli & enrofloxacin
Per cent isolates
Inhibition zone diameters, mm
R≤ 16 - S≥ 22
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 17-21
Interpret as R, resistant R ≤ 16
NRI wild-type population based on the 2.5 peak adjustment
NRI wild-type population based on the 2 peak adjustment

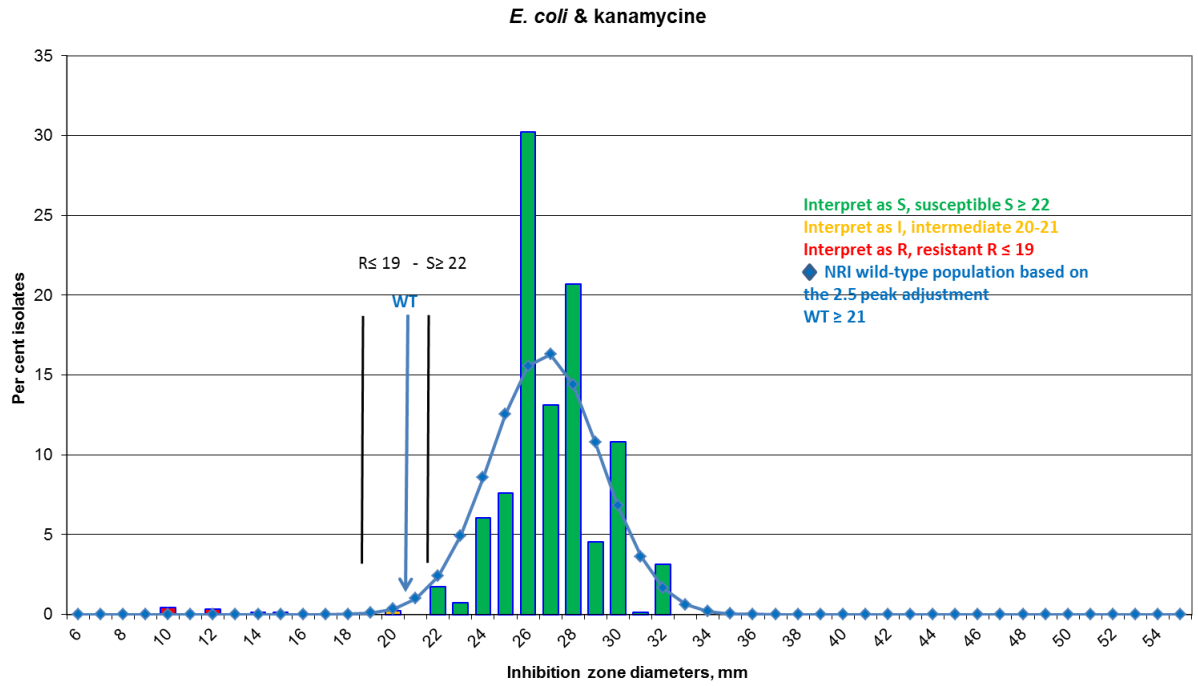
(f)


E. coli & florfenicol
WT
R≤ 18 - S≥ 21
Interpret as S, susceptible S ≥ 21
Interpret as I, intermediate 19-20
Interpret as R, resistant R ≤ 18
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 18
Per cent isolates
Inhibition zone diameters, mm

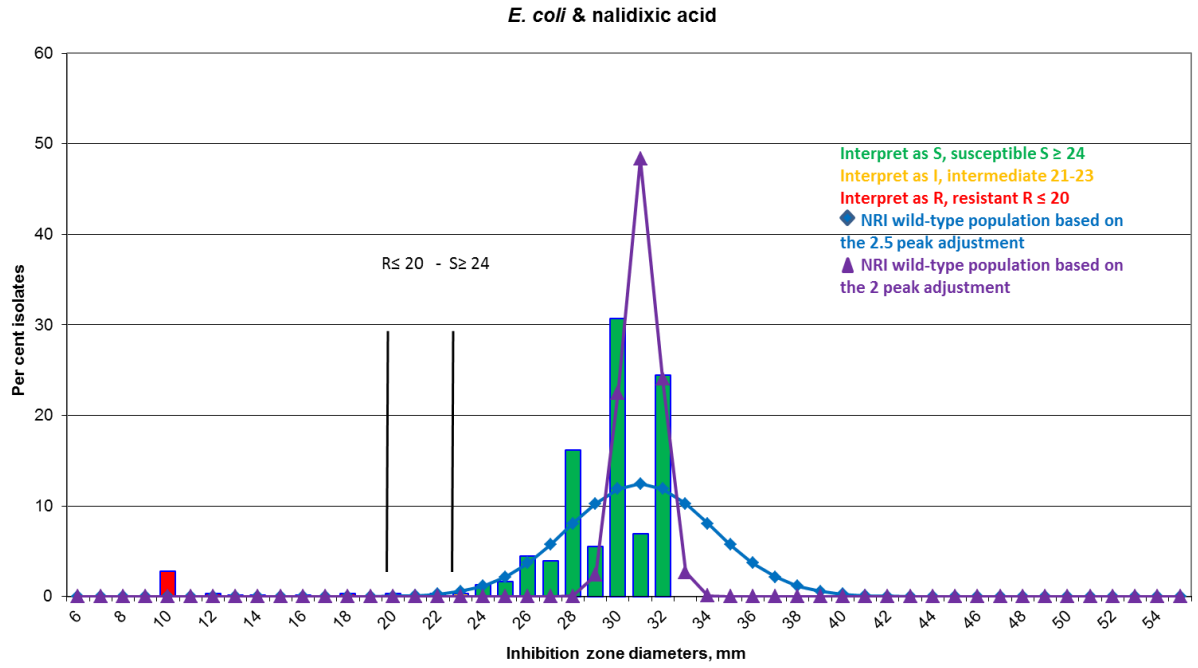
(g)


E. coli & gentamicin
R≤ 20 - S≥ 24
WT
Interpret as S, susceptible S ≥ 24
Interpret as I, intermediate 21-23
Interpret as R, resistant R ≤ 20
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 22
Per cent isolates
Inhibition zone diameters, mm

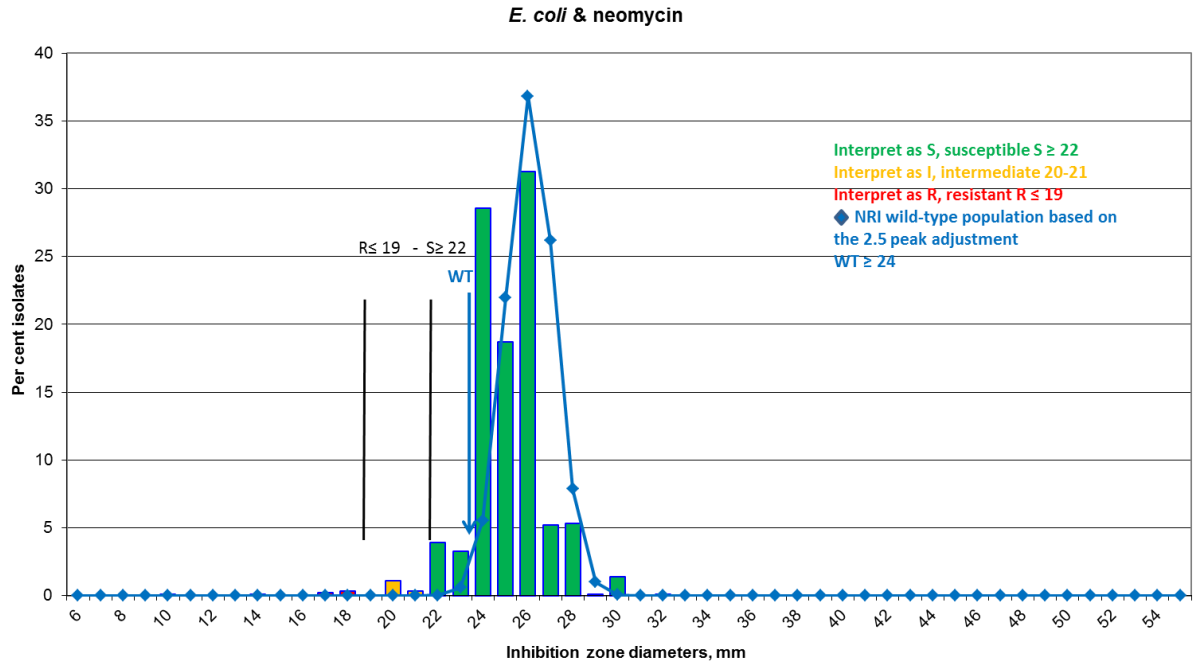
(h)


E. coli & kanamycine
Per cent isolates
Inhibition zone diameters, mm
R≤ 19 - S≥ 22
WT
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 21

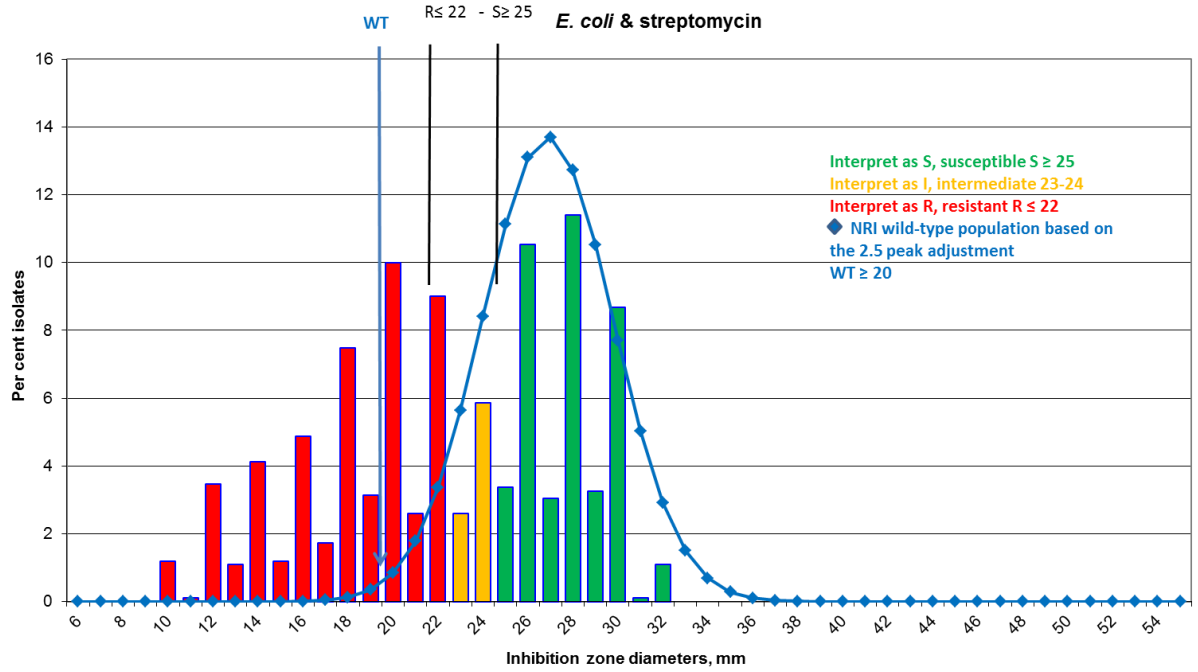
(i)


E. coli & nalidixic acid
Per cent isolates
Inhibition zone diameters, mm
R≤ 20 - S≥ 24
Interpret as S, susceptible S ≥ 24
Interpret as I, intermediate 21-23
Interpret as R, resistant R ≤ 20
NRI wild-type population based on the 2.5 peak adjustment
NRI wild-type population based on the 2 peak adjustment

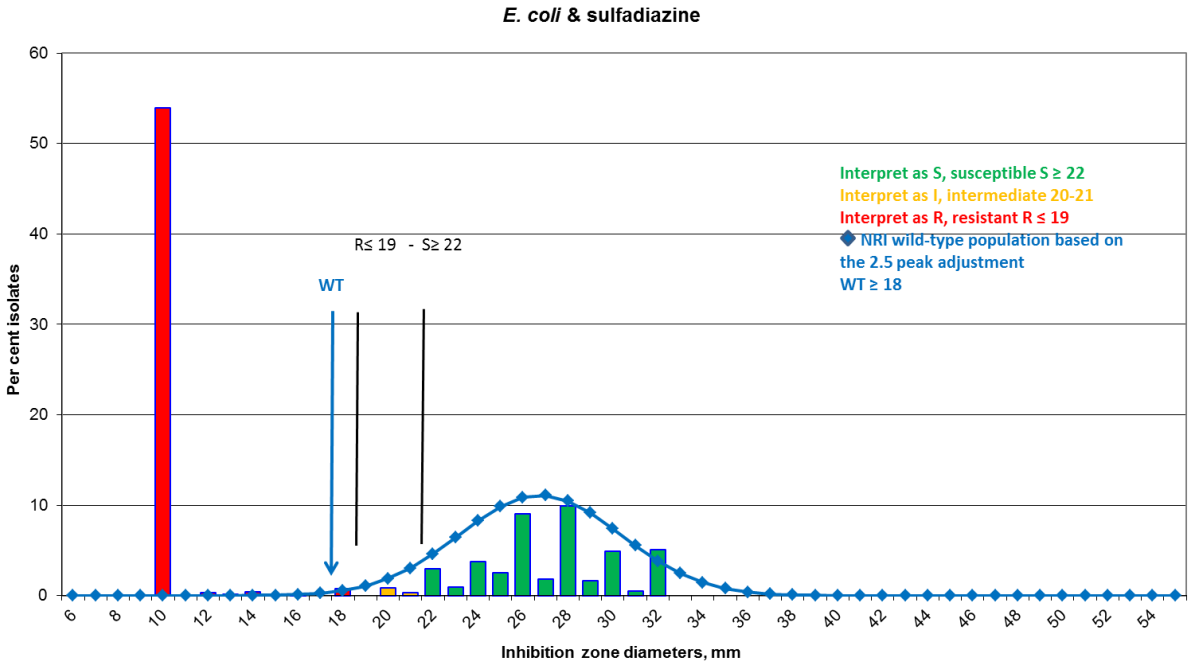
(j)


E. coli & neomycin
Per cent isolates
Inhibition zone diameters, mm
R≤ 19 - S≥ 22
WT
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 24

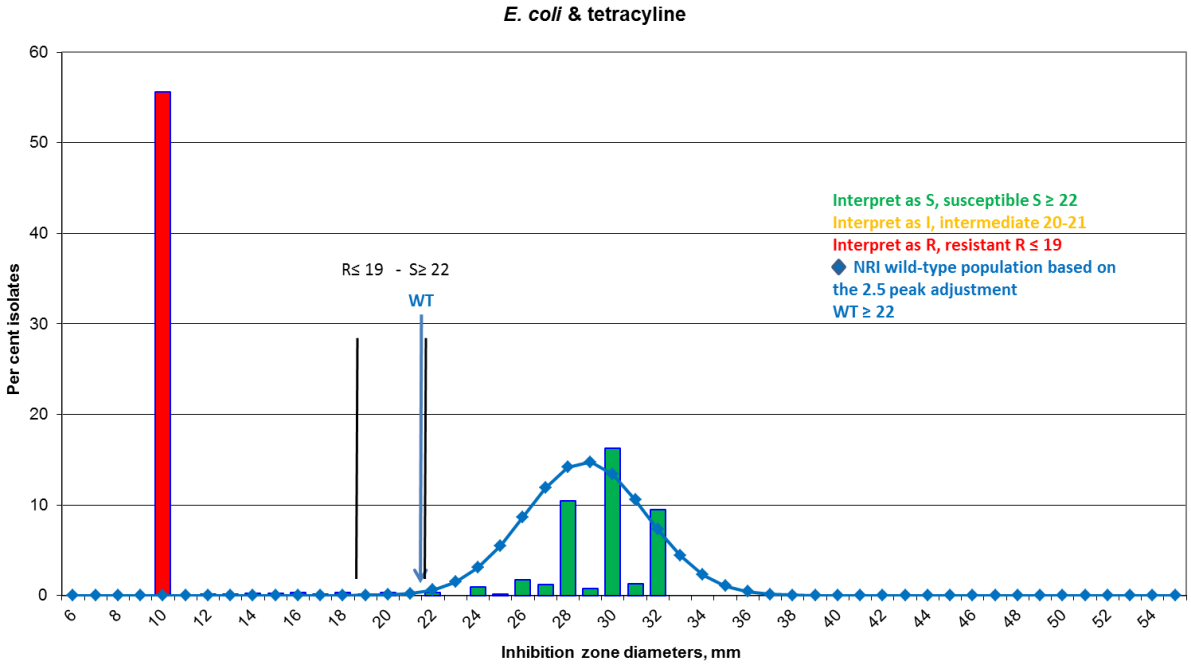
(k)


WT
R≤ 22 - S≥ 25
E. coli & streptomycin
Per cent isolates
Inhibition zone diameters, mm
Interpret as S, susceptible S ≥ 25
Interpret as I, intermediate 23-24
Interpret as R, resistant R ≤ 22
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 20

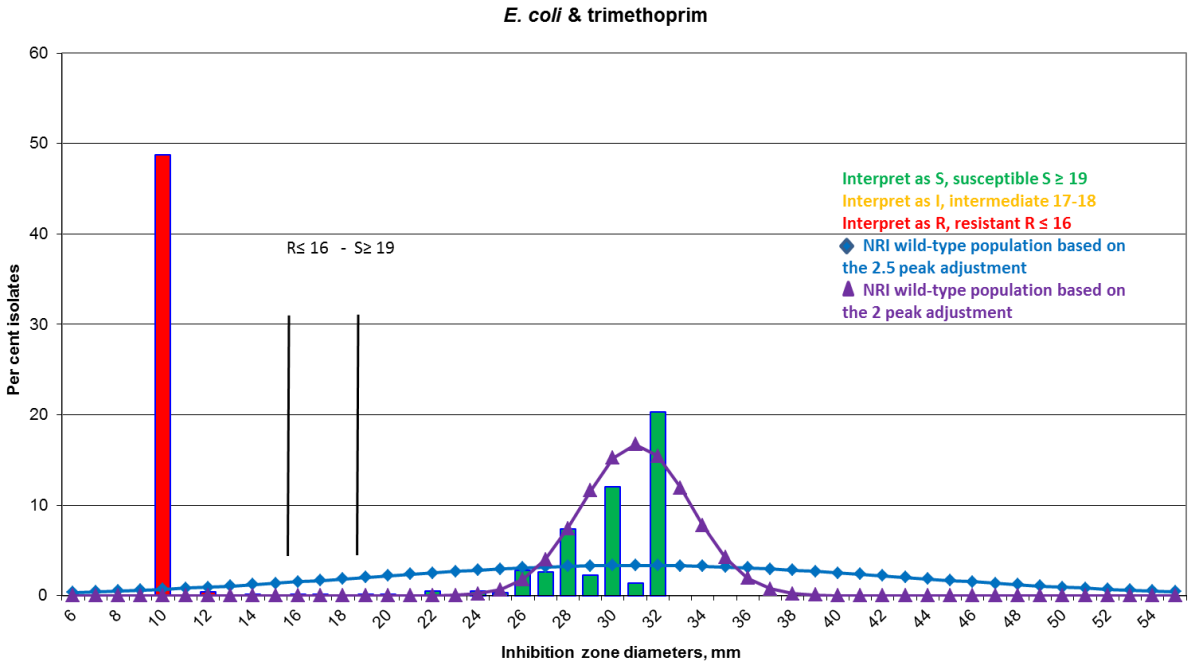
(l)


E. coli & sulfadiazine
Per cent isolates
Inhibition zone diameters, mm
R≤ 19 - S≥ 22
WT
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 18

(m)


E. coli & tetracyline
Per cent isolates
Inhibition zone diameters, mm
R≤ 19 - S≥ 22
WT
Interpret as S, susceptible S ≥ 22
Interpret as I, intermediate 20-21
Interpret as R, resistant R ≤ 19
NRI wild-type population based on the 2.5 peak adjustment
WT ≥ 22

(n)



(o)

Figure 3 (a-o). Histogram of zone diameter values around antimicrobial disks for 921 *Escherichia coli* isolates from pigs at slaughter age (amoxicillin/clavulanic acid (30+15 µg) (a), ampicillin (30 µg) (b), apramycin (40 µg) (c), ceftiofur (30 µg) (d), chloramphenicol (60 µg) (e), enrofloxacin (10 µg) (f), florfenicol (30 µg) (g), gentamicin (40 µg) (h), kanamycin (100 µg) (i), nalidixic acid (130 µg) (j), neomycin (120 µg) (k), streptomycin (100 µg) (l), sulfadiazine (240 µg) (m), oxytetracycline (80 µg) (n) and trimethoprim (5.2 µg) (o)). The normalized resistance interpretation (NRI)-calculated distribution is based on the four-zone average parameter with a peak adjustment of 2.5 zone diameters, and is shown as a line curve (◊). For chloramphenicol, enrofloxacin, nalidixic acid and trimethoprim the NRI-calculated distribution based on the four-zone average parameter but with a peak adjustment of 2 zone diameters is equally presented (Δ).

Clinical breakpoints of the Neo-Sensitabs manufacturer (Rosco, Taastrup, Denmark) were used for the categorization of the tested isolates as susceptible (S), intermediate (I), or resistant (R) and are shown as vertical lines. The Clinical Laboratory Standards Institute (CLSI) standards were followed for inoculum standardization, incubation conditions, and internal quality control organisms (CLSI, 2013). The NRI generated 2.5 SD (standard deviation) limit is shown as a vertical blue arrow. The 2.5 SD limit could not be generated for chloramphenicol, enrofloxacin, nalidixic acid and trimethoprim because of too much irregularities in the normal distribution of zone diameter values.

DISCUSSION

EUCAST recently extended the number of antimicrobial agents for which an epidemiological cut-off value (ECV) is available for *E. coli*, based on inhibition zone diameter distributions (EUCAST, 2015). Yet, a difference in disk content potency for the antibiotics included in the EUCAST database (EUCAST, 2015) and these tested in this study excluded the EUCAST established ECV as interpretive criteria for our results. The Normalized Resistance Interpretation (NRI) method, described by Kronvall et al. in 2003 and applied to clinical human *E. coli* and *Staphylococcus aureus* isolates (Kronvall, 2010), is here for the first time being used for defining the wild type (WT) population of animal related bacteria, i.e. *E. coli* isolated from clinically healthy pigs at slaughter age during a cross-sectional study, after disk diffusion. The NRI method deals with differences in methodology, such as different disk contents, since cut off values are calculated after reconstructing the WT population based on the high zone side of the susceptible peak of the obtained data (Kronvall, 2003a). It also circumvents subjective decisions on an ECV after visual examination of a bacterial population which can be hampered for bacterial species-antibiotic combinations showing an unclear transition between WT and non-WT isolates, equally seen for the *E. coli* isolates from this study.

The NRI methodology is based on the presence of a distribution of WT isolate zone diameters following criteria of normality to a sufficient degree (Kronvall et al., 1999; Kronvall et al., 2003a). In this study, the relevance/applicability/usefulness of the NRI method, using the optimal parameters for quality control strain *E. coli* ATCC 25922 (Joneberg et al., 2003), was demonstrated for several antimicrobials. The calculation of the standard deviations (SD) of the normalized NRI calculated distributions was demonstrated to be useful as a predictor of the appropriateness of the NRI method for the observed disk diffusion results by the detection of outliers in the range of SD for all antibiotics tested. Antibiotics showing deviations in SD of the normalized NRI-calculated distribution, for the 2 and 2.5 zone diameters peak adjustment also showed a steep slope at the high zone value side of the histogram, resulting in non-Gaussian distributions (chloramphenicol, enrofloxacin, nalidixic acid and trimethoprim). For these antibiotics, the method turned out to be none applicable. Non-Gaussian distributions, as a result of the high number of isolates with a zone diameter of 32 mm, e.g. enrofloxacin, can be circumvented either by the use of a lower disk content potency or by enabling the reading of zone diameters > 32 mm. This will allow for the plotting of a normal distribution of the observed zone diameter records.

The high zone side is of particularly interest as it is used as the calibrator to construct the real standard distribution of susceptible isolates. Calculations which include subjectively missing records at the high zone value side will result in NRI distributions wrongly shifted to the left, decreasing the probability of identifying strains as different from the WT population of strains. Identifying the WT populations is of particular interest for surveillance purposes. Therefore, the proportion of isolates classified as WT, but carrying resistance determinants, has to be kept to a minimum. Another subjective modification that has been observed in the

past is the overrepresented number of even zone diameters (Kronvall et al., 2003b), also present in the data from this study. In general, there is a trend of rounding down or up odd numbers in the absence of automatic reading devices.

When results for clinical susceptibility were analyzed and compared, there was a fairly good agreement between the susceptibility levels obtained by clinical interpretive criteria and the NRI calculated levels for most of the antibiotics. Nevertheless, marked differences were noted between clinical breakpoints and the NRI derived ECV for ampicillin and ceftiofur. Clinical breakpoints much lower than the ECV resulted into a gap including isolates categorized as clinically susceptible, but likely carrying acquired resistance determinants. Results based on clinical breakpoints are of significance for the clinician as isolates with decreased susceptibility may still be treatable (Simjee et al., 2008). Yet, as mentioned above, NRI derived ECV identifying them as non-WT is important as it acts as an early warning for an emerging clinical problem (Aarestrup et al., 2007; Silley et al., 2011).

Clinical breakpoints cut through the WT population for several antibiotics tested, categorizing isolates from the WT population as not susceptible. This raises questions on the correctness of the establishment of these breakpoints used for *E. coli* and a certain antibiotic and highlights the need for validated veterinary-specific clinical breakpoints which are useful in the practice of veterinary medicine.

Given the current concerns about the emergence of and trends in antimicrobial resistance in animals, there is an urgent need for a harmonized approach in Europe towards the susceptibility methods used in veterinary medicine (VetCAST, 2014). Nevertheless, until harmonization is achieved, the NRI method can be a valid method to circumvent the differences in the methodology of the routinely used disk diffusion test and to determine the ECV, making antimicrobial susceptibility data comparable. Also, historical data, for instance from previous prevalence studies, might be included in surveillance programs for comparative reasons, provided that zone diameters have been recorded.

In conclusion, in the absence of a clear bimodal distribution of *E. coli* zone histograms and previously established ECV for *E. coli*-antibiotic combinations, an objective setting of ECV was necessary. NRI was applicable to define the wild type population for outcomes with a normal distribution of the susceptible population. Applying NRI in this study highlighted the need for measuring zone diameters in a non-biased way. The increased use of automatic reading devices in the recording of disk diffusion susceptibility test results in large databases which might give rise to a wide application of the NRI method in veterinary medicine.

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CHAPTER 6

ANTIMICROBIAL USE AND RESISTANCE IN *ESCHERICHIA COLI* FROM SOWS AND PIGLETS

PRESENCE OF ANTIMICROBIAL RESISTANCE AND ANTIMICROBIAL USE IN SOWS ARE RISK FACTORS FOR ANTIMICROBIAL RESISTANCE IN THEIR OFFSPRING

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ABSTRACT

This study investigated whether antimicrobial resistant *Escherichia coli* in apparently healthy sows and antimicrobial administration to sows and piglets influenced antimicrobial resistance in fecal commensal *E. coli* from piglets. Sixty sows from three herds and three of their piglets were sampled at several time points. Antimicrobial use data during parturition and farrowing were collected. Clinical resistance was determined for two isolates per sampling time point for sows and piglets using disk diffusion. Only 27.4% of *E. coli* isolates from newborn piglets showed no resistance. Resistance to one or two antimicrobial classes equaled 41.2% and 46.8% in isolates from sows and piglets, respectively, for the overall farrowing period. Multiresistance to at least four classes was found as frequently in sows (15.6%) as in piglets (15.2%). Antimicrobial resistance in

piglets was influenced by antimicrobial use in sows and piglets and by the sow resistance level ($p < 0.05$). Using aminopenicillins and third-generation cephalosporins in piglets affected resistance levels in piglets (odds ratios [OR] > 1; $p < 0.05$). Using enrofloxacin in piglets increased the odds for enrofloxacin resistance in piglets (OR = 26.78; $p < 0.01$) and sows at weaning (OR = 4.04; $p < 0.05$). For sows, antimicrobial exposure to lincomycin-spectinomycin around parturition increased the resistance to ampicillin, streptomycin, trimethoprim-sulfadiazine in sows (OR= 21.33, OR= 142.74, OR= 18.03; $p < 0.05$) and additionally to enrofloxacin in piglets (OR= 7.50; $p < 0.05$). This study demonstrates that antimicrobial use in sows and piglets is a risk factor for antimicrobial resistance in the respective animals. Moreover, resistance determinants in *E. coli* from piglets are selected by using antimicrobials in their dam around parturition.

INTRODUCTION

Since their discovery, antimicrobials have been widely used in animal husbandry for growth promoting, prophylactic, metaphylactic, and therapeutic reasons (Aarestrup, 2005). Despite the ban on growth-promoting antimicrobials in Europe by
2006, it was estimated that in 2007, still 3,500 tons of antimicrobials were used for animal health over 10 different
European countries (ECDC-EFSA-EMEA-SCENIHR, 2009). Antimicrobial administration to sows is common around parturition. Dunlop et al. reported mastitis-metritis-agalactia syndrome and off feed as the most common reasons for non-routine treatments around parturition (1998). Other reasons for the administration of antimicrobials around parturition included the prevention of bacterial diseases in sows and the reduction of possible pathogen transmission to the piglets. In addition, piglets often receive antimicrobials shortly after birth to prevent wound infections or to prevent infections with pathogens such as streptococci, *Escherichia coli*, *Clostridium* species, and staphylococci (Stannarius et al., 2009; Callens et al., 2012). The use of antimicrobials is a concern as it engenders a selection pressure for resistance selection and spread in pathogenic and commensal bacteria to antimicrobials (Dewulf et al., 2007). Regardless of the use of antibiotics, several other factors can be involved in the selection
and spread of resistance determinants. A different type of housing has already been identified as playing a role in the selection and spread of resistance in pigs by Langlois et al. (1988). Exposure of animals to stressors at the farm may enhance the selection and dissemination of resistant determinants (Moro et al., 2000; Mathew et al., 2003). Also, animal categories, such as fattening pigs and sows or different ages, can contribute to diverged resistance rates (Butaye et al., 1999; Dewulf et al., 2007).
Resistance to antimicrobials in sows and piglets between birth and postweaning (Mathew et al., 1998; Stannarius et al., 2009) and the relationship with a low or high antimicrobial have been described (Mathew et al., 1999). Resistant *E. coli* are not restricted to one animal host and they can spread naturally from one animal host to colonize the intestinal tract of another animal host (Levy et al., 1976) or animal species (Marshall et al., 1990). Indeed, transmission of resistant bacteria from sows to piglets has been assumed (Mathew et al., 2005). Thus, one could question to what extent the presence of antimicrobial-resistant bacteria in sows influences the degree of antimicrobial resistance in piglets at birth and during the suckling period, periods with intensive contact between sows and piglets, and within the offspring, which transmission of bacteria is possible (Devriese et al., 1994). Furthermore, the administration of antimicrobials to sows and piglets during this period may increase the presence of antimicrobial resistant bacteria in both sows and piglets. This can be either by a direct selection pressure in the treated animals or by the exposure to antimicrobials due to the shedding by treated animals. The hypothesis of a selection pressure exerted by

antibiotic residues in fecal material and/or feed dust, or dust particles, originating from the treatment period, has been

presented before (Mathew et al., 2005; Alexander et al., 2009). The information available on the interaction between antimicrobial use in sows and their offspring at the one hand and resistance in these animals at the other hand is scarce.

Therefore, the aim of this field study was to investigate whether the presence of antimicrobial resistant *E. coli* in sows and the administration of antimicrobials to sows and piglets during farrowing influenced the antimicrobial resistance in fecal commensal *E. coli* in sows and the offspring by using multilevel models.

MATERIALS AND METHODS

Study population and design

Three different Belgian pig herds were selected and visited between December 2010 and February 2011. The herds were farrow-to-finish herds with at least 200 sows. Piglets were weaned between 21 and 28 days of age. Herds were visited four times to collect samples for antimicrobial resistance profiling of fecal *E. coli* from apparently healthy sows and piglets and to register the antimicrobial drug consumption during the latest production cycle and the entire observation period of the sampled sows and piglets (from birth until weaning).

Twenty sows in gestation were randomly selected from each herd. From each sow, the fecal material was collected after rectal stimulation 1–3 days before parturition and within 12 hr after parturition. Sows were sampled a third time at weaning age of their piglets. For transport to the laboratory, the fecal material was kept in a clean recipient for each individual sow.

The fecal material was also collected from three randomly selected piglets from each sampled sow by means of a rectal swab. The selected piglets were sampled within 12 hr after birth and before any antimicrobial administration. Every piglet was identified by an ear tag and additional samples were taken from the same animal at 2 weeks of age and at weaning. The different sampling times for sows and piglets are presented in Table 1.

Table 1. Sampling scheme on 3 different farrow-to-finish swine herds for the isolation of faecal *Escherichia coli*.

Type of animals (n)	**Prepartum**	**Day of Parturition**	**Postpartum**	**Weaning**
Sows (60)[a]	1-3 days before parturition	Within 12h after delivery (D0)	-	D21-28 after delivery
Piglets (180)[b]	-	Within 12h after delivery Before any antimicrobial administration (D0)	14 days after birth (D14)	D21-28 days after birth (D21-28)

[a] Twenty sows per herd

[b] Three piglets per sows

-: faecal samples were not taken from the respective animals at this sampling moment

Isolation and identification

Immediately after collection, the samples were transported to the laboratory where they were processed within a few hours after arrival. For the isolation of *E. coli*, rectal swabs from the piglets were directly inoculated on Mac-Conkey agar plates (MacConkey Agar No. 3; Oxoid Ltd.), whereas for the sows, a swab was used to inoculate the fecal material from the recipient on the plates. Plates were incubated aerobically for 24 hr at 37°C. From each culture, two suspected *E. coli* colonies were confirmed by means of positive glucose/lactose fermentation, gas production and absence of H_2S production using Kligler Iron Agar (Oxoid Ltd.), indole production (Indole spot on; Becton Dickinson), and the absence of aesculin hydrolysis (Bile Aesculin Azide Agar; Oxoid Ltd.) (Catry et al., 2007).

For antimicrobial resistance profiling, the Kirby-Bauer disk diffusion method was used for susceptibility testing of seven different antimicrobial agents. The Clinical Laboratory Standards Institute (CLSI) standards were followed for inoculum standardization, incubation conditions, and internal quality control organisms (2008). The following antimicrobial tablets (charge in µg) were used: amoxicillin/clavulanic acid (30 µg + 15 µg), ampicillin (33 µg), ceftiofur (30 µg), tetracycline (80 µg), trimethoprim–sulfadiazine (5.2 µg + 240 µg), enrofloxacin (10 µg), and streptomycin (100 µg). After 18 hr of incubation, inhibition zones were read and interpreted according to the manufacturer's guidelines (Rosco, 2007). The following clinical breakpoints were used: for amoxicillin/clavulanic acid R ≤16, for ampicillin R ≤ 16, for ceftiofur R ≤ 19, for tetracycline R ≤ 19, for trimethoprim–sulfadiazine R ≤ 19, for enrofloxacin R ≤ 18, and for streptomycin R ≤ 22. The response measure for the *E. coli* isolates was dichotomized into resistant or susceptible according to the clinical criteria

for resistance. Intermediate responses were allocated to the susceptible ones. To define resistance, the clinical criteria were used. Clinical resistance will be mentioned as ‘‘resistance.’’

Quantification of antimicrobial drug consumption

To investigate the influence of antimicrobial drug use on the prevalence of antimicrobial resistance in fecal *E. coli* in the piglets, data concerning antimicrobial use in the sows were collected from the start from the latest production cycle (prepartum) until weaning (between 21 and 28 days of age) and for the piglets from birth until weaning.

Antimicrobial drug consumption was quantified as treatment incidences (TI) based on the used daily dose pig (UDD_{pig}). The treatment incidence (TI_{UDD}) is defined as the number of days per 1,000 that one pig is treated with one UDD_{pig}. The UDD_{pig} is defined as the administered dose of a drug per day per kilogram pig. For each of the herds, a standardized growth table was used to estimate the body weight at the time of antimicrobial administration. This estimated body weight was used to calculate the UDD_{pig}. For the sows, per herd, one out of the 20 selected sows was weighed. The body weight of the particular sow was used as the standard weight for all selected sows in one herd. Treatment incidences based on the UDD_{pig} (TI_{UDD}) were calculated by means of the following formula, described by Timmerman et al (2006).

$$\frac{Total\ amount\ of\ antimicrobial\ administered\ (mg)}{UDD\ or\ ADD\ \left(\frac{mg}{kg}\right) * number\ of\ days\ at\ risk * kg\ pig}$$

For sows, the number of days at risk was taken for three time periods. The number of days at risk was taken (1) from day of insemination until 1–3 days before parturition (112–114 days), (2) from insemination until parturition (115 days), and (3) from insemination until weaning (136–143 days). For piglets, the number of days at risk was taken from birth until weaning (21–28 days).

Statistical model

The proportion of resistance (PR) was defined for each individual antimicrobial agent as the number of isolates to which resistance against this specific antimicrobial was measured as compared to the total number of isolates tested. In this study, multiresistance was defined as resistance to three or more antimicrobials.

Statistical models were built in SAS® 9.4 and used to estimate the PR for each individual antimicrobial agent. Let Y_{ijt} be the number of isolates to which resistance was found for animal j in nest i at measurement t, and n_{ijt} the corresponding number of isolates being tested. In this study, a total of 60 nests were available (i = *1,.,60*), and the number of animals per nest was 4, with j = *1,2,3* corresponding to the piglets and j = *4* corresponding to the sow. From every nest, 6 measurements were taken, as summarized in Table 1 (t = 1, 2,

3 correspond to the subsequent measurements taken from the piglets, *t* = 4, 5, 6 correspond to the measurements from the sows). Note that measurements are possibly associated because (1) measurements were taken at different time points from the same animals and (2) measurements are taken from animals in the same nest. To model the possible association between the resistance profiles of piglets and their sows, as well as the association of the resistance outcomes of the same animals at different measurements, a generalized linear mixed model was used. More specifically,

it was assumed that Y_{ijt} is given by a binomial process with n_{ijt} the number of trials and p_{ijt} the PR for animal *j* in nest *i* at time point *t*. The probability p_{ijt} was modeled as follows:

$$ln\left(\frac{p_{ijt}}{1-p_{ijt}}\right) = \zeta X_{ijt} + u_i + v_{j(i)} + \varepsilon_{ij}$$

where ζ is the vector of unknown fixed effect (regression parameters) and u_i and $v_{j(i)}$ are normally distributed random effects with mean 0 and variances σ^2 and τ^2, respectively. The parameters u_i are nest-specific intercepts, measuring the deviation of the resistance-profile of each nest from the average resistance-profile. In a similar way, the parameters $v_{j(i)}$ are animal-specific parameters, measuring the deviation of the resistance-profile of each animal from the average. By the use of nest- and animal-specific parameters, associations of the outcomes within nests and within animals were taken into account. In the fixed effects structure, differences with respect to measurements, herd and the extent of exposure to antimicrobials were taken into account. The fixed effects structure can be written as:

$$\zeta X_{ijt} = \alpha_0 + \sum_{k}^{2} \alpha_k I(herd = k) + \sum_{k=1}^{5} \beta_k I(t = k)$$
$$+ \left(\sum_{k}^{5} \gamma_k UDD_{kijt}\right) I(piglet)$$
$$+ \left(\sum_{k}^{5} \delta_k UDD_{kijt}\right) I(sow) + \varepsilon$$

where *I(.)* is an indicator function taking the value 1 if the expression within the brackets is correct and 0 otherwise. The function *I(piglet)* indicates whether animal j in nest *i* is a piglet or not, and a similar definition is used for *I(sow)*. The α-parameters correspond with the differences amongst 3 herds, the β-parameters model the 6 time effects, the γ-parameters measure the effect of the extent of antimicrobial exposure on the resistance of the piglet, and the δ-parameters measure the effect of the extent of antimicrobial exposure on the resistance of the sow. The model was simplified in a stepwise way, keeping only the significant terms in the model (using Akaike's Information Criterion). A correction for multiple testing was inserted for the fixed effect time. For every resistance outcome in an animal at one of the sampling time points, the model was run with the accordingly effect of the extent of antimicrobial exposure of the piglet and the sow for the time period

preceding the sampling. Thus, the model was rerun for every time point a resistance outcome was obtained to link the outcome with the preceding antimicrobial exposure.

To study whether the resistance of the sow has an impact on the resistance of the piglets, a similar model has been used. More specifically, the same generalized linear mixed model was used, but where now p_{ijt} denotes the PR for piglet *j* in nest *i* at time *t* and the fixed effects structure was specified as

$$\zeta X_{ijt} = \alpha_0 + \sum_{k}^{2} \alpha_k \, I(herd = k) + \sum_{k=1}^{3} (\beta_k + \gamma_k \, Y_{i4t}) I \, (t = k)$$

where γ_k represents the effect of the resistance of the sow at time t' on the resistance of the piglets at time k. This analysis is performed for each antibiotic and each time point t' of measurement in the sow, separately.

RESULTS

Descriptive statistics

E. coli antimicrobial resistance profile.

Susceptibility testing was performed on 294 and 863 *E. coli* isolates from sows and piglets, respectively. Overall, 23.5% of the isolates from sows and 26.3% of the isolates from piglets were susceptible to all antimicrobials tested. Resistance to one or

two antimicrobials was reported for 41.2% of the isolates in sows and 46.8% of the isolates in piglets. Resistance to at least three antimicrobials was found more frequently in isolates from sows (35.4%) than from piglets (26.9%) ($p < 0.05$). Resistance to all of the seven antimicrobials tested appeared in piglets for 2 isolates (total of 863 isolates), whereas in sows, the highest number of antimicrobials to which resistance was found in the same isolate was 6 (2 isolates of a total of 294 isolates tested) (Fig. 1). Herd 1, 2, and 3 differed significantly for the level of resistance to ampicillin, ceftiofur, enrofloxacin, streptomycin, trimethoprim-sulfadiazine, and tetracycline ($p < 0.05$) (Table 2).

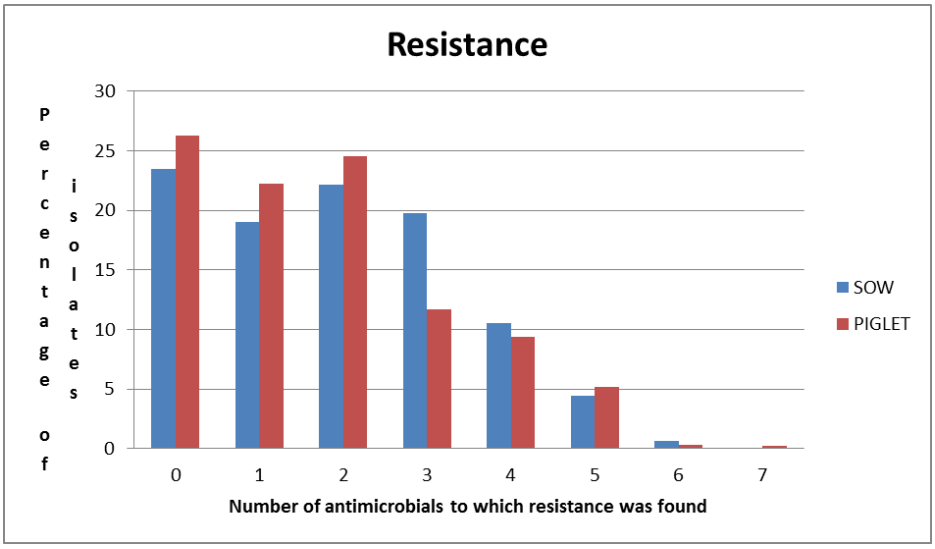

Figure 2. Resistance results for 294 and 863 *Escherichia coli* isolates from sows and piglets in three pig herds.

Table 2. Results of the generalized linear mixed model for the identification of the risk of antimicrobial exposure for the prevalence of antimicrobial resistant faecal *Escherichia coli* isolates in sows and piglets during farrowing (total of 294 and 863 isolates for sows and piglets respectively).

Antimicrobial agent tested	Risk factor		Category	n	Resistance prevalence %	OR	95% CI	*P-value*
Amoxicillin/clavulanic acid	**Herd**		1	1	3.0	>999	<0.01->999	0.96
			2	1	2.5	>999	<0.01->999	0.96
			3	1	0	1	Ref	-
	Antimicrobial use	*Effect on sow*	No sign. effects					
		Effect on piglet	No sign. effects					
Ampicillin	**Herd**		1	1	68.6	14.39	4.36-47.56	<0.0001
			2	1	29.6	0.96	0.29-3.21	0.94
			3	1	55.0	1	Ref	-
	Antimicrobial use	*Effect on sow*	No use	264	48.1	1	Ref.	-
			Lincomycin-spectinomycin *in sow*	30	80	21.33	5.42-83.85	<0.0001
		Effect on piglet	No use	856	49.3	1	Ref.	-
			Amoxicillin *in piglet*	7	100	6.55	2.58-16.64	<0.0001
			No use	65	33.6	1	Ref.	-

				5				
			Ceftiofur$_{in\ piglet}$	298	52.4	4.15	2.16-7.97	<0.0001
			No use	821	48.5	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	42	73.8	5.42	2.63-11.14	<0.0001
			No use	750	48.7	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	113	56.6	8.12	2.40-27.47	<0.01
Ceftiofur	**Herd**		1	1	3.2	23.20	1.09-492.23	<0.05
			2	1	2.4	4.32	0.16-113.15	0.38
			3	1	0.2	1	Ref.	-
	Antimicrobial use	*Effect on sow*	No sign. effects					
		Effect on piglet	No use	565	0.2	1	Ref.	-
			Ceftiofur$_{in\ piglet}$	298	4.4	>999	10.34->999	<0.01
			No use	821	0	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	42	1.7	702.34	39.63->999	<0.0001
Enrofloxacin	**Herd**		1	1	15.0	12.67	1.80-	<0.05

							89.36	
			2	1	5.9	0.36	0.03-4.69	0.44
			3	1	10.1	1	Ref	-
		Effect on sow	No use	286	6.3	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	8	25	4.04	1.34-12.20	<0.05
	Antimicrobial use	*Effect on piglet*	No use		3.2	1	Ref.	-
			Ceftiofur$_{in\ piglet}$		25.2	31.08	4.78-202.27	<0.01
			No use	821	7.1	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	42	47.6	26.78	8.12-88.29	<0.0001
			No use	750	7.5	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	113	19.5	7.50	1.13-49.87	<0.05
Streptomycin	**Herd**		1	1	76.0	24.29	7.19-82.06	<0.0001
			2	1	62.8	3.97	1.23-12.78	<0.05
			3	1	31.3	1	Ref	-
	Antimicrobial use	*Effect on sow*	No use	234	62.1	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	60	90	142.74	31.78-641.13	<0.0001
		Effec	No use	56	18.6	1	Ref.	-

		t on		5			
		piglet	Ceftiofur$_{in\ piglet}$	298	27.9	2.08	1.07-4.02	<0.05
			No use	750	49.7	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	113	79.6	56.98	16.13-201.30	<0.0001
Trimethoprim-sulfadiazine	**Herd**		1	1	25.4	13.14	1.48-116.78	<0.05
			2	1	22.4	6.90	0.77-61.88	0.08
			3	1	18.2	1	Ref	-
	Antimicrobial use	*Effect on sow*	No use	234	18.9	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	60	26.7	18.03	1.91-170.20	<0.05
		Effect on piglet	No use	565	12.4	1	Ref.	-
			Ceftiofur$_{in\ piglet}$	298	26.8	4.72	2.27-9.83	<0.0001
			No use	821	22.5	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	42	40.5	3.07	1.46-6.43	<0.01
			No use	750	22.5	1	Ref.	-
			Lincomycin-spectinomycin$_{in\ sow}$	113	29.2	16.17	1.88-139.21	<0.05

Tetracycline	**Herd**		1	1	45.8			<0.05
			2	1	31.5			0.06
			3	1	34.9	1	Ref	-
	Antimicrobial use	*Effect on sow*	No sign. effects					
		Effect on piglet	No use	856	36.0	1	Ref.	-
			Amoxicillin$_{in\ piglet}$	7	42.9	2.49	1.20-5.18	<0.05
			No use	565	28.9	1	Ref.	-
			Ceftiofur$_{in\ piglet}$	298	34.2	2.11	1.17-3.79	0.01
			No use	821	35	1	Ref.	-
			Enrofloxacin$_{in\ piglet}$	42	57.1	2.72	1.45-5.10	<0.01

Antimicrobial use data in sows and piglets.

Table 3 shows the TI_{UDD} for the three herds at individual animal level (sows and piglets) during the prepartum period (from insemination until 1–3 days before parturition), within 24 hr after birth and at weaning (21–28 days after parturition). No antimicrobials at all were used in sows at herd 2 during gestation (prepartum) or lactation (postpartum). Herds 1 and

3 used antimicrobials in sows within 24 hr after birth. The highest use was reported in herd 3, where all of the 20 sows were treated with lincomycin-spectinomycin in the feed. All of the three herds used antimicrobials in piglets between birth and weaning age (21 or 28 days). For piglets, the highest use was reported at herd 2. No use in piglets was registered between 14 days of age and weaning.

Table 3. Treatment incidences (TI) at animal level (sows and piglets) prepartum, at birth and at weaning for the three sampled herds.

Treatment at sow level					Treatment at piglet level				
Herd	Time point	Number of sows treated[a]	Antimicrobial used	TI_{UDD}[b]	Herd	Time point	Number of piglets treated	Antimicrobial used	TI_{UDD}[b]
1	Prepartum[c]	0	-	0	1	Birth[d]	0	-	0
	Parturition[d]	5	Marbofloxacin	8.7		Weaning	1	Amoxicillin	142.9
	Weaning[e]	5	Marbofloxacin	7.0			1	Amoxicillin	107.1
							2	Amoxicillin	71.4
							6	Enrofloxacin	35.7
2	Prepartum	0	-	0	2	Birth	0	-	0
	Parturition	0	-	0		Weaning	11	Enrofloxacin	35.7
	Weaning	0	-	0			60	Ceftiofur LA	178.6
3	Prepartum	0	-	0	3	Birth	0	-	0
	Parturition	20	Lincomycin-spectinomycin	52.2		Weaning	6	Enrofloxacin	95.2
	Weaning	20	Lincomycin-spectinomycin	44.1			6	Enrofloxacin	142.9

[a] Total number of sows per herd = 20; total numbers of piglets per herd = 60.

[b] TI_{UDD} = Treatment Incidence at the individual animal level (sow or piglet). The treatment incidence is defined as the number of days per 1,000 that one pig is treated with one UDD_{pig}. The UDD_{pig} is the administered dose of a drug per day per kilogram pig.

[c] The number of days at risk = from day of insemination until 1–3 days before parturition (112–114 days).

[d] The number of days at risk = from insemination until parturition (115 days).

[e] The number of days at risk = from insemination until weaning (136–143 days).

[f] The number of days at risk = not applicable.

[g] The number of days at risk = from birth until weaning (21–28 days).

TI, treatment incidences; UDD_{pig}, used daily dose pig.

–, no antimicrobials were used.

Fixed effects

Differences in resistance prevalence were observed between herds for all tested antimicrobials, except for amoxicillin/clavulanic acid. Herd 1 showed the highest levels of antimicrobial resistance for all animals tested ($p < 0.05$) (Table 2).

For amoxicillin/clavulanic acid, where only very low levels of resistance were observed, no effect was seen on the resistance outcome after antimicrobial exposure in sows and piglets. In general, antimicrobial exposure to the piglets solely resulted in higher odds for a positive resistance outcome in the piglets themselves (higher odds for ampicillin, ceftiofur, enrofloxacin, streptomycin, trimethoprim-sulfadiazine, and tetracycline; $p < 0.05$; Table 2) and did not result in higher odds for a positive resistance outcome in their dam at weaning, except for the administration of enrofloxacin to the piglets (enrofloxacin use in piglets versus no enrofloxacin use in piglets resulted in odds ratio for enrofloxacin resistance in sows = 4.04, $p < 0.05$). Furthermore, antimicrobial exposure to lincomycin-spectinomycin in sows resulted in higher odds for a positive resistance result in the sows themselves (for ampicillin, streptomycin, and trimethoprim–sulfadiazine; $p < 0.05$; Table 2) and in their piglets (for ampicillin, enrofloxacin, streptomycin, trimethoprim–sulfadiazine, and tetracycline; $p < 0.05$; Table 2). In contrast, no effect at all was seen for the administration of marbofloxacin to the sows, neither for sows nor for piglets, however, only five sows were treated with this antimicrobial agent.

A positive resistance outcome for streptomycin and ampicillin in sows before parturition and at parturition, respectively, resulted in higher odds for resistance to the respective antimicrobials in their piglets at birth ($p < 0.05$). Positive resistance outcomes for tetracycline in sows at parturition resulted in higher odds for resistance to tetracycline in their piglets at 14 days after birth ($p < 0.05$).

Random effects

The chance on a positive resistance outcome significantly ($p < 0.05$) differed between nests (sow with three piglets) for ampicillin, enrofloxacin, trimethoprim-sulfadiazine, and tetracycline. The correlation of the resistance outcome between animals within a nest was 4.6%, 38.9%, 14.4%, and 6.8% for ampicillin, enrofloxacin, trimethoprim-sulfadiazine, and tetracycline, respectively. Furthermore, individual animal variances of outcomes measured at different time points were seen for ampicillin, enrofloxacin, streptomycin, trimethoprim–sulfadiazine, and tetracycline (significant random intercept on the individual animal level $p < 0.05$). The correlation of the resistance within an animal was 7.7%, 5.6%, 15.3%, 9.1%, and 10.9%, respectively, for ampicillin, enrofloxacin, streptomycin, trimethoprim–sulfadiazine, and tetracycline.

DISCUSSION

The occurrence of clinically resistant *E. coli* isolates in piglets between birth and weaning may be called substantial with almost half of the isolates being resistant to one or two antimicrobials and more than a quarter of them being resistant to at least three antimicrobials.

During farrowing, *E. coli* isolates from sows were found more often resistant to at least three antimicrobials compared with piglet isolates. However, resistance to at least four antimicrobials was found as frequently in sows as in piglets (15.6% and 15.2% of the isolates in sows and piglets, respectively). High levels of antimicrobial resistance in young animals have been reported before (Langlois et al., 1988; Mathew et al., 2003; Dewulf et al., 2007; Stannarius et al., 2009). The presence of resistant *E. coli* strains in newly born piglets before any direct selection pressure, might put the sow forward as a resistance reservoir for her offspring, since young piglets are continuously exposed to the sow's microflora. Similarly, resistant *E. coli* have been found in young calves not previously exposed to antimicrobials (Berge et al., 2005). The individual treatment of the dam has been proposed as a risk for the persistence of a resistant microflora, acting as a commensal resistance pool for the offspring.

Results from this research show a higher resistance frequency in isolates from pigs suckling sows with exposure to in-feed lincomycin-spectinomycin, although only used in one herd. Again, this might be the result of the transfer of resistant bacteria from the dam to her offspring. However, also, as the majority of orally administered spectinomycin is excreted unchanged in the feces (Giguère, 2006), this might form a direct selection pressure for suckling piglets. Treating sows and/or piglets involves a disturbance of the *E. coli* population, without inventing de novo or changing the presence of the antimicrobial resistance determinants itself, but by the selection of the less susceptible subpopulation.

The use of intramuscular marbofloxacin in the sow did not appear to affect resistance in *E. coli* isolates in piglets or in *E. coli* isolated from the sows themselves. Marbofloxacin is mainly excreted in the urine and the excreted residues are in an active form (Walker and Dowling, 2006). As a result, the residues might engender a selection pressure on the bacteria of animals in the surroundings. Yet, no link was found between the administration of marbofloxacin in sows and resistance in either the

treated sows or in their piglets. This might be because of the treatment of too little sows (n = 5), which lowers the power

for detection of any trend.

The use of antimicrobials in piglets increased the prevalence of resistance in the piglets themselves. In this study, no effect of use in piglets (amoxicillin and ceftiofur) on the presence of resistance in their dam was seen, except for enrofloxacin use in piglets toward enrofloxacin resistance in the sows. Resistance to quinolones is mainly determined by a mutation process, which is enhanced by antibiotic concentrations within

the mutant selection window (Smith et al., 2003). Small amounts of excreted enrofloxacin in urine from piglets might engender a selection pressure and select for mutants less susceptible to enrofloxacin. Resistance to amoxicillin and cephalosporins, antimicrobials which are equally excreted in urine (Prescott, 2006), have other resistance mechanisms, for which the mutant selection window is less appropriate (Smith et al., 2003).

One of the herds showed significantly higher resistance outcomes compared with the other herds, whereas the extent to which antimicrobials were used in the currently observed sows in the current production cycle was not proportionally higher in comparison to the other herds. Yet, also the antimicrobial use in the other age categories and in the previous production rounds is likely to influence the resistance levels, as it maintains a commensal resistance pool, as previously described. In this study, no other herd-related factors than antimicrobial use, which also might influence the selection and spread of resistance determinants, were investigated.

For most antimicrobials, a significant correlation between the occurrences of resistance in animals in one nest was observed, indicating that the resistance level in animals within a nest is more alike than from animals in different nests. Only amoxicillin/clavulanic acid and ceftiofur were not consistent with these results. Yet, the low resistance percentages noticed for these antimicrobials may partly explain this divergence. Although the observed correlations are significant for most antimicrobials, they are not always very high, illustrating that substantial different results can be found between animals in the same nest. This is likely the result of the fact that only two isolates are tested per animal, whereas a variation of *E. coli* populations is expected to be present within the gut of each animal.

CONCLUSION

In conclusion, the current results suggest that sows act as a reservoir for their newborns and that the administration of antimicrobial agents to sows during lactation is a risk factor for the persistence of resistant *E. coli* not solely for themselves, both also for their newborn offspring. Also, the administration of antimicrobials in the piglets exerts a direct selection pressure in the piglets themselves during lactation.

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CHAPTER 7

GENERAL DISCUSSION

MONITORING ANTIMICROBIAL USE AND RESISTANCE

The emergence of antimicrobial resistance in commensal, zoonotic, and pathogenic bacteria from pigs, mainly as a result of antimicrobial consumption, and potentially threatening treatment options in both human and veterinary medicine, is worrisome. Closely monitoring antimicrobial use and antimicrobial resistance levels are both important first steps in strategies aiming to contain antimicrobial resistance. Commensal bacteria, commonly present in animals, are exposed to any antimicrobial administration and therefore good indicators for antimicrobial resistance. The first aim of this thesis was to quantify group use of antimicrobial agents in Belgian pig herds.

ANTIMICROBIAL USE IN BELGIAN PIG PRODUCTION

WHAT HAS (NOT) CHANGED SINCE 2003 AND WHAT HAS TO BE DONE?

A first estimation on the use of antimicrobials in Belgian swine production was made in 2003. Since then, no antimicrobial use data have been collected through single monitoring studies or on a continued basis. Therefore, at the onset of this thesis in 2009, it was questioned how this situation did evolve. The objective of the study on antimicrobial use in the Belgian pig production was to determine the quantitative and qualitative group use at herd level for pigs between birth and slaughter for a representative number of herds of the national population (***Chapter 3***). These data were preferably compared to the 2003 antimicrobial use data on a similar study population, including equal sample size. Currently, there is no consensus on the best indicator to be selected to quantify antimicrobial consumption in animals (OIE, 2014a). Treatment incidences (TI) based on the defined daily dose animal ($DDDA_{pig}$) and on the used daily dose animal ($UDDA_{pig}$), used in the former Belgian study on antimicrobial usage, allowed for the quantification of the number of animals being treated in a herd and for a trend analysis of antimicrobial consumption over time. TI based upon $DDDA_{pig}$ take into account variation in potencies (e.g. higher in frequently used 3^{th} and 4^{th} generation cephalosporins) and were thus more suitable as a technical unit of measurement than weighing active substances used relative to the animal population at risk (Jensen et al., 2004). Information on indications, correctness of dosing, prophylactic or metaphylactic use, active substances and administration routes applied, were all part of the primary objective of this study. In order to quantify the truly administered doses, i.e. $UDDA_{pig}$, detailed data such as the numbers of pigs at risk of being treated, the weight at treatment and the treatment length were needed. This required direct access to the herd data. Since at that time no organized structures were present that facilitated the monitoring of antimicrobial usage patterns at the individual herd level, a questionnaire during a face-to-face on site interview with the farmer was performed.

Conclusions of the report on antimicrobial use data collection in 2003 included a high prophylactic group level use and incorrect dosing of most treatments (Timmerman et al., 2006). In the beginning of the 2000s, first

available antimicrobial usage data for pigs equally showed a high antimicrobial use in the Netherlands and Denmark, as Belgium, countries with substantial levels of pork production (LEI, 2011; DANMAP, 2013). Concerns about the risk for antimicrobial resistance selection and spread related to the use of antimicrobials in pigs, as well as other food animals have prompted several institutions, including veterinary specialty practice organizations, to develop and publish judicious use guidelines (RUMA, 2013; EPRUMA, 2015; FVE, 2015). Every use of antimicrobials should be preceded by a proper clinical examination of the animal. Both clinical experience of the veterinarian and diagnostic laboratory information (pathogen isolation and identification) should be part of the final diagnosis. The above excludes the use of antimicrobials as prophylaxis, as animals are then treated at times, it is expected that they will become affected but do not (yet) exhibit symptoms of a disease. Laboratory confirmation of the bacterial pathogen as well as an accurate assessment of its *in vitro* antimicrobial susceptibility form the ideal basis for choosing the most appropriate antimicrobial agent (as narrow as possible). Also, clinical experience of the veterinarian will determine the expected efficacy of the treatment, if this treatment is initiated before the results of laboratory testing are available. For this, the herd veterinarian should be guided by the epidemiological history of the pig herd, particularly in relation to the antimicrobial resistance profiles of the pathogens involved. Regarding this antimicrobial choice, special attention has to be paid to the OIE and WHO list of antimicrobial agents of veterinary and human importance, which both indicate quinolones and $3^{rd}/4^{th}$ generation cephalosporins as critically important (WHO, 2011; OIE, 2014b). Their use should be restricted to final resort and after susceptibility testing. Finally, the appropriate dosage/duration regimen, as mentioned on the prescription leaflet, should be followed.

Compliance between results from ***chapter 3*** and the above described guidelines, can be assessed in order to verify to what extend these guidelines were implemented in Belgian pig production.

Usage data collected in 2010 (***Chapter 3***) revealed an average TI, based on the $DDDA_{pig}$ and on the $UDDA_{pig}$, equal to 235.8 and 200.7, respectively. These numbers indicate that Belgian pigs were being administered antimicrobials (for preventive or metaphylactic reasons) on average during 20% of their life time. Numbers for 2010 were substantially higher than what was found in 2003 ($TI_{DDDApig}$ and $TI_{UDDApig}$ equaled 178.1 and 170.3 respectively) suggesting that, in between, there was a clear rise in the average antimicrobial usage in pigs. Moreover, results for 2010 showed that only one out of the 50 questioned herds (2%) did not administer antimicrobials in group (***Chapter 3***), where six out of the 50 herds (12%) did not use group treatments in the 2003 study (Timmerman et al., 2006). A random sampling of the population may suffer from the willingness for farmers to participate, which likely results in a selection bias. Yet, since data collection and quantification of drug consumption was performed in a comparable manner in 2003 and 2010, observed differences are believed to be a true representation of the evolution over these years. Based on these results it became clear that preventive group treatments have become, although not compliant with guidelines on no

preventive antimicrobial use, more than ever, a habit in the daily management of raising pigs. This illustrates that antimicrobials are often considered as a management tool administered for prophylactic reasons to entire groups of animals. Data from our study did not include any antimicrobial administration for curative reasons. Given that pig farmers in Belgium were only required to keep records of antimicrobial treatments for 2 months prior to the slaughter of animals, it was very difficult to obtain reliable retrospective information on the therapeutic use of antimicrobials throughout the production cycle. In a recent study of Postma et al. (2014), also large between herd variation in curative treatments has been observed, indicating that also this curative use may still contribute substantially to the total amount of antimicrobials administered in a herd. Furthermore, no data on antimicrobial usage in breeding animals were included. Recently, for Belgian pig herds, it has been estimated that sows are treated for prophylactic and curative reasons during 7% of the time of one breeding cycle (Postma et al., 2014). Usage in sows is therefore estimated to be much lower than in fattening pigs. Yet, results from ***Chapter 6*** indicate that antimicrobial exposure of sows prior to farrowing and during lactation can increase antimicrobial resistance in the bacteria of their offspring. Although the prevalence of resistant *E. coli* is decreased in elderly pigs, sows can still act as a reservoir for resistant bacteria to their newborns (***Chapter 6***).

From the above, it is clear that antimicrobial usage in pig production is very high and that the reported data from this study (prophylactic and methaphylactic group level use for fattening pigs between birth and slaughter) are still an underestimation of what is truly administered to all pigs on a herd. Therefore, a more precise estimation requires collection of antimicrobial usage data for all reasons via the different administration routes (preventive, metaphylactic and curative treatments via feed and water, parenteral and local) and for all categories on a farm (nursery pigs, weaned pigs, fattening pigs and sows) on a continued basis by a well-established data collection system.

The frequent incorrect dosing reported by Timmerman et al. (2006) was reaffirmed by the fact that 82% of the administered doses in our study were incorrect (79.5% overdosed and 47.3% underdosed for injectable and oral products respectively) and reflects the negligent handling when it comes to antimicrobial drug administration to pigs (***Chapter 6***).

The increased portion of long acting injectable antimicrobial formulations (long acting amoxicillin, ceftiofur and tulathromycin) as well as broad spectrum substances (3rd and 4th generation cephalosporins) in the number of treatments compared to 2003 clearly shows that farmers intend to choose for new and easy-to-use formulations instead of substances who have been licensed for a longer period, as doxycycline and potentiated sulfonamides (trimethoprim-sulfonamides).

Third and 4th generation cephalosporins have antibacterial activity against Gram-positive (aerobic and anaerobic), Gram-negative aerobic and to a lesser extent Gram-negative anaerobic bacteria (Giguère, 2013). Therefore, they belong to the antimicrobial classes with the broadest spectrum. A bacterial diagnosis can be

considered irrelevant, since their use covers almost the entire spectrum of routinely cultured bacteria. However, their use also results in a broad antimicrobial resistance selection pressure on both pathogenic and commensal bacteria. Therefore, the majority of the use of these broad spectrum substances is not in agreement with the concepts of responsible antimicrobial use. It is highly questionable whether they are necessary to control animal disease problems. The tendency towards the use of new, long acting substances should be stopped in order to minimize antimicrobial selection pressure. While old substances, such as tetracyclines, trimethoprim and penicillin have a more narrow spectrum, their use should equally be reserved as they might act as (co-)selectors for LA-MRSA (Kadlec et al., 2012). A restricted use of critically important 3rd and 4th generation cephalosporins and quinolones should result from the implementation of specific recommendations on antimicrobial use for animal species per indication, developed in Belgium by AMCRA, Centre of Expertise on Antimicrobial Use and Resistance in Animals. Active substances within these classes are assigned a red colour code and are always placed as the final choice option for any bacterial infection in all animal species. Substances with a red colour code must only be used when results of the antimicrobial susceptibility testing clearly show that the bacterial isolate is not susceptible to compounds with a spectrum less broad than the one of 3rd and 4th generation cephalosporins and quinolones. From this, it can be concluded that these red molecules are identified 'the last remaining resort', and awareness among users is stimulated. When strictly applying such an approach, the use of the red molecules is expected to be reduced substantially, as examples in other countries have demonstrated (MARAN, 2014).

Results in ***chapter 3*** show that pigs are more often exposed to oral antimicrobial therapy ($TIDDD_{DDDA}$, oral = 183.5 and TI_{UDDA}, oral = 176.5) than injectable administrations (TI_{DDDA}, injectable = 52.3 and TI_{UDDA}, injectable = 24.2). Moreover, 75 % of the oral administrations are in-feed. BelVet-SAC confirms the high use of medicated feed in pigs by reporting 59.74 tons of antibacterial premixes being used in 2010 in veterinary medicine, of which more than 99% is intended for the pig sector (BelVet-SAC, 2015). As a result, as part of the achievement of a substantial antimicrobial use reduction target in pigs, particular attention should be given to the use of antimicrobials in medicated feed. The in feed mixing in the compound feed company is highly controlled, offering advantages regarding the homogeneity and dosing of the antimicrobial substance. The threshold for use of medicated feed is low and one might question whether this is still acceptable in the current climate of sustainable antimicrobial usage, as was discussed in the recent report of the Scientific Committee of the Federal Agency for the Safety of the Food Chain (2013). Between 2011 and 2013, a 10.2% decrease in medicated feed use has been realized, although in 2014, an increase of 0.2% was seen again (BelVet-SAC, 2015). A continuation and solidification of the decrease will be needed to achieve the 50% reduction in 2017 compared to what was used in 2011. The latter is set as an objective in the vision statement, developed by all member sectors of AMCRA in 2014, defining the guidelines of the antimicrobial use and resistance policy among animals in Belgium (AMCRA, 2014). The authorization to prescribe

medicated feed only by the veterinarian responsible for guidance might substantially contribute to this reduction.

In 2003, in Belgium, data on antimicrobial use from other intensive animal production systems were not yet available. Meanwhile, TI have also been reported for the broiler and veal calf production (Pardon et al., 2012; Persoons et al., 2012). Based upon these results it has become clear that the pig production is the second highest user per produced animal, preceded by the veal calf production (TI_{UDDA} equaled 416.8) and followed by broiler chicken production (TI_{UDDA} equaled 121.4). Recently, an attempt to estimate the proportion of use in tons in the whole population of these different species in Belgium was made taking into account the different size of the production sectors (Filippitzi et al., 2014). Based upon this rough estimate it was concluded that the vast majority of antimicrobials used in Belgium were administered to pigs (159.4 tons), followed by broilers and veal calves (26.5 tons and 25.2 tons respectively) (Filippitzi et al., 2014). This clearly suggests that the pig sector can make a significant contribution to the required decline in the tons of antimicrobials used in the veterinary sector and therefore carries a large responsibility.

Data on antimicrobial use from 2010 reinforced the concerns already expressed in 2006 by Timmerman et al. (2006). Previously formulated guidelines and recommendations did not result in action towards a reduced and responsible use.

COLISTIN USE IN ANIMALS

In Belgian pigs, intervening with post-weaning *Escherichia coli* diarrhea and edema disease is one of the main reasons for prophylactic and metaphylactic group treatments during the stressful nursery period (***Chapter 3***). Before the introduction of zinc oxide (ZnO) at pharmacological doses in 2013, *E. coli* diarrhea in nursery pigs in Belgium has been prevented and controlled mainly via the administration of colistin in-feed and to a lesser extent in water (+/-75% and 25% of the administrations respectively) (***Chapter 3***). In 2012, the total colistin consumption in medicated feed for pigs in Belgium was approximately 2500 kg active substance on a total of 55 000 kg active substance used in medicated feed (4,5%) (BelVet-SAC, 2013). The contribution of colistin in the total antimicrobial use for the control of enteric diseases (prophylactic and metaphylactic) in pigs has been estimated at 55% (***Chapter 3***). The high intrinsic susceptibility of *E. coli* and the poor absorption of colistin in the intestine are important criteria to select colistin in the treatment of neonatal or post-weaning *E. coli* infections in food producing animals (Landman et al., 2008). Also, colistin has high stability, relative low toxicity via oral administration, no cross-resistance with other antimicrobials, and slow and unstable emergence of resistance (Dowling, 2013b).

Consequentially, the extended oral use of colistin in pigs and other animals, targeting intestinal pathogens such as *E. coli* and *Salmonella enterica* (Koyama et al., 1950; Timmerman et al., 2006***; Chapter 3***), requests stringent monitoring of colistin susceptibility, including molecular identification of resistance determinants.

Increasing occurrence of colistin resistance in veterinary important bacteria has only recently been reported (Harada et al., 2005; Boyen et al., 2010; de Jong et al., 2012). This might partially be due to relatively recent attention combined with the poor disk diffusion of colistin in agar. The frequently used disk diffusion technique is thus less appropriate for this antimicrobial agent. More appropriate phenotypic susceptibility tests, such as dilution techniques or disk prediffusion are necessary for proper detection (Boyen et al., 2010).

In Belgium, a trend analysis of the colistin susceptibility test results of national monitoring studies in 2011, 2012 and 2013 has been made for commensal *E. coli* isolates, obtained from healthy animals, including pigs (CODA-CERVA, 2014a). Colistin resistance increased slightly in *E. coli* from pigs (0.64%, 0.92% and 2.43% non-wild type strains in 2011, 2012 and 2013 respectively) and fluctuated in *E. coli* from broilers (0.48%, 4.69% and 1.71% non-wild type strains in 2011, 2012 and 2013 respectively). Colistin resistance has equally emerged in beef cattle, but remains at acceptable low levels (CODA-CERVA, 2014a). In veal calves, the % of non-wild type strains was high in 2011 (14.71%), but decreased in 2012 and 2013 (6.08% and 5.94% respectively) (CODA-CERVA, 2014a). Although not statistically significant, the increasing trend in pigs warrants for further monitoring. Compared to *E. coli* from clinically healthy pigs, a higher percentage of colistin resistance (9.6%) was observed for *E. coli*, isolated from clinically affected pigs in Belgium, using the same epidemiological cut-off value (MIC > 2 μg/ml) (Boyen et al., 2010). A pan-European survey across 8 EU countries, including Belgium, during 2009-2012, showed a growth inhibition of at least 90% of the *E. coli* isolates from animals with clinical signs, at a concentration of 4 μg/ml (MIC_{90} value of 4 μg/ml) (Klein et al., 2015).

In the scope of the high colistin use in Belgian pigs (***Chapter 3***), Moore and Elborn (2012) expressed their concerns on the emergence of colistin resistance in *E. coli* in animals and the possible linked failure of colistin therapy in humans, for instance in the treatment of cystic fibrosis infections in humans, mainly caused by *Pseudomonas aeruginosa*. In human medicine, colistin has reemerged as a last resort therapeutic option to treat infections due to multi-resistant bacteria, including non-fermenting Gram-negative pathogens (*e.g. Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and for carbapenemase producing *Enterobacteriaceae* (*e.g. Klebsiella pneumoniae* and *E. coli*) (Li et al., 2006). Therefore it can be questioned whether the use of colistin in the treatment of infections with these multi-resistant *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli* might be jeopardized by the extended colistin use in veterinary medicine (Moore and Elborn, 2012).

Therapeutic failure of a bacterial infection with an antimicrobial in humans, due to the selection of resistant bacteria in the animal microbiota by this same antimicrobial, supposes the spread of the animal-related resistant bacteria to humans, the colonization with resistant bacteria (same clone) or horizontal transfer of resistance genes to other bacteria in the human microbiota. For colistin resistance, one must bear in mind that the resistance mechanisms are defined by chromosomal mutations and until now, horizontal gene

transfer of colistin resistance has not yet been demonstrated (Landman et al., 2008). Thus, there is no evidence that colistin resistant bacteria transfer their resistance genes from animals to human pathogens or vice versa. Also, with the exception of some well-examined clinical strains, many of the mutation mechanisms are not stable after several passages *in vitro* (Moskowitz et al., 2012). Therefore, a potential hazard of colistin resistance in bacteria associated with animals is estimated to be limited and probably only involves zoonotically important bacteria, such as *S. enterica*. As far as we know, there are at present no indications that animal associated bacterial strains are involved in cystic fibrosis in humans. Nevertheless, based on current data, transmission of such resistance cannot be absolutely excluded. For other antimicrobial drug resistant organisms including *E. coli*, the emergence and transfer of resistant bacteria via direct animal contact or via food has been documented (Angulo et al., 2004). Further research on the potential transfer of resistance in which human and animal derived *P. aeruginosa* and *A. baumannii* strains are compared between animal related bacteria and Gram-negative bacteria from humans is warranted. In addition to the use of colistin, other antibiotic classes (*e.g.* glycylcyclines (tigecycline) and phosphonic acid derivates (*e.g.* fosfomycin)) can be used to combat infections caused by multi-resistant Gram-negative bacteria and have been reclassified as critically important antibiotics (WHO, 2011). Fosfomycin is active *in vitro* against multi-resistant *Enterobacteriaceae*, including a high proportion of *P. aeruginosa*. However, clinical studies on parenteral formulation are limited (Falagas et al., 2008; Reffert and Smith, 2014). Tigecycline, which is active against multi-resistant *Enterobacteriaceae* and *A. baumannii*, has shown satisfactory clinical experience (Giamarellou, 2010). Recently, a novel aminoglycoside, ACHN-490, has shown to have *in vitro* activity against multidrug-resistant *K. pneumoniae* isolates (Endimiani et al., 2009).

The veterinary community should be aware that limited options of antibiotics (including colistin) are available to treat multi-resistant Gram-negative infections in human medicine and that at the same time resistance to these agents might be on the rise. As soon as colistin resistance determinants are found on mobile genetic elements in the bacteria of concern from human or animal origin, or a clonal explosion of virulent bacteria takes place, a new risk assessment would be required (EMA, 2013).

MEASURES TO MANAGE

There is an increased societal demand for sustainable animal production and intensive antimicrobial use is negatively perceived by the general public which might hamper pork consumption. Moreover, pig production represents 83% of animal meat production in Belgium and is the main part of the human meat supply inside Belgium (VLAM, 2014). In order to safeguard animal and human health and to maintain its market position, the pig production will need to invest in a more sustainable pork production including, amongst others, using less antimicrobials. A continued high antimicrobial usage jeopardizes both animal and public health through the emergence of resistant bacteria. Action must be undertaken at this level by defining an antimicrobial use policy which includes regulation of and control on implementation of responsible use guidelines. Though in order to succeed, several other factors must accompany this reduced usage. Antimicrobial stewardship is the term increasingly used in acute care medicine to describe the multifaceted approaches required to reduce usage and avoid resistance selection to sustain the efficacy of antimicrobials in the long run. Below, measures to manage antimicrobial use in swine husbandry are discussed.

A CONTROLLED ANTIMICROBIAL USAGE

Monitoring studies from 2003 (Timmerman et al., 2006) and 2010 (***Chapter 3***) on antimicrobial use, along with the estimations of the tons used in the pig sector (Filippitzi et al., 2014) provide insights in the extent to which intensive pig production in Belgium consumes antimicrobials. Yet, the establishment of a data collection system (DCS), providing usage data at herd level on a continues basis, is needed to make further actions possible. This DCS - at the end-user level - enables mapping of antimicrobial use per animal species and production category (nursery pigs, weaned pigs, fattening pigs and sows), the follow-up of evolutions in time on a herd and within an animal category and the benchmarking towards fellow-herds. The collection at the end-user level enables to identify ‘outliers’ for high and/or irresponsible use, and thus takes a central role in tackling undesired quantitative and qualitative aspects of use. The threshold set for defining outliers can be defined as the 5, 10, 15,… % highest users. As the identification of outliers is based on benchmarking, ideally, all pig herds should be included. In Belgium, a first initiative for data collection at the herd level has been taken by the pig private sector, namely the quality label Certus, managed by Belpork vzw. In the framework of this initiative, approximately 60% of the pig herds in Belgium, collection of data on antimicrobial consumption at herd level is mandatory from January 2014 onwards. It is aimed for that other pig holders will adhere voluntarily and that the system grows to a coverage of the whole sector. Identification of herds with a high antimicrobial use based on benchmarking is reliable only when data collection is mandatory for all pig farmers.

The establishment of a data collection system must be well-considered both to assure clear and correct feed-back to farmers and veterinarians in order to increase awareness on usage patterns and to assure a

comparability of the collected data between countries. For these farms with an antimicrobial use above the thresholds, adaptations of the management will be required. These can either be voluntary implemented or might be reinforced through legislative actions.

Communicating precise data on antimicrobial use and if deemed necessary, followed by restrictions imposed by law, should guarantee the current armory of antimicrobials to be sufficient for the foreseeable future. Questions on the development of new antimicrobials have been put forward worldwide. Yet, it must be remembered that most of the antimicrobials used in pigs are already over thirty years old and that the majority of them are still working. Even the more modern antimicrobials such as 3rd and 4th generation cephalosporins, if used carefully and not for widespread prophylaxis, should maintain their efficacy. Furthermore, one must not forget that resistance determinants are naturally present and that they benefit from the use of antimicrobials for their spread in the ecosystem. As a result, the development of new antimicrobials is a 'self-sustaining process' with a high cost and a low rate of return on investment compared with drugs for the treatment of chronic conditions. Focus should be on research in porcine health, increasing the possibilities for new, innovative solutions, such as vaccines, which could have a large potential. Also, better evidence based treatment and improved treatment strategies with less impact on problematic resistance are important objectives. Effective dosing should be seen as targeting bacterial cure rather than clinical cure (Pankey and Sabath, 2004).

More action can be taken in controlling antimicrobial use. The *Summary of Product Characteristics* (SPC) for currently authorized products should be reviewed to ensure consistency for measures to ensure responsible use. For instance, it is considered appropriate to remove all indications for prophylactic use in order to minimize any potential risk associated with a broader use. Yet, from our results (***Chapter 3***), it appears that in practice, the distinction between prevention and treatment is vague. In fact, much of the use of antimicrobials that is claimed to be therapeutic is intended to control disease and often involves mass medication. Furthermore, the inclusion of new indications, formulations or species on the prescription leaflet of currently authorized products should be subject to full antimicrobial resistance risk assessment before approval.

DRIVERS FOR IMPROVED HEALTH

Large between herd variations in antimicrobial use have been reported (***Chapter 3***, Timmerman et al., 2006). Understanding differences in disease incidence, management, husbandry, biosecurity, hygiene, feed, genetics as well as in farmer and veterinarian attitudes at the farm level might help to explain the observed differences in treatment incidences (Hybschmann et al., 2011; Laanen et al., 2013; Visschers et al., 2015). First attempts on quantifying the effect of biosecurity on production results and antimicrobial treatment incidence in breeder-finisher pig herds through a risk-based weighted biosecurity scoring system showed that herds scoring higher in biosecurity equally had a more efficient production (Laanen et al., 2013; Postma et al., 2014; Backhans et

al., 2015). Moreover, higher biosecurity has been shown to be negatively associated with disease treatment incidence (Laanen et al., 2013; Postma et al., 2014). This suggests that improved biosecurity might help in reducing the amount of antimicrobials used prophylactically. In order to reduce antimicrobial resistance in piglets, control measures should be implemented, at first, at the sow level, involving minimal antimicrobial use and high hygienic standards (***Chapter 6***). Given the fact that the youngest animals are the most susceptible for infectious diseases and thus that the majority of strategic group treatments in pigs are given during the farrowing/battery phase (***Chapter 3***), the greatest factor impacting on the frequency of antimicrobial usage is to be expected in this part of the production cycle. In some European countries, ZnO at pharmacological doses has been introduced to control post-weaning *E. coli* infections (FCEC, 2010). Since August-September 2013, Belgium is the 11th country in the EU with a (temporary) approval for the use of pharmacological doses of ZnO. The use of ZnO might have contributed to the decrease in colistin use in medicated feed between 2012 and 2013 (23%). Questions raise about the long term use of ZnO in the field. Although it promotes health and performance in piglets, ZnO also has antibacterial properties and is a heavy metal with a potential environmental burden. In order to control the emission in the environment, an agreement has been reached between the Belgian government, compound feed manufacturers and feeding companies to reduce the amount of zinc used as a feed additive from 150 ppm to 110 ppm in the fattening phase in order to compensate for the high dosage of Zn (2500 ppm) given for maximal 14 days after weaning. Even though the dosing schedule during fattening leads to a total decrease of 4.5% of the environmental Zn burden, the search for alternatives to both antimicrobials and heavy metals should be continued. Resistance to Zn does occur in bacteria. Moreover resistance against this metal may co-select for antimicrobial resistance, as has been described for livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) (Cavaco et al., 2011). Consequently, ZnO might not to be the best alternative. Ideally, as for any compound with antibacterial effects, the use of ZnO at the farm level should be nationally monitored. In the presence of antimicrobial resistance data, a potential link between ZnO use and antimicrobial resistance might then be detected in an early stage. Also, links between ZnO used at pharmaceutical doses and the use of other veterinary medicines might then be analyzed. In conclusion, one might wonder to which extent the replacement of colistin by pharmacological doses of ZnO will contribute to a decreased antimicrobial resistance, the ultimate goal of a reduced antimicrobial use. Its use might in contradiction result in an undesired increased selection pressure on resistance determinants. This might include the risk of compromising porcine health and welfare.

ANTIMICROBIAL RESISTANCE IN BELGIAN PIG PRODUCTION

To assess the impact of the antimicrobial usage, described in ***chapter 3***, on antimicrobial resistance trends in commensal and pathogenic bacteria of pigs the susceptibility of *Streptococcus suis* and *Escherichia coli* of clinically healthy pigs at the end of the fattening period was tested (***Chapter 4, Chapter 5, Chapter 6***).

ANTIMICROBIAL RESISTANCE IN *STREPTOCOCCUS SUIS* STRAINS FROM BELGIAN PIGS

S. suis, mainly associated with meningitis, affecting piglets principally between 6 and 10 weeks old, is one of the most important swine pathogens worldwide (Gottschalk et al., 2000). In the two monitoring studies on antimicrobial use in Belgium (Timmerman et al., 2003; ***Chapter 3***), it was observed that *S. suis* infections are mentioned as one of the predominating reasons for antimicrobial treatment. This is in agreement with antimicrobial administration as currently being the most widespread preventive measure for *S. suis* infections (Staats et al., 1997; Prescott, 2013). *S. suis* can be isolated from diseased as well as from healthy carrier pigs as they can colonize the tonsils, the nasal cavity, the intestines and the reproductive tract of sows without causing clinical disease (Staats et al., 1997). In this respect, *S. suis* is, as colonizing bacterium, subject to any antimicrobial selection pressure from systemically administered antimicrobials. Results from ***chapter 4*** on *S. suis* susceptibility levels based on clinical interpretive criteria could only be documented for erythromycin, tetracycline, penicillin and florfenicol, as no clinical breakpoints are available for the other antimicrobials tested (CLSI, 2013). *S. suis* was shown to be almost entirely susceptible to florfenicol and penicillin (clinical resistance equaled 0.3% and 1%), whereas high levels of resistance to erythromycin and tetracycline were seen (66% and 95% respectively). Genes encoding resistance to macrolides are widespread among *S. suis* isolates (Wisselink et al., 2006; Zhang et al., 2008; Princivalli et al., 2009) and the high-level resistance to 14-, 15-, and 16-membered macrolides is mainly caused by the constitutive expression of the *erm*(B) gene (Martel et al., 2001; Tian et al., 2004). This gene refers equally resistance to lincosamides and streptogramins, due to methylation of the 50S ribosomal subunit, the common target of action for these classes (Martel et al., 2001). In accordance with the widespread resistance to these antimicrobials, in our study, a non-wild type population was observed for all macrolides and lincosamides tested. Currently, the clinical breakpoint used for tetracycline is only available for *S. suis* isolated from swine respiratory disease (CLSI, 2013). However, the high percentage of non-wild type strains (98%) confirmed earlier information on the widespread tetracycline resistance amongst *S. suis* isolates (Wisselink et al., 2006; Zhang et al., 2008; Princivalli et al., 2009). Tetracycline resistance is mainly encoded by the genes *tet*(M) and *tet*(O), but an increasing number of *tet* genes has been discovered in the past few years (Palmieri et al., 2011). Resistance genes to tetracycline (*tet*(M) and *tet*(O/W/32/O)) and macrolides (*erm*(B)) are often found in common mobile elements, such as transposons (Tn1545) (Seral et al., 2001; de Leener et al., 2004) and integrative and conjugative elements (ICE*Ssu32457*) (Palmieri et al., 2012). Tn1545 has a wide host range and it has been

found for instance in streptococci and enterococci from humans, pigs and poultry (Seral et al., 2001; de Leener et al., 2004; Cauwerts et al., 2007). Furthermore, it can be transferred by conjugation to several bacterial species and is therefore probably responsible for a large part of the tetracycline and macrolide resistance observed in streptococci from several hosts (Aarestrup and Schwarz, 2006). Also the ICE*Ssu32457* may be, amongst other, an important genetic vehicle for the spread of *tet*(O/W/32/O) and *erm*(B), as it is transferable between pathogenic *Streptococcus* species, such as *S. suis*, *S. pyogenes*, *S. pneumonia* and *S. agalactiae* (Palmieri et al., 2012). As in pig streptococci, *erm*(B)-encoded resistance is the most important mechanism in human streptococci *S. pneumoniae* and *S. pyogenes* in Belgium (Descheemaeker et al., 2000; Lagrou et al., 2000). Moreover, homologous sequences of the *erm*(B) gene from porcine *S. suis* and human streptococci (*S. pneumonia* and *S. pyogenes*) indicate possible exchange of resistance genes between human and porcine streptococcal strains (Martel et al., 2001). The spread of resistance determinants between streptococcal populations of humans and pigs is of particular interest as *S. suis* is considered as an emerging zoonotic pathogen responsible for septicemia with or without septic shock, meningitis, endocarditis, arthritis, hearing loss and skin lesions in humans (Goyette-Desjardins et al., 2014). *S. suis* infection is acquired from pigs, either during slaughtering or by handling and eating undercooked pork products. Intensive contact between animals and humans, such as for pig farmers and slaughter house workers, might predispose the spread of resistance determinants between bacterial populations of pigs and humans. Currently, the majority of *S. suis* cases in humans occur in South-East Asia (Goyette-Desjardins et al., 2014). Yet, from 2005 onward, an increasing number of *S. suis* infections in humans has been reported worldwide, also in countries where the infection had been rarely or never reported before (Zalas-Wiecek, 2013; Gómez-Zorrilla et al., 2014). The available information on the resistance determinants strongly suggests that *S. suis* is an important antimicrobial resistance reservoir (Palmieri et al., 2011). Therefore, they can contribute to the spread of resistance genes to other, pathogenic, bacterial populations from humans and animals. In order to find possible exchanges of resistance genes, there is an urge for more knowledge on the resistome of *S. suis*. *S. suis* isolates from pigs with pleuritis have previously been used for the continuous monitoring of antimicrobial susceptibility (Hendriksen et al., 2008). Because they are subject to any antimicrobial selection pressure from systemically, both parenteral and oral, administered antimicrobials, *S. suis* from healthy animals may act as a Gram-positive indicator bacterium when monitoring resistance over time.

The preventive or curative use of macrolides and tetracyclines to combat streptococcal infections has been almost entirely excluded due to the wide spread of resistance determinants against these antimicrobials. Belgian therapeutic recommendations, including a wide range of antimicrobials potentially useful for counteracting streptococcal infections, have placed tetracyclines as third and last treatment option in counteracting *S. suis* infections (AMCRA, 2013). Dutch antimicrobial use guidelines report *S. suis* as highly resistant to macrolides and tetracyclines (KNMVD, 2014). Yet, macrolide or tetracycline administration for any

other indication might (co-) select for resistance determinants conferring resistance to the respective antimicrobials, and engender their spread between bacterial populations within and between the host animals and humans. In ***chapter 3***, it has clearly been described that these molecules are still widely used in the Belgian pig production. In the BelVet-SAC report these molecules are presented as 3rd and 4th most frequently used substances in antibacterial premixes, which are designated for more than 99% to the pig sector (BelVet-SAC, 2015). Therefore, a continuous selective pressure is and will be present in the future as long as these substances are used.

Amoxicillin in feed is currently the most frequently administered antimicrobial for the treatment, prevention and control of *S. suis* infections in weaned piglets in Belgian pig production (***Chapter 3***). This is in line with the worldwide suggestion of β-lactams as the primary antimicrobials to treat streptococcal infections (Zhang et al., 2014). Yet, several reasons can be quoted to restrict the use of broad-spectrum aminobenzyl penicillins, a group within the β-lactams, for instance amoxicillin, to a minimum. Its broad spectrum exerts a selection pressure on commonly acquired resistance determinants in Gram-negative bacteria, such as in *E. coli* (***Chapter 5***) or *Salmonella* spp. (EFSA and ECDC, 2014), bacteria commonly colonizing pigs as both commensal and pathogen. More precisely, its use has been shown to select for extended-spectrum-beta-lactamase producing *E. coli* (ESBL) in the intestinal microbiota of pigs (Cavaco et al., 2008). Therefore, Belgian guidelines restrict the use of aminobenzyl penicillins by proposing the implementation of specific conditions for use (AMCRA, 2013).

Instead, parenteral administered procaine benzyl penicillin (penicillin G) and orally administered benzyl penicillin (phenoxymethyl penicillin or penicillin V) have narrow spectrum activity, i.e. against many Gram-positive bacteria, including beta-hemolytic streptococci, but only to a limited number of Gram-negative bacterial species (Prescott, 2013). Narrow spectrum use is in agreement with prudent use recommendations (Staats et al., 1997, AMCRA, 2013; KNMVD, 2014). Nevertheless, in Belgium, the practical implication interferes with these recommendations as currently no orally administered penicillin (penicillin V) is licensed and *S. suis* infections are generally controlled by group administration, for which oral substances are highly time efficient, and therefore preferred by the farmer.

Furthermore, the results on penicillin susceptibility of *S. suis* based on clinical breakpoints differ from the results of the epidemiological interpretive criterion (***Chapter 4***). The Minimum Inhibition Concentration (MIC) population distribution reveals tailing toward higher MIC values (percentage of non-wild-type isolates equaled 47% based on epidemiological cut-off value of 0.25 µg/ml). This reduced susceptibility is the result of the acquisition of stepwise mutations in genes encoding penicillin binding proteins (Aarestrup et al., 2007). A single-point mutation results in isolates with a modest increase in MIC, and infections due to these isolates may still be treatable with penicillins, but they are of great concern as they represent an introductory step to full resistance (Amyes, 2007). Isolates showing higher values of MICs are associated with additional

mutations and most likely lead to therapy failure (Chambers, 1999). As a result, shifts in the MIC population distributions may predict future evolutions in the clinical efficacy of antimicrobials used to counteract bacterial infections.

Mutations resulting in a decreased susceptibility to penicillin are selected by β-lactam use (Chambers, 1999), commonly used in Belgian pig farms (***Chapter 3***). This raises questions on their future clinical use as primary antimicrobials for streptococcal infections. The use of narrow spectrum molecules (penicillin) should always be preferred above broad-spectrum aminopenicillins and the potential selection of mutant strains should be minimized by the implementation of specific conditions for use. The veterinary diagnostic laboratory should not report the full result of the susceptibility testing. It might be beneficial to report a limited number of antimicrobial substances, primarily first-line/small spectrum before second-and third-line/broad spectrum substances, in order to decrease the risk of unnecessary use of these agents. For instance, if *S. suis* shows clinical susceptibility towards broad spectrum 3^{th} and 4^{th} generation cephalosporins, critically important antimicrobials according WHO and OIE (WHO, 2011; OIE, 2014b), as well as towards small spectrum penicillins or potentiated sulfonamides (trimethoprim-sulfonamide), only these latter substances should be reported. In this respect, for veterinary diagnostic laboratories, there is an urgent need for agreements on what antimicrobials should be tested for certain bacteria and how results should be reported.

COMPARING ANTIMICROBIAL SUSCEPTIBILITY OF BELGIAN AND OTHER STREPTOCOCCUS SUIS STRAINS – A CHALLENGE

Interpreting susceptibility testing results of bacterial species, including *S. suis*, can be most challenging. Variations in interpretive criteria (different clinical breakpoints, clinical versus epidemiological criterion), as well as differences in susceptibility testing methodology (diffusion or dilution testing), animal health status (diseased or healthy), or differences in serotypes tested may contribute to apparent differences in antimicrobial susceptibility of bacterial isolates from different studies. These apparent differences hinder the detection of true differences, mainly caused by different selection pressures such as antimicrobial usage. Furthermore, they might result in different interpretations regarding the usage-resistance link. In our study, clinical breakpoints were consulted from the Clinical and Laboratory Standards Institute document VET01-S2 (CLSI, 2013). This document includes the largest collection of internationally recognized clinical breakpoints for bacteria of animal origin currently available, a considerable number of which represent veterinary-specific breakpoints. These breakpoints are mainly based on the pharmacological and clinical criterion and depend on animal species, disease, pathogen, antimicrobial compound and treatment regimen (dose, route, duration, frequency). For *S. suis*, in the VET01-S2 no clinical breakpoints were available for lincomycin, tiamulin, tilmicosin and tylosin. In the absence of the appropriate breakpoint, many studies use breakpoints for bovine or swine respiratory pathogens different from *S. suis* (Wisselink et al., 2006; Zhang et al., 2008) or from human streptococci (Marie et al., 2002; Wisselink et al., 2006; Zhang et al., 2008). Some susceptibility studies

used the CLSI clinical breakpoint for *Actinobacillus* spp. (32 μg/ml), causing respiratory tract disease in pigs, for evaluating tiamulin and tilmicosin resistance in *S. suis* (Wisselink et al., 2006; Zhang et al., 2008). This resulted in respectively 34.4% and 66.7% of the *S. suis* isolates being clinically resistant. When changing one of the regimen specifics related to the approved interpretive criterion, such as a different pathogen as well as a different population of animals or a different disease process, the approved breakpoint may no longer be entirely valid for predicting clinical success after the administration of an antimicrobial compound. As a result, the *Actinobacillus* spp. clinical breakpoint cannot be extrapolated as such to *S. suis* (Schwarz et al., 2010). Hence, clinical resistance in Belgian *S. suis* isolates was not determined for these antimicrobials without the appropriate clinical breakpoint, i.e. lincomycin, tiamulin, tilmicosin and tylosin (***Chapter 4***). For enrofloxacin, florfenicol and tetracycline, the clinical breakpoint was only available for *S. suis* involved in swine respiratory disease (2 μg/ml, 8 μg/ml and 2 μg/ml respectively) and implemented as such in our study (***Chapter 4***). In 2006, Wisselink et al. (2006) used breakpoints for enrofloxacin and florfenicol based on CLSI data of bovine respiratory Gram-negatives (2 μg/ml and 8 μg/ml respectively). Despite different pathogens or animal species, breakpoints equaled with the ones for *S. suis* involved in swine respiratory disease. For tetracycline, the authors used CLSI data for veterinary pathogens, but which were at that time taken by CLSI from data for human streptococci (8 μg/ml). This resulted in a different tetracycline breakpoint and a considerable higher outcome for clinical resistance in our study (95% based on a clinical breakpoint of 2 μg/ml) compared to the study of Wisselink et al. (2006) (75% based on a clinical breakpoint of 8 μg/ml). Also, nowadays, the CLSI-approved clinical breakpoints in document VET01-S2 for erythromycin and penicillin are based on human pharmacokinetics and treatment regimens for human streptococci. These breakpoints are formulated in order to provide clinical breakpoints for veterinary medicine, suggesting that the application of these breakpoints for animal associated bacteria, *e.g. S. suis*, might be justified in our study as well as in other studies as long as no veterinary-specific breakpoints are available (Wisselink et al., 2006; Zhang et al., 2008). Clinical resistance to penicillin in our study was low (1% based on the 2 μg/ml breakpoint) (***Chapter 4***), whereas higher percentages have been reported in the nineties, even using a higher breakpoint (4 μg/ml) (Turgeon et al., 1994). More recently, in Spain, 7% of the isolated strains of diseased pigs showed clinical resistance to penicillin, using the same breakpoint as in our study (2 μg/ml) (Vela et al., 2005). In China, higher resistance levels were reported for penicillin (9.5%, based on the 4 μg/ml breakpoint), meaning that the use of the 2 μg/ml breakpoint might reveal even higher percentages of clinical resistance.

From the above, it is clear that the lack of validated veterinary-specific resistance breakpoints is an important limitation for veterinarians trying to practice evidence-based medicine. Moreover, due to its variability, it often does not allow for proper comparisons between studies. In Europe, the Veterinary Committee on Antimicrobial Susceptibility Testing (VetCAST) has been recently installed by a group of veterinary microbiologists and pharmacologists, as a subcommittee of EUCAST, that aims at defining and approving clinical breakpoints

specific to the veterinary field (VetCAST, 2014). Hereby, the predictive value of susceptibility data for clinical purposes will hopefully improve in veterinary diagnostics.

In the absence of clinical interpretive criteria for pathogens isolated from pigs, isolates should not be classified as susceptible or resistant for the concerned antimicrobial agents tested, and only MIC data should be presented. Unfortunately, only few studies provide MIC distributions (Martel et al., 2001; Vela et al., 2005; Wisselink et al., 2006). Their use ensures the comparability of data over time at country level and also facilitate the comparison of the occurrence of resistance between countries. Increased MICs of *S. suis* for penicillin in our study (***Chapter 4***) underlined the importance of reporting antimicrobial susceptibility testing results based on epidemiological interpretive criteria, as it can act as an early warning for an emerging clinical problem. Also for tiamulin, results from the *S. suis* MIC population distribution, showed a clear shift towards higher MIC values. Unfortunately, today, epidemiological cut-off values, in order to distinguish between the wild-type and the non-wild-type population within a bacterial population, are not available for *S. suis* from EUCAST (EUCAST, 2015). In this study, MIC values without a clear bimodal distribution, seen for some of the antimicrobials tested (penicillin, tilmicosin, erythromycin, lincomycin, tiamulin and tetracycline), made the setting of an epidemiological cut-off value harsh. Several explanations for subpopulations showing differences in susceptibility can be suggested. In our study, a true resistance difference within the *S. suis* population can result from animals being differently treated. Another explanation might be the presence of different serotypes or bacterial strains with different acquired resistance mechanisms within one *S. suis* population. A continued reporting of serotypes or genotypes is currently lacking for many countries, including Belgium, even though, differences in serotype susceptibility of *S. suis* have been described (Aarestrup et al., 1998; Marie et al., 2002; Vela et al., 2005; Wisselink et al., 2006). Therefore, serotype specific data on susceptibility might be an interesting path to investigate, as it can provide valuable information on the activity of antimicrobial agents against the major serotypes associated with disease in pigs.

ANTIMICROBIAL SUSCEPTIBILITY OF *ESCHERICHIA COLI* STRAINS FROM BELGIAN PIGS

Antimicrobial susceptibility of commensal *E. coli* may provide an indication of the magnitude of the selective pressure from the use of antimicrobials in an animal population. Commensal *E. coli* are therefore often included as Gram-negative indicator bacteria for resistance in national and international monitoring studies (de Jong et al., 2009; FINRES, 2011; DANMAP, 2013; MARAN, 2014, EFSA and ECDC, 2015).

High clinical resistance prevalence in commensal *E. coli* from the antimicrobial susceptibility prevalence study in pigs is not surprising, in view of the high antimicrobial use in Belgian pig production (***Chapter 3***, ***Chapter 5***). Results from different studies investigating antimicrobial resistance in porcine *E. coli* strains are often difficult to compare due to differences in study design, particularly the population of swine sampled as well as sampling, testing protocols and interpretive criteria used. Nevertheless, Chantziaras et al. (2014) demonstrated a high level of comparability in clinical resistance results of commensal swine *E. coli* between

the prevalence study of this thesis (***Chapter 5***) and the Belgian national monitoring study, despite a different sampling and susceptibility testing methodology. The high prevalence of resistance against streptomycin, tetracycline, sulfadiazine, trimethoprim and ampicillin (***Chapter 5***) has also been reported frequently in other countries (Boerlin et al., 2005; Varga et al., 2008; EFSA and ECDC, 2015). The common patterns of resistance to streptomycin, tetracyclines, sulfonamides, trimethoprim and ampicillin (and combinations thereof) frequently observed in the monitoring of *E. coli* isolates are probably related to the presence of class 1 or class 2 integrons, which generally carry genes conferring resistance to these antimicrobials (Marchant et al., 2013; EFSA and ECDC, 2015). As a consequence, a substantial number of antimicrobials or antimicrobial classes used in Belgian swine production is likely to select for isolates with this resistance pattern (***Chapter 3***). Also resistance to chloramphenicol is often found to be present in the multi-resistance pattern of streptomycin, tetracyclines, sulfonamides, trimethoprim and ampicillin (EFSA and ECDC, 2015). The most frequently encountered resistance mechanism against chloramphenicol is encoded by the chloramphenicol acetyltransferases (CAT) genes, commonly found on mobile genetic elements carrying additional resistance genes (Dowling, 2013a). Bacteria expressing the CAT genes show lower levels of resistance to florfenicol, sharing its mechanism of action with chloramphenicol, compared to chloramphenicol (Schwarz et al., 2004). Florfenicol resistance in Gram-negative bacteria is related to plasmid transfer of the *floR* gene and promotes the efflux of both forfenicol and chloramphenicol (Blickwede and Schwarz, 2004). Therefore, lower levels of florfenicol than chloramphenicol resistance in *E. coli* from the prevalence study indicate these old CAT resistance genes are still circulating and that the *floR* gene was only limited present (***Chapter 5***). The continued high chloramphenicol resistance level in *E. coli* (***Chapter 5***), despite its prohibition in food producing animals for more than 20 years, is therefore a result of the use of florfenicol and other antimicrobials (cross and co-selection). Moreover, the limited use of florfenicol in Belgian fattening pigs suggests that mainly co-selection is responsible for a persistent chloramphenicol resistance in *E. coli* from these pigs (***Chapter 3***).

Resistance development against quinolones, categorized as critically important for humans and animals, occurs mainly by mutations, even though plasmid mediated quinolone resistance (PMQR) has been described (Strahilevitz et al., 2009). The prevalence study showed susceptibility results based on clinical breakpoints and revealed a higher percentage of *E. coli* clinically resistant to nalidixic acid (4.1%) compared to enrofloxacin (1.4%) (***Chapter 5***). These results were indicative for the development of quinolone resistance by a stepwise manner, as a single mutation in the quinolone resistance-determining region (QRDR) domain of the *gyrA* gene will confer resistance to the first generation quinolone, nalidixic acid, whereas it only decreases susceptibility to second generation quinolones, such as enrofloxacin (Giguère and Dowling, 2013). Only secondary mutations will then lead to clinical resistance in enrofloxacin. Mutational resistance can mask the presence of PMQR genes, as these genes result in a reduced susceptibility, similar to that conferred by first-

step mutations, that is below the CLSI breakpoint for resistance (Strahilevitz et al., 2009). PMQR contributes to enlarge the mutant selection window (MSW), which is the drug concentration range within which QRDR mutants are selectively enriched (Cano et al., 2007). PMQR can therefore contribute to bacteria becoming fully resistant. Most recent results from the national monitoring program revealed a higher percentage of non-WT *E. coli* strains for ciprofloxacin than for nalidixic acid, both quinolones, suggesting the prevalence of PMQR (CODA-CERVA, 2014b). The emergence of PMQR is of concern due to the possibility of horizontal transfer (Strahilevitz et al., 2009). Quinolones, mainly used in Belgium, for individually treatments of pigs during farrowing and sows, might contribute to the selection and spread of PMQR in *E. coli* mutants and therefore take part in therapeutic failure (***Chapter 3***, ***Chapter 6***). Furthermore, PMQR genes were shown to be associated with other resistance elements on the same plasmid, for instance, with various genes encoding extended-spectrum-beta-lactamases (ESBLs) (Szmolka and Nagy, 2013). The use of ceftiofur in piglets as a risk factor for an increased fluoroquinole resistance in *E. coli* as well as the opposite finding (enrofloxacin use as a risk factor for ceftiofur resistance) suggests the presence of PMQR in these *E. coli* strains (***Chapter 6***). Resistance determinant genotyping of these strains is needed to confirm these findings.

Commensal *E. coli* are also useful as representatives of the *Enterobacteriaceae* to monitor the emergence and changes in the proportion of bacteria possessing extended-spectrum-beta-lactamases (ESBL). Several studies have detected ESBL producing *E. coli* in pigs, amongst other production animals (Hammerum et al., 2014). The use of 3rd and 4th generation cephalosporins leads to ESBL-producing *E. coli* among food producing animals (Cavaco et al., 2008; Hammerum et al., 2014). Results from ***chapter 6*** showed associations between 3rd generation cephalosporin use and resistance in young piglets. As described earlier, more stringent measures regarding this critically important antimicrobial class are needed in the light of an increased 3rd and 4th generation cephalosporin use in Belgian pig farms between 2004 and 2010 (***Chapter 3***), as well as for all animals in Belgium from 2010 onwards, described by the national antimicrobial consumption report (BelVet-SAC, 2015). In fattening pigs, resistance to third generation cephalosporin (ceftiofur), indicative of ESBL producing *E. coli*, was present at low levels (2.7%) (***Chapter 5***). Yet, it should be said that monitoring for ceftiofur resistance using selective media can detect ceftiofur resistant *E. coli* present as a minor component of the total bacterial microbiota in the test sample, which might only occasionally be detected by random sampling from non-selective culture plates (EFSA and ECDC, 2015).

E. coli of animal origin with acquired resistance genes can be transmitted to humans via direct contact with animals, via food of animal origin, or via the environment (Hammerum and Heuer, 2009). These bacteria may subsequently colonize humans or may transfer resistance genes to other bacteria during passage through the intestinal tract. Transfer of resistance determinants from a (transiently) colonizing animal *E. coli* strain to human commensal microbiota has been observed *in vivo*, even in the absence of selective pressure (Hammerum and Heuer, 2009). Regarding the zoonotic potential of *E. coli* related to pigs, attention goes to

the extraintestinal pathogenic *E. coli*, causing meningitis, septicemia, urinary tract infections, pneumonia (Tan et al., 2011). In humans, the majority of infections caused by extraintestinal *E. coli* are not life-threatening (*e.g.*, uncomplicated urinary tract infections), whereas other infections (*e.g.*, bloodstream infections) may be lethal. Yet, accumulating data support the likelihood that animal reservoirs, including pigs, could be responsible for contamination of humans with resistant extraintestinal pathogenic *E. coli* strains through direct contact or the consumption of contaminated food (Johnson et al., 2005; Jakobsen et al., 2010). These bacteria may cause infections for which limited therapeutic options are available (Hammerum and Heuer, 2009). In human medicine, urinary tract infections due to extraintestinal pathogenic *E. coli* are traditionally treated with ampicillin or trimethoprim-sulfamethoxazole. Resistance to these agents as well as to extended-spectrum β-lactamases, aminoglycosides, tetracyclines, and fluoroquinolones is now frequently observed (Smith et al., 2007).

The high antimicrobial resistance of *E. coli* in pigs (***Chapter 5, Chapter 6***), its potential to transfer resistance determinants to human bacteria as well as the potential role of animal-related resistant *E. coli* in human infections, emphasize the importance of including *E. coli* in monitoring studies.

In Belgium, susceptibility testing on *E. coli* strains isolated from both clinically healthy and diseased animals is performed regularly (Butaye, 2012; DGZ, 2014). Thereto, micro-broth dilution and agar disk diffusion tests are both used. In general, there is a tendency to give preference to micro-broth dilution over agar disk diffusion since the dilution test is the more accurate test method for detection of acquired resistance (EFSA, 2008). Yet, both zone disk diameters and MICs, results from disk diffusion and dilution tests respectively, can be displayed in a population distribution offering the advantage of detecting non-wild type strains. Yet, more often, zone disk diameters are categorized into 'susceptible', 'intermediate' or 'resistant', suggesting the clinical effectiveness for a certain antimicrobial-bacterium. Differences in disk diffusion methodology or in clinical breakpoints complicates comparisons of susceptibility results and excludes them from monitoring studies for comparative reasons. The harmonization of veterinary antimicrobial susceptibility testing is an objective of VetCAST (2014). In the meantime, due to the use of the Normalized Resistance Interpretation (NRI) method, potential valuable susceptibility data on commensal *E. coli* from a single study can still be used and should not be excluded from these monitoring studies (***Chapter 5***). Moreover, laboratories often provide a huge source of historical antimicrobial susceptibility data, obtained from the routinely performed disk diffusion test. The interpretation of disk diffusion data could be improved in these individual laboratories using NRI, and the obtained data could be useful for comparative reasons during monitoring, provided that zone disk diameters have been recorded and saved. High quality records of the zone disk diameters can be achieved by automatic reading devices, which for instance will prevent the tendency of rounding down or up zone disk diameters to even numbers, typically seen for non-automatized reading. The use of automatic reading devices will equally increase the recording and saving of zone disk diameters. The need for higher quality standards

for susceptibility testing has been expressed by VetCAST, aiming for optimized methods for antimicrobial susceptibility testing of bacterial pathogens of animal origin and zoonotic bacteria (VetCAST, 2014).

Also, veterinary specific breakpoints for *E. coli* from pigs are currently missing from the EUCAST and CLSI database (***Chapter 5***). The veterinary diagnostic microbiology laboratory urgently needs bacterial species-, animal host- and infection-specific breakpoints for the practice of evidence-based antimicrobial therapy by providing culture and susceptibility information to practitioners. Several authors have shown that antimicrobial resistance more frequently occurs in pathogenic than in commensal *E. coli* strains from pigs (Boerlin et al., 2005; Hendriksen et al., 2008; Chantziaras et al., 2014). Although similar qualitative resistance patterns are being observed, i.e. increased levels of resistance to the same antimicrobials, between these commensal and pathogenic *E. coli*, results of clinical resistance of commensal *E. coli* cannot be used to predict the expected levels of resistance prevalence of pathogenic *E. coli* and vice versa.

The harmonization of veterinary antimicrobial susceptibility testing and the setting of veterinary specific breakpoints in the European Union will be in line with the adoption of the EUCAST harmonized method and the establishment of clinical breakpoints for human medicine (Brown et al., 2015). By doing so, the future of the disk diffusion test will be secured for both human and veterinarian medicine (Matuschek et al., 2014).

CONCLUSIONS

The high occurrence of resistance to antimicrobials in commensal *E. coli* and *S. suis* from Belgian pigs is likely to depend on a number of factors, of which the selective pressure exerted by antimicrobial use in pigs is extremely important. In practice the distinction between prevention and treatment is vague and much of the use of antibiotics that is claimed to be therapeutic is intended to control disease and often involves mass medication. By consequence, this use turns out to be at a permanent, even increased level in Belgian pig industry since it first records in 2003, despite the concerns expressed at that time. Proper monitoring of both antimicrobial usage and resistance is valuable as it detects evolutions in antimicrobial use and resistance linked to antimicrobial consumption. This warrants further actions to improve monitoring of antimicrobial use and resistance.

More specifically, it can be recommended that:

- A data collection system is installed covering the entire Belgian pig production in order to collect and quantify herd level-data. These data should be used to monitor use in time, to benchmark farmers and veterinarians, to identify 'outliers' for high and/or irresponsible antimicrobial use and to implement targeted intervention measures.
- Colistin resistance in animal-related bacteria is monitored and that a risk assessment for human health is made in the event resistance determinants get organized on mobile genetic elements.
- *S. suis* from healthy animals are included as a Gram-positive indicator bacterium when monitoring resistance over time.
- Therapeutic failure of penicillin to treat *S. suis* infections is considered, in view of an increasing percentage of non- wild type strains.
- Veterinary antimicrobial susceptibility testing is harmonized and that veterinary-specific resistance breakpoints are validated in order to facilitate the practice of evidence-based medicine.
- Epidemiological cut-off values for both pathogenic and indicator bacteria, *e.g. S. suis*, are established as they make early detection of emerging antibiotic resistance possible.
- The Normalized Resistance Interpretation (NRI) method is used to define the wild type population for disk diffusion results when wild type cut-offs are not available. NRI allows historical disk diffusion susceptibility data to be included in monitoring studies, provided that inhibition zone diameters are available.
- In order to reduce antimicrobial resistance in piglets, control measures are implemented, at first, at the sow level, involving minimal antimicrobial use and high hygienic standards.

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SUMMARY

Since their discovery, antimicrobials have become indispensable tools in countering bacterial diseases in both humans and animals. Yet, their use is overshadowed by a phenomenon that has become increasingly more threatening over the last decades, namely antimicrobial resistance. Antimicrobial resistance in pathogenic and zoonotic bacteria results in decreased efficiency and/or therapeutic failure in both animals and humans. Even though pathogenic bacteria are generally most studied, commensal bacteria may play a key role in the dissemination and persistence of this resistance.

As a general introduction, an overview is given on the antimicrobial use in production animals, with particular attention to the use of antimicrobials in pig production (***Chapter 1.1.***). In this chapter, background information is provided on the reasons for antimicrobial use and which antimicrobial classes and routes of administration are used. Also, the collection of antimicrobial consumption data for quantifying use is discussed. In addition to the collection of antimicrobial consumption data, the monitoring of antimicrobial resistance in bacteria in food-producing animals is unmistakably important in the establishment of strategies for containing resistance. In ***chapter 1.2.***, the role of swine *Escherichia coli* and *Streptococcus suis*, both commensal bacteria potentially involved as pathogen and/or zoonotic bacteria in animals and humans, in monitoring programs is described. Monitoring resistance identifies trends in the emergence, spread and persistence of resistance to antimicrobials and is therefore a prerequisite for understanding the epidemiology of resistance. ***Chapter 1.3.*** reviews the key aspects and drivers of the four stages in the epidemiology of antimicrobial resistance in animal production, i.e. development, selection and spread, persistence and reversion or reduction.

The objectives of this thesis, which are stated in ***chapter 2***, were to determine the level of antimicrobial use on Belgian pig herds, the levels of epidemiological and clinical resistance in *S. suis* and *E. coli* from clinically healthy fattening pigs and to investigate whether the presence of antimicrobial resistant *E. coli* in sows and the administration of antimicrobials to sows and piglets during farrowing influenced the antimicrobial resistance in fecal commensal *E. coli* in sows and their offspring.

In order to get insight in the quantitative (numbers of days a pig is treated over a lifespan of 1000 days) and qualitative (indications, prophylactic or metaphylactic use, active substances and administration routes used) aspects of antimicrobial group-level use at the herd level in Belgian pig production and to assess changes in the antimicrobial use since 2003, a cross-sectional study on 50 closed or semi-closed fattening pig herds was conducted (***Chapter 3***). Ninety-three percent of the group treatments were prophylactic whereas only 7% were metaphylactic. The most frequently used antimicrobials orally applied at group level were colistin (30.7%), amoxicillin (30.0%), trimethoprim-sulfonamides (13.1%), doxycycline (9.9%) and tylosin (8.1%). The most frequently applied injectable antimicrobials were tulathromycin (45.0%), long acting ceftiofur (40.1%) and long acting amoxicillin (8.4%). The treatment incidences (TI) based on the used daily dose pig (UDD_{pig} or the actually administered dose per day per kg pig of a drug) for all oral and injectable antimicrobial drugs was on

average 200.7 per 1000 pigs at risk per day (min = 0, max = 699.0). This means that a pig is treated on average 201 days over a lifespan of 1000 days, or said differently, it is treated on average 20% of its lifespan. The TI based on the animal daily dose pig (ADD_{pig} or the national defined average maintenance dose per day per kg pig of a drug used for its main indication) was slightly higher (average = 235.8, min = 0, max = 1322.1). This indicates that in reality fewer pigs were treated with the same amount of antimicrobials than theoretically possible. Injectable products were generally overdosed (79.5%), whereas oral treatments were often underdosed (47.3%). Antimicrobial use was substantially higher in 2010 compared with what was found in 2003 ($TI_{DDDApig}$ and $TI_{UDDApig}$ equaled 178.1 and 170.3, respectively) suggesting that there was an increase in the average antimicrobial use in pigs in that period. In conclusion, this study shows that prophylactic group treatment was applied in 98% of the visited herds and often includes the use of critically important and broad-spectrum antimicrobials. In 2010, in Belgium, the guidelines for prudent use of antimicrobials were not yet implemented in pig production.

Antimicrobial resistance in bacteria can be monitored by using clinical or epidemiological interpretive criteria. The use of clinical interpretive criteria may be sufficient from the point of view of the clinician as it predicts the clinical outcome of the treatment in the patient at the prescribed drug dose. Yet, epidemiological cut-off values are more valuable to make comparisons between different studies, to monitor the evolution of antimicrobial resistance in time and to detect acquired resistance at an early stage. Therefore, both interpretive criteria were used to calculate the percentage of clinical resistant and non-wild type strains of *S. suis* and *E. coli* from clinically healthy pigs.

In the antimicrobial resistance prevalence study on *S. suis* (***Chapter 4***), non-wild type percentages were high for tetracycline (98%), lincomycin (92%), tilmicosin (72%), erythromycin (70%), tylosin (66%), and low for florfenicol (0%) and enrofloxacin (0.3%). Clinical resistance percentages were high for tetracycline (95%), erythromycin (66%), tylosin (66%), and low for florfenicol (0.3%) and enrofloxacin (0.3%). For tiamulin, for which no clinical breakpoint is available, 57% of the isolates did not belong to the wild type population. Clinical resistance and non-wild type percentages differed substantially for penicillin. Only 1% of the tested *S. suis* strains was considered as clinically resistant, whereas 47% of the strains showed acquired resistance when epidemiological cut-off values were used. In conclusion, Minimum Inhibitory Concentration (MIC) values for penicillin are gradually increasing, compared to previous reports, although pigs infected with strains showing higher MICs may still respond to treatment with penicillin. The high rate of acquired resistance against tiamulin has not been reported before. Results from this study clearly demonstrate that the use of different interpretive criteria contributes to the extent of differences in reported antimicrobial resistance results. The early detection of small changes in the MIC population distribution of isolates, while clinical failure may not yet be observed, provides the opportunity to implement appropriate risk management steps.

In the absence of epidemiological interpretive criteria for *E. coli*-antimicrobial combinations, clinical breakpoints are used to interpret susceptibility test results obtained by disk diffusion. Yet, differences in the standardization of the methodology over time and between laboratories as well as differences between recommended clinical breakpoints result in a lack of comparability between the test results of disk diffusion, and preclude potentially valuable data, collected in different laboratories, from resistance monitoring studies. In ***chapter 5***, Normalized Resistance Interpretation (NRI) was used on *E. coli* isolates from clinically healthy pigs at slaughter age and was shown to be a valid method to define the wild type population for outcomes with a normal distribution of inhibition zone diameters for the susceptible population. Percentages of non-wild type strains could therefore be determined and compared to clinical resistance percentages. In conclusion, until harmonization of the disk diffusion methodology is achieved, the NRI method might give rise to a wide application in monitoring resistance in veterinary medicine. Also, historical data, for instance from previous prevalence studies, might be included in monitoring programs for comparative reasons, provided that zone diameters have been recorded.

The last study of this thesis investigated whether antimicrobial resistant *E. coli* in apparently healthy sows and antimicrobial administration to sows and piglets influenced antimicrobial resistance in fecal commensal *E. coli* from piglets (***Chapter 6***).

Sixty sows from three herds and three of their piglets were sampled at several time points. Antimicrobial use data during parturition and farrowing were collected. Clinical resistance was determined for two isolates per sampling time point for sows and piglets using disk diffusion. Only 27.4% of *E. coli* isolates from newborn piglets showed no resistance. Resistance to one or two antimicrobial classes equaled 41.2% and 46.8% in isolates from sows and piglets, respectively, for the overall farrowing period. Multiresistance to at least four classes was found as frequently in sows (15.6%) as in piglets (15.2%). Antimicrobial resistance in piglets was influenced by antimicrobial use in sows and piglets and by the sow resistance level ($p < 0.05$). Using aminopenicillins and third-generation cephalosporins in piglets affected resistance levels in piglets (odds ratios [OR] > 1; $p < 0.05$). Using enrofloxacin in piglets increased the odds for enrofloxacin resistance in piglets (OR = 26.78; $p < 0.01$) and sows at weaning (OR = 4.04; $p < 0.05$). For sows, antimicrobial exposure to lincomycin-spectinomycin around parturition increased the resistance to ampicillin, streptomycin and trimethoprim-sulfadiazine in sows (OR= 21.33, OR= 142.74, OR= 18.03; $p < 0.05$) and additionally to enrofloxacin in piglets (OR= 7.50; $p < 0.05$). This study demonstrates that antimicrobial use in sows and piglets is a risk factor for antimicrobial resistance in the respective animals. Moreover, resistance determinants in *E. coli* from piglets are selected by using antimicrobials in their dam around parturition.

In the general discussion (***Chapter 7***), first, it was discussed that the establishment of a data collection system (DCS), providing usage data at herd level on a continuous basis, is needed to make further actions possible, more specifically to identify outliers in quantitative and qualitative antibiotic use. *S. suis* is commonly

present in healthy animals and therefore subject to any antibiotic selection pressure from both parenteral and oral administration routes. Therefore, it may act as a Gram-positive indicator bacterium when monitoring resistance over time. The detection of higher levels of epidemiological compared to clinical resistance in *S. suis* from clinically healthy pigs has emphasized the role of epidemiological interpretive criteria in the detection of acquired resistance and in monitoring resistance over time. Thereto, the setting of epidemiological cut-off values for bacterial species, *e.g. S. suis*, is required. Also, validated veterinary-specific resistance breakpoints are needed to facilitate the practice of evidence-based medicine. There is a need for harmonization of veterinary antimicrobial susceptibility testing. Yet, until harmonization is achieved, the NRI method has shown to be valid for defining the wild type population for disk diffusion results. Antimicrobial exposure of sows prior to farrowing and during lactation can increase antimicrobial resistance in *E. coli* of their offspring. Therefore, sows act as a reservoir for resistant *E. coli* to their offspring. In order to reduce antimicrobial resistance in piglets, control measures should be implemented, at first, at the sow level, involving minimal antimicrobial use and high hygienic standards.

SAMENVATTING

Sinds de ontdekking van antibiotica zijn het onmisbaar belangrijke middelen in de bestrijding van bacteriële ziekten bij mens en dier. Hun gebruik is echter overschaduwd door een gedurende de laatste decennia alsmaar toenemende dreiging, namelijk antibioticumresistentie. Antibioticumresistentie in pathogene en zoönotische bacteriën resulteert in een verminderde werking van antibiotica of zelfs in het falen van therapie bij dier en mens. Hoewel resistentie bij pathogene bacteriën het vaakst beschreven wordt, zijn commensalen van belang in de spreiding en persistentie van antibioticumresistentie.

Als algemene inleiding van deze thesis werd een overzicht gegeven van het gebruik van antibiotica bij voedselproducerende dieren, waarbij extra aandacht werd geschonken aan het gebruik van antibiotica bij varkens (***Hoofdstuk 1.1.***). In dit hoofdstuk wordt informatie aangeleverd over de redenen van antibioticumgebruik en welke antibioticaklassen en toedieningswegen hiervoor toegepast worden. De datacollectie van antibioticumgebruik teneinde het gebruik te kunnen kwantificeren komt eveneens aan bod. Samen met het verzamelen van antibioticumgebruiksgegevens is de opvolging of monitoring van antibioticumresistentie bij bacteriën van voedselproducerende dieren van onmiskenbaar groot belang bij het opzetten van strategieën om antibioticumresistentie te beheersen. In ***hoofdstuk 1.2.*** wordt de rol van *Escherichia coli* en *Streptococcus suis*, beide commensale bacteriën die betrokken kunnen zijn als pathogeen en/of zoönotisch agens bij dieren en mensen, in monitoring programma's beschreven. Het monitoren van resistentie spoort tendensen op in het ontstaan, de spreiding en de persistentie van antibioticumresistentie en is omwille daarvan een noodzaak om de epidemiologie van antibioticumresistentie te begrijpen. ***Hoofdstuk 1.3.*** geeft een overzicht van de belangrijkste aspecten en drijfveren van de vier stappen in de epidemiologie van antibioticumresistentie in voedselproducerende dieren, met name, de ontwikkeling, de selectie en spreiding, de persistentie en de terugkeer naar gevoeligheid of reductie van resistentie.

De doelstellingen van deze thesis, vermeld in ***hoofdstuk 2***, zijn het bepalen van de mate waarin antibiotica gebruikt worden in Belgische varkensbedrijven, de mate waarin epidemiologische en klinische antibioticumresistentie voorkomen in *S. suis* en *E. coli* van gezonde vleesvarkens en het onderzoeken of antibioticumresistente *E. coli* bij zeugen en de toediening van antibiotica aan zeugen en biggen gedurende de kraamperiode invloed hadden op het voorkomen van antibioticumresistentie bij fecale commensale *E. coli* van zeugen en haar biggen.

Om een idee te krijgen over de kwantitatieve (aantal dagen dat een varken behandeld is gedurende 1000 dagen) en kwalitatieve (indicatie, preventief of metafylactisch gebruik, gebruikte actieve substanties en toedieningswegen) aspecten van antibioticumgebruik in groep voor individuele bedrijven in de Belgische varkensproductie werd een dwarsdoorsnede studie uitgevoerd op 50 gesloten of halfgesloten vleesvarkensbedrijven. Op basis hiervan konden ook vergelijkingen gemaakt worden met het antibioticumgebruik in 2003 (***Hoofdstuk 3***). Drieënnegentig procent van de groepsbehandelingen was voor

preventieve redenen en dus slechts 7% diende als metafylaxis. De antibiotica die vaakst oraal in groep werden toegediend waren colistine (30.7%), amoxicilline (30.0%), trimethoprim-sulfonamiden (13.1%), doxycycline (9.9%) en tylosine (8.1%). De vaakst toegediende injecteerbare antibiotica waren tulathromycine (45.0%), lang werkend ceftiofur (40.1%) en lang werkend amoxicilline (8.4%). De behandelingsincidentie (BI) gebaseerd op de werkelijk toegediende dagdosis (UDD_{pig} uitgedrukt in mg per kg varken) voor alle orale en injecteerbare antibiotica bedroeg gemiddeld 200.7 per 1000 varkens (min = 0, max = 699.0). Dit betekent dat een varken gemiddeld 201 dagen op een levensduur van 1000 dagen behandeld wordt met een dergelijke dosis of dat hij 20% van zijn leven onder een antibioticabehandeling staat. De behandelingsincidentie (BI) gebaseerd op de gedefinieerde dagdosis (ADD_{pig} uitgedrukt in mg per kg varken voor de belangrijkste indicatie) was hoger (gemiddelde = 235.8, min = 0, max = 1322.1). Dit betekent dat een lager aantal dieren behandeld werd met een zelfde hoeveelheid antibiotica dan theoretisch mogelijk. Injecteerbare producten werden overwegend overgedoseerd (79.5%), terwijl oraal toegediende middelen vaak ondergedoseerd werden (47.3%). Er kan een stijging in het gebruik van antibiotica verondersteld worden tussen 2003 en 2010 ($TI_{DDDApig}$ and $TI_{UDDApig}$ bedroegen respectievelijk 178.1 and 170.3). Tot slot kon geconcludeerd worden dat 98% van de bezochte bedrijven antibiotica preventief in groep toedient en dat het vaak om kritisch belangrijke en breed werkende antibiotica gaat. Er kan worden gesteld dat in 2010 in België de richtlijnen voor voorzichtig antibioticumgebruik niet werden toegepast in de varkensproductie.

Antibioticumresistentie bij bacteriën kan opgevolgd worden door het gebruik van klinische of epidemiologische interpretatieve criteria. Het gebruik van klinische interpretatieve criteria voldoet aan de eisen van de clinicus aangezien deze de klinische uitkomst van een antibioticabehandeling aan de voorgeschreven dosis in een patiënt trachten te voorspellen. Epidemiologische interpretatieve criteria daarentegen, zijn van nut voor het vergelijken van antibioticumresistentie tussen verschillende studies, voor het opvolgen van evoluties in antibioticumresistentie in tijd en voor het ontdekken van nieuwe verworven resistenties op een vroeg stadium. Beide criteria werden daarom gebruikt bij het berekenen van de percentages van klinisch resistente stammen en stammen behorende tot de niet-wild type populatie van *S. suis* en *E. coli* afkomstig van gezonde varkens.

In de prevalentiestudie van antibioticumresistentie bij *S. suis* (***Hoofdstuk 4***) waren percentages van niet-wild type stammen hoog voor tetracycline (98%), lincomycine (92%), tilmicosine (72%), erythromycine (70%) en laag voor florfenicol (0%) en enrofloxacine (0.3%). Percentages van klinische resistentie waren hoog voor tetracycline (95%), erythromycine (66%), tylosine (66%), en laag voor florfenicol (0.3%) en enrofloxacine (0.3%). In de afwezigheid van een klinisch breekpunt voor tiamuline werd enkel het percentage van stammen berekend die niet tot de wild type populatie behoorden (57%). De hoge mate van verworven resistentie tegen tiamuline werd eerder nog niet beschreven. Percentages van klinische resistentie en niet-wild type stammen verschilden aanzienlijk voor penicilline. Slechts 1% van de geteste *S. suis* stammen werd klinisch resistent beschouwd, terwijl 47% van de stammen verworven resistentie vertoonde gebaseerd op epidemiologische

interpretatieve criteria. Hieruit kan besloten worden dat de Minimale Inhibitorische Concentratie (MIC) waarden voor penicilline aan de stijgende hand zijn vergeleken met vorige rapporteringen. Varkens besmet met stammen die hogere MIC waarden tonen, kunnen echter nog succesvol behandeld kunnen worden met penicilline. Gevoeligheidsresultaten van deze studie tonen duidelijk aan dat het gebruik van verschillende interpretatieve criteria bijdragen aan verschillen in gerapporteerde antibioticumresistenties. Het vroegtijdig opsporen van kleine veranderingen in de MIC populatieverdeling van bacteriën biedt de mogelijkheid om stappen te zetten in het beheersen van antibioticumresistentie en aldus het risico voor dier en mens te beperken.

Resultaten van de disk diffusie gevoeligheidstest worden geïnterpreteerd door middel van klinische breekpunten indien epidemiologische interpretatieve criteria voor bepaalde *E. coli*-antibioticum combinaties niet voorhanden zijn. Verschillen in standaardisatie van de gebruikte methodes in tijd en tussen laboratoria en verschillen in aanbevolen klinische breekpunten maakt het vergelijken van resultaten van de disk diffusie gevoeligheidstest echter niet betrouwbaar. Hierdoor kunnen potentieel nuttige gegevens afkomstig van verschillende laboratoria niet gebruikt worden voor het monitoren van resistentie. ***Hoofdstuk 5*** omschrijft het gebruik van de 'Normalized Resistance Interpretation' (NRI) methode om de antibioticagevoeligheid van *E. coli* afkomstig van gezonde varkens voor slachten weer te geven. Er kon worden aangetoond dat de NRI methode waardevol is om de wild type populatie te omschrijven voor resultaten waarbij de inhibitie zonediameters van de gevoelige populatie een normale verdeling vertonen. Percentages van niet-wild type stammen konden hierdoor berekend en vergeleken worden met klinische resistentie percentages. Op basis van deze resultaten kan gesteld worden dat de NRI methode wereldwijd gebruikt kan worden om antibioticumresistentie te monitoren in de diergeneeskunde tot er harmonisatie van de disk diffusie methode bereikt is. Zo kunnen eveneens historische antibioticumresistentiegegevens, afkomstig van vroeger uitgevoerde prevalentiestudies, gebruikt worden in monitoringsprogramma's, op voorwaarde dat de inhibitiezone diameters geregistreerd werden.

De laatste studie van deze thesis had als doel te onderzoeken of antibioticumresistente *E. coli* van gezonde zeugen en het gebruik van antibiotica bij zeugen en biggen risicofactoren zijn voor het voorkomen van antibioticumresistentie bij fecale commensale *E. coli* van biggen (***Hoofdstuk 6***). Hiervoor werden 60 zeugen en 3 biggen per zeug bemonsterd op verschillende tijdstippen. Gegevens over antibioticumgebruik rondom werpen en tijdens de kraamperiode werden verzameld. Resultaten van de disk diffusie gevoeligheidstest werden geïnterpreteerd op basis van klinische breekpunten en dit voor 2 isolaten per bemonstering van zeugen en biggen. Slechts 27.4% van de *E. coli* isolaten van de pasgeboren biggen toonde volledige gevoeligheid aan de geteste antibiotica. Resistentie tegen 1 of 2 antibioticaklassen bedroeg 41.2% en 46.8% bij *E. coli* van respectievelijk zeugen en biggen geïsoleerd tijdens de kraamperiode. Multiresistentie tegen minstens 4 antibioticaklassen werd even vaak gevonden bij zeugen (15.6%) als bij biggen (15.2%).

Antibioticumresistentie bij biggen werd beïnvloed door het gebruik van antibiotica bij zeugen en biggen en tevens door de mate van voorkomen van antibioticumresistentie bij de zeug ($p < 0.05$). Het gebruik van aminopenicillines en derde generatie cefalosporines bij biggen beïnvloedde het voorkomen van antibioticumresistentie bij deze biggen (odds ratios [OR] > 1; $p < 0.05$). Ook het gebruik van enrofloxacine is een risicofactor voor het voorkomen van enrofloxacine resistentie bij biggen (OR = 26.78; $p < 0.01$) en bij zeugen rond speenleeftijd (OR = 4.04; $p < 0.05$). Bij zeugen verhoogde het gebruik van lincomycine-spectinomycine rond werpen het voorkomen van resistentie tegen ampicilline, streptomycine en trimethoprim-sulfadiazine bij de zeugen zelf (OR= 21.33, OR= 142.74, OR= 18.03; $p < 0.05$) en tevens het voorkomen van enrofloxacine resistentie bij biggen (OR= 7.50; $p < 0.05$). De conclusie die hieruit volgt, luidt dat het gebruik van antibiotica bij zeugen en biggen een risicofactor vormt voor het voorkomen van antibioticumresistentie bij deze dieren. Bovendien wordt door het gebruik van antibiotica bij zeugen rond werpen een selectiedruk uitgeoefend op de resistente *E. coli* van de biggen.

In de algemene discussie (***Hoofdstuk 7***) wordt vooreerst de ontwikkeling van een datacollectiesysteem (DCS) besproken. Dit DCS moet het mogelijk maken om op basis van een voortgezette verzameling van antibioticumgebruiksgegevens van individuele bedrijven, gerichte stappen te ondernemen naar bedrijven met een onverantwoord kwantitatief en kwalitatief gebruik. *S. suis* kan als commensaal teruggevonden worden bij gezonde dieren en wordt daardoor blootgesteld aan een antibiotica selectiedruk na zowel parenterale als orale behandelingen. *S. suis* zou omwille daarvan dienst kunnen doen als indicatorbacterie om resistentie op te volgen in tijd. Het vaststellen van hogere percentages van niet-wild type stammen dan van klinische resistentie percentages bij *S. suis* afkomstig van gezonde dieren heeft het belang aangetoond van epidemiologische interpretatieve criteria in het aantonen van verworven resistentie en in de monitoring van resistentie in tijd. Het bepalen van deze epidemiologische interpretatieve criteria voor bacteriesoorten, zoals *S. suis*, verdient daarom aanbeveling. Ook de validatie van klinische breekpunten voor de diergeneeskunde is noodzakelijk om 'evidence-based medicine' te bewerkstelligen. Antibacteriële gevoeligheidstesten voor de diergeneeskunde dienen geharmoniseerd te worden. De NRI methode kan echter gebruikt worden om de wild-type populatie van bacteriesoorten te omschrijven tot wanneer deze harmonisatie bereikt is. Antibioticumgebruik bij zeugen rond werpen en tijdens de kraamperiode verhoogt het voorkomen van antibioticumresistente *E. coli* bij de zeugen. Doordat zeugen als reservoir van resistente *E. coli* fungeren voor hun biggen moeten controlemaatregelen voor resistentie geïmplementeerd worden op het niveau van de zeug. Dit omsluit zowel een verminderd antibioticumgebruik als strikte hygiëne maatregelen.

CURRICULUM VITAE

Bénédicte Callens werd geboren op 12 december 1985 te Gent. Na het behalen van het diploma hoger secundair onderwijs, richting Moderne Talen-Wetenschappen, aan het Sint-Bernarduscollege te Oudenaarde, werd de studie Diergeneeskunde aangevat aan de Universiteit Gent. Het diploma van dierenarts (optie gezelschapsdieren) werd behaald in 2009. Na het beëindigen van deze studie startte zij in oktober 2009 als doctoraatsbursaal bij de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. In samenwerking met de vakgroep Bacteriologie, Pathologie en Pluimveeziekten verrichte zij onderzoek naar antibioticumgebruik en – resistentie bij vleesvarkens in België. Dit driejarig mandaat werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. Tussen oktober 2012 en oktober 2014 zette zij haar onderzoek verder in opdracht van de Universiteit Gent en dit gecombineerd met een deeltijdse aanstelling als wetenschappelijk medewerker van AMCRA. Tijdens haar tewerkstelling aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde stond zij mee in voor de opleiding van studenten diergeneeskunde.

Bénédicte is auteur of medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en presenteerde haar onderzoeksresultaten op verschillende nationale en internationale congressen.

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