Epidemiology of antimicrobial resistance and the effect of interventions in food-producing animals

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Epidemiology of antimicrobial resistance and the effect of interventions in food-producing animals

Epidemiologie van antimicrobiële resistentie en het effect van interventies in voedselproducerende dieren

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General introduction

"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant"

Alexander Fleming,
December 11, 1945

The antibiotic era and the emergence of bacterial resistance

Until the 20th century infectious diseases had been the leading cause of human morbidity and mortality but the greatest developments in the known history of medicine were about to happen. It was in 1899 when Emmerich and Löw realized that prepared extracts from *Pseudomonas aeruginosa* had antibacterial activity against other pathogenic bacteria. Their preparations were used in hospitals with inconsistent results and high toxicity, whereupon the treatment was abandoned. Later, in 1945 further investigations would confirm the production of quinolones by this bacterial species[1,2]. Paul Ehrlich set the next milestone in 1904 initiating a systematic screening of chemical compounds in search of a drug against syphilis. He was the first to formulate the hypothesis of a "magic bullet" that would just target disease-causing microbes without harming the human body[2]. This large-scale systematic search of compounds led Fritz Mietzsch and Josef Klarer to synthesize the first 'sulfa' antimicrobial in the early 1930s. This approach is nowadays the cornerstone of drug discovery used by the pharmaceutical industry[2]. Alexander Fleming made in 1929 his first observations on the antimicrobial properties of *Penicillium* fungi. He persistently tried to encourage the scientific community to purify the active substance until 1945, when penicillin started to be marketed and widely distributed[2,3]. Between 1950s and 1970s most of the antimicrobials we use now, were discovered and practically no new classes have been found since then[2].

Antimicrobials have several mechanism of action including interference with synthesis of cell walls (e.g. β-lactams such as penicillin, cephalosporins and carbapenems), inhibition of protein synthesis (e.g. tetracyclines and macrolides), interference with DNA synthesis (e.g. quinolones and fluoroquinolones), inhibition of metabolic pathways (e.g. trimethoprim-sulfamethoxazole), and bacterial membrane disruption (e.g. polymyxins). Bacteria resistant to these attacks will survive and proliferate when they are exposed to these agents. Microorganisms have adaptive responses to changing environments and selective pressures have been observed in the lab even before the extensive use of penicillins[4]. Overexploitation and misuse of antimicrobials over the last decades has intensified this process. As a result, effectiveness of available antimicrobials has been diminished and new mechanisms of bacterial resistance have arisen or become more visible. However, antimicrobial substances already existed in nature long before their discovery. Phylogenetic reconstruction studies have shown that resistance genes to several antimicrobial classes were already present before the large scale use of antimicrobials[5,6]. Recent microbiome research found functional resistance genes, conferring resistance to even synthetic antimicrobials, in the microbiome of human populations without apparent exposure to modern medicine practices[7].

Antimicrobial resistance can be intrinsic or can be developed de novo via mutations or gene transfer. The resistances based on mutations are mostly vertically propagated during bacterial replication. Development of resistance can also happen through acquisition of resistant genes from other bacteria. In that case, genetic material is horizontally transferred from resistant strains to antimicrobial susceptible bacteria through conjugation, transformation

or transduction, with transposons usually facilitating the multiple incorporation of genes in the bacterial host's genome or plasmids[8]. Traditionally, acquired resistance mechanisms have been explained with the bullet-target concept, referring to mechanisms inactivating antimicrobials directly or indirectly; target mechanisms are the modification of drug's target site (e.g. methylation in 23S ribosomal RNA making it insensitive to macrolides) or the production of an alternative metabolic pathway that bypasses the action of the drug (e.g. ribosomal protection proteins that confer resistance to tetracyclines); bullet mechanisms include the production of enzymes that destroy the antimicrobial agent (e.g. production of β-lactamase enzymes that destroy β-lactam antimicrobials) or the expression of efflux systems that pump the drugs out of bacteria[2,8]. Recent research adds to the complexity of drug-bacteria interactions and suggests population-based resistant mechanisms such as kin selection; where a small number of resistant mutants confers protection to the susceptible population coming at a fitness cost to themselves[9]. Moreover, in complex biofilm systems, resistance is conferred to the whole bacterial community regardless of kinship[2]. The concept of resistance is therefore broadening to the system level and other areas of research, such as biofilm formation, are being considered.

Antimicrobials in animals and humans: two sides of the same coin

Around the time antimicrobials were introduced in human medicine, they also started to become a vital element in intensified food-animal production. Currently, antimicrobials in livestock are widely used around the world and are applied for several purposes. The number of individual antimicrobial treatments applied to diseased animals is exceeded, by far, by group treatments for disease control (e.g. in outbreaks) or prevention. These group treatments are commonly applied through feed or water to animal herds for metaphylactic (i.e. some animals have clinical signs of disease) or prophylactic reasons (i.e. animals have no clinical signs of disease although some may be subclinical)[10]. In addition, around 60 years ago it was first observed that subtherapeutic doses of antimicrobials enabled animals to obtain more energy from feed and gain more weight[11,12]. From that moment onwards, the non-therapeutic use of antimicrobial growth promoters (AGPs) has been widely practiced.

After AGPs became the norm in livestock production and generation of resistance was observed, the first concerns about potential impacts in human health were expressed by Swann in a report to the British Parliament (1969) advocating for banning this practice[13]. In the more recent years several reports were published recommending the same precautionary action that was first applied in Sweden (1985), Norway (1995) and Denmark (1998-1999) and it would be followed by an EU level ban in 2006[14-16]. AGPs traditionally included bambermycin, avoparcin and bacitracin among other antimicrobials, and despite the evidence for its contribution to the resistance problem, substantial amounts are still used in many parts of the world especially in the USA and some parts of Asia[17].

Accurate information on the volume of antimicrobials administered to animals has not

been available until recent years and in most parts of the world these data are very limited. There is more transparency in Europe, where sales of antimicrobials in animals and humans are reported annually by the European Medicines Agency[18, 19]. In any case, quantities of antimicrobials in animals account for a large proportion over the total use (i.e. including use in humans). For instance, in the USA antimicrobial consumption in animals in 2010 was estimated to account for around 80% of the annual total consumption[20]. It is also worrisome that conservative estimates have projected a global increase of 67% on veterinary antimicrobial use by 2030 as a consequence of the growing livestock production sectors in middle-income countries[21].

Resistance to antimicrobials used in animal and human medicine is determined by the same mechanisms and this is of extreme importance if we consider that, with a few exceptions, the same antimicrobial classes are used in human and veterinary medicine[17]. Thus, informed guidance on the decision to restrict the use of certain drugs in animals is a key element for the preservation of the benefits of antimicrobials for people. This is the reason why the World Health Organization ranked antimicrobials according to their importance for human treatments, availability of alternatives, cross-resistance selection and frequency of use[22]. This ranking facilitates focusing management efforts, such as restrictions on usage or bans for animal use of critically important drugs for humans (e.g. fluoroquinolones, macrolides and 3rd-4th generation cephalosporins). These recommendations are starting to be implemented in a few countries and help to reduce overlap between animal and human use of antimicrobials. Whether this will result in changes in resistance patterns and newly emerging risks in animals needs further assessment.

Emerging bacterial resistance in animals transmittable to humans: the case of MRSA and ESBLs

Using antimicrobials in livestock production entails a wide spectrum of identified or potential, yet difficult to quantify, hazards for human health, food safety, animal health and welfare, and for the environment. Several factors have favoured a worldwide and rapid dissemination of resistance traits not only in pathogenic bacteria from animals but also in commensal flora[23]. Namely, the way by which antimicrobials are administered to animals, high animal densities in production systems, the globalization of trade of animal products and the constant human and animal movements. Based on temporal, geographical, genetic and epidemiological associations, causal relationships can be assessed between antimicrobial use and resistance in animals and the subsequent transmission to humans[24-28]. Resistant bacterial clones or resistant genes can reach humans via numerous routes including food (e.g. multidrug resistant [MDR] *Campylobacter* spp. and Enterobacteriaceae species), direct contact and/or potentially the air (e.g. methicillin-resistant *Staphylococcus aureus* [MRSA]) and the environment (e.g. MDR Enterobacteriaceae). The possible transmission routes of antimicrobial resistance between

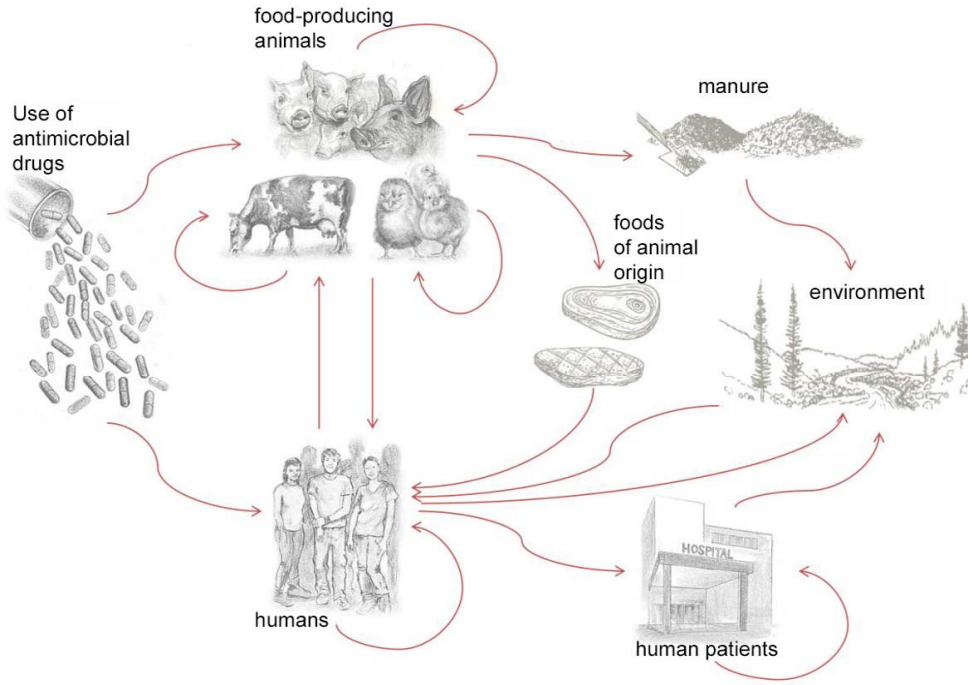
humans, animals and the environment are depicted in the figure below.

This thesis describes the recent emergence of new antimicrobial resistances in animals. It focuses on commensal bacteria *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) and particularly on two types of resistant species of importance for human health: livestock-associated MRSA (LA-MRSA) and extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-*E. coli*). Description on resistance emergence will be limited to these species although it conceptually applies to other bacterial taxa. The different mechanisms of acquired resistance in these two bacteria strongly influences their epidemiology and risk profile.

The emergence of MRSA

The upper respiratory tract is the primary habitat of *S. aureus* in humans and animals. The use of β-lactam antibiotics selected for methicillin-resistant strains (MRSA) turning this bacterium into a public health problem[29]. The gene conferring this methicillin resistance was called *mecA*. Located in the staphylococcal cassette chromosome *mec* (SCC*mec*), it encoded a modified antibiotic target (penicillin-binding protein 2a) not present in susceptible strains[30]. MRSA was first identified in hospital isolates in 1960, only one year after the introduction of methicillin[31]. After 1970 incidence of hospital-acquired MRSA (HA-MRSA) progressively

Figure. Main transmission routes of antimicrobial resistant bacteria and resistance traits between animal, humans and the environment. (credit: Angel Dorado Candela)



increased in several countries over 30%, becoming one of the leading causes of nosocomial infections such as bacteraemia, pneumonia, endocarditis and skin infections[23]. In the late 1990s, genetically distinct strains with new virulence characteristics started to be recovered in isolates from people outside hospitals. These new strains mainly caused soft tissue infections in younger patients and were named community-associated MRSA strains (CA-MRSA)[32-34].

Occurrence in animals began in 2005 when MRSA transmission from pigs to humans was first reported in the Netherlands[35]. Since then, MRSA was increasingly isolated in samples from pigs, veal calves and to a lesser extent poultry across the world. Livestock-associated strains (LA-MRSA) were non-typeable by routinely used pulsed-field electrophoresis (PFGE) with the restriction enzyme Sma I and they mainly belonged to the sequence-type (ST) 398[29,36,37]. In 2008, it was estimated that prevalence of LA-MRSA-positive pig holdings was at least 23% in the EU, with wide variations between member states (from 0% to 51%)[38]. Currently, LA-MRSA is widely spread around the world and does not show pronounced host specificity. As a result, the farming community with direct animal contact is exposed and frequently colonized or contaminated[29]. Transmission between people seems to be infrequent and LA-MRSA infections are relatively rare[39]. Nevertheless LA-MRSA human cases have become more visible in countries with a very low overall MRSA prevalence, whereupon receiving heightened public attention. Notwithstanding the low prevalence, human infections are a matter of worldwide concern because of the potential risks. Genes with new resistance mechanisms or new variants of *mecA* may emerge and spread (e.g. MDR plasmid gene *cfr; mecC* gene variants)[40, 41] which brings attention to the need for close surveillance of LA-MRSA.

The emergence of ESBLs

Enterobacteriaceae (e.g. *Salmonella*, *Escherichia*, *Enterobacter*, *Klebsiella* and *Yersinia*) are a large family of Gram-negative bacteria genera inhabiting the intestinal tract of animals and humans. *E. coli* and *K. pneumoniae* are common causes of intraabdominal, respiratory, urinary tract, and bloodstream infections in hospitals[42]. Antimicrobial resistance in the causative agents of these infections has dramatically increased in the last decades and nowadays is one of the greatest public health concerns. The emergence of ESBLs illustrates how horizontal transfer of genes contributes to rapid propagation of resistance across different bacterial species. Bacterial plasmids containing MDR genes equip these species with a flexible and interchangeable resistance tool[23]. The production of β-lactamase enzymes is the most important resistance mechanism among these bacilli and also in this case, the extensive use of antimicrobials accelerated the expansion of resistance. The first plasmid-mediated β-lactamases, TEM-1 and SHV-1, were identified 60 years ago in *E. coli* and *K. pneumoniae* clinical isolates[43,44]. Later on, cephalosporins (extended spectrum β-lactam antimicrobials) were introduced and extensively used, given their stability against TEM-1 and SHV-1. As a consequence, the first extended-spectrum β-lactamase (ESBL) enzyme, SHV-2, hydrolysing

cephalosporins, emerged in 1985[45]. Subsequently mutant derivatives from TEM and SHV rapidly spread throughout the world and nowadays there are more than 130 different TEMs and 50 SHVs ESBLs[46]. In the early 1990s a new ESBL with greater activity against cefotaxime, CTX-M-1, was identified in Germany[47]. Shortly after, a great dissemination of cefotaxime-resistant *Salmonella* started in South America and CTX-M-2 was described[48,49]. CTX-M-encoding plasmids were acquired by *E. coli* and *K. pneumoniae* from different *Kluyvera* species and particular CTX-M genotypes began to expand in different geographical regions[23]. During the last decade, a endemic CTX-M prevalence has been reached in many parts of the world and CTX-M genotypes have become the most dominant ESBLs in Europe[23].

In recent years, increasing number of reports began to show ESBL carriage in livestock and food products like meat, milk and fish[50-53]. The spotlight was put again on animals as a potential source of resistant infections in humans. Clonal dissemination together with horizontal transfer of resistance originates complex links between bacterial populations sharing environments with animals and humans. Consequently, ESBL epidemiological investigations are complicated by different direct and indirect exposure routes and sources. Nonetheless, current evidence clearly indicates that a proportion of human extra-intestinal infections with ESBL-producing bacteria originates from animals[54-59]. The risk for public health attributed to animal and environmental sources will need to be fully assessed while precautionary actions are taken to protect human health.

The Dutch approach to tackle the transmission of antimicrobial resistance from animals to humans

Antimicrobial use in human medicine in the Netherlands is among the lowest in Europe. This is reflected in low resistance levels in health care settings. In particular, MRSA and ESBL are present in less than 1% of the isolates from clinical bacterial infections[60,61]. Contrarily, high volumes of antimicrobials have historically been used in animal husbandry. This contrast in antimicrobial use in human patients and food-producing animals has stimulated a series of measures in the Netherlands to tackle potential problems originating from the animal reservoir[61].

It is essential to know past trends in veterinary use of antimicrobials to understand the most recently initiated Dutch policies. Between 1999 and 2006, annual sales of antimicrobial growth promoters decreased gradually from 250 tons to zero[62]. However, this dramatic change was accompanied by a marked increase in sales of antibiotics for treatment of food-producing animals, from 300 tons in 1999 to 600 tons in 2007[62]. According to the European Surveillance of Antibiotic Use working group (ESVAC), between 2005-2009, the Netherlands was among the EU countries where most antibiotics were used per kg live weight animal produced[62]. The parallel emergence of LA-MRSA and the increasingly reported isolation of ESBL-producing bacteria in animals and food triggered profound changes in the health policy agenda. In

2008, a task force on Antimicrobial Resistance in Food animals was installed by the Dutch government and a new policy was outlined to drastically reduce the use of antimicrobials in animals[61]. Since then, mandatory reduction targets have been set and enforced by the different animal production sectors. Using 2009 as index, the first targeted reductions of 20% in 2011 and 50% in 2013 were abundantly reached[63]. Use of critically important antimicrobials (3rd generation cephalosporins and fluoroquinolones) were also restricted for animal treatments by requiring cultures and susceptibility testing before these drugs could be prescribed[64,65]. The new reduction target of 70% reduction by 2015 will be soon evaluated. Thanks to the integrated surveillance and monitoring system, the first indications of decreasing trends in antimicrobial resistance have been observed[63]. Nevertheless, new measures and approaches will be needed in the future in this obstacle race against antimicrobial resistance.

In this thesis

This thesis provides further insight into antimicrobial resistance in the animal-human interface. Interventions with the ultimate aim of reducing the prevalence of resistance are explored by epidemiological risk factor analyses. The presented results rely on longitudinal data and contribute to establishing causality by considering temporality[66]. Finally, additional knowledge gaps on the relative contribution of animal reservoirs to human health are also addressed.

Part I (chapters 2 and 3) is focused on MRSA in veal calf farming. Chapter 2 explores MRSA carriage dynamics and carriage determinants in humans occupationally exposed to veal calves. A risk factor analysis gives insight into possible farm-level interventions and a potential risk of MRSA transmission through air is for the first time assessed. In chapter 3, a three-arm study evaluates the efficacy over time of two measures believed to reduce MRSA prevalence in veal calves and subsequently in farmers; the interventions consist in the implementation of a protocol to decrease use of antimicrobials and the application of a stringent cleaning and disinfection program. Additionally, the potential 'adverse effects' of a sustained reduction of antimicrobials on animal production is estimated against a set of farm technical performance parameters.

Part II (chapters 4 and 5) present the results of a longitudinal study in pig farming. In chapter 4, the direct relationship between amount of antimicrobials used in animals and MRSA prevalence in pigs and humans in direct contact with pigs is quantified. A risk factor analysis identifies determinants of MRSA carriage in the animal and human populations as a basis for practical interventions. In chapter 5 the same methodology is applied, but now for ESBL-*E. coli* carriage in pigs.

In Part III (chapter 6), an ecological approach is followed to associate a nationwide antimicrobial use reduction in the main livestock sectors to resistance levels in commensal *E. coli.*

Lastly, Part IV (chapter 7), describes a dimensionality reduction study by which molecular similarities are being evaluated for ESBL-producing *E. coli* from different human, animal and environmental reservoirs. The study uses gene and plasmid replicon typing information from several studies. A meta-collection of isolates has been created to identify reservoirs that may constitute a burden for ESBL infections and carriage in humans.

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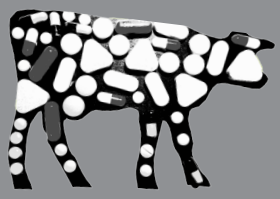
Part I

Livestock-associated methicillin-resistant *Staphylococcus aureus* in veal calf farming



Chapter 2

Risk factors for persistence of livestock-associated MRSA and environmental exposure in veal calf farmers and their family members: an observational longitudinal study

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Abstract

Objectives: Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) emergence is a major public health concern. This study was aimed at assessing risk factors for persistently carrying MRSA in veal calf farmers and their family members. We also evaluate the dynamics of MRSA environmental load during the veal-calf production cycle.

Design: Observational, longitudinal, repeated cross-sectional study.

Setting: 52 veal calf farms in the Netherlands.

Participants: From the end of 2010 to the end of 2011, a total of 211 farmers, family members and employees were included in the study.

Primary outcome and secondary outcome measures: Nasal swabs were taken from participants on days 0, 4, 7 and week 12. A persistent MRSA carrier was defined as a person positive for MRSA on days 0, 4 and 7. Participants filled in an extensive questionnaire to identify potential risk factors and confounders. For estimation of MRSA prevalence in calves and environmental contamination, animal nasal swabs and Electrostatic Dust Collectors were taken on day 0 and week 12.

Results: The presence of potential animal reservoirs (free-ranging farm cats and sheep) and the level of contact with veal calves was positively associated with persistent MRSA carriage. Interestingly, at the end of the study (week 12), there was a twofold rise in animal prevalence and a significantly higher MRSA environmental load in the stables was found on farms with MRSA carriers.

Conclusions: This study supports the hypothesis that environmental contamination with MRSA plays a role in the acquisition of MRSA in farmers and their household members and suggests that other animal species should also be targeted to implement effective control strategies.

Introduction

In recent years, livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA), specifically sequence type (ST) 398, has emerged in food-producing animals and people in contact with these animals[1-4]. Illness associated to ST398 in humans is rare and only a small proportion of MRSA infections can be attributed to LA-MRSA[5,6]. Nonetheless, invasive infections and hospital outbreaks of MRSA ST398 have been reported in Europe, the USA and Asia[5,7,8].

LA-MRSA strains have been found mainly in pigs and veal calves, but they have the capacity to colonize a wide spectrum of hosts, including sheep and poultry[9]. Farmers are easily contaminated and in general the carriage prevalence in farmers is high. Frequency of transmission between farmers and their family members and among hospitalized humans appears to be low[2,10,11]. However, this belief might be contradicted by recently described LA-MRSA transmission events in Dutch patients with neither risk factors nor livestock contact[12]. The potential public health threat posed by these strains is emphasized in a recent meta-population model in which the likelihood of persistent carriage in the livestock-exposed population was the key parameter for LA-MRSA spreading to the community[13].

Previous studies have been mainly based on cross-sectional designs and have shown that intensity of animal contact and MRSA prevalence among animals are positively associated to LA-MRSA human carriage[14]. Associations between animal carriage and farm hygiene and antimicrobial use have also been shown[15,16]. A longitudinal study including periods of high and low exposure to animals showed that LA-MRSA carriage was mainly transient. It was suggested that LA-MRSA is a poor persistent colonizer in humans, which was confirmed by a study on short-term occupational exposure[10,14]. However, risk factors for persistent LA-MRSA carriage and for a possible true colonization have not been thoroughly assessed. Furthermore, little is still known about the dynamics of environmental contamination with MRSA in the farm and its role in transmission to humans. A recent study showed a steep increase in prevalence among calves and in MRSA air load during the production cycle[17].

The aim of the current study was twofold. First, to assess risk factors and dose–response relationships for persistently carrying MRSA over a period of 1 week at the beginning of the production cycle in veal calf farmers and their family members. Second, to evaluate the deposition of MRSA-containing dust inside the farm and its relationship with animal and human MRSA carriage.

Materials and methods

Study design and population

A longitudinal cohort study was performed over a period of 12 weeks in 52 veal calf farms starting at the beginning of the production cycle. All farms were visited from the end of 2010 to the end of 2011. All farms met the following inclusion criteria: implemented all-in-all-out

system; no other livestock in large scale apart from veal calves; a unique location for all the stables or farm; veal calf farmers not working in another animal sector (e.g., transport of pigs) and not operating in other farms. Preference for selection was given to farms in the proximity of Utrecht, the Netherlands. On each farm there were two sampling periods for animal and environmental samples (day 0 and week 12) and four sampling periods for human samples (days 0, 4, 7 and week 12). Nasal swabs from both anterior nares of calves were taken and analysed in 10 pools of six swabs each (60 animals per farm). Swabs were also collected from farmers, family members and employees (n=211). On day 0, quantitative nasal and throat swabs were taken by field workers in the majority of participants or by self-sampling. On days 4, 7 and on week 12, dry cotton swabs (Copan, Brescia, Italy) were used to self-sample the nose. Swabs were given to participants with instructions including photographs in case of self-sampling. Nasal swabs in animals and humans were introduced in the nostril and rotated once. Throat swabs in humans sampled the area of the inner cheek including the tonsils. The swabs were immediately taken to the laboratory or sent by post and processed within 24 h after arrival. Furthermore, environmental samples were taken by placing four Electrostatic Dust Collectors (EDCs; Zeeman, Utrecht, The Netherlands) on different surfaces inside the stables and one on the highest cupboard in the living room or kitchen of the house. The EDCs were left in place during a period of 2 weeks and sent by post to the laboratory. On arrival, EDC samples were stored at –20°C until quantitative analysis[18].

All participants completed an informed consent form and filled in an extensive questionnaire including items related to individual health status, household and farm characteristics, activities performed on the farm and hygiene practices. The protocol of the study was approved by Medical Ethical Committee of Utrecht University. The collection of animal samples was in compliance with the Dutch Law on Animal Health and Welfare.

For the assessment of MRSA-persistent carriage, we selected the beginning of the veal-calf production cycle, just after the stables were empty and when animal prevalence is lower. In this period, deposition of MRSA-containing dust particles in human nasal cavities and mechanical carriage was assumed to be less likely. Therefore and for the purpose of this study, a person was defined to be a persistent MRSA carrier when each of the nasal swabs collected on days 0, 4 and 7 were positive for MRSA presence.

Laboratory analysis

Swabs in liquid transport medium (ESwab, Copan, Brescia, Italy) were used for quantitative cultivation. Serial dilutions (1:10) of the transport medium (concentration 100) were made by adding 100 µL sample to 900 µL phosphate buffered saline (PBS) to a final concentration of 10−4 of the original sample. Each dilution was cultured on chromID *S. aureus* and chromID MRSA agar plates (BioMérieux, La Balme Les Grottes, France) at 37°C for 18–24 h. Plates with 10–100 colony-forming units (CFU) were used to calculate the original amount of CFU per swab. In order to detect positive samples without bacterial growth in the first day, the

remaining transport medium and swab were enriched overnight in Mueller Hinton broth with 6.5% NaCl (MH+), and consequently cultured on chromID *S. aureus* and chromID MRSA agar plates. The theoretical lower limit of quantification (LLOQ) of MRSA CFU was 10. Dry cotton swabs (Copan) were inoculated directly onto chromID *S. aureus*, chromID MRSA and MH+. Confirmation of MRSA presence in the three sampling moments was carried out using real-time (RT) PCR targeting *mecA, femA* and *nuc* genes[19,20]. Methicillin-susceptible *S. aureus* (MSSA) presence was tested when the bacterial growth on chromID *S. aureus* was higher than on chromID MRSA. For this purpose, 10 colonies were screened for methicillin susceptibility by using the Cephoxitin disk diffusion method. Confirmation of MSSA was carried out using RT-PCR. Nasal swabs from calves were analysed in pools following standard procedures previously described[21].

To obtain an estimate of exposure in CFU per EDC, EDCs were analysed using RT-quantitative PCR (qPCR). EDC samples were suspended in 10 mL EDTA saline buffer (150 mM NaCl, 1 mM EDTA) and mixed in a Stomacher (Seward Ltd., London, UK) for 10 min. Two millilitre of the resulting suspension was stored at –20°C for the analysis. For DNA isolation, 200 µL of the suspension was incubated at 95°C for 15 min. PBS was added and a Versant kPCR molecular system (Siemens Healthcare Diagnostics, The Hague, The Netherlands) was used for DNA purification with an elution volume of 50 µL. Five microliter of the purified sample were used for detection of *mecA, femA* and *nuc* genes by the means of a LightCycler 480-II system (Roche Diagnostics, Almere, The Netherlands). For MRSA quantification, a standard curve was established for all targets. A standard control sample was included in each run to correct the curve for run-to-run variation. For interpretation of the results, CFU counts per PCR were transformed to CFU counts per EDC (1 CFU/PCR=200 CFU/EDC). The theoretical limit of detection was 20 CFU/EDC.

RT-PCR targeted at *C01* gene was carried out for confirmation of ST398 in all MRSA-positive human, animal and environmental samples.

Data analysis

Statistical analysis was performed using SAS software V.9.2 (SAS institute Inc, Cary, North Carolina, USA). Descriptive analysis determined the cross-sectional human prevalence on each of the four sampling moments and the longitudinal carriage patterns (persistent, intermittent or non-carriers).

Risk factors for nasal MRSA-persistent carriage were investigated with univariate and multivariate analysis. PROC GENMOD was used for Generalized Estimating Equations modelling to take clustering of data at farm level into account. The mean response was modelled with a Poisson regression with robust SEs. Crude and age-sex adjusted prevalence ratios (PRs) were obtained. Eligibility criteria for variables to be considered in multivariable analysis included univariate p-values below 0.2, less than 10% of missing data in relation with the outcome, and at least two persistent carriers falling in each of the categories of the

explanatory categorical variables. Bivariate correlation structure of all eligible variables was studied with PROC CORR, and Spearman correlation coefficients were obtained. Thereafter, eligible variables were added in a stepwise backward selection approach and retained in the final model when $p<0.15$. A p-value <0.05 was considered statistically significant.

The shape of the relationships between MRSA-persistent carriage and numerical variables was studied by means of non-parametric or semiparametric regression modelling (smoothing) using PROC GAM to relax the assumption of linearity. For this purpose, the number of CFU from quantitative nasal swabs positive for MRSA but below LLOQ was set to 5.

To assess the environmental exposure during the first week, farms were classified in three categories: (1) farms with persistent carrier, when there was at least one MRSA-persistent carrier working and/or living on the farm; (2) farms with intermittent carrier, when there was at least one MRSA-intermittent carrier and there was no persistent carrier on the farm; (3) non-carrier farms, when all people at the farm were MRSA negative on the first three sampling periods. On week 12 farms were classified as carrier and non-carrier farms when there was at least one MRSA carrier on the farm, and when all people on the farm were MRSA negative on week 12, respectively. Proportions of MRSA-positive EDCs were calculated per farm category and sampling moment. For calculation of average exposure levels, CFU counts per EDC were log-transformed since they followed a highly right-tailed distribution. PROC LIFEREG was used for left-censored regression (tobit) modelling to obtain an accurate estimate of the mean exposure level accounting for the large proportion of undetectable values. Thereafter geometric means were calculated.

Results

Descriptive results

Nasal swabs were collected from 211 participants on 52 farms. The average nasal MRSA prevalence for the four sampling moments was twice as high in farmers (29.7%) as compared with family members (13%). Cross-sectional nasal MRSA prevalences per sampling moment are displayed in appendix Figure S1.

Nasal carriage patterns for MRSA, MSSA and *S. aureus* in general (including both MSSA and MRSA) were assessed over the 1-week period. The MRSA and MSSA-persistent carrier prevalence followed opposite directions in farmers as compared with family members. For MRSA-persistent carriage the prevalence in farmers (15.5%) was twice as high as in family members (7.6%). MSSA-persistent carriage prevalence was three times higher in family members than in farmers (15.3% and 5.2%, respectively). Regarding *S. aureus*, there were not significant differences between the subpopulations of farmers and family members and 22.8% of all individuals were persistently carrying the bacteria, 29.6% were intermittent carriers and the remaining 47.6% never carried *S. aureus*. Appendix table S1 shows these longitudinal carriage patterns in more detail.

The RT-PCR targeted at *C01* gene showed that ST398 was present in 90.5% of the human MRSA isolates, in 97.9% of the MRSA-positive animal pools and 90.9% of the MRSA-positive EDCs.

Microbiological status and persistent MRSA nasal carriage

CFU counts were determined in 42 participants from quantitative nasal swabs on day 0. Figure 1 shows the shape of the relationship between the probability of being a persistent MRSA nasal carrier and the log-transformed MRSA concentration (CFU/swab suspension). The median CFU count was 43.65 with an IQR 5.01–1096.48. In addition, the univariate logistic regression analysis in this population resulted in 1.68 times higher risk (95% CI 1.34 to 2.10, p<0.001) for persistent MRSA carriage per 10 CFU increase.

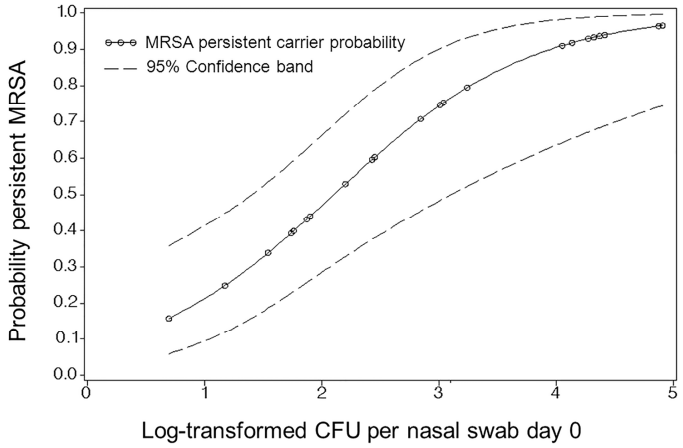

Figure 1. Probability of nasal methicillin-resistant *Staphylococcus aureus* (MRSA) persistent carriage and its relationship with the log-transformed colony-forming units (CFU) from MRSA-positive nasal swabs at day 0. Non-parametric regression modelling.

No MSSA was found in MRSA-positive samples at day 0. In order to obtain an estimation of the PR for the outcome when MSSA is present at day 0, data were manipulated by placing an MSSA-positive result for one of the persistent carriers. This way an adjusted PR of 0.14 (95% CI 0.02 to 1.06, p=0.06) was obtained.

People found positive for MRSA in throat swabs at day 0 were at higher risk for being persistent nasal carriers (adjusted PR=12.2, 95% CI 5.2 to 28.8, p<0.0001). The spearman correlation coefficient between this variable and the outcome was 0.6 (p<0.0001).

A sensitivity analysis restricted to ST398 was carried out and it yielded similar results as described above.

Univariate and multivariate analyses for persistent MRSA nasal carriage

Crude and age-sex-adjusted PRs in determinants meeting the specified criteria are presented in Table 1. Gender and smoking habits were not clearly associated with the outcome (p>0.2).

Although these variables together with age are considered potential confounders, sensitivity analysis was performed with smoking habits added to gender and age for adjustment. This did not result in significant changes in estimates (results not shown) when compared with adjustment without smoking habits.

Statistically significant risk factors for persistent MRSA carriage were identified (Table 1). Pet ownership showed a PR of 2.7 (p=0.05). The number of working hours per week in the farm was positively associated with the outcome (adjusted PR=2.5 expressed per 20 h/week increase, p=0.001). An increasing probability for MRSA-persistent carriage with number of working hours in the farm was also demonstrated through semiparametric regression modelling (see appendix Figure S2). Administration of antimicrobials to calves through milk and injection in the past month preceding sampling was also a significant risk factor (adjusted PR=3.4, p=0.01). Other associations with the outcome did not show statistical significance. These include protective factors such as people living on farms with a changing room available (adjusted PR=0.5, p=0.07) or on farms where clean towels are used after work (adjusted PR=0.6, p=0.11) and risk factors, such as people living in farms where baby boxes are cleaned at the beginning of the production cycle (adjusted PR=1.3, p=0.54). Other determinants such as the prevalence of MRSA in animals at the farm level did not show an association with persistent human MRSA carriage (PR=1.0, 95% CI 1.0 to 1.0, p=0.96). There was also no association found with variables regarding individual health status.

Results from the multiple logistic regression analysis are presented in Table 2. In model A, all variables meeting the described criteria were eligible to entry. In this model, number of working hours per week showed the most significant association with persistent MRSA carriage (PR=1.8 expressed per 20 h/week increase, p<0.0001). As this variable was a very strong determinant, the result of which potential tasks were not retained, a model was explored (model B) without the number of working hours. In consequence, stable management (sorting calves) was retained in the final model B with a statistically significant PR of 3.1 (p=0.03). In both multivariate models, the presence of cats on the farm was significantly associated with the outcome (PR=2.8, p=0.01 in model A and PR=2.6, p=0.04 in model B).

Specific tasks on the farm were adjusted for number of working hours in a bivariate analysis and the estimates obtained were not statistically significant. Only stable management remained positively associated with the outcome with a PR of 2.5 (95% CI 0.7 to 9.6; p=0.17); however, administration of antibiotics in the month before sampling showed no association with a PR of 1.1 (95% CI 0.2 to 5.9; p=0.91).

A sensitivity analysis restricted to ST398 was carried out and it yielded similar univariate and multivariate results.

Table 1. Crude and adjusted for sex and age prevalence ratios (PR) for nasal MRSA persistent carriage in 195 veal calf farmers and household members from 51 farms.

Determinant	Category	N	No. Persistent carriers† (prevalence %)	PR and 95% CI	Adjusted PR‡ and 95% CI
General characteristics:					
Sex	Female	92	11 (12.0)	1.4 (0.6-3.2)	-
	Male	103	9 (8.7)	Ref.	-
Age (mean=30, range=0.1-81)	per 10 y	195	-	1.3 (1.1-1.6**)	-
	per 1 y	195	-	1.0 (1.0-1.0**)	-
Farm and household characteristics:					
Presence of sheep in farm	Yes	46	8 (17.4)	2.2 (1.1-4.5*)	2.4 (1.2-4.8*)
	No	149	12 (8.1)	Ref.	Ref.
Presence of cats on farm	Yes	99	15 (15.2)	3.0 (1.2-7.1*)	2.7 (1.1-6.6*)
	No	96	5 (5.2)	Ref.	Ref.
Presence of pets	Yes	121	16 (13.2)	2.7 (1.0-7.4*)	2.6 (1.0-6.7§)
	No	74	4 (5.4)	Ref.	Ref.
Tasks performed last 7 days¶ :					
Sorting calves (stable management)	Yes	82	15 (18.3)	4.2 (1.5-12.3**)	4.7 (1.3-16.8*)
	No	113	5 (4.4)	Ref.	Ref.
Healthcare / control††	Yes	63	11 (17.5)	2.6 (1.1-6.1*)	2.3 (0.8-7.3)
	No	132	9 (6.8)	Ref.	Ref.
Feeding calves	Yes	123	18 (14.6)	7.2 (0.9-58.6§)	5.4 (0.6-52.3)
	No	72	2 (2.8)	Ref.	Ref.
Work at farm, hygiene cleaning and disinfection:					
Administration of antibiotics during the last month	Yes	64	12 (18.8)	3.2 (1.4-7.1**)	3.4 (1.3-9.1*)
	No	131	8 (6.1)	Ref.	Ref.
# working hours per week (mean=16.5, range=0-80)	per 1 h	195	-	1.0 (1.0-1.0***)	1.0 (1.0-1.1**)
	per 20 h	195	-	1.8 (1.4-2.4***)	2.5 (1.4-4.2**)
Clean towel	Yes	150	13 (8.67)	0.6 (0.3-1.3)	0.6 (0.3-1.1)
	No	45	7 (16.7)	Ref.	Ref.
Changing room available	Yes	177	17 (9.7)	0.6 (0.3-1.2)	0.5 (0.2-1.0§)
	No	18	3 (16.7)	Ref.	Ref.
Cleaning of baby boxes	Yes	11	2 (18.2)	1.9 (1.0-3.5*)	1.3 (0.6-2.8)
	No	184	18 (9.8)	Ref.	Ref.

†A person is considered a persistent carrier when all nasal swabs at days 0, 4 and 7 are positive for MRSA.

‡Prevalence ratios adjusted for sex and age.

§Non-significant trend (p-value 0.05–0.10). * p-value 0.01–0.05. **p-value 0.0001–0.01. ***p-value <0.0001.

¶Tasks performed in the week before time 0.

††The task healthcare and control includes the administration of antibiotics.

MRSA, methicillin-resistant *Staphylococcus aureus*; PR, prevalence ratio.

Table 2. Results from multiple logistic regression analysis for nasal MRSA-persistent carriage in veal calf farmers and their household members (N=195).

Determinant	Category	PR	95% CI	p-value
Model A				
Number of working hours per week	per 1 hour	1.03	1.02-1.04	0.000*
	per 20 hours	1.81	1.49-2.19	
Presence of cats on farm	Yes	2.80	1.23-6.36	0.014*
	No	Ref.		
Presence of sheep in farm	Yes	1.83	0.89-3.77	0.100
	No	Ref.		
Changing room available	Yes	0.48	0.20-1.13	0.094
	No	Ref.		
Cleaning of baby boxes	Yes	3.96	1.59-9.90	0.003*
	No	Ref.		
Model B				
Age	per 1 year	1.02	1.00-1.05	0.037*
	per 10 years	1.26	1.01-1.56	
Presence of cats on farm	Yes	2.57	1.05-6.33	0.040*
	No	Ref.		
Presence of sheep in farm	Yes	1.78	0.88-3.59	0.107
	No	Ref.		
Sorting calves	Yes	3.10	1.14-8.47	0.027*
	No	Ref.		

Model A: final model in which all variables meeting eligibility criteria were added to the automatic selection. Model B: final model in which all the variables in model A were added to the automatic selection except number of working hours. *p-value statistically significant (i.e., <0.05). MRSA, methicillin-resistant *Staphylococcus aureus*; PR, prevalence ratio.

Contamination of the environment with MRSA

At the beginning of the production cycle, MRSA was detected in only 4.6% of all EDCs placed in stables and on six farms. Differences in environmental exposure across persistent, intermittent and non-carrier farms were not significant (Table 3). None of the EDCs placed inside the houses were found to be positive for MRSA.

In week 12, MRSA was detected in 50.6% of all EDCs placed in the stables and on 39 farms. There was a significantly higher proportion of EDCs positive for MRSA and a trend for higher CFU counts per EDC in farms where MRSA carriers were found in week 12 (table 4). Stratified analysis was performed in farmers and family members. The same trends for higher MRSA environmental load were found only in farmers, however not statistically significant (results not shown). MRSA was found in EDCs from 10 houses (table 4).

The mean pooled MRSA prevalence in calves rose from 18.7% at day 0 to 46% in week

12. A simple linear regression between the EDC MRSA levels (maximum log-transformed MRSA CFU/EDC per farm) and animal prevalence showed a positive and significant association (β=0.006, p=0.0014). Furthermore, there was a 60% increased probability for detecting an MRSA-positive EDC in farms where animal prevalence in week 12 was above the mean (PR=1.6, 95% CI 1.09 to 2.38, p=0.02). With regard to human carriage in relation to animal prevalence, no association between being an MRSA carrier and the prevalence

Table 3. Environmental MRSA samples (EDCs) taken in stables at the beginning of the production cycle in 51 farms with persistent, intermittent or non-MRSA carrying veal calf farmers and household members.

	Persistent*	Intermittent*	Non-carrier*	p-value†
Number of farms with MRSA-positive EDCs / total number of farms (%)	2/18 (11.11)	2/12 (16.67)	2/21 (9.52)	0.86
Number of MRSA positive EDCs / total number of EDCs (%)	2/69 (2.90)	4/47 (8.51)	3/78 (3.85)	0.38
GM MRSA CFU/EDC (p Value) ‡	<1 (0.75)	<1 (0.29)	<1 (ref.)	

*A farm was categorized as persistent when there was at least one persistent carrier living and/or working on the farm, non-carrier farms had no individual positive for MRSA in nasal swabs on days 0, 4, 7 and intermittent farms were the remaining.

†p-values among proportions were calculated with Fisher's exact test. Mean values had not an overall assigned p value since they could not be tested with non-parametric tests.

‡Geometric mean (antilogged results from tobit regression). p-values indicate the difference with the reference category (non-carrier farm).

CFU, colony-forming units; EDC, Electrostatic Dust Collector; GM, geometric means; MRSA, methicillin-resistant *Staphylococcus aureus.*

Table 4. Environmental MRSA samples (EDCs) taken in stables and houses on week 12 in 49 farms with MRSA carriers and non-carriers.

	Location EDC	Carrier farms*	Non-carrier farms*	p-value†
Number of farms with MRSA-positive EDCs / total number of farms (%)	Stable	22/25 (88.00)	17/24 (70.83)	0.14
	House	3/25 (12.00)	7/24 (29.17)	0.17
Number of MRSA-positive EDCs / total number of EDCs (%)	Stable	54/90 (60.00)	35/86 (40.70)	0.01
	House‡	-	-	-
GM§ MRSA CFU/EDC	Stable	27.54	16.98	0.06
	House	2.29	5.5	0.29

*A farm was categorized as carrier when there was at least one carrier on week 12 living and/or working on the farm, non-carrier farms were the remaining.

†p-values among proportions were calculated with $\chi 2$ test and Fisher's exact test when 20% of the expected cell values were <5. p-values for the GM indicate the difference with the reference category (non-carrier farms).

‡There was one EDC per house, thus the values in this line are the same as the ones in 'Number of farms with MRSA-positive EDCs/total number of farms (%)'.

§Geometric mean (antilogged results from tobit regression).

CFU, colony-forming units; EDC, Electrostatic Dust Collector; GM, geometric means; MRSA, methicillin-resistant *Staphylococcus aureus*.

in calves was found on day 0. On week 12 there was a slight increase in prevalence among farmers as compared with the previous sampling period (see Appendix Figure S1) and individuals from farms with MRSA prevalence in calves above the mean were at two times higher risk of carrying MRSA (PR=2.12, 95% CI 1.12 to 4.01, p=0.02).

Discussion

The associations found during the first week after arrival of the animals on the farm show that the level of exposure to veal calves and the presence of potential animal reservoirs (pets, free-ranging farm cats and sheep) are risk factors for persistent MRSA carriage in farmers and household members. Additionally, persistent MRSA carriers seem to have a different microbiological profile when compared with intermittent and non-carriers, which is characterized by higher MRSA load in nose, presence of MRSA in throat and absence of MSSA. This study shows that as the production cycle advances, there is a rise in MRSA prevalence in calves that leads to higher contamination of the air and higher probability for human MRSA carriage.

Descriptive results confirm that high MRSA carriage prevalence (17.6%) is observed among individuals living on farms, as seen in other studies[2,16]. This percentage represents a carriage burden in countries where estimated MRSA prevalence in the community is below 1% such as the Netherlands and Scandinavian countries. The large difference in prevalence between farmers and family members can be attributed to the different intensity of animal contact and is again an indication of a low LA-MRSA human-to-human transmission[16,22]. Swabs in liquid transport medium were used only on day 0 for the purpose of quantification. The fact that a higher prevalence is observed on day 0 as compared with days 4 and 7 might be due to highest sensitivity for MRSA detection as compared with dry cotton swabs (see online supplementary figure S1). The carriage patterns of *S. aureus* presented are similar to those described by Wertheim et al,[23] in which they found percentages of 20%, 30% and 50% for persistent, intermittent and non-carriers, respectively, among healthy individuals. The lower MRSA-persistent carrier prevalence in the total study population (9.7%) as compared with the average cross-sectional MRSA prevalence (17.6%) indicates that carriage of LA-MRSA is fleeting and varies within individuals.

Confirmation of only ST398 was carried out in the laboratory and it was predominant (higher than 90%) among the MRSA isolates from humans, animal pools and EDC samples. MRSA-positive subjects negative for ST398 did not visit a hospital during the previous 12 months of the study and there were other elements than ST398 MRSA present in animal and environmental samples. All MRSA was considered to be circulating and transmitted in the farm since it is very likely that other livestock-associated STs were present as in previous studies[14,16].

Owing to culturing techniques, MSSA was detected with difficulty when there was a predominant MRSA growth. The possible underestimation of MSSA asks for a cautious

interpretation of the results. Nevertheless it is remarkable that no persistent MRSA carrier was positive for MSSA at day 0. This suggests that the presence of MSSA in the nose might be a protective factor for MRSA-persistent carriage. Moreover, a negative association between MSSA and MRSA has been recently found in a study[14].

In the first week of the production cycle the MRSA environmental load was lower and it can be assumed that nasal contamination with MRSA-containing dust particles and transient mechanical carriage was less likely to occur as compared with further time points in the production cycle. As shown in Figure 1, there is an increased probability for persistent MRSA carriage associated with higher MRSA CFU counts in nasal swabs. Moreover, isolation of MRSA in throat swabs at day 0 was significantly associated to the outcome (PR=12.2). These findings suggest that there might be a true colonization in persistent MRSA carriers as defined here. Furthermore a recent study has shown that ST398 is capable of adequately competing for a niche with a human strain and survives in the human nose for longer periods[24].

Direct association between administration of antibiotics and MRSA-persistent carriage in farmers and their family members, as defined in our study, was shown in univariate results (PR=3.2). It is known that when antimicrobials are administered to animals, substantial quantities of these drugs can be present in manure, on surfaces of animal houses and in dust as a potential risk source[25]. We could hypothesize that respiration of dust containing antibiotics, either from a contaminated environment or directly from a powder formulation, would exert a selective pressure in the anterior nares leading to higher risk for MRSA-persistent carriage in people occupationally exposed. However, this association was not confirmed in multivariate models and it needs further exploration. Number of working hours and other tasks were correlated and may have more influence on persistent carriage. This was also shown when adjustment for number of working hours was carried out in a bivariate fashion.

This study supports that close contact with animals is a major risk factor for persistent LA-MRSA carriage in humans. This is made clear by the final set of variables retained in the multivariate models. The number of working hours was most strongly associated with persistent carriage as indicated by the model A and by the smoothed exposure–response relation shown in appendix Figure S2. Moreover, when the number of working hours was removed for model B, another variable representing close contact with animals (stable management) was retained by the backward procedure.

In recent years, several reports have suggested a potential role for pet animals, specifically cats and dogs, in household MRSA transmission and relapse of human MRSA infections. This transmission seems to be of anthropozoonotic origin. Thus, pets can acquire human strains from humans and they can cause colonization or infection in human cohabitants[26–31]. In most cases, the distribution of the clones in pet animals has mirrored the epidemiology of human clones and mainly shared hospital-associated and community-associated MRSA strains have been reported. It is remarkable that in this study, having a pet in the household was strongly associated with MRSA carriage in veal farmers and household members.

Moreover, there is a demonstrated spread of LA-MRSA between animal species, humans and the farm environment[32]. In this study no other animal apart from veal calves were sampled; however, the presence of free-ranging farm cats and sheep were significantly associated and retained in multivariate models. A previous large cross-sectional study sampled 35 cats from 25 farms, 26 of them came frequently in the veal stables. Only one of these cats was found to be MRSA positive with a spa type t011 (ST398)[33]. Cats might act as reservoirs but this is more suggestive of cats acting as mechanical vectors. These animals might represent an intermittent source of LA-MRSA that might contribute to LA-MRSA-persistent carriage in humans.

Other farm characteristics and hygiene practices were also associated with persistent MRSA carriage, although not significantly. Having a changing room in the farm and using a clean towel after working in the stables were found as protective factors. This might give a direction to specific preventive strategies. On the other hand, cleaning of baby boxes at the beginning of the production cycle was a risk factor for the outcome (PR=4 in multivariate model A and PR=1.9 in univariate analysis). This hygiene practice could give rise to transitory spread in the air of accumulated MRSA.

Environmental contamination with dust particles containing MRSA is much lower in veal calf farming as compared with pig farming and associations are less evident[34]. As shown in table 3, no difference in the environmental MRSA load was found across persistent, intermittent and non-carrier farms at the beginning of the production cycle. However, the twofold rise in animal prevalence at the end of the study was associated with a considerably higher environmental MRSA load and a significantly higher proportion of MRSA-positive EDCs were found on farms with MRSA carriers on week 12. This finding supports that contamination of the environment plays a role in the acquisition of MRSA in people living or working in the farm.

A possible limitation of the study is the self-sampling of nose and throat by individuals which might be lacking in accuracy for MRSA detection. This is however believed to be a minor bias. A recent pilot study has shown high degree of agreement between self-samples and investigator samples (93% agreement, κ 0.85 for nasal swabs and 83% agreement, κ 0.60 for throat swabs)[35]. Another limitation is the previously described underestimation of MSSA presence but this is of negligible impact in the results because detection of MRSA and *S. aureus* remains unaffected. Finally, there were many missing values in some variables and they were excluded from the analysis. There were five individuals out of the 211 with missing nasal samples but sensitivity analysis did not reveal significant changes in estimates.

In conclusion, people living and/or working in veal calf farms who persistently carry MRSA seem to be defined by a differential microbiological profile. The associations found here with the presence of free-ranging farm cats and multispecies farming ask for improved internal and external biosecurity measures. Detailed molecular-epidemiological analysis of MRSA specimens on the farm in various animal species and humans is also essential

to identify reservoirs and transmission routes for LA-MRSA. Finally, environmental contamination with MRSA has to be thoroughly studied to assess the extent of its importance in the transmission of MRSA within the veal-calf farming community.

Footnotes

Preliminary results from this study were presented at the annual meeting of the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM), 20-22 March, 2013, Madrid, Spain; oral presentation title: *Determinants for persistent Livestock-Associated MRSA carriage in veal calf farmers and their family members.*

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Contributors

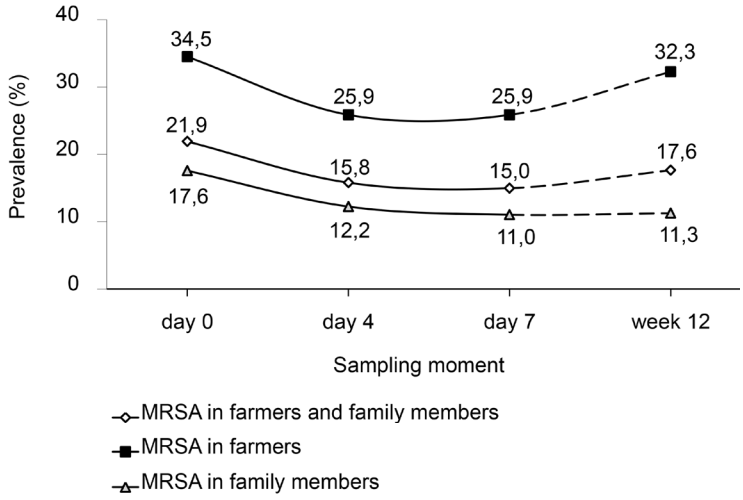
AD-G performed the statistical analyses and interpretation of the data, and drafted the manuscript. MEHB collected the data, contributed to the interpretation of the data, and contributed to the critical revision of the manuscript. HG participated in the conception and design of the study, collected the data and contributed to the critical revision of the manuscript. BAGLVC and JAJWK contributed to the critical revision of the manuscript. KMV carried out the laboratory analysis. JAW conceived the study and contributed to the critical revision of the manuscript. DJJH conceived the study and contributed to the interpretation of the data and the critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

None.

Supplemental material

Appendix Figure S1. Nasal MRSA prevalence per sampling moment in farmers and family members from 51 veal calf farms.



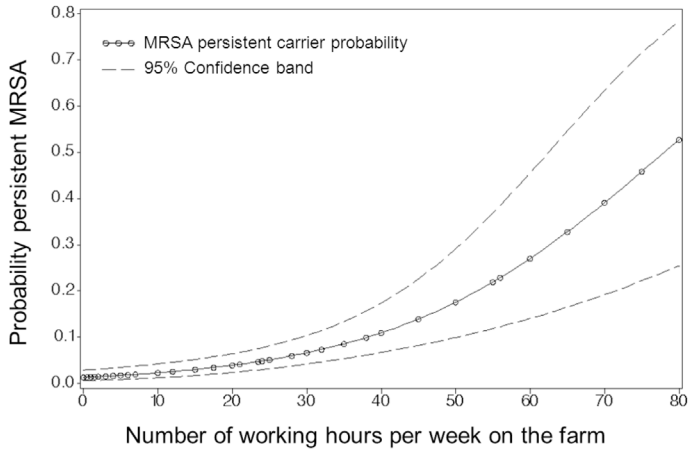
Appendix Table S1. Patterns for one week nasal carriage of *S. aureus*, MRSA and MSSA in the total study population and subpopulations of farmers and household members.

	No. persistent (%)†	No. intermittent (%)†	No. non-carrier (%)†	Total no.
MRSA in nose:				
Total population§	20 (9.7)	35 (17.0)	151 (73.3)	206
Farmers	9 (15.5)	15 (25.9)	38(61.3)	62
Family members	11 (7.6)	20 (13.9)	113 (78.5)	144
MSSA in nose:				
Total population§	25 (12.1)	36 (17.5)	145 (70.4)	206
Farmers	3 (5.2)	14(22.6)	45(72.5)	62
Family members	22 (15.3)	22 (15.3)	100 (69.4)	144
S. aureus in nose:				
Total population§	47 (22.8)	61 (29.6)	98 (47.6)	206
Farmers	14 (24.1)	22(35.5)	26(41.9)	62
Family members	33 (22.9)	39 (27.1)	72 (50.0)	144

†A person was persistent carrier when each of the nasal swabs collected on days 0, 4 and 7 was positive for MRSA or MSSA; For *S. aureus* carriage patterns, people intermittently positive to MRSA or MSSA were also considered as persistent as long as they were carriers of the resistant or susceptible strains on days 0,4 and 7. Non-carriers had no positive swabs; intermittent carriers were the remaining persons.

§there were 5 missing values (total study population=211).

Appendix Figure S2. Probability of nasal MRSA persistent carriage and its relationship with number of working hours in the farm. Semiparametric regression modelling setting sex and age as parametric components for adjustment.



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Chapter 3

Effects of reducing antimicrobial use and applying a cleaning and disinfection program in veal calf farms: experiences from an intervention study to control livestock-associated MRSA

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Abstract

With the ultimate aim of containing the emergence of resistant bacteria, a Dutch policy was set in place in 2010 promoting a reduction of antimicrobial use (AMU) in food-producing animals. In this context, a study evaluated strategies to curb livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA). Fifty-one veal calf farms were assigned to one of 3 study arms: *RAB* farms reducing antimicrobials by protocol; *RAB-CD* farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; and *Control* farms without interventions. MRSA carriage was tested in week 0 and week 12 of 2 consecutive production cycles in farmers, family members and veal calves. Interventions were validated and a cyclic rise in MRSA-prevalence in animals was shown with a more moderate increase in *RAB* farms. Prevalence in humans declined parallel over time in the study arms but *RAB* farms were at the lowest MRSA levels from the beginning of the study. In *RAB-CD* farms, human and animal prevalence did not differ from *Control* farms and MRSA air loads were significantly higher than in the other study arms. Mimicking the national trend, an overall AMU decrease (daily dosages per animal per cycle (DDDA/C)) was observed over 4 pre-study and the 2 study cycles; this trend did not have a significant effect on a set of evaluated farm technical parameters. AMU was positively associated with MRSA across study arms (ORs per 10 DDDA/C increase=1.26 for both humans (p=0.07) and animals (p=0.12 in first cycle)). These results suggest that AMU reduction might be a good strategy for curbing MRSA in veal calf farming, however the specific cleaning and disinfecting program in *RAB-CD* farms was not effective. The drop in MRSA prevalence in people during the study could be attributed to the observed long-term AMU decreasing trend.

Introduction

Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) and specifically sequence type (ST) 398 has emerged in food producing animals with pigs and veal calves as the biggest animal reservoir[1–3]. The public health concern rose in 2005 when LA-MRSA was seen to be transmitted to farmers and family members with the implicit risk of introduction into community and hospitals[2]. Although illness attributed to LA-MRSA in humans appears to be uncommon, this reservoir can significantly contribute to the overall MRSA carriage, especially in countries with low MRSA prevalence[4–6]. In the Netherlands, of all new MRSA isolated through screening of patients in 2013, 33% were attributed to have livestock origin (i.e. ST398)[7].

Dynamics of MRSA carriage in veal calves appears to be cyclic; an initial low MRSA prevalence after the empty barn period, at the beginning of a new production cycle, is followed by a steep rise in prevalence as the cycle continues[8]. In general, rose veal calf farms are at lower risk for MRSA carriage than white veal calf farms[9]. Identified risk factors for MRSA prevalence in calves include farm hygiene, group treatment with antimicrobials and age of the calves[9]. In humans, direct and intensive animal contact seems to be the major force driving MRSA carriage[3,10]. Higher environmental contamination with MRSA has also been associated to higher MRSA levels in animals and exposed humans[10,11]. Nonetheless, the multifactorial nature of MRSA dynamics results in an intricate web of risk factors that complicate the identification of straightforward measures to curb MRSA prevalence in animals and humans[9].

The use of antimicrobials as growth promoters in animals was banned by the EU in 2006 in an approach to diminish the risk of emerging antimicrobial resistant bacteria. The Dutch government also launched a policy in 2010 to reduce antimicrobial use (AMU) in animals nationwide[12]. The target was set for a 50% AMU reduction by 2013 compared to 2009 together with a transparent benchmarking at the farm level to identify persistent high consumers[12–14.] In the veal calf sector, a decreasing trend already started in 2007, since then consumption levels were reduced by 48% in 2013[13].

This work presents the results and experiences from an intervention study aimed at reducing MRSA in animals and humans in veal farms. The effect on MRSA carriage of the sole reduction in AMU, or its combination with a cleaning and disinfection program between cycles, was compared with a control group over a 12-week period in two consecutive production cycles. Additionally, a risk factor analysis was done to identify associations with MRSA that could give shape to other interventions in the future. Finally, a long term effect of AMU reduction on several farm technical and production parameters was evaluated.

Materials and Methods

Study design overview

A total of 51 farms (3x17) were followed up over a period of 12 weeks starting at the beginning of 2 consecutive production cycles and they were assigned to three different study arms. All farms were visited from the end of 2010 to the end of 2012 and met the following inclusion criteria: implemented all-in-all-out system; no other livestock in large scale apart from veal calves; one unique location for all the stables or farm; veal calf farmers not working in another animal sector and not operating in other farms; preference for selection was given to farms in the proximity of Utrecht, the Netherlands. A more detailed description on the veal calf production chain for a better understanding of the sector is given by Bos et al[9].

Two intervention groups and a control group composed of 17 farms each were followed up during the study period. The farms were recruited in triplets to ascertain comparability. Each triplet was selected at a same time point within the same cooperative having the same breed of calves and comparable production parameters (mainly mortality and AMU in previous cycles). Each farm within a triplet was randomly assigned to one of the 3 following study arms: intervened farms reducing AMU by a protocol (named *RAB* arm); intervened farms reducing AMU by a protocol and applying cleaning and disinfection program of stables (*RAB-CD* arm); and control group of farms where no interventions were implemented (*Control* arm).

Guided reduction of antimicrobial use in *RAB* and *RAB-CD* arms

The protocol for AMU reduction in *RAB* and *RAB-CD* arms promoted individual treatments and focused on limiting group treatments by favoring a transition from treating whole herds to treating herds partially. When applying start treatments (i.e. prophylactic mass treatment at the start of a production cycle—after this study no longer allowed according to Dutch regulations), intervened farms could not use combinations of more than one antibiotic. They were only allowed to initiate any group treatment (including start treatments) in the following conditions: i) more than 4% of new cases of illness occurring in the herd within 24 hours; ii) at least 10% rise in new cases of illness happening during 5 consecutive days; iii) treatments under the supervision of a veterinarian; iv) milk feeding circuits cleaned by circulating 1 kg of detergent (SUN powder) per 250L of water at 50°C through the feeding tubes between administrations of antimicrobials. Veterinarians guided farmers to implement the AMU reduction protocol and reported compliance to the researcher by using a checklist on the aforementioned conditions for each of the group treatments applied.

Cleaning and disinfection program in *RAB-CD* arm

A professional cleaning and disinfection program was applied in *RAB-CD* farms before the start of the study production cycles. The goal was to reduce or eliminate MRSA and other

bacteria present in the stables. Cleaning was done by soaking with water (6 hours to wet dirt), applying foam gel (Biogel, CID LINES N.V., Ieper, Belgium) (10–60 minutes of contact time) with a high pressure foam lance (50–150 bar) and clearing with high pressure water jet. The day after, disinfection was done with VIROCID (Alkyl dimethyl benzyl ammonium chloride, didecyl dimethyl ammonium chloride, Glutaraldehyde) (CID LINES N.V., Ieper, Belgium). In natural ventilated stables, 0.3L water/m^2 with 0.25–0.5% of VIROCID were sprayed and, in mechanical ventilated stables, 4L water/m^3 with 1-2L of VIROCID were fogged. After drying, slake lime was spread in the stables. The feed kitchen and buckets were also cleaned and new artificial nipples were provided in farms using this system for milk supply. Since professional companies were contracted for application of the protocol, compliance with each of the steps for cleaning and disinfection was not formally assessed. Surfaces in each of the farm stables were stamped with an agar plate to check the level of contamination after application of the program. In total 20 stamped samples were taken in each stable of the 17 farms (4 from the grid floors, 2 from floor in walk ways, 1 from walls of walk ways, 2 inside feeding buckets, 1 in the bottom of feeding buckets, 2 in the fences of the pens, 2 in the feed fence, 3 from the walls of pens, 1 in the floor of feeding kitchen, 1 in the wall of feeding kitchen and 1 outside the mixer). Three farms from the other study arms were randomly selected as controls from the *RAB* (n=1) and *Control* arms (n=2) to validate the cleaning and disinfection program. These farms applied their routine cleaning procedure and the level of bacterial contamination was likewise checked in each of their stables, just before the first production cycle.

Evaluation of MRSA levels in veal calves, in the human study population and in environmental dust

MRSA carriage was assessed at 4 sampling moments (week 0 and week 12 in the 2 consecutive cycles). The timing of sampling was motivated by an earlier study which had shown that in veal farming there is a marked increase in the prevalence of MRSA carriage in calves, which levels off around week 12 and stabilizes for the remained of the production cycle[8]. Nasal swabs in veal calves and humans were inserted in the nostril and rotated once. During the first production cycle (first 2 sampling moments) animal swabs were taken and analyzed in 10 pools of 6 swabs each (60 animals per farm). During the second production cycle (last 2 sampling moments), the study protocol changed and animal samples were analyzed individually to obtain more precise prevalence estimates. As a rule of thumb, the square root of number of animals in the farm was considered as an appropriate sample size in this second cycle (n=13 to 40 animals). An extensive questionnaire on farm characteristics was filled out at the beginning of the study and items on sorting of calves and on cleaning and disinfection of stables were collected at the beginning of the study cycles. Swabs from farmers, family members and employees were also collected (n=206). On day 0 of the first production cycle, swabs in liquid transport medium (ESwab, Copan, Brescia, Italy) were taken by field workers

in the majority of participants or by self-sampling. Dry cotton swabs (Copan, Brescia, Italy) were used to self-sample the nose of humans during all other sampling moments. Instructions including photographs in case of self-sampling were provided to participants. All swabs were immediately taken to the laboratory or sent by surface mail and processed within 24 hours after arrival. All participants signed an informed consent form. The protocol of the study was approved by the Medical Ethical Committee of the University Medical Center Utrecht (permit number: 10-471/K). Approval from an animal ethics committee was not required. The collection of nasal swabs from animals was in compliance with the Dutch law for animal welfare and did not fall under the Dutch Experiments on Animals Act (1996) or Directive 2010/63/EU.

Passive dust samples were taken on day 0 and week 12 of the first production cycle by placing 4 Electrostatic Dust Collectors (EDCs) (Zeeman, Utrecht, The Netherlands) on different surfaces inside the stables[15]. The EDCs were left in place during a period of 2 weeks and sent by surface mail to the laboratory. Upon arrival, EDC samples were stored at -20°C until DNA extraction and quantitative analysis[15].

Laboratory analysis

Nasal swabs from calves were tested for MRSA and confirmed by PCR following standard procedures previously described[16]. Estimates of MRSA air loads (i.e. dust deposition) were given in colony-forming units (CFU) per EDC and nasal swabs from humans were analyzed as previously described by PCR[10]. Real-time (RT) PCR targeted at C01 gene was done for confirmation of ST398 in all MRSA-positive animal, human and dust samples.

Stamped samples for assessment of compliance with the cleaning and disinfection program in the *RAB-CD* arm were analyzed by an external commercial laboratory (Silliker Netherlands BV); samples were taken using RODAC (Replicate Organism Detection and Counting) plates, containing Trypticase Soy Agar with Lechitin and Polysorbate 80. When ready to use, the lid of the plate was removed and gently touched the surface or area/equipment to be sampled. After incubation at 37°C for 21 ± 3h hours, all colonies were counted. The level of bacterial contamination of the surfaces was ranked from 0 to 4 meaning: 0=no colonies found (named *excellent* rank), 1=1–40 colonies found (named *good* rank), 2=41–120 colonies found (named *moderate* rank), 3=121–400 colonies found (named *poor* rank), 4≥400 colonies found (named *heavily contaminated* rank). For quality control, a RODAC plate not being stamped was used as negative control and a RODAC plate stamped in a surface certainly dirty was used as positive control.

Data on antimicrobial use

Information on AMU was available for 4 consecutive pre-study production cycles and for the 2 study cycles. In each farm, AMU was calculated as Defined Daily Dosages per Animal per Cycle (DDDA/C) for each of the 4 baseline and 2 study cycles. A detailed description of this

calculation is given by Bos et al.[12] with the difference that in the present work, daily dosages are expressed per cycle and total animal mass present in the farm (weight*no. animals) uses weights at the moment of prescription obtained from growth curves. For interpretation of the results, a DDDA/C of 1 means that the average animal in the population was exposed to antimicrobials for one day during the cycle (approximately 6 months).

Data on technical and production parameters

Four different sector integrations provided farm-based data during the 4 consecutive pre-study cycles and the 2 study cycles. The following parameters were available for evaluation: i) percentage of mortality in calves; ii) final weight of carcasses in kg; iii) costs of veterinary care; iv) duration of the production cycle in days. Additionally, a parameter on age of death calves was available only in farms from 3 integrations. All parameters were mean standardized from each specific integration average. Despite slight differences in data processing and calculations made by the integrations, all results presented in this work are product of a joint analysis. A sensitivity analysis by integration yielded similar results and justified this approach (not shown).

Data analysis

All statistical analyses were performed using SAS software version 9.4 (SAS institute Inc., Cary, North Carolina, USA). Baseline comparability of study arms was checked on all items of the questionnaires and also in technical parameters (in the 4 pre-study cycles) by ANOVA or Chi squared / Fisher's exact tests. Descriptive analysis determined the mean cross-sectional prevalence on each of the 4 sampling moments per arm of intervention in the human study population and in animals.

AMU was evaluated in the 3 study arms during 4 pre-study baseline production cycles and during the 2 study cycles. Compliance with the cleaning and disinfection program was checked with the mean rank of bacterial contamination in the 20 samples/stable taken from *RAB-CD* farms against the 3 randomly selected farms from the other arms and against the positive and negative controls taken in each stable. The mean ranks by sampled surface in the two consecutive study cycles were also compared through ANOVA.

MRSA presence/absence in animals was modelled per cycle (at pool level in first cycle and individual level in second cycle), and in humans it was modelled with the 2 cycles together. Evaluation of the effects of study arms for MRSA in animals and humans, and the risk factor analysis for MRSA in animals, accounted for clustering at farm level and the repeated measurements design. For this purpose, PROC GLIMMIX was used to fit univariate generalized linear mixed models with random intercept for farm and simple covariance structure adjusting for sampling moment (included as factor). Interaction terms between study arm and sampling moment were considered to test for intervention effects over time. This approach allowed to observe temporal differences in a change in prevalence in each of

the intervention arms, in comparison with the *Control* arm. Determinants presented in the risk factor analysis for a pooled sample or an animal to be MRSA-positive complied with the following criteria in at least one study cycle: i) less than 10% missing observations; ii) more than 10% of observations in each of the categories of a variable; iii) p-value ≤ 0.10. A multiple regression model for animal MRSA-positivity was built by backward elimination from 2 full models containing variables with p<0.2 in the univariate analysis for each of the production cycles.

The MRSA-AMU association (independently of study arm) was evaluated in animals and humans as described above. AMU was normally distributed and no transformation was needed for analysis. For this sub-analysis, two different strata were considered in humans: i) people working 20 or more hours per week in the farm (defined as farmers for the purpose of this paper); ii) people working less than 20 hours (defined as family members).

Environmental contamination with MRSA was explored by calculating proportions of MRSA-positive EDCs and means CFU/EDC for each study arm that were tested with Chi squared/Fisher's exact tests or ANOVA respectively. CFU counts were log transformed since they followed a right-tailed distribution. PROC LIFEREG was used for left-censored tobit regression to obtain a more accurate estimate of average levels in the form of geometric means (GMs) of CFU/EDC accounting for the large proportion of undetectable values[10].

The effects of study arm during the study period and long term AMU (considering the 4 pre-study cycles) were evaluated for each technical parameter by fitting univariate linear mixed models with random intercept for farm and simple covariance structure. Significant interactions between AMU, study arm and time were presented.

Results

Baseline comparability of study arms

All the 51 farms assigned to one of the three study arms had comparable baseline characteristics and production parameters. Chi-squared or Fisher's exact test in categorical variables or ANOVA in numerical variables did not show significant differences by arm of intervention for most of the questionnaire items (n=61) at baseline. On each farm, only white calves were present and the average farm had 800 calves (SD=301.5, min=180 max=1670). All farms applied treatments with antimicrobials at the beginning of the production cycle as routine (n=42) or if necessary (n=8) mostly using oxytetracycline (n=49). However, administration of colistin as initial treatment significantly differed by study arm; there were more farms using colistin in the *RAB-CD* arm (n=15) in comparison with *RAB* and *Control* arms (n=5 and 7 respectively).

Characteristics of the human study population are presented in Table 1. *RAB-CD* and *Control* farms had significantly higher proportion of farmers (from 45 to 47%) as compared to *RAB* farms (27%) (Chi-squared test p-value=0.02).

Table 1. Characteristics of the human population from an intervention study performed in 51 veal calf farms to reduce MRSA carriage, the Netherlands 2010–2012.

Descriptive statistic		Arm of study			All farms
		RAB	*RAB-CD*	*Control*	
Mean age (standard deviation)		26.1 (18.1)	32.4 (18.1)	30.8 (18.2)	29.5 (18.3)
Median number of working hours (IQR)		5.0 (0-30)	10.0 (0-35)	8.5 (0-32)	7.0 (0-32)
Total no. of people		78	63	65	206
By working hours*	Farmers†	23 (27%)	27 (47%)	26 (45%)	76 (43%)
	Family members†	40 (63%)	30 (53%)	32 (55%)	102 (57%)
By sex	Male	46 (59%)	35 (55%)	29 (45%)	110 (53%)
	Female	32 (41%)	28 (45%)	36 (55%)	96 (46%)
By sex and working hours	Male farmers†	19 (50%)	17 (52%)	17 (63%)	53 (54%)
	Male family members†	19 (50%)	16 (48%)	10 (37%)	45 (46%)
	Female farmers†	4 (16%)	10 (42%)	9 (71%)	23 (29%)
	Female family members†	21 (84%)	14 (58%)	22 (29%)	57 (61%)

MRSA, livestock-associated methicillin resistant *Staphylococcus aureus*; *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control,* farms without interventions.

*Farmers: 20 or more working hours per week in the farm; family members: less than 20 working hours per week in the farm. †Note that numbers in strata of working hours do not sup up to total numbers because there are missing values for the variable number of working hours per week in the stables.

Validation of interventions in *RAB* and *RAB-CD* arms: use of antimicrobials during the study and cleaning and disinfection program

Overall, a marked downward trend in AMU (i.e. DDDA/C) was observed during the 4 pre-study and the 2 study production cycles. The reduction in AMU was explained by a reduction in the number of group treatments. The number of individual treatments remained at low levels without significant changes over time (Figure 1).

Study arms had similar AMU before the beginning of the study (Figure 1); *Control* farms had a slightly, but statistically non-significantly higher AMU (mean DDDA/C during pre-study cycles=44) than intervened farms (mean DDDA/C during pre-study=42). The level of compliance with the protocol for AMU reduction in intervened farms was satisfactory; against a background of a general decline in AMU, *RAB* and *RAB-CD* arms achieved an additional reduction during the study compared to *Control* farms. A more drastic reduction in DDDA/C was observed in *RAB-CD* and *RAB* arms (-35% and -24% respectively) compared to *Control* arm (-15%), when the last pre-study period of AMU was compared to mean AMU during the study (Figure 1).

Moreover, the four conditions for applying antimicrobial group treatments in intervened

farms were met at least in 75% of the applications. A significant transition for treating the herd partially instead as a whole was observed during the study in all farms; in the first cycle 11% of all group treatments were used for just part of the herd while this proportion in the second cycle was 19% (Chi-squared test p=0.03). This transition over time was observed especially in intervened farms but did not significantly differ from *Control* farms. Considering the whole study period, a significantly higher proportion of partial herd group treatments was observed in *RAB-CD* farms followed by *RAB* and *Control* farms (24, 11 and 10% respectively, Chi-squared test p<0.01). The mean number of group treatments (excluding starting routine treatments) per farm and per cycle during the study was 4 (SD=1.92) and it was not significantly different by production cycle or by study arm. 65% of group treatments were administered for respiratory problems, 19% for treating digestive disorders and 16% for treating other diseases.

Proportions for each of the antimicrobial classes were comparable between study arms and are provided in supplemental Figure S1. Considering the pre-study and study periods, 45.4% of the total antibiotic use originated from the administration of tetracyclines, 13.9% from colistin, 12.7% from macrolides/lincosamides, 9.0% from trimethoprim/sulfonamides, 8.0% from aminoglycosides, 5.9% from penicillins and 5.2% from other classes including florfenicol (2,6%), fluoroquinolones (2.3%) and cephalosporins (0.3%). Percentages remained similar during the 4 pre-study cycles, but a manifest decrease was observed specially in colistin during the 2 study cycles (17.1% in pre-study and 4.9% in study period).

Cleaning of stables with only high pressure water between cycles is a common practice that was done by farmers in 14 *RAB* farms and in 16 *Control* farms; the regular cleaning in these farms never included the use of soaking agents, disinfectants or cleaning of baby boxes. Farms in the *RAB-CD* arm complied with the cleaning and disinfection program and, according to the level of bacterial contamination assessed by the stamp samples, were cleaner

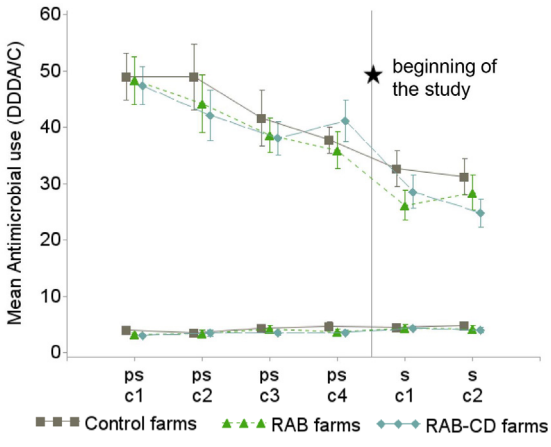

Figure 1. Mean antimicrobial use (as Defined Daily Dosages per Animal and Cycle (DDDA/C)) and 95% confidence interval in 51 veal calf farms during 4 pre-study production cycles (ps-c1 to ps-c4) and the 2 study cycles (s-c1 and s-c2) for group treatments (3 upper lines) and individual treatments (3 lower lines), the Netherlands 2009–2012. For assessing baseline comparability, study arms are also shown during the pre-study cycles before assignment to any intervention. *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions.

at the start of a new cycle. The 3 farms used as controls in the bacteriological assessment (from *RAB* and *Control* arms), evaluated in the first cycle, were ranked as *poor* (mean CFU rank=2.8; SD=1.25), while *RAB-CD* farms were ranked as *good* (mean CFU rank=1.10, SD=0.45) (ANOVA p<0.01). The mean CFU rank in all the stamped samples from *RAB-CD* farms accounting for the 2 consecutive cycles was 1.02 (SD=0.45). All negative controls in the 2 cycles had a CFU rank of 0, and 71 positive controls ranked 4 except one with a rank of 2. When the cleaning and disinfection program was applied the second time, before the beginning of the second cycle, stables were slightly cleaner (mean CFU rank after first cleaning and disinfection=1.10, SD=0.45; mean CFU rank after second cleaning and disinfection=0.94, SD=0.44; ANOVA p=0.12). There was a significant difference (ANOVA p=0.02) between the level of bacterial contamination after the first cleaning and disinfection for grid floor samples (mean CFU rank=1.25, SD=0.59), which was higher than after the second time (mean CFU rank=0.88, SD=0.72). The same trend was observed among samples taken in floor and walls of walk ways, walls of pens, and walls of the stable kitchen (ANOVAs p-values<0.12).

MRSA prevalence in veal calves and intervention effects

The MRSA prevalence considerably increased from the entrance of animals in the farm to week 12 (Figure 2) and this pattern was reproduced in both study cycles. The rise in prevalence over time was significantly flattened in *RAB* farms as compared to *Control* farms while *RAB-CD* farms showed an intermediate trend. Mixed models including study arm and sampling moment showed no significant differences between study arms at baseline in each of the cycles. However, the likelihood of an MRSA-positive sample (expressed as Odds Ratio (OR)) was 2 to 3 times higher in *Control* and *RAB-CD* farms than in *RAB* farms in week 12 of both cycles (Table 2). Overall, MRSA prevalence was lower in the second production cycle. This is at least partially explained by the different sampling strategy (pooled samples in the first cycle versus individual samples in the second cycle) but can also be the result of the observed AMU reduction.

Large differences in prevalence change between the study arms were also shown by statistically significant interactions between sampling moment and intervention (overall p-value of 0.05 in first cycle and <0.01 in second cycle). The higher precision and thus stronger statistical significance for the second cycle is likely the result of the use of individual samples. The differences between study arms were statistically significant for the comparison between *RAB* and *Control* farms in week 12. *RAB-CD* farms did not significantly differ from *Control* farms.

From all the MRSA-positive pooled samples (n=323 during cycle 1) and individual samples (n=554 during cycle 2) retrieved in the study, 97.6% were sequence type (ST) 398.

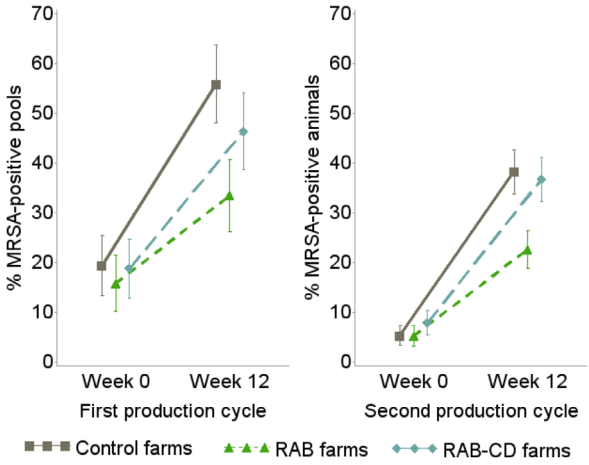

Figure 2. Mean MRSA prevalence and 95% confidence interval in veal calves from 51 farms during an intervention study to reduce MRSA carriage, the Netherlands 2010–2012. Prevalence is estimated using pooled samples in the first production cycle and individual samples in the second cycle. *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions; MRSA, methicillin resistant *Staphylococcus aureus*.

Table 2. ORs for a pooled sample (in the first cycle) and an individual animal (in the second cycle) to be MRSA-positive in an intervention study performed in 51 veal calf farms to reduce MRSA carriage, the Netherlands 2010-2012.

	1st production cycle: ORs for a pooled sample to be MRSA-positive				2nd production cycle: ORs for an individual animal to be MRSA-positive			
Sampling moment-Arm of study	MRSA-positive / N total*	OR	95% CI	Wald p value†	MRSA-positive / N total#	OR	95% CI	Wald p value†
Week 12-*Control*	95/170	9.70	3.62-26.03	<0.01	183/478	13.7	4.07-46.00	<0.01
Week 12-*RAB-CD*	79/170	5.97	2.24-15.87	<0.01	171/465	14.7	4.69-49.31	<0.01
Week 12-*RAB*	57/170	3.08	1.76-5.39	<0.01	111/488	6.35	3.95-10.20	<0.01
Week 0-*Control*	33/170	1.17	0.42-3.25	0.77	26/482	0.56	0.15-2.02	0.37
Week 0-*RAB-CD*	32/170	1.31	0.48-3.62	0.60	37/465	1.44	0.41-5.03	0.57
Week 0-*RAB*	27/170	Ref.			26/488	Ref.		

Results from generalized linear mixed models accounting for clustering at farm level in which study arm and sampling moment were grouped in a single determinant to evaluate interaction effects (i.e. differential effects by arm of intervention and time). MRSA, methicillin resistant *Staphylococcus aureus*; *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions; Ref., reference category of the variable.
*17 farms per arm of intervention, 2 sampling moments and 10 pooled samples per farm. †Overall p<0.01.
#17 farms per arm of intervention, 2 sampling moments and a mean of 28 animals sampled per farm.

MRSA prevalence in the human study population and effect of study arms

Out of the 206 people in the study, 193 were assessed during the 4 sampling moments. There was an overall decreasing trend for human MRSA carriage from the first to the second study cycle (Figure 3). During the first production cycle there was a downward parallel trend for MRSA prevalence in all study arms (Figure 3). The same was true for the second cycle except

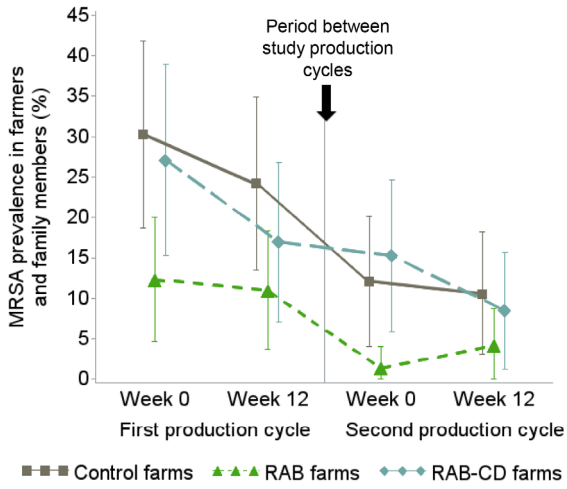

Figure 3. Mean MRSA prevalence and 95% confidence interval in farmers and family members from 51 veal calf farms during an intervention study to reduce MRSA carriage, the Netherlands 2010–2012.
RAB, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control,* farms without interventions. MRSA, methicillin resistant *Staphylococcus aureus*.

for *RAB* farms, were there was an increase in MRSA prevalence from the beginning of the cycle to week 12 (Figure 3). Overall, the proportion of MRSA-positive people in *Control* farms was the highest (20.9%) closely followed by *RAB-CD* farms (17.0%) and in *RAB* farms it was the lowest (7.2%) (Figure 3).

Trends in MRSA prevalence over time between the study arms during the study were not significantly different (i.e. no significant interaction between sampling moment and intervention). A significantly lower MRSA prevalence was observed at the start of the study in *RAB* farms, indicative of potential selection bias. Evaluating the whole study, the probability for carrying MRSA was 81% lower in people living and/or working in *RAB* farms as compared to *Control* farms (OR=0.19, 95% confidence interval (CI)=0.07–0.53, Wald p<0.01) (main effect of study arms p<0.01). The differences in human MRSA probability between *RAB-CD* and *Control* arms were, however, only significant in the group of farmers (working 20 or more hours per week) (main effect of study arms p<0.01). Associations were not statistically significant in those working less than 20 hours per week (OR for being MRSA-positive in *RAB-CD* arm=0.78, 95%CI=0.31–1.96, Wald p=0.59). From all the MRSA-positive human samples (n=120) retrieved during the study, 90% were ST398.

Environmental contamination with MRSA

Data on the presence of MRSA in settled dust, assessed by the EDCs, was only available for the first study production cycle.

Indications of a possible negative effect of the cleaning and disinfection protocol in *RAB-CD* farms were observed. MRSA air load dramatically increased from the beginning of the cycle to week 12 in all study arms but, interestingly, CFU counts in EDCs were at least twice as high in the *RAB-CD* farms as compared to *RAB* and *Control* farms (Table 3). ST398 was present in 90.9% of all the MRSA-positive EDCs.

Table 3. Environmental contamination with MRSA in veal calf stables during the first production cycle of an intervention study performed in 51 farms to reduce MRSA carriage, the Netherlands 2010-2012.

		RAB farms	*RAB-CD* farms	*Control* farms	P-value
No. MRSA-positive EDCs/ total EDCs (%)	Complete cycle	21/114 (18.4)	44/130 (33.9)	29/118 (24.6)	0.02*
	Time 0	0/60 (0)	7/66 (10.6)	2/64 (3.1)	0.01*
	Week 12	21/54 (38.9)	37/64 (57.8)	27/54 (50.0)	0.12*
Mean MRSA CFU/EDC (SD†)	Complete cycle	17.2 (43.3)	61.8 (155.4)	24.1 (51.4)	<0.01#
	Time 0	0 (0)	28.6 (82.8)	7.9 (43.0)	0.01#
	Week 12	35.4 (56.9)	93.9 (197.9)	42.2 (54.3)	0.03#
GM‡ MRSA CFU/ EDC (p-value§)	Complete cycle	2.9 (0.24)	9.1 (0.04)	4.6 (Ref.)	0.01¶
	Time 0	0 (0.19)	0.4 (0.07)	0.1 (Ref.)	0.01¶
	Week 12	14.5 (0.31)	30.8 (0.18)	20.4 (Ref.)	0.06¶

P-values <0.05 are in boldface. MRSA, methicillin resistant *Staphylococcus aureus*; *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions; CFU, colony-forming units; EDC, electrostatic dust collector; Ref., reference category of the variable.

*p-values among proportions are calculated with Chi-squared / Fisher's exact tests; †Standard deviation.

#p-values for the difference in means obtained from ANOVA.

‡Geometric mean (antilogged results from tobit regression).

§p-values in brackets indicate the difference with the reference category (control farms).

¶overall p-values from tobit regression.

AMU-MRSA association in veal calves and humans evaluated independently of study arm

During the first production cycle, the overall association between AMU and MRSA in veal calves had an OR of 1.26 per 10 DDDA/C increase (95% CI=0.90–1.63, p=0.12). At the first time point of this cycle, the association was stronger with an OR of 1.48 (95%CI=1.06–2.06), while in week 12 the association was weaker with an OR of 1.11 (95% CI=0.82–1.52). AMU was not associated to MRSA in the second cycle (OR per 10 DDDA/C=1.15, 95%CI=0.78–1.69, p=0.48) (Table 4).

Interestingly, higher AMU was also related to increased probability for human MRSA carriage. The univariate OR for a person to be MRSA-positive per 10 DDDA/C increase during the whole study was 1.26 (95% CI=0.99–1.62, p=0.07). The association was less significant in the stratified analysis for farmers (OR=1.20, 95% CI=0.87–1.67, p=0.27) and for family members (OR=1.39, 95% CI=0.85–2.28, p=0.19). The number of hours working in the farm was the strongest determinant for MRSA in humans (OR per 10 hours increase=1.68, 95% CI=1.42–1.98, p<0.01). Nonetheless, a bivariate model adjusting for number of working hours did not significantly changed the estimate for AMU on human MRSA prevalence (OR adjusted per 10 DDDA/C increase=1.27, 95% CI=0.96–1.69, p=0.10). The effect size of AMU on human MRSA carriage did not change by sampling moment (i.e. interactions time-

AMU were never significant). Overall association during the study was not found between prevalence in calves and MRSA in humans (OR per 10% increase in animal prevalence=1.06, 95% CI=0.94–1.18, p=0.34).

Farm management and characteristics associated to MRSA in calves during the study

Herd size-effect (i.e. number of calves) was not associated to MRSA (Table 4). This was the only variable forced into the models, because of a priori indications of the relevance of herd size.

The following were risk factors for MRSA with statistical significance in at least one study cycle: origin of the calves in the Netherlands or neighboring countries instead of imported from not neighboring countries, longer duration of initial treatments with antimicrobials, higher number of stables, lower minimum temperatures in stables, natural ventilation instead of mechanical, and presence of sheep and free-ranging cats (Table 4). Borderline significant determinants increasing the probability for MRSA in calves were: decreased weight when calves enter the baby boxes, longer periods before releasing calves from baby boxes, and presence of pets in the farm (Table 4). Determinants presented in Table 4 were independent from each other, pairwise correlation between them revealed a low level of correlation (all Spearman and Pearson rho's<0.5). The final multiple regression models for both production cycles indicated that origin of the calves, presence of free-ranging cats, ventilation and number of stables were the most relevant determinants for MRSA positivity (Table 5).

Evaluation of the effects of long term AMU reduction on farm technical parameters

With regard to baseline, only mean veterinary costs were slightly higher in the *Control* arm than in the intervention arms (ANOVA p=0.10, mean standardized veterinary costs of 0.8, 0.1, -0.3 for *Control*, *RAB-CD* and *RAB* arms respectively). The trends in technical parameters by study arm over the pre-study and study cycles are presented in Figure 4.

During the study period, production parameters did not significantly differ by study arm. Only duration of the production cycle showed a trend towards increased values in intervened farms compared to *Control* farms. Veterinary costs significantly increased with higher AMU (Figure 4 and Table 6 (models 1 and 2).

Modelled longitudinal long term trends (i.e. over the pre-study and study periods together) are displayed in Table 6 (model 3) showing the following: i) a significant increase in mortality over time, but not associated to the decreasing AMU trends (i.e. neither DDDA/C main effect nor DDDA/C-time interaction were significant); ii) veterinary costs positively associated to AMU and time; iii) significantly increased duration of the cycles as the DDDA/C decreased over time (i.e. significant and negative DDDA/C-time interaction).

Table 4. Farm characteristics associated to MRSA in veal calves during the study period in the 51 farms, the Netherlands 2010–2012.

		1st cycle: ORs for a pooled sample to be MRSA-positive				2nd cycle: ORs for an individual animal to be MRSA-positive			
Variable *	Category	N†	OR	95% CI	P ‡	N†	OR	95% CI	P ‡
Number of veal calves §	Per 300 animals	1020	1.23	0.86-1.77	0.26	2866	1.05	0.67-1.64	0.82
Origin of veal calves ¶	Just from the NL	160	2.98	0.93-9.58	0.07	361	2.47	0.58-10.47	0.22
	The NL or neighbor country	640	3.57	1.46-8.73	0.01	1738	6.96	2.46-19.71	
	Not neighbor country	220	Ref			713	Ref.		
Weight (Kg) when calves enter the baby boxes (mean=46.5)	Per 5 kg	1020	0.73	0.50-1.09	0.13	2866	0.62	0.37-1.02	0.06
No. days that calves remain in the baby boxes (mean=34.5)	Per 5 days	980	1.16	0.98-1.34	0.08	2543	1.28	0.98-1.69	0.07
No. days of initial treatment with antimicrobials	7 to 10 days	320	2.09	1.02-4.31	0.05	987	1.47	0.56-3.84	0.44
	5 days	680	Ref.			1827	Ref.		
AMU during the cycle (DDDA/C) §	Per 10 DDDA/C	980	1.26	0.90-1.63	0.12	2800	1.15	0.82-1.63	0.48
Number of stables	4 to 6	100	3.31	1.04-10.55	0.04	329	1.90	0.42-8.73	0.41
	1 to 3	920	Ref.			2537	Ref.		
Minimum temperature in stables#	<10°C	120	1.46	0.43-5.03	0.54	342	5.67	1.21-26.43	0.03
	Between 10 to 15°C	640	0.63	0.28-1.46	0.28	1794	1.24	0.43-3.59	0.69
	Between 15 to 20°C	260	Ref.			730	Ref.		
Ventilation of stables	Mechanical	240	0.63	0.28-1.39	0.25	2077	0.32	0.12-0.83	0.02
	Natural	780	Ref.			789	Ref.		
Presence of sheep in the farm	Yes	240	1.59	0.69-3.69	0.28	705	2.79	0.99-1.85	0.05
	No	780	Ref.			2161	Ref.		
Presence of free-ranging cats in the farm	Yes	540	2.47	1.25-4.88	0.01	1549	3.29	1.36-7.94	0.01
	No	480	Ref			1317	Ref.		
Presence of pets in the farm	Yes	660	1.95	0.93-4.07	0.08	1890	2.15	0.83-5.55	0.11
	No	360	Ref.			976	Ref.		

Footnotes Table 4.
Univariate results from generalized linear mixed model accounting for clustering at farm level and adjusting for sampling moment. P-values <0.05 are in boldface. MRSA, methicillin resistant Staphylococcus aureus; Ref., reference category of the variable; AMU, antimicrobial use; DDDA/C, Defined Daily Dosages per Animal per Cycle.
*Presented variables comply with the following criteria in at least one study cycle: i) less than 10% missing observations; ii) more than 10% of observations in each of the categories of a variable; iii) $p \leq 0.10$.
†Maximum number of observations: i) in first cycle n=1020 pools (10 pools in 51 farms in 2 sampling moments); ii) in the second cycle n=2866 animals (mean of 28 animals sampled per farm in 51 farms in 2 sampling moments).
‡Wald P-value. §Considered relevant to be evaluated irrespective of significance.
¶Neighboring countries from which animals are imported: Luxemburg (8 farms) , Belgium (11 farms) and Germany (29 farms). Not neighboring countries were mostly Poland (16 farms), Latvia (5 farms) and Lithuania (13 farms). Overall p=0.02 in first cycle and p<0.01 in second cycle. #Overall p=0.25 in first cycle and 0.06 in second cycle.

Discussion

This intervention study showed that lower levels of antimicrobial consumption significantly reduced the probability for MRSA carriage in veal calves. Contrarily to the *RAB-CD* and *Control* arms, the *RAB* arm showed a clearly flattened prevalence rise in animals that was reproduced over the 2 study cycles. In people living and/or working in veal farms, MRSA prevalence gradually decreased parallel in all study arms but this reduction was not associated to any specific intervention effect. Animal and human MRSA carriage in *RAB-CD* farms did not significantly differ from *Control* farms. Thus, the specific cleaning and disinfection program used in this study was not shown to be successful, possibly because it resulted in increased MRSA air loads. A positive quantitative trend between AMU and MRSA (independent of study arm) in humans and animals was also demonstrated, but the study period was relatively short to establish solid links between AMU changes and MRSA dynamics. Long term trends, considering the pre-study period, showed a significant decrease in AMU comparable to the nationwide trend. The interventions were not associated to observed changes in technical parameters such as mortality or carcasses weight. However, the long term trend in reduction of AMU was associated with an increase in the length of a production cycle. Finally, a set of determinants for MRSA in calves were disclosed longitudinally to possibly give shape to more refined additional future interventions.

During the 4 pre-study production cycles, a steady reduction in antimicrobial group treatments was observed in participating farms. This mirrors the nationwide decrease in AMU in food-producing animals enforced by the Dutch Government[13,14]. Unexpectedly, this change in group treatments did not parallel an increase in individual treatments which remained at low and almost unchanged levels. The beginning of the study marked a steeper reduction in AMU compared to the pre-study period. This was especially true in the intervened farms, and mainly the result of the transition to partial treatment of herds instead of treatment of herds as a whole. The proportions of AMU by families of antibiotics are similar to the ones already described for the veal calf sector[17].

Table 5. Most relevant farm characteristics associated to MRSA in veal calves obtained from the multiple regression models during the study period in the 51 farms, the Netherlands 2010-2012.

	1st production cycle: ORs for a pooled pig sample to be MRSA-positive				2nd production cycle: ORs for an individual animal to be MRSA-positive			
Variable*	N †	OR	95% CI	P-value‡	N †	OR	95% CI	P-value‡
Sampling time								
Week 0	510	0.20	0.15-0.28	<0.01	1408	0.09	0.07-0.12	<0.01
Week 12	510	Ref.			1404	Ref.		
Origin of veal calves #								
Just from the NL	160	2.36	0.78-7.11	0.13	361	3.63	0.93-14.20	0.06
The NL or neighbor country	640	3.11	1.33-7.23	0.01	1738	7.07	2.58-19.40	<0.01
Not neighbor country	220	Ref.			713	Ref.		
Number of stables								
4 to 6	100	3.18	1.09-9.22	0.03	NA	NA	NA	NA
1 to 3	920	Ref.			NA	NA	NA	NA
Ventilation of stables								
Mechanical	NA	NA	NA	NA	2023	0.23	0.10-0.53	<0.01
Natural	NA	NA	NA	NA	789	Ref.		
Presence of free-ranging cats in the farm								
Yes	540	2.14	1.11-4.12	0.02	1495	2.25	1.01-5.02	0.05
No	480	Ref.			1317	Ref.		

Multiple regression associations after backward elimination from 2 full models containing variables with p<0.2 in the univariate analysis (Table 4) for each of the production cycles. P-values <0.05 are in boldface. MRSA, methicillin resistant *Staphylococcus aureus*; Ref., reference category of the variable; NA, variable not retained in the final model.

*Presented variables comply with the following criteria in at least one study cycle: i) less than 10% missing observations; ii) more than 10% of observations in each of the categories of a variable; iii) p ≤ 0.10.

†Maximum number of observations: i) in first cycle n=1020 pools (10 pools in 51 farms in 2 sampling moments); ii) in the second cycle n=2812 animals (mean of 28 animals sampled per farm in 51 farms in 2 sampling moments).

‡Wald p-value.

#Neighboring countries from which animals are imported: Luxemburg (8 farms) , Belgium (11 farms) and Germany (29 farms). Not neighboring countries were mostly Poland (16 farms), Latvia (5 farms) and Lithuania (13 farms). Overall p=0.03 in first cycle and p<0.01 in second cycle.

Figure 4. Mean and standard error for each of the standardized (STD) technical parameters in 51 veal calf farms during 4 pre-study production cycles (ps-c1 to ps-c4) and the 2 study cycles (s-c1 and s-c2), the Netherlands 2009–2012.
For assessing baseline comparability, study arms are also shown during the pre-study period before randomization to any intervention. *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions.

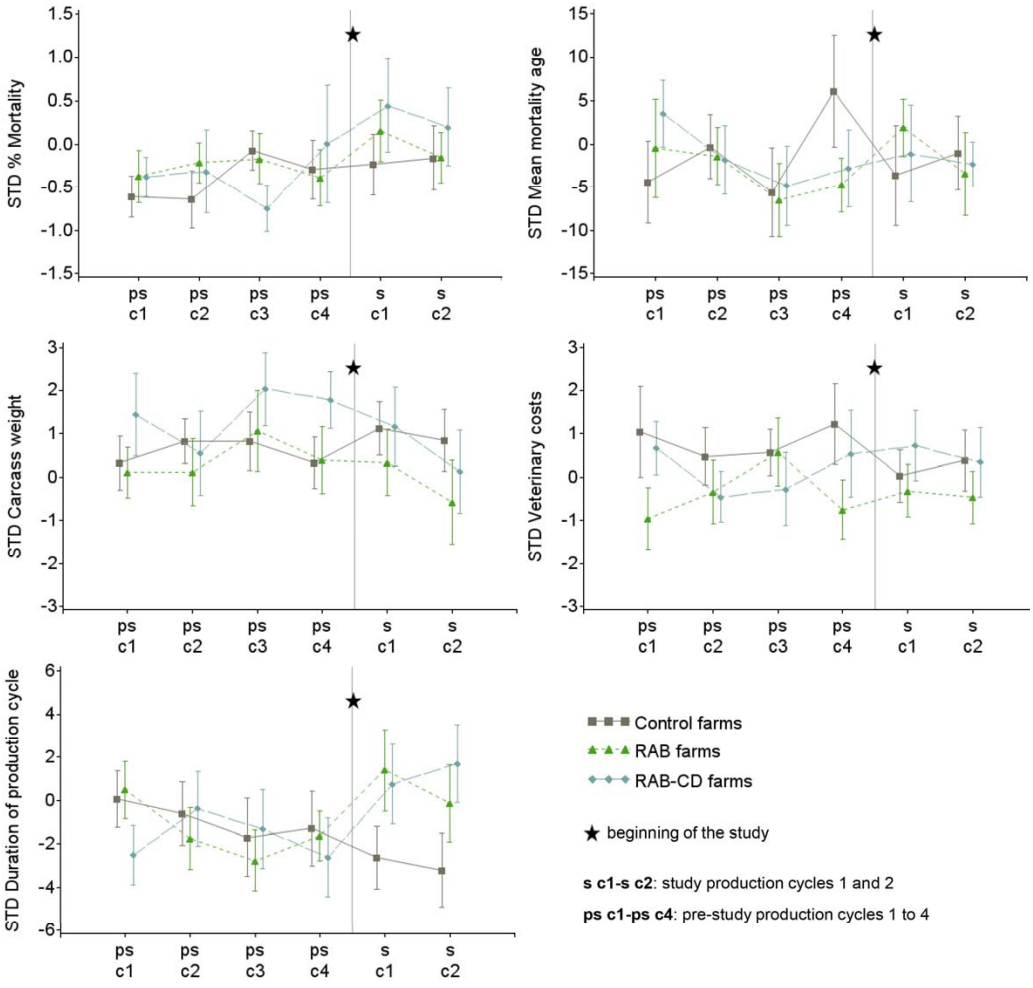

Table 6. Relations between different technical production parameters and interventions (model 1) and antimicrobial use (model 2) during the study period in the 51 veal calf farms. Model 3 relates the technical production parameters to antimicrobial use during all the 6 available production cycles (4 pre-study and 2 study cycles). The Netherlands 2009-2012.

Standardized parameter		Models with the 2 study cycles						Model with 4 pre-study + 2 study cycles		
		Model 1 with study arms			Model 2 with DDDA/C			Model 3 with DDDA/C		
	Effects	Est.*	SE†	P	Est.*	SE b	P	Est.*	SE†	P
Mortality	Intercept	-0.63	1.66	0.71	0.42	1.72	0.81	-0.8	0.4	0.04
	Cycle (num)	-0.15	0.3	0.61	-0.14	0.30	0.64	0.12	0.05	0.02
	DDDA/C	NA	NA	NA	0.01	0.01	0.41	0.00	0.01	0.53
	RAB	0.19	0.45	0.68	NA	NA	NA	NA	NA	NA
	RAB-CD	0.52	0.44	0.24	NA	NA	NA	NA	NA	NA
	Control	Ref.			NA	NA	NA	NA	NA	NA
Mortality age ‡	Intercept	6.78	15.74	0.67	11.94	16.46	0.47	1.98	4.80	0.68
	Cycle (num)	-1.66	2.78	0.55	-1.65	2.81	0.56	-0.38	0.64	0.55
	DDDA/C	NA	NA	NA	-0.13	0.15	0.38	-0.06	0.07	0.43
	RAB	2.06	5.48	0.71	NA	NA	NA	NA	NA	NA
	RAB-CD	0.62	5.46	0.91	NA	NA	NA	NA	NA	NA
	Control	Ref.			NA	NA	NA	NA	NA	NA
Veterinary costs	Intercept	0.27	2.37	0.91	-3.15	2.33	0.18	-4.22	0.68	<0.01
	Cycle (num)	-0.01	0.42	0.98	0.07	0.39	0.85	0.30	0.09	<0.01
	DDDA/C	NA	NA	NA	0.09	0.02	<0.01	0.08	0.01	<0.01
	RAB	-0.66	0.85	0.44	NA	NA	NA	NA	NA	NA
	RAB-CD	0.33	0.84	0.70	NA	NA	NA	NA	NA	NA
	Control	Ref.			NA	NA	NA	NA	NA	NA
Mean weight carcass	Intercept	4.78	2.82	0.10	5.06	3.00	0.10	1.37	0.79	0.09
	Cycle (num)	-0.69	0.50	0.17	-0.70	0.51	0.17	-0.12	0.10	0.23
	DDDA/C	NA	NA	NA	-0.02	0.03	0.51	-0.01	0.01	0.65
	RAB	-1.18	1.01	0.25	NA	NA	NA	NA	NA	NA
	RAB-CD	-0.34	0.99	0.74	NA	NA	NA	NA	NA	NA
	Control	Ref.			NA	NA	NA	NA	NA	NA
Duration of the cycle	Intercept	-0.90	4.90	0.86	5.28	5.04	0.30	-5.70	2.21	0.01
	Cycle (num)	-0.37	0.85	0.66	-0.47	0.83	0.58	1.43	0.50	0.01
	DDDA/C	NA	NA	NA	-0.09	0.05	0.06	0.11	0.04	0.01
	Interaction#	NA	NA	NA	NA	NA	NA	-0.04	0.01	<0.01
	RAB	3.56	2.24	0.12	NA	NA	NA	NA	NA	NA
	RAB-CD	4.16	2.20	0.07	NA	NA	NA	NA	NA	NA
	Control	Ref.			NA	NA	NA	NA	NA	NA

Results from linear mixed models accounting for clustering at farm level for each of the production parameters. Antimicrobial use as DDDA/C, Defined Daily Dosages per Animal per Cycle.
P-values <0.05 are in boldface. Ref., reference category of the variable. *RAB*, farms reducing antimicrobials by protocol; *RAB-CD*, farms reducing antimicrobials by protocol and applying a cleaning and disinfection program; *Control*, farms without interventions; NA, variable not retained in the final model.
*Mean estimates obtained from mixed model for technical parameters with production cycle and DDDA/C or study arm as determinants.
†Standard error of mean estimates.
‡Mortality age was only available from 2 sector integrations.
#Interaction between DDDA/C and production cycle; interaction terms are only presented when there is statistical significance (p<0.05).

With regard to the validation of interventions, the three study arms were comparable in terms of farm characteristics and production parameters, and both intervention arms complied with the protocol for AMU reduction. The cleaning and disinfection program in *RAB-CD* arm significantly reduced overall bacterial contamination on farm surfaces, especially after the second application, but paradoxically, it was associated to increased MRSA in the environment later on during the cycle. It should be remarked that the interpretation of the results from *RAB-CD* farms can be complicated; there was not an efficacy measure for cleaning of the milk tubes and the application of the cleaning and disinfection protocol was not formally assessed since it was delivered by a professional company.

One of the key findings is the clear success in curbing MRSA prevalence in animals in *RAB* farms. Increasing MRSA levels in calves during the progress of a production cycle have been previously described and it is confirmed by our results[8]. This increase over time has been attributed to changes in the contact structures between animals, when the move from individual to group housing, and environmental contamination. The fact that this increase in prevalence is more limited in the intervention group indicates that MRSA lost some of its ecological advantage in veal herds because of a lower use of antimicrobials. The significant interaction term between time and study arm showed that prevalence rise in *RAB* arm was least pronounced. On the contrary, *RAB-CD* and *Control* arms had more similar MRSA levels and dynamics. Like in the *RAB* arm, *RAB-CD* farms also applied the AMU reduction protocol and, although the MRSA reduction was still considerable, it was statistically not significant. The power of this study was such that only strong effects of AMU reduction on MRSA could be detected and a larger study might pick up smaller effects.

Interestingly, *RAB-CD* farms were associated to MRSA air loads twice as high as the other 2 study arms. It has to be acknowledged that applying a professional program for cleaning and disinfection was not a common practice on most farms included in this study. A thorough application of this program in *RAB-CD* might loosened dust and debris which had accumulated over a long period of time and led to increased dustiness later on in the cycle serving as a vehicle for MRSA transmission. In fact environmental contamination with MRSA has been already indicated as a route of transmission in humans in a meta-analysis

including data from the present study[10,11]. Another hypothesis for the relative lack of success in curbing MRSA in the *RAB-CD* arm is that application and long residual action of biocides might have led to co-selection of biocide resistant genes and MRSA[18] ;alkyl dimethyl benzyl ammonium chloride is a quaternary ammonium compound that was used in the protocol for disinfection. This group of biocides have been associated to selection of genes (e.g. *qacA/B*, *smr, qacG, qacJ*) encoding for drug efflux proteins that confer multidrug resistance in *S. aureus* of bovine and caprine origin[19,20]. Although these genes have not been yet associated to co-selection of MRSA in animals, a recent research showed an increased proportion of *qac* genes in methicillin-resistant strains colonizing humans[21]. The effects of cleaning and disinfection should be explored in greater detail and farmers should not decide against more cleaning and disinfection on the basis of this study alone. These results should not be interpreted as arguments against application of disinfection programs. Longer term or different programs for cleaning and disinfection might need to be introduced.

MRSA carriage dynamics during the study markedly differed between veal calves and the human study population. Contrarily, MRSA carriage in humans steadily decreased and the overall association over the study with MRSA in animals was not strong; this mainly is because of the low prevalence in animals at the beginning of the production cycle that diluted the association of higher animal prevalence levels on week 12 with MRSA in humans. The lack of parallel animal-human dynamics might indicate the presence of other important determinants for MRSA carriage and supports that livestock strains might be truly colonizing and not merely contaminating humans[10]. As a previous study has shown, ST398 is capable of adequately competing for a niche with a human strain and survive in human nose for longer periods[22].

This study proves a positive quantitative association between DDDA/C and MRSA in veal calves and humans. This quantitative relationship has also been recently shown in the pig sector[23]. Working hours per week in the farm remained the strongest determinant for MRSA in the human study population as it has been widely reported[3,10]. However, AMU appeared also as a clear determinant preserving its effect size even when modelled together with working hours. Univariate associations between administration of antimicrobials and MRSA in veal calf farmers and family members have been shown in the past[10], but not quantitatively and after adjustment for working hours. Antimicrobial residues remain in the farm environment after treatments and the aspiration of dust containing these residues together with direct exposure to powder formulations could favor the emergence of resistance[10,24].

The overall decreasing MRSA levels in humans during the study are more likely attributable to the long term AMU decreasing trend. However, the absence of a control group of farms free of this trend made impossible to clearly link MRSA and AMU dynamics. Nevertheless, we can hypothesize that the sustained reduction in AMU in previous cycles had a parallel effect on MRSA delayed on time. Additionally, the proportion of MRSA-positive people in *RAB* farms was significantly lower at the beginning of the study indicating

a possible selection bias that could not be further assessed at the time of the present analysis.

Long term trends from pre-study cycles showed a slight increase in mortality which was not statistically associated to the AMU reduction (i.e. non-significant time-AMU interaction). There is no other data available to unravel the reasons behind this undesirable trend. Veterinary costs had a slight increasing trend over time, difficult to observe in the graphs, but revealed by the models. This is possibly explained by other costs arising from worsening health performance in farms or by increased use of vaccines. Results suggest that the expected reduction in direct costs, as a product of the sustained reduction in antimicrobials purchases, did not outweigh other costs. This association does require further detailed investigation in future studies. Lastly, the models showed that duration of the cycle increased as the AMU decreased, this phenomenon was in particular observed at the beginning of the intervention period of the study. This effect might be an artefact of the implementation of the study itself, but this remains unclear.

A set of risk factors for MRSA carriage in calves were identified in the univariate analysis; higher duration in initial treatments with antimicrobials increased the probability for MRSA together with a set of factors that could be related to worsened health in animals such as low temperatures or low animal weights; young animals are more susceptible for colonization by bacteria which could also explain why we found associations with variables regarding baby boxes. The multiple regression emphasized the importance of internal and external biosecurity for MRSA control. Increased risk for MRSA carriage was found in farms with animals from the Netherlands or neighboring countries, possibly indicative of higher prevalences or different sector structures in these regions compared with Eastern Europe. The presence of free-ranging cats was also a consistent risk factor in the models suggesting their role as MRSA vectors, carriers or just proxies for the level of biosecurity in farms[10]. Finally, farms with more stables and natural ventilation were associated with increased MRSA rates, once more exposing biosecurity related risk factors.

More than 90% of the MRSA-positive samples retrieved from animals, humans and settled dust were confirmed to be ST398 (i.e. livestock-associated). Nonetheless, the outcome used only confirmation on MRSA regardless the sequence type because all MRSA was assumed to be circulating and transmitted within the farm. We based this assumption in the plausible presence of less prevalent livestock-associated STs as it has been already described[25,26.] Moreover, it was confirmed that MRSA-positive non-ST398 people did not visit a hospital during in previous 12 months before the study, thus presence of hospital-acquired MRSA strains is unlikely.

A limitation of the study was the different sampling approach for animals in each of the study cycles, pooled samples in the first, individual samples in the second cycle. Nevertheless, the authors consider this to have a minor impact on the results. Pool sample testing is a low-cost alternative but various factors such as the well-known dilution effect have a negative impact on sensitivity and specificity for prevalence estimation when using

pooled samples[27,28]. Frequentist and Bayesian methods of estimating individual prevalence from pooled samples were reviewed[29] in an attempt to make a combined analysis of the 2 study cycles together with MRSA prevalence as outcome. Results were not fundamentally different and for simplicity we made separate analyses per cycle with the binary outcome (MRSA presence/absence).

Conclusions

The set of risk factors found for MRSA in calves outlines possible future interventions and asks for a deeper engagement between different countries to tackle the problem of emergent resistant bacteria. Controlled intervention arms plainly showed that further reduction in AMU could be a good strategy for decreasing MRSA levels in veal calf farms. However, the application of the described cleaning and disinfection program could have initial negative effects. The study indicates that the long term AMU decrease is likely to lower MRSA levels in people living and/or working in veal farms. Nevertheless, future studies including longer follow-up periods are strongly encouraged to evaluate the observed complex dynamics. Dutch policies aimed at decreasing AMU in food-producing animals might be already beginning to bear fruit but more research is needed.

Footnotes

Preliminary results from this study were presented at the 3rd International Conference on Responsible Use of Antibiotics in Animals, 29 Sep-1 Oct 2014, Amsterdam, the Netherlands; poster presentation title: *Can livestock-associated MRSA levels in veal calf farms be modulated by reducing antimicrobial use and by applying a cleaning and disinfecting program?: experiences from an intervention study*.

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Conceived and designed the experiments: DJJH JAW BAGLVC JAJWK HG. Performed the experiments: HG MEHB. Analyzed the data: ADG. Contributed reagents/materials/analysis tools: KMV. Wrote the paper: ADG HG.

Competing Interests

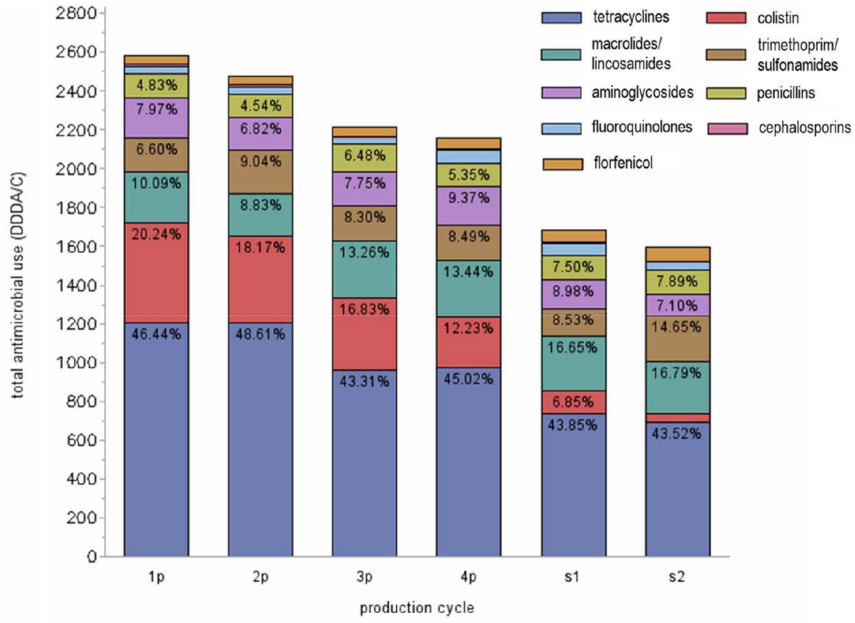
The authors have declared that no competing interests exist.

Data Availability Statement

Data owned by the authors cannot be made freely available due to approved research protocol restrictions and because it contains identifying information. None of the authors is affiliated to the farm cooperatives and their data were obtained as part of a “Data Transfer Agreement”; thus, permission from third parties is required. All relevant data are available after positive evaluation by the institutional review board upon request by contacting Professor Dick Heederik (d.heederik@uu.nl).

Supporting material

Supplemental Figure S1. Total use of antibiotics (as defined daily dosages per animal and cycle (DDDA/C)) in 51 veal calf farms by antibiotic class during 4 pre-study (1p-4p) and the 2 study cycles (s1,s2), the Netherlands 2009–2012. Percentages for each antibiotic class over the total antimicrobial use per cycle are indicated inside the bars.



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Part II

Livestock-associated methicillin-resistant *Staphylococcus aureus* and extended-spectrum β-lactamase-producing *Escherichia coli* in pig farming

Chapter 4

Dose-response relationship between antimicrobial drugs and livestock-associated MRSA in pig farming

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Abstract

The farming community can be a vehicle for introduction of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in hospitals. During 2011–2013, an 18-month longitudinal study aimed at reducing the prevalence of LA-MRSA was conducted on 36 pig farms in the Netherlands. Evaluations every 6 months showed a slight decrease in MRSA prevalence in animals and a stable prevalence in farmers and family members. Antimicrobial use, expressed as Defined Daily Dosages per Animal per Year (DDDA/Y), decreased 44% during the study period and was associated with declining MRSA prevalence in pigs. MRSA carriage in animals was substantially higher at farms using cephalosporins. Antimicrobial use remained strongly associated with LA-MRSA in humans regardless of the level of animal contact. A risk factor analysis outlined potential future interventions for LA-MRSA control. These results should encourage animal and public health authorities to maintain their efforts in reducing antimicrobial use in livestock and ask for future controlled intervention studies.

Introduction

In 2005, sequence type (ST) 398 of methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in Europe with proven transmission between pigs and humans[1,2]. Since then, pigs, veal calves, and (to a lesser extent) poultry were increasingly found to harbor livestock-associated MRSA (LA-MRSA)[3]. ST398 is widely spread across Europe, and ≈70% of pig farms in the Netherlands test positive[4]. After transfer to humans, it can be introduced into hospitals and the community[5–8]. In 2011, ST398 accounted for 39% of all new MRSA detected through screening of patients in the Netherlands[9]. To our knowledge, no intervention studies have been undertaken to assess the efficacy of MRSA-reducing measures on farms. Trade of animals is a major risk factor for introducing MRSA into a negative herd[10-12]. Larger herds have been associated with higher antimicrobial use[4]. Antimicrobial use could not be identified as a clear determinant for MRSA[4]. Transmission dynamics within herds vary by animals' ages and phase of production, potentially leading to endemicity[13].

In 2006, the European Union banned the use of antimicrobial drugs as growth promoters. In the Netherlands the most noticeable change started in 2010, when the government set objectives for a 50% reduction in antimicrobial use by 2013 and 70% by 2015, compared with 2009. This policy was combined with benchmarking of farms, and later veterinarians, to identify persistently high users of antimicrobial drugs[14]. As part of this national program, farm treatment and health plans have to be drafted and reviewed annually[15], which has resulted in an almost 60% reduction for the major livestock industry sectors[16,17].

Against the background of nationwide reduction of antimicrobial use, during 2011-2013, we evaluated MRSA carriage changes in pigs and humans and study the effect of introduction of an additional range of preventive measures on MRSA carriage in animals, and humans living and/or working on the farms.

Materials and Methods

Study Design, Sample Collection, and Laboratory Analysis

Thirty-six pig farms were enrolled in and completed the study; 15 were recruited from farmer cooperatives in the Netherlands, 20 were recruited by veterinarians in the cooperatives, and 1 was recruited by a farm health consultant. Farms were visited at the start of the study during March-September 2011. A questionnaire was completed during a walk-through survey with the farm veterinarian. The questionnaire contained items on farm characteristics, biosecurity, animal management and hygiene practices (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/6/14-0706-Techapp1. pdf). Then, tailor-made interventions were developed with the farmer for each farm to be implemented from the beginning of the study. Interventions focused on 1) further reducing antimicrobial use, 2) improving personnel and farm hygiene, and 3) changing animal contact structures.

Each farm was assessed 4 times during the 18-month period (6-month intervals). At

each sampling time, the farm questionnaire was filled out again to monitor changes. Human participants completed another questionnaire (online Technical Appendix Table 2) focused on tasks performed, animal contact, and individual health status. Dry cotton nasal swabs (Copan, Brescia, Italy) were used to obtain samples from humans and animals. Persons self-sampled their nostrils, and veterinarians swabbed both anterior nares of 60 pigs per farm. Animal swab samples were analyzed in 10 pools of 6 animals. Each pool comprised pigs of the same age group in the same pen (suckling piglets, weaned piglets, gilts, sows, and finishing pigs). All animal and human samples were sent by courier to the Infectious Diseases and Immunology Department (Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands). The Medical Ethical Committee of the University Medical Centre Utrecht approved the study protocol, and all participants gave written informed consent.

Swab samples were pre-enriched in Mueller Hinton broth, followed by selective enrichment with ceftizoxime and aztreonam and culture on Brilliance MRSA agar (Oxoid, Badhoevedorp, the Netherlands)[18]. Suspected colonies were subcultured on Columbia agar with sheep blood (Oxoid) and confirmed by using real-time PCR targeting *mecA, femA, nuc*, and *C01* genes[19,20].

Farm Types

We classified production types as farrowing and farrow-to-finish. Farrowing farms did not produce fatteners and delivered growers (25 kg) to finishing farms (with the exception of 1 farm that delivered gilts for farrowing). Farrow-to-finish farms integrated farrowing and finishing production and delivered fattening pigs to the abattoir. A farm was defined as open when it received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when gilts were not supplied externally.

Data on Antimicrobial Use

In the Netherlands, all antimicrobial drug deliveries to each farm are compiled in national databases. Owners of the study farms gave written consent for retrieval of these antimicrobial use data over a 2-year period. Antimicrobial use was expressed as Defined Daily Dosages per Animal per Year (DDDA/Y) per farm for the 4 periods preceding each sampling time. The DDDA/Y is a standard weighted measure indicating the number of days of antimicrobial drug use per year for an average animal on the farm. A more detailed description of the calculation of DDDA/Y has been described[14,16].

Data Analysis

We conducted all statistical analyses in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). We explored changes in MRSA carriage in animals and humans and antimicrobial use over time using simple descriptive statistics. DDDA/Y was $\log_2$ transformed because of its right-skewed distribution. A total of 134 variables in the farm questionnaire and 59 in

the human questionnaire were selected for longitudinal analysis together with antimicrobial use (criteria of <10% missing values and ≤10% of farms in each category). Odds ratios (ORs) for MRSA positivity in a pig or a human sample in the presence or absence of a determinant were obtained by using random intercept generalized linear mixed models (PROC GLIMMIX; SAS Institute, Inc.). Only associations from the selected variables with $p \leq 0.10$ in pigs (adjusting for age group of the pool) and $p \leq 0.20$ in humans (adjusting for hours worked on the farm) were presented. Goodness-of-fit of the models was described by using -2 log residual pseudo-likelihood estimation, and model assumptions were checked with diagnostic plots. Generalized additive mixed modeling (gamm4 package in R 3.0.2; R Foundation for Statistical Computing, Vienna, Austria) was used to assess the shape of the relationship between antimicrobial use and MRSA in human and animals.

Results

The number of farms was unequally distributed by type of farm (Table 1). Characteristics among persons from different farm types did not differ significantly (Table 2). All MRSA isolated from animals and humans was ST398.

Antimicrobial Use Reduction and Assessment of Particular Interventions

During the 4 periods, tetracyclines were the most used antimicrobial drugs (37.6% of total DDDA/Y), followed by penicillins (30.2%), trimethoprim/sulfonamides (12.3%), macrolides/lincosamides (12.0%), and polymyxins (4.6%). The remaining 3.3% corresponded mainly with cephalosporins, amphenicols, pleuromutilines, and fluoroquinolones. Most antimicrobial classes decreased in parallel during the study; only macrolides slightly increased in DDDA/Y

Table 1. Characteristics of farms in a study of the dose–response relationship between antimicrobial drug use and livestock-associated methicillin-resistant *Staphylococcus aureus* in pig farming, the Netherlands, 2011–2013.

Type of farm*	No. farms	Median no. (interquartile range)	
		Sows	Fatteners
All	36	350 (270–550)	773 (0–1,950)
Open	22	337 (300–500)	500 (0–1,300)
Farrowing†	9	533 (350–800)	0
Farrow-to finish	13	314 (242–380)	1,100 (600–2,010)
Closed	14	407 (232–698)	1,400 (450–2,725)
Farrowing†	3	439 (239–905)	0
Farrow-to finish	11	367 (200–673)	1,892 (1,025–2,950)

*Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.

†No fattening pigs present.

Table 2. Characteristics of persons followed during the entire period of a study of the dose–response relationship between antimicrobial drug use and livestock-associated methicillin-resistant *Staphylococcus aureus* in pig farming, the Netherlands, 2011–2013*

Characteristic	Total study population	Farmers, employees	Partners	Children
Age, y (SD)	33.0 (17.8)	44.0 (13.6)	45.2 (8.9)	14.4 (5.6)
Mean time worked, h (SD)	21.8 (25.2)	46.0 (19.9)	10.1 (14.0)	2.2 (6.6)
Total no.	158	66	32	60
Sex				
M	91	58	0	33
F	67	8	32	27
Open farm	91	34	17	40
Farrowing†	26	11	5	10
Farrow-to finish	65	23	12	30
Closed farm	67	32	15	20
Farrowing†	14	8	3	3
Farrow-to finish	53	24	12	17

*Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.
†No fattening pigs present.

(9.9% to 16.5% from the first to the fourth period), and tetracyclines and trimethoprim/sulfonamides decreased slightly (from 37.0% to 32.7% and from 14.9% to 11.2%, respectively). Overall, 86% of the DDDA/Y were administered as batch or group treatment (i.e., animals were treated in groups mainly orally for prophylactic or metaphylactic reasons) and 14% as individual treatment (mainly by injection). These percentages did not significantly differ by type of farm. During the study, overall DDDA/Y decreased 44%, comparable with the national trend, across all farm types except open farrowing farms (Figure 1). Open and/or farrowing farms used at least twice as many antimicrobial drugs as closed and farrow-to-finish farms (Figure 1).

Farm management changes over time captured from the questionnaires were modest; just 10% of the intervention variables (median 9.7%, interquartile range [IQR] 6.0%–12.3%) per farm changed during the study. Thus, 27 farms had <12 of the 134 variables that changed. The median number of farms within a single change was 3 (IQR 1-4). Thus, 75% of the changes occurred in ≤4 farms. Changes over time did not differ by different farm type. Because of these limited and heterogeneous changes, an intervention effect could not be evaluated and we performed only a risk factor analysis.

MRSA in Pigs

The number of MRSA-positive farms decreased slightly during the study (from 31 to 29 positive farms). Twenty-eight farms were MRSA-positive at all sampling times. Most were

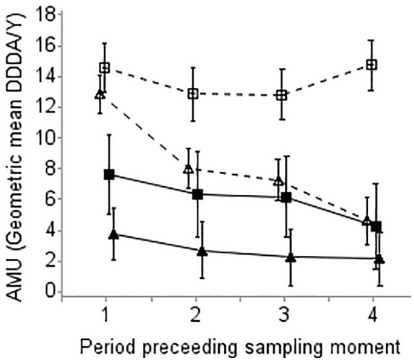


Figure 1. Antimicrobial use by type of farm during the 4 periods before each sampling time in a study of the dose-response relationship between antimicrobial drug use and livestock-associated methicillin-resistant *Staphylococcus aureus* on pig farms, the Netherlands, 2011–2013. GM and 95% CI from log2 DDDA/Y.

Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts. Closed triangles indicate closed farrow-to-finish farms; closed squares indicate closed farrowing farms; open triangles indicate open farrow-to-finish farms; open squares indicate open farrowing farms. AMU, antimicrobial use; DDDA/Y, Defined Daily Dosages Animal per Year; GM, geometric mean. Error bars indicate 95% Cis.

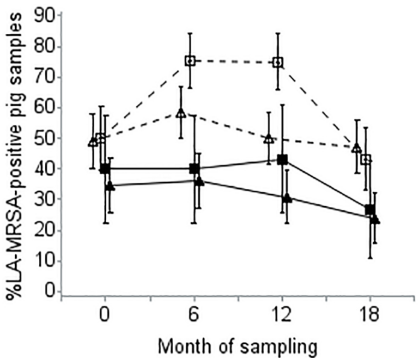


Figure 2. Prevalence of LA-MRSA–positive pooled samples from pigs on farms in a study of the dose-response relationship between antimicrobial drug use and LA-MRSA on pig farms, the Netherlands, 2011–2013.

Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts. Closed triangles indicate closed farrow-to-finish farms; closed squares indicate closed farrowing farms; open triangles indicate open farrow-to-finish farms; open squares indicate open farrowing farms. LA-MRSA, livestock-associated methicillin-resistant *Staphylococcus aureus*. Error bars indicate 95% Cis.

open (21 farms; 13 farrow-to-finish and 8 farrowing farms), and 7 were closed (5 farrow-to-finish and 2 farrowing). Four closed farrow-to-finish farms remained MRSA-negative during the entire study. From the remaining 4 farms, 3 became negative and 1 became positive during the study.

Overall pool-prevalence per sampling time decreased slightly on all farms. Open and farrowing farms remained at higher prevalences than closed and farrow-to-finish farms (Figure 2).

MRSA carriage differed notably between different age groups. The average pool-prevalence was 45.6% for finishing pigs; it was highest for suckling and weaned piglets (52.2% and 66.2%, respectively) and lowest for gilts and sows (30.2% and 30.8%, respectively). These prevalences did not significantly differ by farm type.

MRSA in Humans

MRSA prevalence in humans did not change significantly over time (Figure 3, panels A, B). Prevalence and carriage dynamics differed by number of hours worked on the farm. Prevalence for persons working ≥20 hours per week was 5 times higher than for persons

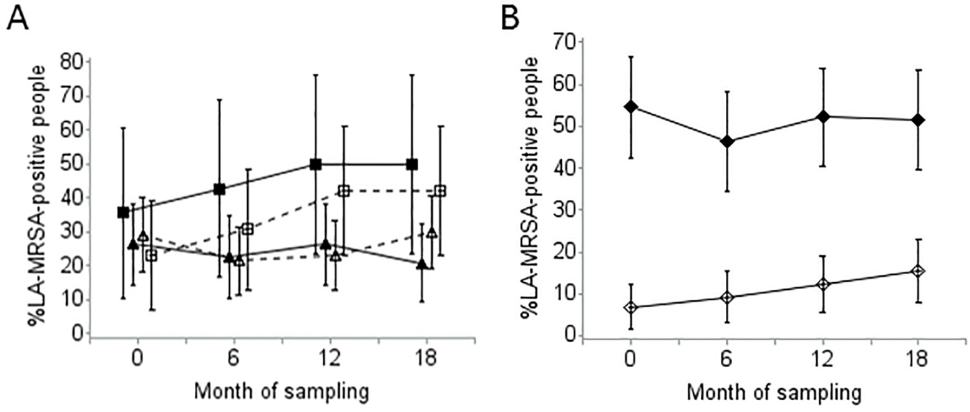
working <20 hours (Figure 3, panel B). Persons working ≥20 hours more frequently tested positive for MRSA at all sampling times (25%) or at least at 1 sampling time (48%), compared with those working <20 hours (2% and 24%, respectively). MRSA carriage dynamics did not significantly differ by level of antimicrobial use (data not shown) or by farm type (see overlap of 95% CIs in Figure 3, panel A).

Antimicrobial Use and MRSA Carriage in Pigs and Humans

Farms with higher antimicrobial use were more likely to have MRSA-positive pigs (Figure 4). The odds that a pool would be MRSA positive was 16% higher for a 2-fold increase in DDDA/Y (Table 3). MRSA in pigs from open and from farrowing farms (high users of antimicrobial drugs) showed a positive trend and a significant association, respectively, with antimicrobial use (Table 3). The odds for testing LA-MRSA positive was higher when the proportion of group treatments with antimicrobial drugs was ≥0.5 (odds ratio [OR] 1.79, 95% CI 1.12–2.88; p=0.02). This association was also found on open and on farrow-to-finish farms but was stronger in farrowing farms (OR 2.9, 95% CI 0.98–8.60; p=0.05). Changes in MRSA carriage in pigs over time were significantly associated with changes in antimicrobial use; the odds for a 2-fold increase in antimicrobial use per sampling time (antimicrobial use-time interaction) decreased from the second to the last sampling (ORs 0.94, 1.27, 1.26, and 1.14 in the 4 consecutive samplings; p=0.01). The same was found in an analysis restricted to open farms (ORs 0.86, 1.33, 1.18, and 1.06; p=0.01). In farrowing farms (with little reduction

Figure 3. Prevalence of LA-MRSA in humans (n=158) during a study of the dose-response relationship between antimicrobial drug use and LA-MRSA on pig farms, the Netherlands, 2011–2013. Results are stratified by type of farm (A) and number of hours worked on the farm (B).

Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts. Closed triangles indicate closed farrow-to-finish farms; closed squares indicate closed farrowing farms; open triangles indicate open farrow-to-finish farms; open squares indicate open farrowing farms; open diamonds indicate persons working <20 hours per week; closed diamonds indicate persons working ≥20 hours per week. LA-MRSA, livestock-associated methicillin-resistant *Staphylococcus aureus*. Error bars indicate 95% Cis.



in antimicrobial use), the antimicrobial use–time interaction was also significant, but ORs increased over time (ORs 1.04, 1.38, 1.62, 1.62; p=0.03).

We also observed a positive trend between antimicrobial use in animals and human MRSA carriage (Figure 4); the unadjusted OR for a 2-fold increase in DDDA/Y was 1.17 (95% CI 0.98–1.39; p=0.09). The antimicrobial use-MRSA association did not significantly change after adjustment for hours worked (OR_{adj}) (Table 3). When stratified by working hours, antimicrobial use remained especially associated with MRSA for persons working ≥20 hours per week (OR_{adj} 1.25, 95% CI 1.01–1.54; p=0.04), compared with those working <20 hours (OR_{adj} 1.21, 95% CI 0.92–1.59; p=0.18). A similar trend was observed across farrow-

Table 3. ORs for livestock-associated MRSA in pigs and in humans with increasing use of antimicrobial drugs, the Netherlands, 2011–2013*

Characteristic	ORs for a 2-fold increase in DDDA/Y							
	Pooled pig samples				Farmers and family members			
	No.†	OR‡ (95% CI)	p value	-2 log RSPL§	No.¶	OR# (95%CI)	p value	-2 log RSPL§
All farms	1,421	1.16 (1.02–1.33)	0.03**	6937.5	626	1.22 (1.01–1.48)	0.04	3196.9
Supply of gilts††								
Open	867	1.11 (0.97–1.27)	0.12**	3828.9	365	1.08 (0.85–1.38)	0.53	1806.9
Closed	554	0.86 (0.69–1.33)	0.79	3132.2	261	1.31 (0.94–1.81)	0.11	1424.3
Production type								
Farrowing	476	1.38 (1.03–1.86)	0.03**	2399.2	158	1.28 (0.85–1.94)	0.24	784.3
Farrow-to-finish	954	1.11 (0.95–1.30)	0.18	4621.4	468	1.19 (0.95–1.5)	0.13	2439.8

*Farm antimicrobial use was defined as 1 unit increase in the log2 DDDA/Y. Results from the random intercept generalized linear mixed models accounting for the repeated measurements design and adjusting for confounders. DDDA/Y indicates the number of days of antimicrobial use per year for an average animal on the farm. It was determined by dividing the total number of kilograms treatable with a single mass unit of the antimicrobial drug concerned, according to the package insert information, by the average number of animal kilograms on the farm. The denominator comprised sows and fatteners. DDDA/Y, defined daily dosages animal per year; MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odd ratio; RSPL, residual pseudo-likelihood. Bold type indicates significance (p<0.05).

†Number of observations at all sampling times together (10 pooled pig samples per farm on 36 farms in 4 sampling times). Values are missing for 19 observations.

‡For analysis in pigs, a farm random intercept was included in the mixed models and adjustment of ORs was made for sampling time and age group of pigs in the pool.

§RSPL from the generalized linear mixed models. Models per stratum of external supply or type of production are not nested and -2 log RSPL cannot be used for comparison.

¶Number of observations in all sampling times together (158 persons, 4 sampling times). Values are missing for 6 observations.

#For analysis in humans, a farm and a person random intercept were included in the mixed models, and number of hours worked on the farm and sampling time were used for adjustment of ORs.

**These models additionally showed significant antimicrobial use–time interaction indicating parallel change in antimicrobial use and livestock-associated MRSA prevalence over the study period (see extended explanation in text).

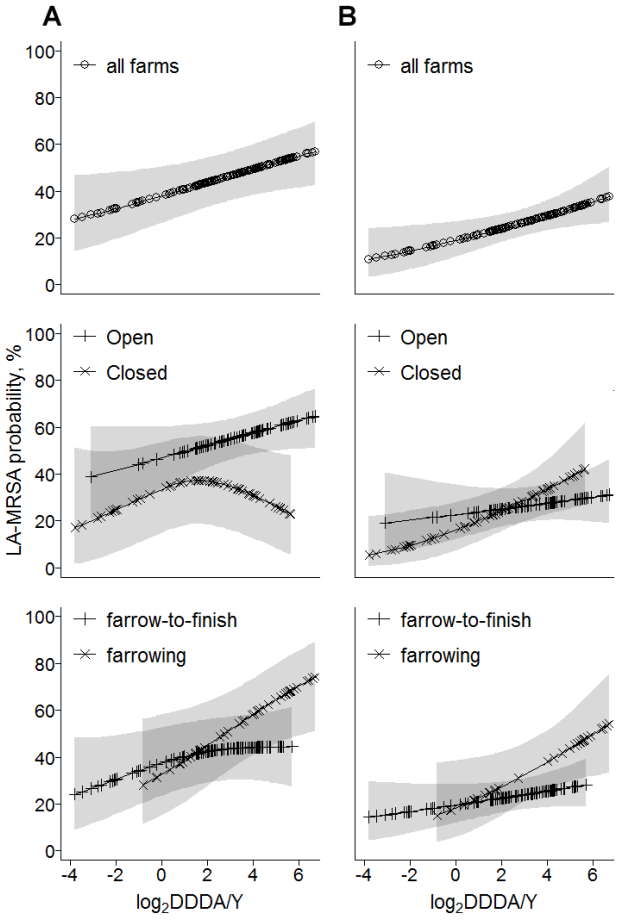
††Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.

to-finish, farrowing, and closed farms (Table 3).

The probability of LA-MRSA carriage was higher when the proportion of antimicrobial group treatments was ≥0.5 (OR_{adj} 1.76, 95% CI 0.79–3.90; p=0.17). Reduction in antimicrobial use over time was not associated with any change in MRSA carriage in humans.

Specific levels of DDDA/Y for tetracyclines and penicillins were positively associated (p values from 0.06 to 0.23) with MRSA in pigs and humans (data not shown). The use of cephalosporins (on 7 farms, 6 of them open) during the first sampling time, was strongly associated with MRSA carriage in pigs (OR 2.94, 95% CI 1.45–5.87; p=0.002). This association was not found for humans. Associations with other antimicrobial classes were weaker and often not statistically significant.

Figure 4. Dose–response relationships between antimicrobial use (log_2 DDDA/Y) and livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) predictive probabilities in pigs (A) and humans (B), the Netherlands, 2011–2013.



DDDA/Y was determined by dividing the total number of kilograms treatable with a single mass unit of the antimicrobial drug concerned, in accordance with the package insert information, by the average number of animal kilograms at the farm. Splines were obtained from generalized additive mixed models with random intercepts for farms in the analysis for pigs and humans. Models accounted for the repeated measurements design and were adjusted for age group of pigs and for animal contact (i.e., hours worked) for humans. Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts. P values and maximum-likelihood (ML) scores for the splines in the models for pigs: all farms (p=0.03; ML 1433.5); open farms (p=0.09; ML 991.3); closed farms (p=0.09; ML 407.9); farrowing farms (p=0.02; ML 438.5); farrow-to-finish farms (p=0.39; ML 936.5). P values and ML scores for the splines in the models for humans: all farms (p=0.01; ML 573.9); open farms (p=0.41; ML 337.8); closed farms (p=0.01; ML 229.9); farrowing farms (p=0.03; ML 170.3); farrow-to-finish farms (p=0.17; ML 398.2). DDDA/Y, Defined Daily Dosages Animal per Year; ML, maximum likelihood. Shaded areas indicate 95% CIs.

Other Factors Determining MRSA in Humans and Pigs

Number of hours worked on the farm per week was strongly associated with MRSA in the human study population (univariate OR 1.82/10 hours worked increase, 95% CI 1.58–2.06; p<0.0001). Except for antimicrobial use, tasks related to animal contact and touching pigs from other farms were identified as risk factors for MRSA carriage in humans (Table 4). All variables in Table 4 were moderately or highly correlated (Spearman/Pearson r>0.5), and no multivariable model was built. We found no correlation between farm size, antimicrobial use, and hours worked.

More biosecurity items reducing MRSA carriage in pigs were found on closed farms (e.g., different compartments per production phase, boarding platform for sows, washing overalls) (Table 5, http://wwwnc.cdc.gov/EID/article/21/6/14-0706-T5.htm). Some variables had a similar effect on open and closed farms, increasing risk for MRSA (e.g., injection of antimicrobial drugs, clipping of teeth, and vaccination of piglets) or decreasing MRSA carriage (e.g., presence of a medication pipe separated from the water pipe, delivery room for materials, and keeping the sows in stable groups [i.e., not mixing]) (Table 5). However, other effects showed conflicting directions between strata (e.g., farm treatment plan, cleaning and disinfecting the carcass barrels, source of water supply) (Table 5). Low-level correlation existed between some variables (pairwise Spearman r<0.5) and with antimicrobial use or cephalosporin use (Table 5). A full multivariable model (online Technical Appendix Table 3) was fitted by using the significant determinants from Table 5 together with the use of antimicrobials and cephalosporins; results from the backward elimination of non-significant terms are presented in Table 6. The presence of external supply of animals, overall antimicrobial use, and use of cephalosporins were significant risk factors retained through all elimination steps.

Table 4. ORs for determinants of livestock-associated MRSA in humans, adjusted for number of hours worked per week on the farm, the Netherlands, 2011–2013*

Variable	No.†	OR‡ (95% CI)	p value§	-2 log RSPL¶
Age, per 10 y increase	632	1.14 (0.93–1.41)	0.20	3204.1
MRSA prevalence in pigs, %, per 10% increase	632	1.08 (0.97–1.21)	0.16	3190.9
MRSA-negative farm				
Yes	114	0.06 (0.01–0.27)	<0.01	3288.1
No	518	Ref		
Touching dogs in past 6–12 mo				
Yes	446	0.51 (0.27–0.96)	0.04	3173.7
No	180	Ref		
Touching pigs from other farms in past 6–12 mo				
Yes	86	2.82 (1.35–5.91)	0.01	3205.3
No	546	Ref		
Sorting of sows in past 7 d				
Yes	221	1.91 (0.97–3.77)	0.06	3144.5
No	392	Ref		
Sorting of suckling piglets in past 7 d				
Yes	159	2.21 (1.16–4.22)	0.02	3169.5
No	455	Ref		
Sorting of weaned piglets in past 7 d				
Yes	174	1.63 (0.83–3.20)	0.16	3162.9
No	439	Ref		
Feeding sows in past 7 d				
Yes	220	2.03 (0.99–4.17)	0.05	3126.0
No	390	Ref		
Cleaning and disinfecting weaned piglets section in past 7 d				
Yes	81	1.70 (0.76–3.80)	0.20	3157.8
No	538	Ref		

*Results from the random intercept generalized linear mixed models accounting for the repeated measurements design and adjusted for number of hours worked. MRSA, methicillin-associated *Staphylococcus aureus*; OR, odds ratio; Ref, reference category; RSPL, residual pseudo-likelihood. Bold type indicates p values <0.05.

†Number of observations in all sampling times together (158 persons, 4 sampling times). Some variables have missing observations.

‡For analysis in humans, a farm and a person random intercept were included in the mixed models, and number of hours worked on the farm and sampling time were used for adjustment of ORs.

§Only variables with p<0.2 in the mixed models are presented in the human analysis.

¶RSPL from the generalized linear mixed models.

Table 5. ORs for determinants of livestock-associated MRSA positivity in pooled samples from pigs, the Netherlands, 2011–2013*

Characteristic	All farms		Open farms		Closed farms	
	No.†	OR (95% CI)	No.†	OR (95% CI)	No.†	OR (95% CI)
Farm						
No. sows, 300 increase§	1,421	1.4 (0.7–2.7)	867	1.3 (0.8–2.2)	554	2.6 (0.7–9.7)
External supply of gilts						
Open	867	6.6 (2.3–19.0)¶	867	Not computable	0	Not computable
Closed	554	Ref	0		554	
Type of production						
Farrow-to-finish	945	0.4 (0.1–1.6)	511	0.7 (0.3–1.6)	434	0.4 (0.0–17.7)
Farrowing	476	Ref	356	Ref	120	Ref
Farm treatment plan						
Yes	1,157	0.7 (0.4–1.3)	723	0.6 (0.3–1.1)	434	2.1 (0.6–7.1)
No	190	Ref	110	Ref	80	Ref
Water supply for animals						
Public, from tap	452	2.8 (1.3–6.0)#	218	0.8 (0.4–1.8)	234	7.7 (2.5–24)#
Private source	929	Ref	619	Ref	310	Ref
Separate medication pipe						
Yes	920	0.4 (0.2–0.7)#	526	0.4 (0.2–0.7)#	394	0.8 (0.2–3.7)
No	441	–	311	Ref	130	Ref
Biosecurity						
Different compartments per production phase						
Yes	880	0.9 (0.5–1.6)	600	1.7 (0.8–3.8)	280	0.4 (0.2–1.1)
No	521	Ref	257	Ref	264	Ref
Boarding platform for sows						
Yes	512	0.7 (0.4–1.4)	358	1.3 (0.7–2.6)	154	0.2 (0.1–1.0)
No	909	Ref	509	Ref	400	Ref
Clearly defined border of boarding platform						
Yes	989	0.7 (0.4–1.3)	569	1.1 (0.6–1.9)	420	0.2 (0.1–0.6)#
No	432	Ref	298	Ref	134	Ref
Carcass barrels cleaned and disinfected after emptied						
Yes	527	0.5 (0.3–1.0)**	317	0.4 (0.2–0.8)#	210	1.6 (0.5–5.1)
No	864	Ref	530	Ref	334	Ref

Characteristic	All farms		Open farms		Closed farms	
	No.†	OR (95% CI)	No.†	OR (95% CI)	No.†	OR (95% CI)
Delivery room for materials						
Yes	1,031	0.4 (0.2–0.7)**	677	0.5 (0.2–1.0)**	354	0.3 (0.1–0.6)#
No	320	Ref	140	Ref	180	Ref
Pigs go outside when moved						
Yes	627	0.8 (0.4–1.6)	367	1.4 (0.8–2.6)	274	0.2 (0.1–0.8)**
No	744	Ref	470	Ref	260	Ref
Workers' overalls washed						
Yes	687	0.8 (0.5–1.4)	317	1.2 (0.7–2.1)	370	0.3 (0.1–1.2)
No	734	Ref	550	Ref	184	Ref
Removal of manure in winter						
Manure stays <6 mo	1,007	1.2 (0.7–2.0)	647	0.8 (0.5–1.5)	360	2.9 (1.0–8.9)
Manure stays >6 mo	380	Ref	186	Ref	194	Ref
Animal management and contact structure						
Injection of piglets with antimicrobial drugs during the first week.						
Yes	830	2.0 (1.2–3.3)#	610	1.4 (0.8–2.5)	220	3.7 (1.6–8.6)#
No	571	Ref	257	Ref	314	Ref
Tooth clipping in piglets						
Yes	516	3.2 (1.4–7.0)**	346	3.0 (1.5–6.2)#	170	4.0 (0.5–30.6)
No	875	Ref	501	Ref	374	Ref
Vaccination of piglets and/or fatteners						
Yes	1,090	2.5 (1.4–4.5)**	690	2.0 (1.1–3.4)**	400	7.2 (1.6–32)**
No	311	Ref	167	Ref	144	Ref
Needles for vaccination renewed per compartment						
Yes	848	1.9 (1.2–3.1)**	508	1.7 (1.0–2.7)**	340	2.1 (0.4–12.1)
No	456	Ref	312	Ref	144	Ref
Some piglets reared motherless						
Yes	385	1.3 (0.7–2.3)	311	1.6 (0.9–2.7)	74	0.2 (0.0–0.9)**
No	1,026	Ref	546	Ref	480	Ref
Sows in stable groups						
Yes	772	0.5 (0.3–0.8)#	432	0.6 (0.3–1.0)	340	0.5 (0.2–1.1)
No	619	Ref	405	Ref	214	Ref

Characteristic	All farms		Open farms		Closed farms	
	No.†	OR (95% CI)	No.†	OR (95% CI)	No.†	OR (95% CI)
Hygiene						
In the piglet section						
Disinfectant	189	0.3 (0.2–0.7)#	139	0.3 (0.1–0.7)#	50	0.9 (0.1–5.9)
Soaking agent	280	2.0 (1.0–4.4)	180	3.1 (1.3–7.5)**	100	0.1 (0.0–0.8)**
Disinfectant + soaking	698	1.2 (0.6–2.3)	408	1.4 (0.6–3.1)	290	0.8 (0.3–2.5)
None	254	Ref	140	Ref	114	Ref
In the mating section						
Disinfectant + soaking	239	0.6 (0.3–1.1)	89	0.3 (0.1–0.8)**	150	2.2 (0.6–7.7)
None	1,182	Ref	778	Ref	404	Ref
In the gilt section						
Soaking agent	220	1.0 (0.5–2.0)	100	1.5 (0.7–3.6)	120	0.3 (0.1–1.1)
Disinfectant + soaking	585	1.0 (0.6–1.6)	335	1.0 (0.6–1.7)	250	1.3 (0.5–3.9)
None	616	Ref	432	Ref	184	Ref

Fits for the univariate adjusted models in all farms: -2 log RSPL estimations ranged from a minimum of 6386.56 to a maximum of 7016.07.

*Results from the longitudinal analysis with generalized linear mixed models taking into account the repeated measurements design and adjusted for age group of the pool. Variables with p<0.1 in the overall analysis or in at least 1 stratum (open or closed) are presented. OR and p values are in bold type when p<0.1. Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts. MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odds ratio; Ref, reference category; RSPL, residual pseudo-likelihood.

†Number of observations at all sampling times together (10 pooled pig samples per farm in 36 farms in 4 sampling times). Some variables have missing observations.

§Items evaluated irrespective of significance.

¶p<0.001.

#p<0.01.

**p<0.05.

Table 6. ORs for the most important determinants of livestock-associated MRSA positivity in pooled pig samples (n=1,054) from 32 farms (multivariable final model), the Netherlands, 2011–2013*

Characteristic	No.†	OR (95% CI)	p value
Sampling time			
0 mo	262	0.83 (0.48–1.43)	<0.001
6 mo	290	2.05 (1.25–3.37)	
12 mo	259	1.96 (1.20–3.20)	
18 mo	243	Ref	
Age group			
Gilts	212	1.08 (0.65–1.80)	<0.001
Finishers	140	4.09 (2.30–7.25)	
Suckling piglets	212	3.87 (2.34–6.39)	
Weaned piglets	280	9.89 (5.96–16.39)	
Sows	210	Ref	
External supply of gilts‡			
Open	630	5.54 (1.56–19.27)	0.008
Closed	424	Ref	
Delivery room for materials			
Yes	804	0.29 (0.13–0.62)	0.001
No	250	Ref	
Sows housed in stable groups			
Yes	594	0.53 (0.29–0.96)	0.038
No	460	Ref	
Antimicrobial drug use, per 2-fold increase (log_2 DDDA/Y)	1,054	1.22 (1.03–1.44)	0.024
Use of cephalosporins			
Yes	84	3.15 (1.47–6.74)	0.003
No	970	Ref	

*Model fit: -2 log RSPL estimation=5331.7. Multivariable final model after backward elimination of non-significant variables from a full model (online Technical Appendix Table 3, http://wwwnc.cdc.gov/EID/article/21/6/14-0706-Techapp1.pdf) containing the significant associations (p<0.05) presented in Table 5 (http://wwwnc.cdc.gov/EID/article/21/6/14-0706-T5.htm) for all farms, together with antimicrobial drug use, use of cephalosporins, sampling time, and age group of the pool. MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odds ratio; DDDA/Y, defined daily dosages animal per year; Ref, reference category; RSPL, residual pseudo-likelihood.
†Multiple variables had missing values in the full model reducing the number of observations in the final model.
‡Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.

Discussion

We found a quantitative association between antimicrobial use and MRSA in pigs and humans living and/or working on pig farms. Our findings indicate that a reduction in antimicrobial use is likely to be effective in reducing MRSA carriage in pigs. Risk for MRSA is higher for increased use of tetracyclines and penicillins but more so for use of cephalosporins. Except for the change in antimicrobial use over time, overall changes in farm management were modest and not sufficient to contribute to decreasing MRSA levels. Nevertheless, several factors were identified as possible candidates for future intervention studies.

The extent of representativeness of a convenient sample is difficult to evaluate. Nonetheless, descriptive results show the heterogeneity of farms included; the decreasing trend in use of antimicrobial drugs and the proportions by antimicrobial classes and by group and individual treatments mirror national data[16,17].

Levels of antimicrobial use differed considerably by farm type. Open and/or farrowing farms were high users of antimicrobial drugs and showed a strong positive dose-response relationship between antimicrobial use and MRSA in pigs. In particular, the use of cephalosporins was related to higher carriage rates of MRSA. The literature shows that selective pressure favors transmission and spread of MRSA in pigs[13,21]. MRSA ST398 isolates have shown high diversity of resistance genes, and all of them are resistant to penicillin and tetracycline[22]; the DDDA/Y of these antimicrobial classes was related to MRSA in our results. Although the use of cephalosporins represented a small proportion of total antimicrobial use, it was strongly associated with MRSA in pigs. These antimicrobial drugs are known to be important for generation and propagation of resistance in *S. aureus* and other microorganisms[23]. The fact that they were administered before the first sampling time might be related to the initial increase in MRSA prevalence in pigs. We refrained from presenting detailed associations by antimicrobial classes because mostly all classes were used on all the farms and were correlated; thus, effects of individual classes of antimicrobial drugs were difficult to disentangle and require cautious interpretation. The higher risk posed by administering group treatments confirms previous findings in the literature[4,12]. Interaction between antimicrobial use and time was significant, suggesting a decrease of MRSA prevalence in pigs over time with decreasing antimicrobial use. These associations were not found on closed and farrow-to-finishing farms, indicating that below a certain level, antimicrobial use contributes less to MRSA prevalence. Nevertheless, it is important to consider that other studies have reported high MRSA transmission in the absence of antimicrobial agents[24,25]. Thus, antimicrobial use should not be the only target for intervention.

Direct contact with positive animals has been widely reported as the major force driving MRSA carriage in persons living and/or working on farms[26-28]. In our study, higher risk for MRSA in the human study population was strongly associated with the number of hours worked on the farm and to the variables related to tasks performed on the farm. However, antimicrobial use also showed a significant positive dose-response relationship to MRSA

human carriage during the study, even after adjustment for hours worked. When antimicrobial drugs are administered to animals, substantial quantities of these drugs remain in manure, on surfaces of barns, and in dust as a potential risk source[29]. The selective pressure exerted by exposure to dust containing antimicrobial drugs or directly to antimicrobial powder formulations would explain the higher risk for MRSA carriage in persons living or working on pig farms. However, this independent effect of antimicrobial use on susceptible bacteria in humans is difficult to disentangle from direct MRSA transmission from animals to humans.

The role of animal trade in introducing and spreading MRSA has been reported[4,10-13], but information about carriage status of animals entering the farm was not available in this study. Nevertheless, our results corroborate that external supply of animals is significantly associated with higher MRSA levels. A higher selective pressure for MRSA might also occur on open farms because they had higher overall antimicrobial use and 6 of them used cephalosporins. However, external supply of animals appeared to be a risk factor, even when evaluated together with antimicrobial use and cephalosporin use in the multivariate model.

A previous study in the Netherlands found that the prevalence of MRSA-positive pig farms steeply increased from 40% in 2007 to 70% in 2008[4]. Our results show that this prevalence remains high (>80%) but the slight increase since 2008 indicates that MRSA carriage in pigs might have reached a steady state. Herd size was identified as a risk factor when MRSA was emerging in livestock[12]; however, we found no such association.

Several determinants could be targeted for specific interventions in the near future. Factors regarding biosecurity considerably reduced the risk for MRSA, especially on closed farms. It is remarkable that mostly variables related to management of piglets were associated with MRSA. Piglets are more susceptible to infection, and they receive larger amounts of antimicrobial drugs. Tooth clipping in piglets increased the probability for MRSA carriage; MRSA transmission from piglet to piglet might be higher when the same plier is used or through the worker. Unexpected risk factors could be the product of reverse causality such as vaccination of piglets, fatteners, or both and frequent change of needles. These possibilities need to be explored in other, independent studies. Observations for cleaning and disinfection were not consistent. It has been previously reported that disinfection has a short-lasting positive effect for MRSA reduction[30]. Keeping the groups of sows stable was an interesting protective factor that might reduce MRSA spread within the farm. Animals that drank water from the public supply instead of from a private source had increased probability for MRSA. Zinc oxide specifically co-selects for MRSA ST398[31,32], and concentrations can be higher in tap water as a result of leaching from pipes. A higher zinc intake in animals might have led to higher selection for MRSA, but this association needs further research.

Pooling of animal samples leads to less precise prevalence estimates[33,34] but is a low-cost alternative for individual sampling that enabled enlargement of the number of farms tested. Individual testing, however, would not be expected to lead to different outcomes.

This study shows the inherent difficulty in evaluating pragmatic interventions for MRSA

control in pig farms under field conditions over a relatively short period. More farms and controlled interventions, together with longer follow-up periods to capture prevalence changes, are needed to assess intervention effects over time. Despite the limitations, we identified factors that can define attainable future interventions (e.g., avoiding tooth clipping, keeping sows in stable groups). Finally, we demonstrated that antimicrobial use has a strong and positive dose-response relationship with MRSA in pigs and humans living and/or working on pig farms. In particular, use of cephalosporins resulted in increased MRSA carriage rates in pigs. Animal and public health authorities should continue to promote the reduction of antimicrobial use. Different approaches for MRSA control might be needed in light of the differences by type of production and external supply of animals.

Footnotes

Preliminary results from this study were presented at 3rd American Society of Microbiology (ASM-ESCMID) Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, 4-7 November 2013, Copenhagen, Denmark; oral presentation title: *Intervention measures reducing livestock-associated MRSA on pig farms in the Netherlands: a longitudinal study*. And at the Society for Veterinary Epidemiology and Preventive Medicine (SVEPM), 26-28 March 2014, Dublin, Ireland; poster presentation title: *Strong dose-response relationship between antimicrobial use and livestock-associated MRSA in pig farming: results from a pragmatic intervention study*. The authors have declared that no competing interests exist.

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Supporting material

Appendix Table 1. Farm questionnaire used in each of the four sampling times in a risk factor analysis for livestock-associated methicillin-resistant *Staphylococcus aureus* in pig farming, the Netherlands, 2011-2013.

Question	Possible answers
General farm characteristics:	
1. Farm size: mean number of sows present per year ¶†	No.
2. Type of production¶	Farrowing / Farrow-to-finish
3. Mean number of fattener pigs present per year	No.
4. Frequency of gilts supply per year	No.
5. Number of farms from which pigs are supplied per year	No.
6. External supply of gilts ≥1 time a year from at least one supplier (aggregated from questions 4 and 5) ¶	Open / Closed
7. Complete all in-all out system is applied for closed farm¶	Yes / No
8. Frequency of removal of piglets	No.
9. Frequency of removal of fattener pigs	No.
10. Frequency of removal of rearing gilts	No.
11. Frequency of removal of sows	No.
12. Percentage of loss of weaned piglets per year	No.
13. Average lactation period (days)	No.
14. Mean number of weaned piglets per sow per year	No.
15. Mean number of weaned piglets per litter	No.
16. Percentage of loss of fattener pigs per year	No.
17. Percentage of loss of sows per year	No.
18. Mean growth per piglet per day	No.
19. Mean growth per fattener pig per year	No.
20. After delivering the leftover piglets are placed together	Yes / No
21. After delivering there is a leftover piglets department	Yes / No
Biosecurity and hygiene status	
22. Hygiene status of the farm	A/ B/ C/ D/ E/ F
23. The farm owns an Specific pathogen free (SPF) status¶	Yes / No
24. SPF status for	App/ Aujeszky/ M.Hyo/ PRRSv/ None
25. Housing of the gestating sows ¶	Cubicle / Groups
26. Group size of the gestating sows¶	Yes / No
27. There are other animals present on the farm¶	Yes / No
28. There are also sheep present on the farm¶	Yes / No
29. There are also goats present on the farm¶	Yes / No
30. There are also cattle present on the farm¶	Yes / No

Question	Possible answers
31. There are also horses present on the farm¶	Yes / No
32. There are also poultry present on the farm¶	Yes / No
33. No other farming animals are present on the farm	Yes / No
34. Cats are able to enter the shed	Yes / No
35. Dogs are able to enter the shed¶	Yes / No
36. Number of people working on the farm (including assisting family members)	Yes / No
37. Biosecurity score (aggregated sum from questions 38, 42, 45,49, 90, 136 where yes=1 and no=0)¶	0 to 6
38. There is only one entrance to the farm, which is the hygiene lock, other doors are locked¶	Yes / No
39. A doorbell or phone number of the owner is clearly visible at the entrance of the farm. In this way it is possible to contact the people of the farm¶	Yes / No
40. The farm's terrain is paved and cleaned up ¶	Yes / No
41. Silos are filled from the side of the dirty road¶	Yes / No
42. Pigs and personnel go outside during working activities¶	Yes / No
43. The hygiene lock consists of a clean and dirty part, separated by a corridor shower¶	Yes / No
44. The lock does not contain a shower, but it does consist of a clearly separated clean and dirty part	Yes / No
45. Showering is mandatory¶	Yes / No
46. If showering is not mandatory, everyone washes his or her hands before entering the farm	Yes / No
47. Showering is not mandatory, however wearing farm-issued clothing is (pants and shirt	Yes / No
48. Farmer and his co-workers use the hygiene lock in the same way visitors do¶	Yes / No
49. The farmer and his co-workers wash their hands before entering the farm¶	Yes / No
50. There is warm water available¶	Yes / No
51. There is soap available	Yes / No
52. There is a clean towel present	Yes / No
53. There are clean boots and overalls available¶	Yes / No
54. Workers' overalls are washed daily¶	Yes / No
55. Overalls are washed	Daily/Weekly / Monthly/ Less than monthly
56. On average, for how many months does the manure stay in the pits during the summer?¶	<6 / ≥6

Question	Possible answers
57. On average, for how many months does the manure stay in the pits during the summer?¶	<3 / 3-6 / 6-9 / >9
58. On average, for how many months does the manure stay in the pits during the winter?¶	<6 / ≥6
59. On average, for how many months does the manure stay in the pits during the winter?¶	<3 / 3-6 / 6-9 / >9
60. Delivered animals are placed in quarantine for a certain period of time. This part has its own entrance and is not a part of the rest of the farm	Yes / No
61. The quarantine has its own lock and clothing	Yes / No
62. The quarantine is visited at the end of the day	Yes / No
63. After delivery, the gilts arrive at an empty and cleaned section. This is not a quarantine	Yes / No
64. When gilts are delivered, these animals do not arrive at an empty section or quarantine	Yes / No
65. Piglets are delivered on the same day as fatteners	Yes / No
66. Sperm is delivered on the dirty road, the cooling box is not brought on the farm terrain¶	Yes / No
67. There is a delivery room for materials and bagged goods. Materials are not delivered directly to the farm¶	Yes / No
68. Pest control is handed over to a professional organization¶	Yes / No
69. Birds are able to enter the sheds¶	Yes / No
70. Is there presence of rats and/or mice?¶	Yes / No
71. Animals have access to an outdoor run (e.g. after weaning)	Yes / No
72. When pigs are moved, they have to go outside¶	Yes / No
73. There is a boarding platform for the sows, preventing the truck from parking directly against the shed¶	Yes / No
74. There is a boarding platform for the piglets, preventing the truck from parking directly against the shed¶	Yes / No
75. There is a boarding platform for the fatteners, preventing the truck from parking directly against the shed ¶	Yes / No
76. There is boarding platform for piglets and/or fatteners¶	Yes / No
77. The boarding location is not situated directly next to or beneath an air inlet¶	Yes / No
78. The border for the delivery of animals is a 100% clear and is also implemented this way¶	Yes / No
79. After delivery of the animals, the delivery platform is cleaned and disinfected immediately¶	Yes / No
80. The driver does not enter the clean road¶	Yes / No
81. Transport trucks are clean, empty and disinfected when they arrive on the farm to load the sows¶	Yes / No
82. The carcass storage is cooled and locked¶	Yes / No

Question	Possible answers
83. The carcass cooler is situated on the dirty road¶	Yes / No
84. Small destruction materials can be thrown into the cooler from the clean road¶	Yes / No
85. There is a double number of barrels on the farm. So there is a surplus of barrels¶	Yes / No
86. After the destructor emptied the barrels, the barrels are cleaned and disinfected before retrieved¶	Yes / No
87. Rinsing water of cleaning barrels is discharged into the sewer	Yes / No
88. When handling carcasses, gloves are always worn¶	Yes / No
89. When treating sick animals, gloves are always worn¶	Yes / No
90. When treating piglets, gloves are always worn¶	Yes / No
91. After someone entered a pen of the weaned piglets or the fatteners, hygienic measures are taken	Yes / No
Animal health management	
92. During gestation, vaccinations are implemented	Yes / No
93. During lactation, vaccinations are implemented	Yes / No
94. The piglets and/or fatteners are vaccinated¶	Yes / No
95. PRRSv vaccination is implemented	Yes / No
96. Mycoplasma hyponeumoniae vaccination is implemented	Yes / No
97. PCV2 vaccination is implemented	Yes / No
98. APP vaccination is implemented	Yes / No
99. Glässer vaccination is implemented	Yes / No
100. The piglets are vaccinated without the use of a needle	Yes / No
101. The teeth of the new-born piglets are clipped¶	Yes / No
102. The tails of the piglets are docked¶	Yes / No
103. The boar piglets are castrated	Yes / No
104. All piglets are given an injection of antibiotics in their first week of life¶	Yes / No
105. When treating the piglets, gloves are worn	Yes / No
106. The gloves are renewed:	After each litter/ After each section /Each day
107. When treating the piglets, other hygiene measures are taken in order to prevent the transfer of infection from one to the other litter¶	Yes / No
108. Needles for vaccination of sows are renewed:¶	Once a day / Once a week / When necessary
109. Needles for vaccination of piglets and/or fatteners are renewed per pen¶	Yes / No
110. Needles for vaccination of piglets and/or fatteners are renewed per section¶	Yes / No

Question	Possible answers
111. At the end of the day, the syringes are cleaned:	Daily, rinsing with cold water / Taken apart and with water and soap / Dishwasher / Not cleaned
112. There is a sick-bay present¶	Yes / No
113. The sick-bay is used as a sick-bay	Yes / No
114. In the sick-bay, different ages are present	Yes / No
115. Animals enter and exit a sick-bay (back to the farm)	Yes / No
116. The sick-bay is visited at the end of the day	Yes / No
117. There is a care option for sick and cripple animals at their own group/section¶	Yes / No
Animal contact structure	
118. The sows are housed in stable groups¶	Yes / No
119. Piglets are placed per litter¶	Yes / No
120. Some piglets are reared motherless¶	Yes / No
121. After the third day, piglets can still be switched¶	Yes / No
122. Foster sows are used¶	Yes / No
123. When creating foster sows, different litters of piglets are moved up to a different sow	Yes / No
124. Separation between piglet cages is open¶	Yes / No
125. Separation between piglet cages is taken up by the feeder and/or trough, which is shared between the animals¶	Yes / No
126. Supervision of the animals from the central hall way	Yes / No
127. Separation between cages fatteners is open¶	Yes / No
128. Separation between cages fatteners is taken up by the feeder and/or trough, which is shared between the animals¶	Yes / No
129. Separation between cages for sows is open¶	Yes / No
130. Separation between cages for sows is taken up by the feeder and/or trough, which is shared between the animals¶	Yes / No
131. Carcasses are placed on the ground in the section¶	Yes / No
132. Carcasses are placed on the ground in the central hall way¶	Yes / No
133. Cadaver bags are used¶	Yes / No
134. Considering hygienic measures, direction of work is from young to old ¶	Yes / No
135. Sows, piglets and fatteners are different components within the farm. Each component makes use of different clothing and materials¶	Yes / No
Cleaning and disinfection	
136. All farm sections are cleaned and disinfected ¶	Yes / No
137. All farm sections are disinfected ¶	Yes / No

Question	Possible answers
138. All farm sections are cleaned with soaking agents¶	Yes / No
139. Farrow section hygiene (aggregated variable from questions 143 and 144)¶	Disinfection and or soaking / None
140. Farrow corridor hygiene (aggregated variable from questions 148 and 149)¶	Yes / No
141. Farrowing section is cleaned with cold water	Yes / No
142. Farrowing section is cleaned with warm water	Yes / No
143. Farrowing section is cleaned with soaking agent	Yes / No
144. Farrowing section is cleaned with disinfection agent	Yes / No
145. After cleaning farrowing section, there is a dry period of at least 24 hours	Yes / No
146. Farrowing section is cleaned by sweeping	Yes / No
147. Farrowing corridor is cleaned with soaking agent	Yes / No
148. Farrowing corridor is cleaned with disinfection agent	Yes / No
149. Piglets section hygiene (aggregated variable from questions 153 and 154)¶	Disinfection and soaking / Just soaking / Just disinfection / None
150. Piglets corridor hygiene (aggregated variable from questions 157 and 158)¶	Disinfection and soaking / Just soaking / None
151. Piglets section is cleaned with cold water	Yes / No
152. Piglets section is cleaned with warm water	Yes / No
153. Piglets section is cleaned with soaking agent	Yes / No
154. Piglets section is cleaned with disinfection agent	Yes / No
155. After cleaning piglets section, there is a dry period of at least 24 hours	Yes / No
156. Piglets section is cleaned by sweeping	Yes / No
157. Piglets corridor is cleaned with soaking agent	Yes / No
158. Piglets corridor is cleaned with disinfection agent	Yes / No
159. Fatteners section hygiene (aggregated variable from questions 163 and 164)	Disinfection and soaking / Just soaking / Just disinfection / None
160. Fattener corridor hygiene (aggregated variable from questions 167 and 168)	Disinfection and or soaking / None
161. Fattener section is cleaned with cold water	Yes / No
162. Fatteners section is cleaned with warm water	Yes / No
163. Fatteners section is cleaned with soaking agent	Yes / No

Question	Possible answers
164. Fatteners section is cleaned with disinfection agent	Yes / No
165. After cleaning fatteners section, there is a dry period of at least 24 hours	Yes / No
166. Fatteners section is cleaned by sweeping	Yes / No
167. Fatteners corridor is cleaned with soaking agent	Yes / No
168. Fatteners corridor is cleaned with disinfection agent	Yes / No
169. Gilts section hygiene (aggregated variable from questions 173 and 174)¶	Disinfection and or soaking / Just soaking / None
170. Gilts corridor hygiene (aggregated variable from questions 177 and 178)¶	Disinfection and or soaking / None
171. Gilts section is cleaned with cold water	Yes / No
172. Gilts section is cleaned with warm water	Yes / No
173. Gilts section is cleaned with soaking agent	Yes / No
174. Gilts section is cleaned with disinfection agent	Yes / No
175. After cleaning gilts section, there is a dry period of at least 24 hours	Yes / No
176. Gilts section is cleaned by sweeping	Yes / No
177. Gilts corridor is cleaned with soaking agent	Yes / No
178. Gilts corridor is cleaned with disinfection agent	Yes / No
179. Mating section hygiene (aggregated variable from questions 183 and 184) ¶	Disinfection and or soaking / None
180. Mating corridor hygiene (aggregated variable from questions 187 and 188)¶	Disinfection and or soaking / None
181. Mating section is cleaned with cold water	Yes / No
182. Mating section is cleaned with warm water	Yes / No
183. Mating section is cleaned with soaking agent	Yes / No
184. Mating section is cleaned with disinfection agent	Yes / No
185. After cleaning mating section, there is a dry period of at least 24 hours	Yes / No
186. Mating section is cleaned by sweeping	Yes / No
187. Mating corridor is cleaned with soaking agent	Yes / No
188. Mating corridor is cleaned with disinfection agent	Yes / No
189. Gestation shed section hygiene (aggregated variable from questions 193 and 194)¶	Disinfection and or soaking / None
190. Gestation shed corridor hygiene (aggregated variable from questions 197 and 198)¶	Disinfection and or soaking / None
191. Gestation shed section is cleaned with cold water	Yes / No
192. Gestation shed section is cleaned with warm water	Yes / No
193. Gestation shed section is cleaned with soaking agent	Yes / No
194. Gestation shed section is cleaned with disinfection agent	Yes / No

Question	Possible answers
195. After cleaning gestation shed section, there is a dry period of at least 24 hours	Yes / No
196. Gestation shed section is cleaned by sweeping	Yes / No
197. Gestation corridor is cleaned with soaking agent	Yes / No
198. Gestation corridor is cleaned with disinfection agent	Yes / No
Workflow, feed and water supply	
199. Work is visibly done with a week planner¶	Yes / No
200. Work is visibly done with a day planner¶	Yes / No
201. There are protocols present in the shed (work flows) ¶	Yes / No
202. The date of placement is present on the section doors ¶	Yes / No
203. Farm treatment plan recorded and stored¶	Yes / No
204. A medical prescription with dosage is present on the farm¶	Yes / No
205. Farrowing sows are fed with broth¶	Yes / No
206. Farrowing sows are fed with dry feed¶	Yes / No
207. Farrowing sows are fed with milk	Yes / No
208. Farrowing sows are fed with mush/pulp	Yes / No
209. Dry and gestating sows are fed with broth¶	Yes / No
210. Dry and gestating sows are fed with dry feed¶	Yes / No
211. Dry and gestating sows are fed with milk	Yes / No
212. Dry and gestating sows are fed with mush/pulp	Yes / No
213. Gilts are fed with broth¶	Yes / No
214. Gilts are fed with dry feed¶	Yes / No
215. Gilts are fed with milk	Yes / No
216. Gilts are fed with mush/pulp	Yes / No
217. Piglets with sow are fed with broth¶	Yes / No
218. Piglets with sow are fed with dry feed¶	Yes / No
219. Piglets with sow are fed with milk¶	Yes / No
220. Piglets with sow are fed with mush/pulp¶	Yes / No
221. Weaned piglets are fed with broth¶	Yes / No
222. Weaned piglets are fed with dry feed¶	Yes / No
223. Weaned piglets are fed with milk	Yes / No
224. Weaned piglets are fed with mush/pulp¶	Yes / No
225. Fatteners are fed with broth¶	Yes / No
226. Fatteners are fed with dry feed	Yes / No
227. Fatteners are fed with milk	Yes / No
228. Fatteners are fed with mush/pulp	Yes / No
229. Animals get water mainly from¶	Public source, tap/ Private source
230. Water medication is possible via a dosator¶	Yes / No

Question	Possible answers
231. Water medication is possible per section¶	Yes / No
232. A separate medication pipe is present on the farm¶	Yes / No
233. The water pipe is cleaned¶	Yes / No
234. In the farrowing section drinking water is just supplied via a nipple¶	Yes / No
235. In the farrowing section drinking water is mainly supplied via a nipple¶	Yes / No
236. In the farrowing section drinking water is mainly supplied via a water bowl¶	Yes / No
237. In the piglet section drinking water is just supplied via a nipple¶	Yes / No
238. In the piglet section drinking water is mainly supplied via a nipple¶	Yes / No
239. In the piglet section drinking water is just supplied via water bowl¶	Yes / No
240. In the piglet section drinking water is mainly supplied via a water bowl¶	Yes / No
241. In the fattener section drinking water is mainly supplied via a nipple¶	Yes / No
242. In the fattener section drinking water is just supplied via water bowl¶	Yes / No
243. In the fattener section drinking water is mainly supplied via a water bowl¶	Yes / No
244. In the (rearing) gilt section drinking water is mainly supplied via a nipple¶	Yes / No
245. In the (rearing) gilt section drinking water is mainly supplied via a water bowl¶	Yes / No
246. In the mating section drinking water is mainly supplied via a nipple¶	Yes / No
247. In the mating section drinking water is mainly supplied via a water bowl¶	Yes / No
248. In the gestation shed drinking water is mainly supplied via a water bowl¶	Yes / No

Bold type indicates variables presented in table 5 with p≤0.1 in the overall analysis or in at least one stratum (open or closed).

¶Variables with less than 10% missing values, at least 10% of farms in each category. Random intercept generalized linear mixed models were fitted for each variable by using a macro statement in SAS.

†Farm size had a p>0.10 but was evaluated and presented irrespective of significance.

Appendix Table 2. Human questionnaire used in each of the four sampling times in a risk factor analysis for livestock-associated methicillin-resistant *Staphylococcus aureus* in pig farming, the Netherlands, 2011-2013.

Question	Possible answers
General characteristics:	
1. Sex: ¶	M / F
2. Age¶	Years
3. Relation to pig farmer¶	Farmer / Worker / Partner/ Children
4. I live on the pig farm ¶	Yes / No
5. Number of household members: ¶	No.
Animal contact	
6. Average number of hours worked on the pig farm: ¶	No.
7. Did you touch someone else's pigs in the last 6/12 months? ¶	Yes / No
8. Did you touch dairy cattle in the last 6/12 months? ¶	Yes / No
9. Did you touch any calves in the last 6/12 months? ¶	Yes / No
10. Did you touch any horses in the last 6/12 months?	Yes / No
11. Did you touch any sheep in the last 6/12 months? ¶	Yes / No
12. Did you touch any goats in the last 6/12 months? ¶	Yes / No
13. Did you touch any dogs in the last 6/12 months? ¶	Yes / No
14. Did you touch any cats in the last 6/12 months? ¶	Yes / No
15. Did you touch any poultry in the last 6/12 months?	Yes / No
16. Do you have pets? ¶	Yes / No
a. How many dogs? ¶	No.
b. How many cats? ¶	No.
c. How many rabbits?	No.
d. How many rodents?	No.
e. How many fishes?	No.
17. Are your pets allowed in the living room? ¶	Yes / No
Tasks performed in the farm:	
18. Sorting the sows in the past 7 days ¶	Yes / No
19. Sorting the suckling piglets in the past 7 days ¶	Yes / No
20. Sorting the weaned piglets in the past 7 days ¶	Yes / No
21. Sorting the fattener pigs in the past 7 days ¶	Yes / No
22. Feeding sows in the past 7 days ¶	Yes / No
23. Feeding suckling piglets in the past 7 days ¶	Yes / No
24. Feeding weaned piglets in the past 7 days ¶	Yes / No
25. Feeding fattener pigs in the past 7 days ¶	Yes / No
26. Washing sows in the past 7 days ¶	Yes / No
27. Washing suckling piglets in the past 7 days ¶	Yes / No

Question	Possible answers
28. Washing weaned piglets in the past 7 days ¶	Yes / No
29. Washing fattener pigs in the past 7 days ¶	Yes / No
30. Healthcare tasks in sows in the past 7 days ¶	Yes / No
31. Healthcare tasks in suckling piglets in the past 7 days ¶	Yes / No
32. Healthcare tasks in weaned piglets in the past 7 days ¶	Yes / No
33. Healthcare tasks in fattener pigs in the past 7 days ¶	Yes / No
34. Birth assistance of sows in the past 7 days ¶	Yes / No
35. Birth assistance of suckling piglets in the past 7 days ¶	Yes / No
36. Birth assistance of weaned piglets in the past 7 days ¶	Yes / No
37. Birth assistance of fattener pigs in the past 7 days ¶	Yes / No
38. Removing the manure of sows in the past 7 days ¶	Yes / No
39. Removing the manure of suckling piglets in the past 7 days ¶	Yes / No
40. Removing the manure of weaned piglets in the past 7 days ¶	Yes / No
41. Removing the manure of fattener pigs in the past 7 days ¶	Yes / No
42. Cleaning and disinfecting sows in the past 7 days ¶	Yes / No
43. Cleaning and disinfecting suckling piglets in the past 7 days ¶	Yes / No
44. Cleaning and disinfecting weaned piglets in the past 7 days ¶	Yes / No
45. Cleaning and disinfecting fattener pigs in the past 7 days ¶	Yes / No
46. Did you yourself administer antibiotics to the pigs in the last month? ¶	Yes / No
Personal hygiene:	
47. Do you use a mouth mask while working in the sheds? ¶	Yes / No
48. Do you wash your hands directly before entering the sheds? ¶	Yes / No
49. Do you wash your hands directly after leaving the sheds? ¶	Yes / No
Individual health condition:	
50. Were you hospitalized in or did you visit a hospital in the Netherlands in the last 6/12 months? ¶	Yes / No
51. Were you hospitalized in another Dutch health institution or did you visit one in the last 6/12 months?	Yes / No
52. Were you hospitalized in a foreign hospital or did you visit one in the last 6/12 months?	Yes / No
53. Have you received homecare in the last 6/12 months?	Yes / No
54. Did you visit your general practitioner in the last 6/12 months? ¶	Yes / No
55. Did you visit a specialist on the outpatient clinic in the last 6/12 months?	Yes / No
56. Have you had allergies in the last 6/12 months? ¶	Yes / No
57. Have you had eczema in the last 6/12 months? ¶	Yes / No
58. Have you had psoriasis in the last 6/12 months? ¶	Yes / No
59. Have you had impetigo in the last 6/12 months?	Yes / No
60. Have you had open wounds in the last 6/12 months? ¶	Yes / No

Question	Possible answers
61. Have you had an abscess in the last 6/12 months?	Yes / No
62. Have you had a furuncle in the last 6/12 months?	Yes / No
63. Have you had a cold in the last 6/12 months?	Yes / No
64. Have you had laryngitis in the last 6/12 months?	Yes / No
65. Have you had inflammation of the ear in the last 6/12 months?	Yes / No
66. Have you had sinusitis in the last 6/12 months?	Yes / No
67. Have you had pneumonia in the last 6/12 months?	Yes / No
68. Have you had COPD in the last 6/12 months?	Yes / No
69. Have you had blood poisoning in the last 6/12 months?	Yes / No
70. Have you had arthritis in the last 6/12 months?	Yes / No
71. Have you had diabetes in the last 6/12 months?	Yes / No
72. Have you had surgery in the last 6/12 months?	Yes / No
73. Have you been fitted with a joint prosthesis in the last 6/12 months?	Yes / No
74. Have you had an external fixator in the last 6/12 months?	Yes / No
75. Have you had an IV in the last 6/12 months?	Yes / No
76. Have you had an stomach tube in the last 6/12 months?	Yes / No
77. Have you had an intestinal tube in the last 6/12 months?	Yes / No
78. Have you had an trachea fistula in the last 6/12 months?	Yes / No
79. Have you had artificial respiration in the last 6/12 months?	Yes / No
80. Have you had a bladder catheter in the last 6/12 months?	Yes / No
81. Have you had dialysis in the last 6/12 months?	Yes / No
82. Have you had acupuncture in the last 6/12 months?	Yes / No
83. Have you had medication with a needle in the last 6/12 months?	Yes / No
84. Are you currently treated with medication?	Yes / No
a. Name medication.	text
b. Indication	text
c. Since date	date
85. Have you ever smoked or do you currently smoke? ¶	Yes / No
Contact with other people	
86. Did persons living in a foreign country stay at your place in the last 6/12 months? ¶	Yes / No
87. In which country do these persons live?	Country
88. Did you go to another country for holidays?	Yes / No
89. If more than 6/12 months ago, when was the last time?	Date
90. If less than 6/12 months ago, when was the last time?	Date
Contact with meat	
91. How many times per week do you eat meat? ¶	No.
92. How many times per week do you have contact with unheated/unprocessed meat (for examples during cooking or packaging)? ¶	No.

Footnotes Appendix Table 2.
Bold type indicates variables in boldface are presented in table 4 with p≤0.2 in the longitudinal analysis with mixed models.
¶Variables with less than 10% missing values, at least 10% of farms in each category. Random intercept generalized linear mixed models were fitted for each variable by using a macro statement in SAS.

Appendix Table 3. ORs for determinants of livestock-associated MRSA positivity in pooled pig samples (n=1,054) from 32 farms (full multivariable generalized linear mixed model), the Netherlands, 2011-2013*

Variable	N¶	OR (95%CI)	P-value
Sampling moment			
0 months	262	0.89 (0.47-1.71)	<0.001
6 months	290	2.30 (1.27-4.15)	
12 months	259	1.99 (1.15-3.42)	
18 months	243	Ref.	
Age group			
Gilts	212	1.11 (0.66-1.85)	<0.001
Finishers	140	4.25 (2.37-7.59)	
Suckling piglets	212	3.99 (2.40-6.63)	
Weaned piglets	280	10.40 (6.22-17.38)	
Sows	210	Ref.	
External supply of gilts†			
Open	630	4.87 (1.24-19.16)	0.023
Closed	424	Ref.	
Water supply for animals			
Tap water	424	2.42 (0.81-7.25)	0.112
Private source	630	Ref.	
Separate medication pipe			
Yes	750	0.76 (0.31-1.90)	0.559
No	304	Ref.	
Carcass barrels cleaned and disinfected after emptied			
Yes	398	0.62 (0.30-1.31)	0.214
No	656	Ref.	
There is a delivery room for materials			
Yes	804	0.37 (0.15-0.90)	0.028
No	250	Ref.	
Injection of antimicrobials in piglets during the first week			
Yes	601	1.45 (0.77-2.73)	0.249
No	453	Ref.	

Variable	N¶	OR (95%CI)	P-value
Tooth clipping in piglets			
Yes	416	1.83 (0.61-5.46)	0.280
No	638	Ref.	
Piglets and/or fatteners are vaccinated			
Yes	870	1.37 (0.59-3.16)	0.460
No	184	Ref.	
Needles renewed per compartment			
Yes	738	1.38 (0.68-2.78)	0.370
No	316	Ref.	
Sows in stable groups			
Yes	594	0.61 (0.31-1.18)	0.141
No	460	Ref.	
Hygiene in piglet compartment			
Disinfection	149	0.81 (0.24-2.69)	0.773
Soaking	270	1.54 (0.53-4.51)	
Soaking and disinfection	431	1.28 (0.52-4.51)	
None	204	Ref.	
Hygiene in the mating section			
Soaking and disinfection	169	0.50 (0.18-1.36)	0.173
None	885	Ref.	
Antimicrobial use per2-fold increase (log2 DDDA/Y)	1054	1.21 (1.00-1.45)	0.049
Use of cephalosporins			
Yes	84	2.76 (1.24-6.14)	0.013
No	970	Ref.	

Model fit: -2 Log RSPL= 5397,87

*Full multivariable model with random intercept for farm, sampling time and animal age group as terms of adjustment. All variables from the longitudinal risk factor analysis in pigs with p≤0.0.05 (table 5) are fitted in the model together with overall antimicrobial use (log2 DDDA/Y) and use of cephalosporins. MRSA, methicillin-resistant *Staphylococcus aureus*; OR, odds ratio; DDDA/Y, defined daily dosages animal per year; Ref, reference category; RSPL, residual pseudo-likelihood.

¶Multiple variables had missing values in the full model reducing the number of observations in the final model (table 6).

†Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.

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Chapter 5

Farm management practices associated with ESBL-producing *Escherichia coli* in pigs: a longitudinal study in the context of reduced use of antimicrobials

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Abstract

Livestock may serve as a reservoir for transmission of extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-*E. coli*) to humans. A reduction in antimicrobial use (AMU) in animals, especially cephalosporins, is hypothesized to curb the emergence of this resistance. We evaluated the prevalence of ESBL-*E. coli* in pigs from 36 farms longitudinally (4 sampling times in 18 months) during 2011-2013, under the context of a sector-wide initiative to reduce AMU. An extensive questionnaire on farm characteristics and AMU as Defined Daily Dosages per Animal Year (DDDA/Y) was available for the 6-month periods before each sampling moment. Presence of ESBL-*E. coli* was determined by selective plating and ESBL genes were identified and characterized by microarray, PCR and gene sequencing. Associations between the likelihood for a farm to have ESBL-*E. coli*-positive pigs and farm management practices were modelled with logistic regression. During the study period, the number of farms with ESBL-*E. coli* carrying pigs decreased from 16 to 10 and the prevalence of ESBL-*E. coli*-positive samples halved from 27% to 13%. The presence of ESBL-*E. coli* differed between animal age groups. Overall, the most detected ESBL genes were $bla_{\text{CTX-M-1}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{CTX-M-14}}$. The presence of ESBL-*E. coli* carrying pigs was not related to total AMU but it was strongly determined by the use of 3rd/4th generation cephalosporins at any time in the study (OR=46.4, p=0.006). Other farm management factors, related with improved biosecurity, were also plausibly related to lower probabilities for ESBL-*E. coli*-positive farms (e.g. presence of a hygiene lock, pest control delivered by a proffesional). This study suggests that restricting the use of 3rd/4th generation cephalosporins is a good control strategy for ESBL-*E. coli* in pigs. The risk factors identified highlight that there is still room for improving biosecurity and animal contact structures in order to control the emergence of resistant bacteria.

Introduction

A variety of extended-spectrum beta-lactamases (ESBLs) have been identified in Enterobacteriaceae derived from food-producing animals worldwide[1]. High antimicrobial use (AMU) and inappropriate use of cephalosporins in livestock production are considered to be associated with the emergence and high prevalence of ESBL-producing *Escherichia coli* (ESBL-*E. coli*) in animals[2]. Transmission of ESBL-*E. coli* from animals to humans can occur through food or direct contact[3,4]. Infections with ESBL-*E. coli* are a major global public health concern[5].

Several European studies reported high proportions of farms where ESBL were present. In Spain, ESBL-*E. coli* were detected in faecal samples collected from stable floors of 8 out of 10 farms[6]. Two German studies found ESBL-*E. coli* in faecal samples collected from pigs on 15 out of 17 and 26 out of 35 farms respectively[7,8]. In a Danish study ESBL was detected in pigs on 15 out of 19 pig farms with high consumption of cephalosporins versus 4 out of 20 pig farms with no cephalosporin use[9].

A reduction in AMU, more specifically cephalosporins, has been suggested to decrease ESBL-*E. coli* on pig farms[2,9,10]. Because of demands regarding reduction in AMU in livestock production by the Dutch government, the total consumption of antimicrobials by animals dropped drastically in the Netherlands since 2011[11-14]. Moreover, in 2011 the Dutch pig farm sector introduced an initiative to reduce the use of 3rd/4th generation cephalosporins. Additionally, from January 2013, veterinarians were legally required to limit the use of 3rd/4th generation cephalosporins and fluoroquinolones to individual treatments in confirmed infections by bacteriological culture and susceptibility tests. As a consequence, most of the pig farms did not use this group of antimicrobials anymore since 2011[12-15]. Although not studied until now, other management practices besides reduction in AMU might have an effect on the presence of ESBL-*E. coli* on pig farms as well.

The objectives of this longitudinal study were to determine prevalence of ESBL-*E. coli* on pig farms and to assess the effect of reduced AMU and other farm management practices on the presence of ESBL-*E. coli* on pig farms.

Materials and Methods

Study design

The design of the study has been described elsewhere[4,16]. Briefly, 36 multiplier pig farms (sows and piglets present), with or without finishing pigs, completed the study. Production types were classified in *farrowing* and *farrow-to-finish farms*. Farrowing farms did not produce fatteners and they delivered piglets to finishing farms (with the exception of one farm delivering gilts for farrowing). Farrow-to-finishing farms integrated farrowing and finishing pig production and delivered fattening pigs to the abattoir. Additionally, a farm was defined as open when receiving external supply of gilts for at least once a year from at least

one supplier, and as closed when there was no external supply of gilts.

Farms and veterinarians were visited at the start of the study by the researcher between March 2011 and September 2011. At four sampling moments over a period of 18 months (6-month intervals), rectal samples from 60 pigs were collected by the farm veterinarian, using sterile cotton-wool swabs (Cultiplast®) and sent refrigerated to the laboratory by courier. All animal age groups present were sampled (sows, gilts, suckling piglets, weaning piglets and finishing pigs). Rectal swabs were combined in 10 pools of 6 pigs. Each pool consisted of an age group in the same pen. At the first sampling moment (baseline measurements), a questionnaire was completed during a walk through survey by the farm veterinarian to identify which management aspects could be improved to reduce antimicrobial resistant bacteria. The questionnaire contained items on farm characteristics, biosecurity, animal management and hygiene practices and can be found elsewhere (http://wwwnc.cdc.gov/EID/article/21/6/14-0706-Techapp1.pdf, Appendix Table 1 in Chapter 4)[4]. A tailor-made intervention protocol was developed by the veterinarian and the farmer. Interventions were focused on improving personnel and farm hygiene, changing animal contact structures, and reducing AMU (in a background of decreasing AMU nationwide due to government demands) . At each sampling moment the farm questionnaire was filled out again to monitor changes in farm practices.

Laboratory analysis

All samples were analysed as described previously, namely pooled swabs were analysed for the presence of ESBL-*E. coli* by selective plating[4]. Samples were suspended in 10 ml peptone water and incubated overnight at 37°C. For screening of ESBL-*E. coli*, suspensions were cultured on selective agar plates (*Brilliance*™ ESBL Agar, Oxoid®) and incubated overnight at 37°C. When no growth was seen, plates were incubated another night at 37°C. Morphologically different colonies suspected of ESBL production were cultured individually on a blood agar plate (Oxoid®) and incubated overnight at 37°C. In case of morphological uncertainty an oxidase test was performed before culturing. Bacterial species identification of the isolates was performed by MALDI/TOF (Bruker®). For phenotypical confirmation of ESBL-*E. coli*, a 0.5 McFarland suspension was inoculated on a Mueller Hinton agar and a combination disc test (ROSCO®) including cefotaxime, cefotaxime+clavulanate, ceftazidime, ceftazidime+clavulanate, cefepime, and cefepime+clavulanate (*Neo-Sensitabs*™) was used to confirm the presence of ESBL-*E. coli* (EUCAST guidelines, 2012). Isolates were stored at -80°C.

All ESBL-producing *E. coli* were selected for further molecular analysis to confirm the presence of ESBL genes. DNA was isolated using UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) or DNeasy 96 Blood & Tissue Kit (Qiagen). Real-Time PCR (SybrGreen, Life Technologies, conventional PCR (BioMix Red, Bioline) and a $bla_{\text{CTX-M}}$ group 1 specific PCR[17] was used to detect presence of the ESBL gene groups $bla_{\text{CTX-M-1}}$, bla_{TEM}, bla_{SHV} and $bla_{\text{CMY-2A}}$. Isolates with a negative PCR result were analysed using ESBL

microarray (Check-MDR CT101, Checkpoints, Wageningen) to detect other ESBL gene groups. DNA from PCR or ESBL microarray positive isolates was sequenced with group-specific primers to determine the exact gene type. DNA sequences were interpreted with Basic Local Alignment Search Tool (National Center for Biotechnology Information).

Data on antimicrobial use

Data on AMU from the farms of this study has been described elsewhere[16]. In short, all antimicrobial deliveries made to each farm were retrieved from the sector quality system national databases. AMU was expressed as Defined Daily Dosages per Animal per Year (DDDA/Y) per farm for the four periods preceding each sampling moment. The DDDA/Y is a standard weighted measure which can be interpreted as the number of days of antibiotic use per year for an average animal or animal place. A more detailed description on the calculation of DDDA/Y is described in the Netherlands Veterinary Medicines Authority report and by Bos et al.[11,18]. Also, a separate variable regarding 3rd/4th generation cephalosporin use was created. Since the use of cephalosporin was incidental during the study period, a farm was classified as no cephalosporin use or as any cephalosporin use during the period on which AMU data was available.

Data analysis

Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Farms were classified as ESBL-positive if an ESBL gene was detected in an isolate from at least one pooled pig sample. Changes in presence of ESBL-producing *E. coli* on a farm and AMU over time were explored using simple descriptive statistics. DDDA/Y was $\log_2$ transformed because of its right-skewed distribution. Variables in the farm questionnaire and AMU were selected for univariate longitudinal analysis when meeting criteria of <10% missing values and ≤10% of farms in each category. A total of 134 variables in the farm questionnaire were selected together with AMU and cephalosporin use. The associations between presence of ESBL-producing *E. coli* on the farm and AMU, cephalosporin use and other farm variables was calculated with generalized linear mixed models (PROC GLIMIX; SAS Institute, Inc.) with random intercept for farms, taking into account the dependency of data and repeated measurements design. The univariate analysis was done for all the farms and for open and close farms separately; only associations from all farms with $p \leq 0.2$ from the questionnaire were presented. Pairwise Spearman correlations in questionnaire variables from the univariate analysis with $p \leq 0.1$ together with AMU and sampling time were checked to construct a full model. The final multivariate model was the result of a backward elimination from the full model, except for sampling time and AMU in DDDA/Y which were forced in the model during all elimination steps. The final model retained variables significant at $p \leq 0.05$, again except for sampling moment and AMU. Model assumptions were checked with diagnostic plots. Variables from the full model at farm level were used to make a model

at pooled sample level (i.e. modelling probabilities for a pig pooled sample to test ESBL-positive); this way we adjusted for age group of the animals. The latter model accounted for clustering at the farm level.

Results

Farms characteristics and prevalence of ESBL-*E. coli*

A description of the 36 farms is presented in Table 1. The number of farms where ESBL-*E. coli* carrying pigs were present decreased significantly from 16 farms at the beginning of the study (month 0) to 10 positive farms in the last sampling moment. Nineteen farms were negative to ESBL-*E. coli* during the whole study (8 farrow-to-finish closed, 5 farrowing open, 4 farrow-to-finish open and 2 farrowing closed). Eight farms were ESBL-*E. coli*-positive in all sampling moments (6 farrow-to-finish open, 1 farrow-to-finish closed and 1 open farrowing farm). Seven farms became negative during the study (3 farrowing open, 1 farrow-to-finish open, 2 farrow-to-finish closed and 1 farrowing close). One farrow-to-finish open farm became ESBL-*E. coli*-positive during the course of the study.

A pronounced significant drop in prevalence of ESBL-*E. coli* was observed during the study. The proportion of ESBL-*E. coli*-positive samples in all farms halved from 27% in the first to 13% in the last sampling moment. Farrow-to-finish open farms were at clear higher prevalences as compared to the rest of farm types (Figure 1).

ESBL-*E. coli* carriage significantly differed between the sampled age groups. Overall ESBL-*E. coli* prevalence in pooled samples ranged from 11.7% in (rearing) gilts to 24.2% in sucking piglets (Table 2). The prevalence decreased parallel across all age groups (results not shown). Mostly $bla_{\text{CTX-M-1}}$ genes were detected in pig isolates. Other ESBL genes found were $bla_{\text{TEM-52}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-32}}$ (Table 3).

Table 1. Characteristics of farms in a study of determinants for carriage of Extended-Spectrum Beta-Lactamases in *E. coli* from pigs, the Netherlands, 2011-2013.

Type of farm*	No. farms	Median no. (interquartile range)	
		Sows	Fatteners
All	36	350 (270–550)	773 (0–1,950)
Open	22	337 (300–500)	500 (0–1,300)
Farrowing†	9	533 (350–800)	0
Farrow-to finish	13	314 (242–380)	1,100 (600–2,010)
Closed	14	407 (232–698)	1,400 (450–2,725)
Farrowing†	3	439 (239–905)	0
Farrow-to finish	11	367 (200–673)	1,892 (1,025–2,950)

*Farms were defined as open when they received external supplies of gilts ≥1 time per year from at least 1 supplier and as closed when they received no external supply of gilts.

†No fattening pigs present.

Figure 1. Prevalence of ESBL-*E. coli*-positive pooled samples from pigs per farm type in a study of determinants for ESBL carriage, the Netherlands, 2011-2013. ESBL-*E. coli*, genotypically confirmed extended-spectrum beta-lactamase-producing *Escherichia coli*. Error bars indicate 95% CIs.

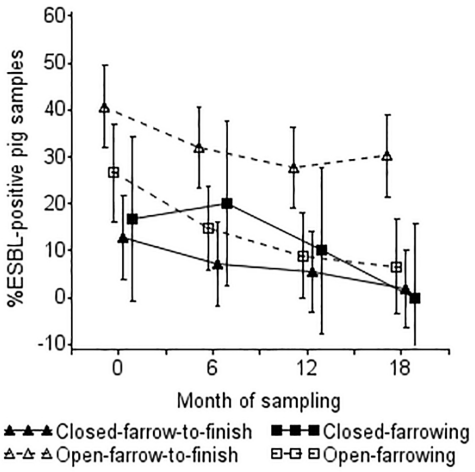


Table 2. Overall prevalence of ESBL-*E. coli* in pooled samples from pigs in different age groups in a study of determinants for ESBL carriage, the Netherlands, 2011-2013. ESBL-*E. coli*, genotypically confirmed extended-spectrum beta-lactamase-producing *Escherichia coli*.

Age group	Pooled samples† (n)	Pooled samples with presence of ESBL-*E. coli* (n and %)
Sows	283	60 (21.2)
(Rearing) gilts	281	33 (11.7)
Suckling piglets*	285	69 (24.2)
Weaned piglets	318	66 (17.2)
Finishing pigs	183	31 (16.9)

*Suckling piglets=pooled sample contained rectal swabs from one mother sow and five of her suckling piglets.

†No age group was reported for eight pools in one farm. On two farms, only nine pools were analysed.

Table 3. Distribution of ESBL genes in pig isolates in a study of determinants for carriage of extended-spectrum beta-lactamases in *E. coli* from pigs, the Netherlands, 2011-2013.

Sampling time	CTX-M-1	TEM-52	CTX-M-14	CTX-M-15	Other*	Total
0 mo	87†	32	18	11	6	154
6 mo	66	15	3	3	2	89#
12 mo	61	15	3			79
18 mo	48	20	3		1	72

*Other: CTX-M-2/97 (n=4), TEM-3 gr (further specification by sequence analysis was unsuccessful) (n=1), CTX-M-2 (n=3), CTX-M-32 (n=1).

†CTX-M-1 isolates were not tested for additional genes in the first sampling moment.

#One isolate was harbouring 2 ESBL genes.

Evaluation of interventions: marked antimicrobial use reduction and minor changes in farm management

Farms considerably reduced AMU as a result of the national benchmarking program for farms. A steady downward trend in $\log_2$ DDDA/Y, mirroring the overall national trend, was observed in all farm types except in farrowing open farms with a 0.7% increase in AMU (Figure 2). The AMU reduction was the highest in farrow-to-finish open farms (64%) and in closed farms (farrow-to-finish and farrowing) there was around a 40% reduction (Figure 2). Open farms used three times more antimicrobials as compared to closed farms (overall

DDDA/Y of 9.7 and 3.1 respectively). The difference in overall AMU between open and closed farms was independent of the presence or absence of fattening pigs as shown by a non-significant interaction term between external supply and type of production. Being a farrowing farm had a multiplicative effect with a two-fold increase in DDDA/Y in the strata of open and closed farms (overall ADDD/Y of 13.7, 7.7, 6.0 and 2.6 for open farrowing, open farrow-to-finish, closed farrowing and closed farrow-to-finish respectively).

During the whole study period, tetracyclines were the most consumed antimicrobial (37.6% of the total DDDA/Y), followed by penicillins (30.2%), trimethoprim/sulfonamides (12.3%), macrolides/lincosamides (12.0%) and polymyxins (4.6%). The last 3.3% corresponded mainly to combinations of antibiotics but also included cephalosporins , amphenicols, pleuromutilines and fluoroquinolones. Six farms used 3rd/4th generation cephalosporins in the period preceding the first sampling moment, two of these farms also used cephalosporins in the period between the first and second sampling moment. One farm only used 3rd/4th generation cephalosporins in the period between the first and second sampling moment. DDDA/Y for 3rd/4th generation cephalosporins varied from 0.06 to 0.39.

Almost all families of antibiotics had a parallel decrease during the study having similar DDDA/Y percentages across all the periods preceding each sampling moment (Figure

Figure 2. Antimicrobial use by type of farm during the 4 periods (≈6 months) before each sampling moment in a study of determinants for carriage of Extended-Spectrum Beta-Lactamases in *E. coli* from pigs, the Netherlands, 2011-2013. GM and 95% CI from log2 DDDA/Y. AMU, antimicrobial use; DDDA/Y, defined daily dosages animal per year. Error bars indicate 95% CIs.

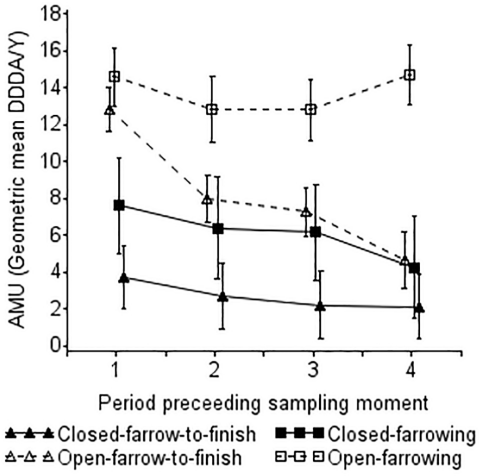
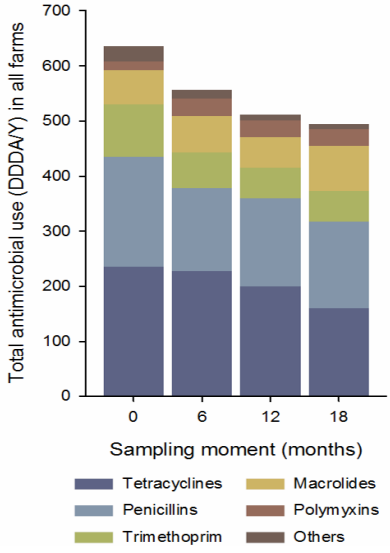


Figure 3. Proportions of antimicrobials used over the total DDDA/Y per farm type during the 4 periods (≈6 months) before each sampling moment in a study of determinants for carriage of Extended-Spectrum Beta-Lactamases in *E. coli* from pigs, the Netherlands, 2011-2013. AMU, antimicrobial use; DDDA/Y, defined daily dosages per animal per year.



3). Only macrolides had a slight increase in percentage of DDDA/Y during the study accompanied by a slight decrease in tetracyclines and trimethoprim/sulfonamides (Figure 3). Overall, 86% of the DDDA/Y were administered as (partial) herd treatment and 13.4% as individual treatment and these percentages did not significantly differ by period of study or type of farm (not shown).

Farm management changes over time were modest; just 10% of the intervention variables (median 9.7%, interquartile range (IQR)=6.0-12.3) changed during the study per farm. Thus, 27 farms had less than 12 variables out of the 134 that changed. The median number of farms within a single change was 3 (IQR=1-4). Thus 75% of the changes occurred in four or less farms. No differences in changes over time were observed by the different farm types. Because of these limited and heterogeneous changes, an intervention effect could not be evaluated and we performed only a risk factor analysis.

Antimicrobial use and farm management practices related to presence of ESBL-*E. coli* in pig farms

Univariate Odds Ratios (ORs) for the presence of ESBL-*E. coli* carrying pigs on a farm are presented in Table 4. The probability for a farm to have ESBL-*E. coli* carrying pigs was 24% higher per twofold increase in DDDA/Y, but this association was not statistically significant. This quantitative relationship did not change during the course of the study (i.e. non-significant interaction between sampling moment and AMU for a farm to test ESBL-positive). Stratified analysis showed this positive relation in closed farms as well, but not in open farms. Apart of the DDDA/Y, other variables regarding AMU were associated with ESBL-posity of farms. When the proportion of group treatments was above 0.5 the odds of being ESBL positive was four times higher. The use of 3rd/4th generation cephalosporins at any time in the maximum 6 months preceding and during the study period was significantly positively associated with the presence of ESBL-*E. coli* carrying pigs on a farm (OR=12.6, CI=1.1-144.4) (Table 4).

The presence of ESBL-*E. coli* carrying pigs was significantly less likely when water for the pigs was supplied from a public source instead of a private source (OR=0.1, CI=0.0-0.9),when a hygiene lock was the only entrance on the farm (OR=0.2, CI=0.0-1.0) and when pest control was carried out by a proffesional company (OR=0.1, CI=0.0-0.8). There was a trend (p-value between 0.5 and 0.1) for the presence of ESBL-*E. coli* carrying pig for the following determinants: external supply of gilts, presence of goats in the farm, drivers do not enter the clean road, dogs can enter the shed, sick and cripple animals are taken care of in their own section and tooth clipping in piglets (Table 4).

The results from the final multivariable model at farm and pool level are presented in Table 5. Presence of goats in the farm and the use of 3rd/4th generation cephalosporins before and during the study period were risk factors for the presence of ESBL-*E. coli* carrying pigs on the farm (OR=49.2, CI=1.7->999.9 and OR=46.4 CI=3.1-393.1 respectively). A hygiene

lock as the only entrance to the pig farm was a protective factor (OR=0.1 CI=0.0-0.5). The same factors were found in the model at the pooled pig sample level. Thereby, a significant decrease of ESBL-*E. coli* positive samples from the first to the last sampling moment was found. The presence of ESBL-*E. coli* was significantly different between the separate age groups in the final model at the pooled sample level.

Table 4. Univariate ORs for a pig farm to be ESBL-*E. coli*-positive in a study of determinants for ESBL carriage, the Netherlands, 2011-2013.

Determinant		All farms		Open farms		Closed farms
Category	N¶	OR (95% CI)	N¶	OR (95% CI)	N¶	OR (95% CI)
Farm characteristics:						
No. sows						
per 100 increase	144	0.7 (0.5-1.1)**	88	0.6 (0.3-1.1)**	56	0.8 (0.4-1.7)†
External supply of gilts						
Open	88	6.0 (0.7-48.8)**	0	nc	56	nc
Closed	56	Ref	88		0	
Type of production§						
Farrow-to-finish	96	3.1 (0.4-25.4)†	52	10.3 (0.8-135.4)**	44	0.6 (0.0-72.3)†
Farrowing	48	Ref	36	Ref	12	Ref
Water supply for animals						
Public, from tap	46	0.1 (0.0-0.9)***	22	0.2 (0.0-2.6)*	24	0.2 (0.0-5.6)†
Private source	94	Ref	63	Ref	31	Ref
Presence of goats in the farm						
Yes	17	15.1 (0.8-271.8)**	10	27.2 (0.4-1863.5)*	7	28.7 (0.1-7904.0) †
No	127	Ref	78	Ref	49	Ref
MRSA pool prevalence						
per 10% increase	144	1.2 (0.9-1.6)*	88	1.2 (0.8-1.7)†	56	1.3 (0.8-2.2)†
Biosecurity:						
Hygiene lock is the only entrance						
Yes	81	0.2 (0.0-1.0)***	51	0.2 (0.0-1.2)**	30	0.2 (0.0-5.6)†
No	62	Ref	37	Ref	25	Ref
Drivers do not enter the clean road						
Yes	96	0.2 (0.1-1.2)**	50	0.2 (0.0-1.6)*	46	0.6 (0.0-18.4)†
No	45	Ref	37	Ref	8	Ref
Dogs can enter the shed						
Yes	29	5.0 (0.9-28.7)**	27	4.7 (0.7-34.0)*	2	nc
No	115	Ref	61	Ref	54	

Determinant	All farms		Open farms		Closed farms	
Category	N¶	OR (95% CI)	N¶	OR (95% CI)	N¶	OR (95% CI)
Removal of manure in summer						
Manure stays <6 mo	123	0.2 (0.0-1.5)*	72	0.2 (0.0-1.4)**	51	nc
Manure stays >6 mo	18	Ref	14	Ref	4	
Pest control is handed over to a professional organization						
Yes	99	0.1 (0.0-0.8)***	60	0.3 (0.0-2.4)†	39	0.0 (0.0-0.3)***
No	44	Ref	28	Ref	16	Ref
Animal management and contact structure:						
Foster sows can have pigs from more than one litter						
Yes	75	2.5 (0.6-9.5)*	45	3.7 (0.7-19.5)*	30	1.0 (0.0-24.3)†
No	57	Ref	34	Ref	23	Ref
Housing of gestating sows						
Cubicle	69	3.3 (0.6-19.1)*	43	8.2 (1.0-68.7)***	26	0.4 (0.0-19.1)†
Groups	69	Ref	41	Ref	28	Ref
Sick and cripple animals are taken care of in their own section+						
Yes	29	4.7 (1.0-23.5)**	18	7.8 (1.0-59.5)***	11	0.7 (0.0-33.8)†
No	103	Ref	59	Ref	44	Ref
Gloves always used when treating piglets						
Yes	39	3.0 (0.6-15.9)*	19	4.0 (0.4-41.2)†	20	5.4 (0.2-141.9)†
No	104	Ref	69	Ref	35	Ref
Tooth clipping in piglets						
Yes	52	5.1 (0.9-29.0)**	35	5.0 (0.5-54.2)*	17	8.3 (0.2-337.6)†
No	89	Ref	51	Ref	38	Ref
Antimicrobial use:						
Antimicrobial use (log2DDDA/Y)§						
per twofold increase	144	1.2 (0.8-1.8)†	88	0.9 (0.5-1.5)†	56	1.9 (0.7-4.7)*
Use of 3rd/4th generation cephalosporins at any sampling						
Yes	28	12.6 (1.1-144.4)***	24	3.9 (0.2-72.5)†	4	nc
No	116	Ref	64	Ref	52	
Proportion of group treatments#						
Above 0.5	100	4.0 (0.8-19.2)**	72	1.7 (0.2-13.6)†	28	7.5 (0.3-221.1)†
Below 0.5	44	Ref	16	Ref	28	Ref

ESBL-*E. coli*, genotypically confirmed extended-spectrum beta-lactamase-producing *Escherichia coli*; OR, odds ratio; Ref, reference category; nc, non-computable.

§Items evaluated irrespective of significance. ¶ Number of observations at all sampling times together (10 pooled pig samples per farm in 36 farms in 4 sampling times). Some variables have missing observations.

+Variable is not selected for multivariable analysis because of having >5% of missing values over the total number of possible observations n=144). #Variable is not selected for multivariable analysis because of high correlation with antimicrobial use (spearman rho=0.7). †P>0.2; *p≤0.2; **p≤0.1; ***p≤0.05.

Table 5. Multivariate ORs for the most important determinants for a pig farm to be ESBL-*E. coli*-positive (Model A) and for a pig pooled sample to be ESBL-*E. coli*-positive (Model B) during the 4 sampling moments of a longitudinal risk factor analysis in 36 pig farms, the Netherlands, 2011–2013.

	Model A (farm level)			Model B (pig pool level)		
Variable	N	OR (95%CI)	p-value	N	OR (95%CI)	p-value
Age group						
gilts	NA	NA	NA	279	0.27 (0.14-0.52)	<0.001
finishers				183	0.48 (0.24-0.94)	
suckling piglets				283	1.64 (0.89-3.02)	
weaned piglets				380	0.59 (0.33-1.04)	
sows				281	Ref	
Sampling time						
0 mo	36	3.0 (0.5-18.0)	0.498	352	5.4 (2.8-10.2)	<0.001
6 mo	36	1.1 (0.2-6.5)		356	1.8 (1.0-3.2)	
12 mo	36	1.1 (0.2-6.5)		358	1.1 (0.6-1.9)	
18 mo	35	Ref		340	Ref	
Presence of goats in the farm						
yes	17	49.2 (1.70->999.9)	0.024	169	4.0 (1.1-15.3)	0.042
no	126	Ref		1237	Ref	
Antimicrobial use (log2DDDA/Y)						
per twofold increase	134	1.35 (0.86-2.13)	0.192	1406	0.99 (0.76-1.30)	0.943
Use of 3rd/4th generation cephalosporins at any sampling						
yes	28	46.4 (3.1-393.1)	0.006	271	72.0 (5.75-903.1)	0.001
no	115	Ref		1135	Ref	
Hygiene lock is the only entrance						
yes	81	0.1 (0.0-0.5)	0.007	797	0.1 (0.0-0.3)	<0.001
no	62	Ref		609	Ref	

All variables in the full model were weakly correlated (spearman rho<0.4) . ESBL-PE, genotypically confirmed extended-spectrum beta-lactamase-producing Escherichia coli; OR, odds ratio; Ref, reference category; NA, not applicable.

Discussion

This study suggests that the restriction in the use of 3rd/4th generation cephalosporins is likely to result in a decrease of ESBL-*E. coli* carriage on pig farms. ESBL-*E. coli* carriage in pigs significantly decreased during the study period. The observed steady reduction in total AMU did not explain these changes but the incidental use of 3rd/4th generation cephalosporins was shown to be the most influential factor for ESBL-*E. coli* carriage of animals on farms. Additional farm management practices focused on improved biosecurity were also shown to play a role on ESBL transmission on pig farms.

In terms of ESBL-*E. coli* prevalence and gene types, other European studies have reported higher numbers of positive farms while $bla_{CTX\text{-}M\text{-}1}$ gene is the most commonly found type in livestock in Europe[1,6-9].

Despite a parallel decrease of total AMU and ESBL-*E. coli* prevalence during the study, AMU was not significantly associated with an increased likelihood of ESBL-*E. coli*-positive farms. Remarkably, when 3rd/4th generation cephalosporins had been applied before or during the study, the probability for a farm to have ESBL-*E. coli*-positive pigs was dramatically increased. Although we have to acknowledge that the confidence interval of this association was wide, its significance directly calls for the well-known causal evidence attributed to the use of these drugs for the emergence of ESBLs[2,19]. Most of the farms in this study did not use 3rd/4th generation cephalosporins, which is comparable to the use in the Dutch pig farm population in the same period (2011-2013)[11,18]. We can conclude that for curbing ESBL numbers, reducing or restricting the use of 3rd/4th generation cephalosporins is more decisive that an overall AMU reduction. Thereby, it can be hypothesized that the overall decrease of ESBL-*E. coli* carriage in pigs in this study is also a delayed result of the possible reduction in the use of 3rd/4th generation cephalosporins before 2011. This is in line with the fact that the farms that did use 3rd/4th generation cephalosporins in this study only used it in the first two sampling moments. Cephalosporins are relatively new drugs and unlike other historically long used drugs such as tetracyclines or penicillins, resistance to cephalosporins seems not to be permanently established in bacterial communities[20]. This means that ESBL resistance might be more rapidly reverted in comparison with other resistances, as suggested by the observed lack of monotonic trend in the development of cephalosporin resistance[21].

To our knowledge, evidence for risk factors other than AMU is very limited. A recent cross-sectional study in Germany showed that some farm management and hygienic factors could be tackled to control cefotaxime resistant *Escherichia coli*[22]. In our study, the set of selected determinants in the univariate analysis showed that apart of the restricted use of cephalosporins, additional measures focused on improving biosecurity and animal management measures could be an aid to control ESBL-*E. coli* occurrence in pig farms. The introduction of new animals on pig farms has been reported as a risk factor for antimicrobial resistance[16,23]. In this study, a trend was seen for higher probability of ESBL-*E. coli* in farms with an external supply of pigs. In terms of animal age groups, the presence of ESBL-*E. coli* decreases over the production cycle; from suckling piglets to weaned piglets and finishing pigs, as it has been already reported by a Danish study[24]. The presence of goats in the farm as a risk factor was retained in the final model; the plausibility of this causal relationship is very doubtful and this could be just an incidental finding resulting from these farms being less strict in management and biosecurity practices (i.e. a proxy for a moor poorly managed pig farm). A more specific protective factor in the multivariate model for ESBL-*E. coli*-positive farms was the hygiene lock as only entrance to the farm; it is quite plausible that this biosecurity measure might prevent the entrance of ESBLs in the farm as suggested for

other drug resistances[25]. Changes in management practices not regarding AMU were minor, therefore risk factors were probably detected more because of contrast between farms than contrast within farms over time.

We consider that our sample of farms fairly served for the purpose of generalizability of results to a greater level. The descriptive results showed that farms in the study contained different production types, and more importantly, their AMU was very close to national data in terms of total volumes, proportions of different antimicrobial families and proportions of individual and group animal treatments[26]. However, the differences between open and close farms need to be cautiously interpreted since we might be lacking of statistical power for a stratified analysis. The statistical power was also seriously compromised to assess a quantitative association with cephalosporins; because of the limited use of these drugs during the study, we just evaluated their associations with ESBL-*E. coli* qualitatively. Also, we hypothesize that the use of cephalosporins at any point is a proxy for the cephalosporin use before 2011. Therefore excluding time variation in the use of 3rd/4th generation cephalosporins was justified.

Human ESBL carriage and direct contact with ESBL-*E. coli* carrying pigs is associated as shown by previous work[4]. This may pose a health risk for farmers and potentially for other humans with regular contact with this working population. Thereby ESBL-*E. coli* may be transmitted into the general population through pork[27]. The decreased ESBL-*E. coli* prevalence and the effect of 3rd/4th generation cephalosporins, next to improved biosecurity and changes in other farm management practices, shows that reduction of ESBL-*E. coli* on pig farms is possible. This might lead to reduced transmission of ESBL-*E. coli* from pigs to humans, which could be beneficiary to public health.

Conclusion

ESBL-*E. coli* prevalence decreased in pigs during 2011 and 2013 in the Netherlands. On pig farms, the use of 3rd/4th generation cephalosporins is associated with the presence of ESBL-*E. coli* carrying pigs.

Footnotes

Preliminary results from this study were presented in a poster presentation at the 13th International Society for Veterinary Epidemiology and Economics Conference in Maastricht, The Netherlands, 2012 (abstract 12787), in a poster presentation at the 23rd European Congress of Clinical Microbiology and Infectious Diseases in Berlin, Germany, 2013 (P1471), in an oral presentation at the 23rd Conference on Epidemiology in Occupational Health in Utrecht, The Netherlands, 2013 (abstract 328), in a poster presentation at the 53th Interscience Conference on Antimicrobial Agents and Chemotherapy in Denver, USA, 2013 (C2-1610), and in an oral presentation at the 3rd International One Health Congress in Amsterdam, The Netherlands, 2015 (abstract 148).

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Competing Interests

The authors have declared that no competing interests exist.

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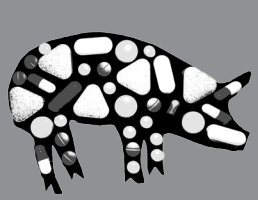
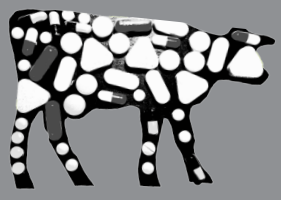
Part III

Commensal indicator *Escherichia coli* in animal production sectors





Chapter 6

Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands

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Abstract

Objectives: to quantify associations between antimicrobial use and acquired resistance in indicator *Escherichia coli* over a period of time which involved sector-wide antimicrobial use reductions in broilers and pigs (years 2004 to 2014), veal calves (2007-2014) and dairy cattle (2005-2014). Prevalence estimates of resistance were predicted for an hypothetical further decrease in antimicrobials use.

Methods: data reported annually for the resistance surveillance program in the Netherlands were retrieved. Two multivariate random-effects logistic models per animal sector were used to relate total and class-specific antimicrobial use (as defined daily dosages per animal per year, DDDA/Y) with the probability of *E. coli* resistance to a panel of 10 antimicrobial agents.

Results: positive dose-response relationships (ORs) were obtained from all models. Total antimicrobial use was more associated with the resistance phenotypes than class-specific use. Associations were remarkably robust in pigs and veal calves. Resistance to historically widely used antimicrobials (e.g. penicillins, tetracyclines) was, in relative terms, less influenced by drug use changes over time than resistance to newer or less prescribed antimicrobials (e.g. 3rd/4th generation cephalosporins, fluoroquinolones). In pigs and veal calves, prevalence estimates to most common resistance phenotypes were projected to decline ≈5-25% during 2014-2016 if total antimicrobial use reduction reached 80%; projections for poultry and dairy cows were more modest.

Conclusions: epidemiological evidence indicated that drug use history and co-selection of resistance are key elements for perpetuation of resistance. Recent Dutch policies reducing total use of antimicrobials seem to have decreased resistance, in particular in pig and veal calf production sectors.

Introduction

The use of antibiotics is the major driver for the emergence of bacterial resistance, which can be transmitted from food-producing animals to humans[1-8]. During the last years, many efforts have been made in Europe for a more prudent veterinary use of antimicrobials, such as the EU-wide ban on the use of growth promoters in 2006, and the development of comprehensive antimicrobial use and resistance monitoring programs[9-13].

In the Netherlands, regulations for farmers and veterinarians have also changed considerably in recent years[14,15]. In 2007, antimicrobial sales for food animals positioned the country among the EU members with highest consumption[13,16] and the government, animal sectors and veterinarians initiated concerted action to tackle this situation. In 2010, an ambitious policy defined mandatory targets for veterinary antimicrobial use, aiming at reductions of 50% by 2013 and 70% by 2015 compared with the index year 2009. The first target was amply reached in 2013[17]. Additionally, from that year on, the use of 3rd and 4th generation cephalosporins and fluoroquinolones was restricted by law for infections demonstrated by bacterial culture and susceptibility test results[18]. In recent years, resistance levels in *Campylobacter spp.* and commensal *Escherichia coli* in the main livestock sectors have progressively decreased, which is interpreted as evidence of these measures having a positive impact[19].

The purpose of this study was to quantify the association between use of antimicrobials in animals and resistance levels in commensal indicator *E. coli* over a period with a major reduction in antimicrobial use within the four major livestock production sectors (broilers, pigs, veal calves and dairy cattle) in the Netherlands. Moreover, we explored a potential future scenario by predicting resistance rates that would result from a further reduction in antimicrobial use.

Materials and methods

Antimicrobial use and resistance data

A more detailed description of the data used for this study can be found in the Appendix, along with additional references[20-24]. Briefly, we retrieved the annual Defined Daily Dosages per Animal per Year (DDDA/Y) reported by the Netherlands Veterinary Medicines Authority (SDa) for total use and specific antimicrobial classes until 2014 per animal sector[17]. Additionally, we used the results for resistance in *E. coli* communicated annually in the *Monitoring Antimicrobial Resistance and Antibiotic Use in Animals in the Netherlands* (MARAN) reports[19]. Consistent with MARAN reports, the terms 'resistance' or 'resistant' in this work refer to non-wild-type isolates defined by epidemiological cut-off values (www.eucast.org) [19]. Multidrug-resistant referred to isolates with non-wild-type susceptibility to 3 or more antimicrobial classes[25].

Years in which both antimicrobial use and resistance data were available were matched.

Similarly, use in antimicrobial classes was matched with susceptibility tests for specific antimicrobials. This resulted in 8 used antimicrobial classes matching 10 antibiotics tested: usage of penicillins, tetracyclines, 3rd/4th generation cephalosporins, fluoroquinolones, quinolones and amphenicols matched with acquired resistance to ampicillin, tetracycline, cefotaxime, ciprofloxacin, nalidixic acid and chloramphenicol respectively; aggregated information by the SDa on use of trimethoprim/sulphonamides was matched with both trimethoprim and sulfamethoxazole resistance, and use of aminoglycosides with resistance to both gentamicin and streptomycin.

Statistical analysis

Resistance patterns were assessed during the study period for the panel of 9 antimicrobials excluding streptomycin, which was not tested all years. The same pattern evaluation was made for the period including streptomycin. Percentages of isolates resistant to each antimicrobial, among isolates exhibiting a certain number of resistance phenotypes (from 0, fully susceptible; to 9 or 10, pan-resistant to all agents) were calculated. We defined *1-[pan-susceptibility]* as the proportion of isolates resistant to at least one of the antimicrobials (i.e. 1 - proportion of isolates susceptible to all agents). Trends for annual antimicrobial use and for prevalence of resistance were plotted using smoothed lines passing through the point estimates, and changes between the years 2009-2014 (from the implementation of the antimicrobial policies) were described.

Logistic regression analysis for grouped data (number of resistant isolates over the total tested) was used to obtain odds ratios (ORs) for an *E. coli* isolate to be resistant to each antimicrobial agent (or to any agent, represented by total resistance) per 1 unit increase in total antimicrobial use (total DDDA/Y, which included also occasional use of 1st-2nd generation cephalosporins, combinations of antibiotics, macrolides/lincosamides, pleuromutilines and polymyxins) or homologous use (i.e. DDDA/Y corresponding to the same antimicrobial class as the agent tested). Two multivariate random-effects generalized linear mixed models were fitted per animal sector. Models were adjusted for year and included a random intercept to account for the correlation between the different antimicrobial resistance phenotypes per year. The first model explored the associations between total antimicrobial use and resistance (agent-specific and *1-[pan-susceptibility]*). The second model assessed the relationships between homologous antimicrobial use and corresponding agent resistance. For the latter, only classes with DDDA/Y>0.5 in all years were modelled to obtain model convergence and reliable estimates. A categorical explanatory variable with the resistance types (agent-specific and *1-[pan-susceptibility]*) was included and its interaction with DDDA/Y was used to separate the different model outcomes. In veal calves, an extra variable was included in the model to adjust the OR estimates for 2 different sampling frames used in this sector (i.e. until 2011 in farms and from 2012 at slaughterhouses). Model assumptions were checked with diagnostic plots. ORs and 95% confidence intervals (CI) for the different associations

were plotted. Finally, predicted *E. coli* resistance prevalence, related to a hypothetical total antimicrobial use reduction of 80% by 2016 from the index year 2009, were made per animal sector. Only the models with total antimicrobial use as a determinant were used for making the predictions. All models were fitted with PROC GLIMMIX in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Sigma Plot software version 12.5 (Systat Software Inc., San Jose, CA, USA) was used to create the graphs.

In a sensitivity analysis, we also explored the effect of a 1-year lag of antimicrobial use on resistance with the same models (e.g. relating antibiotic use in year 2013 with prevalence of resistance in 2014). These results were generally similar and are not presented.

Results

Resistance patterns

Percentages of antimicrobial resistance to each of the 9 antimicrobials tested during 2004-2014 (Appendix Tables S1 to S4) did not fundamentally differ from those including streptomycin (i.e. ten agents) in the period 2007-2013 (Table 1). Susceptibility to all antimicrobials was highest among isolates from dairy cattle (93%) while it was relatively low in veal calves and slaughter pigs (38 and 22% respectively), and very low in broilers (12%) (Table 1). The highest level of multidrug resistance was observed in broilers (75% of isolates), followed by pigs (55%), veal calves (45%) and dairy cattle (4%) (Table 1). Patterns of resistance visualized by shaded cells were comparable between animals; resistance phenotypes to ampicillin, tetracycline, sulfamethoxazole, trimethoprim and streptomycin dominated among most of the multi-resistant isolates, except for broilers, which showed an additional dominance of ciprofloxacin and nalidixic acid resistance (Table 1).

A stratified analysis per period of time in all animal sectors (Appendix Tables S1 to S4) showed that susceptibility to all antimicrobials was higher in the last 4 years of the study, when compared with the previous periods, and multidrug resistance was reduced. Nonetheless, resistance patterns did not fundamentally change over time; resistance to commonly used antimicrobials dominated in all linked phenotypes and cefotaxime resistance was related to isolates with the highest number of resistance phenotypes.

Table 1. Antimicrobial resistance patterns for all *E. coli* isolates obtained from the Dutch antimicrobial resistance monitoring (MARAN) in broilers, slaughter pigs, veal calves and dairy cows during the period 2007-2013.*

Animal species	No. of resistance phenotypes†	No. of isolates‡	% of the total isolates	% of antimicrobial agent resistances§								
				AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	STM	GEN
Broilers	Fully susceptible	227	12	0	0	0	0	0	0	0	0	0	0
	Resistant to 1	98	5	37	21	8	1	1	0	0	0	29	3
	Resistant to 2	151	8	32	19	18	3	48	49	1	3	24	4
	Resistant to 3	160	8	44	24	38	24	49	49	5	4	62	3
	Resistant to 4	230	12	82	48	67	50	39	39	7	10	53	6
	Resistant to 5	245	13	82	67	87	74	40	41	13	12	76	8
	Resistant to 6	259	13	84	67	95	80	80	80	22	11	72	8
	Resistant to 7	263	14	94	86	98	92	98	98	18	15	90	13
	Resistant to 8	221	11	99	96	100	91	99	100	80	25	99	11
	Resistant to 9	71	4	100	94	100	99	100	100	90	48	100	69
	Pan-resistant	9	0	100	100	100	100	100	100	100	100	100	100
	Total of isolates	1934	100	67	54	66	55	57	57	21	12	62	10
Slaughter pigs	Fully susceptible	406	22	0	0	0	0	0	0	0	0	0	1
	Resistant to 1	216	12	4	62	1	2	0	0	1	0	28	2
	Resistant to 2	218	12	11	65	32	19	0	0	3	0	66	3
	Resistant to 3	247	14	22	84	72	45	1	0	5	0	69	2
	Resistant to 4	269	15	42	77	93	86	0	0	14	3	80	4
	Resistant to 5	344	19	86	98	100	98	1	1	17	1	95	2
	Resistant to 6	88	5	95	99	100	99	8	9	80	6	98	7
	Resistant to 7	14	1	86	100	100	86	86	86	43	21	93	0
	Resistant to 8	12	1	100	100	100	100	100	100	67	33	92	8
	Resistant to 9	0	0	0	0	0	0	0	0	0	0	0	0
	Pan-resistant	0	0	0	0	0	0	0	0	0	0	0	0
	Total of isolates	1814	100	33	63	53	46	2	2	11	2	57	2

Animal species	No. of resistance phenotypes†	No. of isolates‡	% of the total isolates	% of antimicrobial agent resistance§									
				AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	STM	GEN
Veal calves	Fully susceptible	510	38	0	0	0	0	0	0	0	0	0	1
	Resistant to 1	158	12	2	94	1	0	0	0	0	0	3	1
	Resistant to 2	69	5	35	90	9	13	3	3	3	1	39	4
	Resistant to 3	81	6	31	90	58	25	11	11	6	0	68	0
	Resistant to 4	93	7	59	89	88	60	5	5	14	1	76	1
	Resistant to 5	181	14	91	99	97	77	4	4	33	2	91	2
	Resistant to 6	108	8	67	99	99	94	33	34	69	2	94	9
	Resistant to 7	50	4	86	98	100	86	86	78	56	2	88	20
	Resistant to 8	45	3	91	98	98	98	96	93	84	11	100	31
	Resistant to 9	40	3	100	100	100	100	100	100	98	5	100	98
	Pan-resistant	4	0	100	100	100	100	100	100	100	100	100	100
	Total of isolates	1339	100	35	59	42	34	14	14	20	1	41	6
Dairy cattle	Fully susceptible	1320	93	0	0	0	0	0	0	0	0	0	1
	Resistant to 1	33	2	9	52	3	0	0	3	3	0	12	18
	Resistant to 2	6	0	33	67	17	0	17	17	17	0	33	0
	Resistant to 3	23	2	43	78	61	13	0	0	9	0	87	9
	Resistant to 4	10	1	70	90	100	50	0	0	0	10	80	0
	Resistant to 5	12	1	83	100	100	83	8	8	25	0	92	0
	Resistant to 6	7	0	57	100	100	71	29	29	43	29	100	43
	Resistant to 7	2	0	100	100	100	100	100	100	0	0	100	0
	Resistant to 8	5	0	100	80	100	80	100	100	80	40	100	20
	Resistant to 9	4	0	100	100	100	100	100	100	100	25	100	75
	Pan-resistant	1	0	100	100	100	100	100	100	100	100	100	100
	Total of isolates	1423	100	3	5	4	2	1	1	1	0	4	1

*The period 2007-2013 included streptomycin in the panel of agents for antimicrobial susceptibility testing. Resistance patterns during the whole study period 2004-2014 (excluding streptomycin) are presented in Appendix Tables S1-S4, available as Supplementary data at JAC Online.
†Number of resistance phenotypes (0-10) to the 10 antimicrobial agents tested.
‡Number of isolates collected between 2007-2013 and resistant to 0-10 antimicrobial agents.
§Percentage of resistance to each of the 10 antimicrobials by the number of resistance phenotypes they exhibit (from 0 to 10). Cells are gradually shaded in grey according to percentage (i.e. the larger percentage, the darker the cell and vice versa). AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; STM, streptomycin; GEN, gentamicin.

Long-term trends and changes in antimicrobial use and prevalence of resistance

Trends in antimicrobial use and resistance by animal sector over the study period are displayed in Figure 1. In broilers and slaughter pigs, there was a period of increasing antimicrobial use until 2009, followed by a period of marked decrease until 2014; prevalence of acquired resistance moderately paralleled trends in antibiotic use. In veal calves, antimicrobial use and resistance decreased until 2014 but an abrupt difference in resistance was evident before and after 2012, likely because of the change in sampling strategy. Use of antimicrobials in dairy cattle was stable at a low level while the low resistance prevalence sharply increased in 2009. Animal sectors had different regimes of drug prescription, but in general, tetracyclines, penicillins and trimethoprim/sulphonamides were the most frequently used drugs in all sectors, with a substantial contribution of quinolones in broiler production(Figure 1).

Changes in antimicrobial use and resistance prevalence from the index year 2009 to 2014 are shown in Table 2. In broilers and slaughter pigs, relative decreases in use of the most commonly administered antimicrobials were the most dramatic (from -57 to -70% in broilers for total use and specific use of tetracyclines and quinolones; and from -54 to -63% in pigs for the total and the specific use of tetracyclines and trimethoprim/sulphonamides). However, in broilers, the relative decrease in prevalence of resistance to these drugs was more limited (from -8 to -31%) than in pigs (from -22 to -43%). In the veal calf sector, slightly more moderate decrease in use of the most common antimicrobials (from -40 to -44%) was paired with the most dramatic drop in prevalence of common resistance phenotypes (from -25 to -46%, partially explained by the change in sampling strategy). In dairy cattle antimicrobial use and resistance levels remained very low except for the unexpected sharp increase in 2009 that made the interpretation of relative changes difficult and unreliable. In all sectors, as a result of the implemented restriction policies, use of 3rd/4th generation cephalosporins and fluoroquinolones was almost completely absent in 2014, and this was accompanied by noticeable changes in resistance (-83% reduction of cefotaxime resistance and -19% reduction of ciprofloxacin resistance in broilers, -86 and -100% in pigs, -41 and -64% in veal calves and total absence of resistance to both agents in dairy cattle) (Table2).

Figure 1. Antimicrobial drug use (as Defined Daily Dosages per Animal Year - DDDA/Y) (left) and percentages of resistant *E. coli* isolates (right) for broilers, pigs, veal calves and dairy cows before and after the implementation of a policy for antimicrobial use reduction (index year 2009 represented as the vertical dotted line), the Netherlands 2004-2014.

Antimicrobial classes with use below 0.5 DDDA/Y in all years are not shown. *1-[pan-susceptibility]* refers to isolates resistant to at least one of the agents of the susceptibility testing panel (i.e. 1 - proportion of fully susceptible isolates).

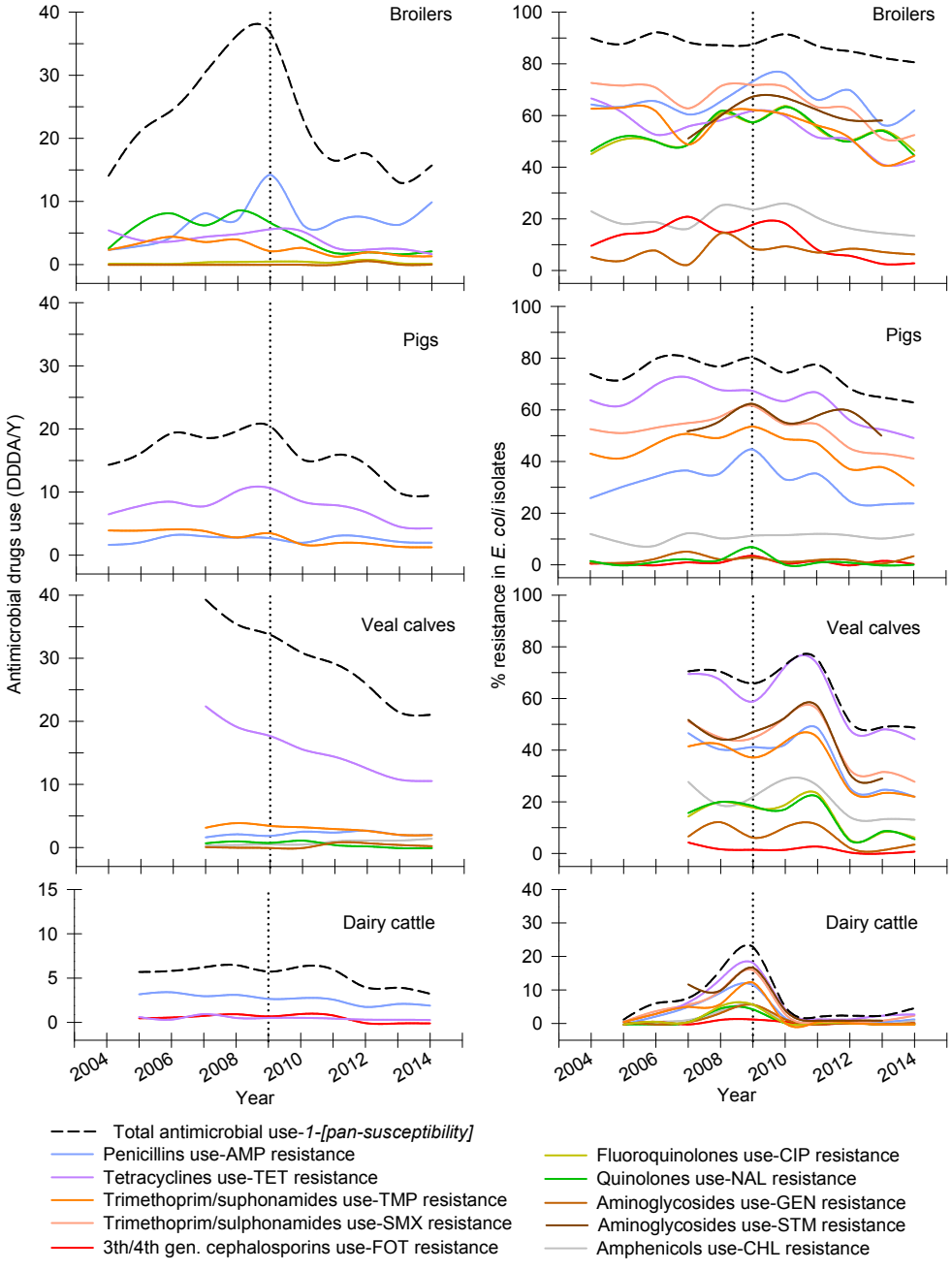


Table 2. Changes in antimicrobial drug use (as Defined Daily Dosages per Animal Year - DDDA/Y) and prevalence of resistance in *E. coli* isolates for broilers, pigs, veal calves and dairy cows during a reduction in antimicrobial use from index year 2009 to year 2014 in the Netherlands.

Animal species	AMU* (DDDA/Y)			AMR* (%)			Absolute change 2009-2014		Relative change 2009-2014	
	AM class	Year 2009	Year 2014	AM agent†	2009	2014	AMU* (DDDA/Y)	AMR* (%)	AMU* (DDDA/Y)	AMR* (%)
Poultry	Total AMU	36.8	15.8	*1-PS*	87.6	80.6	-21.0	-7.0	-57.1	-8.0
	Tetracyclines	5.6	1.7	TET	61.9	42.4	-3.9	-19.4	-69.8	-31.4
	Penicillins	14.3	9.9	AMP	73.2	62.1	-4.4	-11.1	-30.5	-15.2
	Trimethoprim/ sulphonamides	2.2	1.3	TMP	62.2	44.6	-0.8	-17.6	-37.7	-28.4
				SMX	71.8	52.5				
	Amphenicols	0.0	0.0	CHL	23.7	13.5	0.0	-10.2	0.0	-42.9
	Fluoroquinolones	0.5	0.2	CIP	57.4	46.4	-0.3	-11.0	-64.7	-19.1
	Quinolones	6.7	2.1	NAL	57.4	44.6	-4.5	-12.8	-68.0	-22.3
	3rd/4th gen. cephalosporins	0.0	0.0	FOT	17.9	2.9	0.0	-15.0	0.0	-83.7
	Aminoglycosides	0.0	0.0	STM	67.4	n.a.	0.0	n.a.	0.0	n.a.
				GEN	8.6	6.4		-2.2		-25.9
Pigs	Total AMU	20.5	9.5	*1-PS*	80.4	63.0	-11.0	-17.4	-53.6	-21.6
	Tetracyclines	10.7	4.3	TET	67.6	49.2	-6.4	-18.3	-59.4	-27.1
	Penicillins	2.8	2.1	AMP	44.9	24.0	-0.7	-21.0	-25.9	-46.6
	Trimethoprim/ sulphonamides	3.6	1.3	TMP	53.7	30.9	-2.2	-22.8	-62.6	-42.5
				SMX	61.8	41.3				
	Amphenicols	0.0	0.2	CHL	11.5	12.0	0.1	0.5	278.6	4.4
	Fluoroquinolones	0.0	0.0	CIP	7.1	0.0	0.0	-7.1	-100.0	-100.0
	Quinolones	0.0	0.1	NAL	7.1	0.3	0.0	-6.8	45.8	-96.4
	3rd/4th gen. cephalosporins	0.1	0.0	FOT	3.7	0.5	-0.1	-3.2	-100.0	-86.3
	Aminoglycosides	0.0	0.0	STM	62.5	n.a.	0.0	n.a.	0.0	n.a.
				GEN	3.0	3.6		0.5		17.5

Animal species	AMU* (DDDA/Y)			AMR* (%)			Absolute change 2009-2014		Relative change 2009-2014	
	AM class	Year 2009	Year 2014	AM agent†	2009	2014	AMU* (DDDA/Y)	AMR* (%)	AMU* (DDDA/Y)	AMR* (%)
Veal calves	Total AMU	33.8	21.2	*1-PS*	66.1	49.0	-12.7	-17.1	-37.4	-25.9
	Tetracyclines	17.8	10.7	TET	59.1	44.5	-7.1	-14.5	-40.0	-24.6
	Penicillins	1.5	2.2	AMP	41.5	22.3	0.7	-19.3	44.3	-46.4
	Trimethoprim/ sulphonamides	3.6	2.1	TMP	37.4	22.3	-1.5	-15.2	-41.4	-40.5
				SMX	45.0	28.1				
	Amphenicols	0.6	1.5	CHL	22.2	13.4	0.9	-8.9	145.2	-39.9
	Fluoroquinolones	0.9	0.0	CIP	18.1	6.5	-0.8	-11.6	-97.7	-64.1
	Quinolones	0.2	0.5	NAL	18.7	5.8	0.3	-12.9	133.3	-68.9
	3rd/4th gen. cephalosporins	0.4	0.0	FOT	1.8	1.0	-0.4	-0.7	-100.0	-41.4
	Aminoglycosides	0.1	0.3	STM	47.4	n.a.	0.3	n.a.	580.0	n.a.
				GEN	6.4	3.8		-2.7		-41.4
Dairy cattle	Total AMU	5.8	3.3	*1-PS*	23.1	4.9	-2.5	-18.3	-43.0	-79.0
	Tetracyclines	0.6	0.4	TET	18.4	3.0	-0.2	-15.4	-37.1	-83.8
	Penicillins	2.8	2.0	AMP	11.8	1.5	-0.8	-10.3	-27.4	-87.3
	Trimethoprim/ sulphonamides	0.2	0.2	TMP	12.5	0.0	0.0	-12.5	14.3	-100.0
				SMX	16.2	2.6				
	Amphenicols	0.0	0.1	CHL	5.9	1.1	0.0	-4.8	100.0	-81.0
	Fluoroquinolones	0.1	0.0	CIP	4.5	0.0	-0.1	-4.5	-100.0	-100.0
	Quinolones	0.0	0.0	NAL	5.9	0.0	0.0	-5.9	0.0	-100.0
	3rd/4th gen. cephalosporins	0.8	0.0	FOT	1.5	0.4	-0.8	-1.1	-100.0	-74.6
	Aminoglycosides	0.0	0.0	STM	16.9	n.a.	0.0	n.a.	0.0	n.a.
				GEN	5.9	0.4		-5.5		-93.7

*AMU, antimicrobial use; AMR, antimicrobial resistance. Decreasing changes are gradually shaded in grey (the darker, the biggest the decrease).
†AM, antimicrobial agent; *1-PS*, *1-[pan-susceptibility]*, resistance to at least one of the agents of the susceptibility testing panel (i.e. 1 - proportion of fully susceptible isolates); AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; GEN, gentamicin. STM, streptomycin was only used for testing during 2007-2013 and calculations for changes in 2009-2014 are not applicable (n.a.).

Associations between antimicrobial use and resistance

Dose-response antimicrobial use-resistance associations in the form of ORs and 95% CIs are shown in Figure 2. The numerical outcomes of the models are presented in Table 3. As a rule, the probability of resistant isolates was higher with increased used of antimicrobials. Total antimicrobial use was more significantly associated with the different resistance phenotypes (Figure 2a) than with homologous use (figure 2b). Associations in dairy cattle were never significant (Figure 2a-2b).

In broilers, the probability of an isolate to be resistant to any of the antibiotics was from 1 to 5% higher per unit increase in total DDDA/Y; these associations were statistically significant or borderline significant (i.e. CIs for ORs ≥1), except for *1-[pan-susceptibility]*, ampicillin and streptomycin resistance. Increased use in homologous antimicrobial classes was related to higher probabilities (e.g. fluoroquinolones use-ciprofloxacin resistance, OR=1.42). However these homologous associations were less significant (Figure 2a-2b, Table 3).

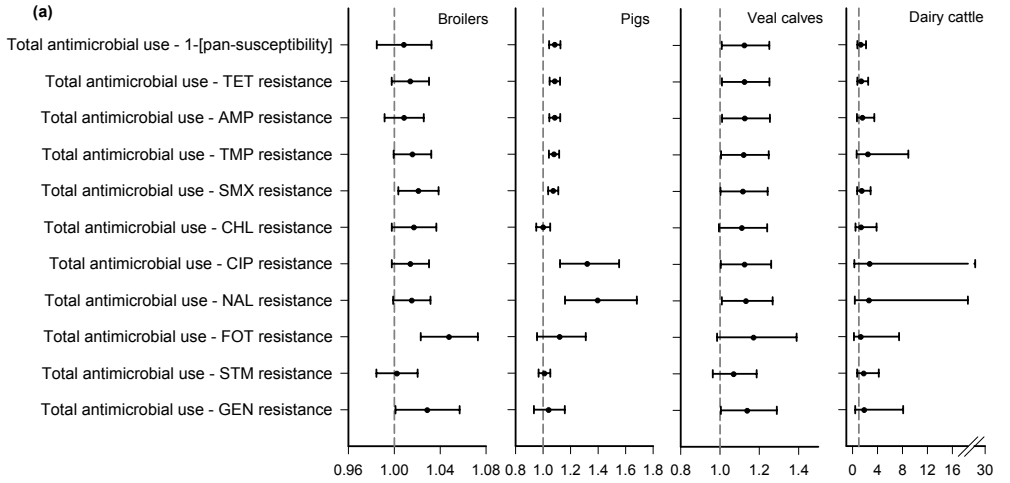
In pigs, total antimicrobial use was positively related to resistance with higher probabilities than in broilers. One unit increase in DDDA/Y was associated with a ≈8% increased odds of total, tetracycline, ampicillin, trimethoprim and sulfamethoxazole resistance, and with a ≈30 to 40% increased probability of ciprofloxacin and nalidixic acid resistance. As per the models with homologous use, the ORs were never significant (Figure 2a-2b, Table 3).

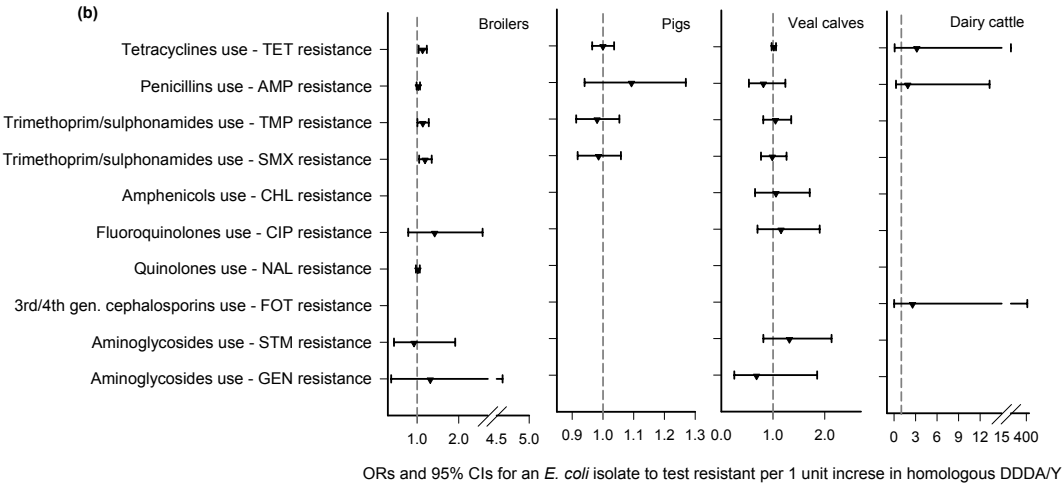
In veal calves, the ORs for the relationships between total antimicrobial use and resistance were similar and statistically significant for most of the resistance phenotypes (ORs between 1.07-1.17). Statistical significance was lost in models accounting for the change in sampling strategy. Estimates from the models with homologous antimicrobial use were not significant (Figure 2a-2b, Table 3).

In dairy cattle, the antimicrobial use-resistance associations were the strongest (i.e. higher ORs), but they were not statistically significant (Figure 2a-2b, Table 3).

Occasionally, the models in the different species generated negative estimates which we deemed are likely implausible (i.e. increased antimicrobial use associated with reduced resistance). These were probably resulting from the intrinsic nature of our data (e.g. potential misclassifications) or from having less data points (e.g. for streptomycin, which was tested only during 2007-2013, ORs < 1 in broilers) (Figure 2a-2b, Table 3).

Figure 2. Probabilities (ORs and 95% CIs) for an *E. coli* isolate to test resistant per 1 DDDA/Y increase in total antimicrobial use (a, filled circles) or in homologous antimicrobial class use (b, filled triangles) in the different food-producing animal sectors, the Netherlands 2004-2014.*





*Results from the 2 multivariate random-effects generalized linear mixed model (logistic regression) fitted with total antimicrobial use as determinant (panel a) and homologous antimicrobial use (panel b, only classes with DDDA/Y>0.5 in all years were modelled) (Table 3). The plotted estimates for veal calves are originated from the model not accounting for change in sampling strategy. Vertical blue reference line is used to assess significance (i.e. statistically significance at 95% confidence level if OR and CI are greater than 1). AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; STM, streptomycin (only tested from 2007-2013), GEN, gentamicin. *1-[pan-susceptibility]* refers to isolates resistant to at least one of the agents of the susceptibility testing panel (i.e. 1 - proportion of fully susceptible isolates).

Table 3. Probabilities (ORs and 95% CIs) for an *E. coli* isolate to test resistant per 1 DDDA/Y increase in antimicrobial use for broilers, slaughter pigs, veal calves and dairy cows, the Netherlands 2004-2014.*

Animal species	Model with total antimicrobial use as determinant			Model with homologous antimicrobial use as determinant		
	AMU†	AMR‡	OR and 95%CI	AMU†	AMR‡	OR and 95%CI§
Broilers	Total AMU	*1-PS*	1.01 (0.98-1.03)			n.a.
		TET	1.01 (1.00-1.03)	Tetracyclines	TET	1.13 (1.04-1.24)#
		AMP	1.01 (0.99-1.03)	Penicillins	AMP	1.03 (0.99-1.07)
		TMP	1.02 (1.00-1.03)#	Trimethoprim/ sulphonamides	TMP	1.14 (1.01-1.28)#
		SMX	1.02 (1.00-1.04)#		SMX	1.19 (1.05-1.35)#
		CHL	1.02 (1.00-1.04)	Amphenicols	CHL	n.c.
		CIP	1.01 (1.00-1.03)	Fluoroquinolones	CIP	1.42 (0.79-2.57)
		NAL	1.02 (1.00-1.03)	Quinolones	NAL	1.02 (0.97-1.07)
		FOT	1.05 (1.02-1.07)#	3rd/4th gen. cephalosporins	FOT	n.c.
		STM	1.00 (0.98-1.02)	Aminoglycosides	STM	0.93 (0.45-1.92)
		GEN	1.03 (1.00-1.06)#		GEN	1.32 (0.38-4.59)
Pigs	Total AMU	*1-PS*	1.08 (1.04-1.13)#			n.a.
		TET	1.08 (1.05-1.12)#	Tetracyclines	TET	1.00 (0.96-1.04)
		AMP	1.08 (1.05-1.13)#	Penicillins	AMP	1.09 (0.94-1.27)
		TMP	1.08 (1.04-1.12)#	Trimethoprim/ sulphonamides	TMP	0.98 (0.91-1.05)
		SMX	1.07 (1.04-1.11)#		SMX	0.99 (0.92-1.06)
		CHL	1.00 (0.95-1.05)	Amphenicols	CHL	n.c.
		CIP	1.32 (1.12-1.55)#	Fluoroquinolones	CIP	n.c.
		NAL	1.40 (1.16-1.68)#	Quinolones	NAL	n.c.
		FOT	1.12 (0.96-1.31)	3rd/4th gen. cephalosporins	FOT	n.c.
		STM	1.01 (0.97-1.05)	Aminoglycosides	STM	n.c.
		GEN	1.04 (0.93-1.16)		GEN	n.c.
Veal calves (model not accounting for change in sampling strategy)	Total AMU	*1-PS*	1.12 (1.01-1.25)#			n.a.
		TET	1.12 (1.01-1.25)#	Tetracyclines	TET	1.02 (0.97-1.06)
		AMP	1.13 (1.01-1.25)#	Penicillins	AMP	0.81 (0.53-1.24)
		TMP	1.12 (1.00-1.25)#	Trimethoprim/ sulphonamides	TMP	1.05 (0.81-1.35)
		SMX	1.12 (1.00-1.25)#		SMX	0.98 (0.77-1.26)
		CHL	1.11 (0.99-1.24)	Amphenicols	CHL	1.06 (0.65-1.71)
		CIP	1.12 (1.00-1.26)	Fluoroquinolones	CIP	1.29 (0.44-3.79)
		NAL	1.13 (1.01-1.27)#	Quinolones	NAL	n.c.
		FOT	1.17 (0.99-1.39)	3rd/4th gen. cephalosporins	FOT	n.c.
		STM	1.07 (0.96-1.19)	Aminoglycosides	STM	1.34 (0.84-2.15)
		GEN	1.14 (1.00-1.29)#		GEN	0.71 (0.27-1.89)

Animal species	Model with total antimicrobial use as determinant			Model with homologous antimicrobial use as determinant		
	AMU†	AMR‡	OR and 95%CI	AMU†	AMR‡	OR and 95%CI§
Veal calves (model accounting for change in sampling strategy)¶	Total AMU	*1-PS*	0.99 (0.93-1.06)			n.a.
		TET	1.00 (0.94-1.06)	Tetracyclines	TET	1.01 (0.97-1.17)
		AMP	1.00 (0.94-1.06)	Penicillins	AMP	0.87 (0.56-1.35)
		TMP	0.99 (0.93-1.06)	Trimethoprim/ sulphonamides	TMP	1.03 (0.79-1.34)
		SMX	0.99 (0.93-1.05)		SMX	0.96 (0.74-1.24)
		CHL	0.99 (0.93-1.05)	Amphenicols	CHL	1.06 (0.64-1.74)
		CIP	1.00 (0.94-1.07)	Fluoroquinolones	CIP	1.23 (0.75-2.03)
		NAL	1.01 (0.94-1.08)	Quinolones	NAL	n.c.
		FOT	1.05 (0.95.-1.16)	3rd/4th gen. cephalosporins	FOT	n.c.
		STM	0.95 (0.89-1.01)	Aminoglycosides	STM	1.47 (0.90-2.40)
		GEN	1.02 (0.95-1.09)		GEN	0.68 (0.27-1.70)
Dairy cattle	Total AMU	*1-PS*	1.30 (0.78-2.17)			n.a.
		TET	1.39 (0.77-2.51)	Tetracyclines	TET	3.16 (0.08-119.9)
		AMP	1.57 (0.71-3.49)	Penicillins	AMP	1.90 (0.27-13.32)
		TMP	2.45 (0.67-8.95)	Trimethoprim/ sulphonamides	TMP	n.c.
		SMX	1.46 (0.74-2.9)		SMX	n.c.
		CHL	1.35 (0.47-3.85)	Amphenicols	CHL	n.c.
		CIP	2.72 (0.29-25.57)	Fluoroquinolones	CIP	n.c.
		NAL	2.62 (0.37-18.5)	Quinolones	NAL	n.c.
		FOT	1.30 (0.23-7.46)	3rd/4th gen. cephalosporins	FOT	2.57 (0.02-416.46)
		STM	1.77 (0.74-4.21)	Aminoglycosides	STM	n.c.
		GEN	1.85 (0.42-8.11)		GEN	n.c.

*Two multivariate random-effects generalized linear mixed model (logistic regression) are fitted per animal species; one with total antimicrobial use and the other with homologous antimicrobial usage as determinants. Model outcomes are the frequencies of resistant isolates over total number of isolates. Resistance phenotypes to each antimicrobial agent (and *total R*) are indicated in the model as an explanatory variable and its interaction with antimicrobial use (DDDA/Y total and disaggregated by classes) differentiates the outcomes. ORs presented in the table are extracted from the interaction term.

†AMU, antimicrobial use. Determinant of the model.

‡AMR, antimicrobial resistance. *1-PS*, *1-[pan-susceptibility],* resistance to at least one of the agents of the susceptibility testing panel (i.e. 1 - proportion of fully susceptible isolates); AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; STM, streptomycin (only tested from 2007-2013), GEN, gentamicin.

§Homologous resistance was only modelled in the classes with DDDA>0.5 in all years (n.a. , not applicable; n.c. not computed)

¶A variable indicating the different sampling strategy (until 2011 in farms and from 2012 in slaughterhouses) was included in the model for adjustment of the estimates.

#significant associations at 95% confidence level.

Predicting resistance prevalence associated with an 80% reduction in antimicrobial use

Predicted resistance levels for 2016 in broilers remained similar to the observed ones in 2014; only ciprofloxacin and ampicillin resistance were expected to be reduced by ≈3-4% but resistance to other agents was expected to even increase (e.g. for tetracycline and cefotaxime) (Table 4). In pigs and veal calves, predictions were the most optimistic; *1-[pan-susceptibility]* and resistance to the most commonly used antimicrobials were projected to decrease by ≈5-9% in pigs and by ≈14-28% in veal calves. In dairy cattle, *1-[pan-susceptibility]* was projected to decrease by ≈3% (Table 4).

Table 4. Predicted prevalences (%) of resistance in *E. coli* isolates for year 2016 in the different food-producing animal sectors if their total antimicrobial use was decreased by 80% from index year 2009.*

Animal species	AMR†	Observed resistance prevalence (%) in 2014‡	Predictions for resistance in year 2016 if total antimicrobial use was reduced 80% from index year (2009)	
			Predicted resistance prevalence (%) in 2016	Predicted absolute change prevalence (%) between 2014-2016§
Broilers	*1-PS*	80.6	82.3	1.6
	TET	42.4	44.0	1.5
	AMP	62.1	58.0	-4.1
	TMP	44.6	44.6	0.0
	SMX	52.5	53.0	0.4
	CHL	13.5	13.3	-0.2
	CIP	46.4	43.0	-3.4
	NAL	44.6	42.7	-1.9
	FOT	2.9	4.3	1.4
	STM‡	58.1	55.3	-2.8
	GEN	6.4	4.3	-2.1
Pigs	*1-PS*	63.0	53.7	-9.3
	TET	49.2	40.8	-8.4
	AMP	24.0	16.0	-8.0
	TMP	30.9	25.7	-5.2
	SMX	41.3	33.6	-7.8
	CHL	12.0	11.7	-0.3
	CIP	0.0	0.1	0.1
	NAL	0.3	0.0	-0.2
	FOT	0.5	0.3	-0.2
	STM‡	50.2	52.4	2.2
	GEN	3.6	1.5	-2.1

Animal species	AMR†	Observed resistance prevalence (%) in 2014‡	Predictions for resistance in year 2016 if total antimicrobial use was reduced 80% from index year (2009)	
			Predicted resistance prevalence (%) in 2016	Predicted absolute change prevalence (%) between 2014-2016§
Veal calves¶	*1-PS*	49.0	21.0	-28.0
	TET	44.5	19.0	-25.5
	AMP	22.3	7.7	-14.6
	TMP	22.3	8.0	-14.2
	SMX	28.1	11.7	-16.4
	CHL	13.4	4.9	-8.5
	CIP	6.5	2.3	-4.2
	NAL	5.8	2.0	-3.8
	FOT	1.0	0.1	-0.9
	STM‡	29.3	23.8	-5.5
	GEN	3.8	0.7	-3.0
Dairy cattle	*1-PS*	4.9	1.5	-3.4
	TET	3.0	0.8	-2.2
	AMP	1.5	0.3	-1.2
	TMP	0.0	0.0	0.0
	SMX	2.6	0.5	-2.1
	CHL	1.1	0.3	-0.9
	CIP	0.0	0.0	0.0
	NAL	0.0	0.0	0.0
	FOT	0.4	0.1	-0.3
	STM‡	1.1	0.2	-0.9
	GEN	0.4	0.1	-0.3

*Predictions for % of resistance obtained for year 2016 and the total antimicrobial use corresponding to an 80% reduction from 2009. Only the multivariate random-effects generalized linear mixed models with total antimicrobial use as determinant were used.

†AMR, antimicrobial agent resistance. *1-PS*, *1-[pan-susceptibility],*, resistance to at least one of the agents of the susceptibility testing panel (i.e. 1 - proportion of fully susceptible isolates); AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; STM, streptomycin (only tested from 2007-2013), GEN, gentamicin.

‡The observed prevalence of resistance for STM (streptomycin) indicated in the table are for year 2013 (last year in which STM was included in the susceptibility testing panel).

§Decreasing changes are gradually shaded in grey (the darker, the biggest the decrease).

¶In veal calves only the model not accounting for the change in sampling strategy was used to obtain the prediction.

Discussion

Our results suggest that a progressive reduction in the use of antibiotics during the past five years in the Netherlands might have resulted in lowered resistance levels in livestock. Multi-resistant isolates were very common and usage history and co-selection of phenotypes might explain to a large extent the observed patterns. Positive and significant antimicrobial use-resistance relationships were derived from the analyses, but the associations clearly differed between the animal sectors. A further decrease in use of these drugs was projected to have a bigger impact, resulting in lower predicted levels of resistance, especially in the pig and veal calf industry. However, resistance in broilers was projected to level off because of the weaker antimicrobial use-resistance relationships.

We focused on *E. coli* for various reasons. This bacterium is a widely used indicator of Gram-negative species incorporated in resistance surveillance systems, thus continuous data over several years were readily available[19,26]. Moreover, *E. coli* is highly abundant in the intestinal tract of humans and animals and is an important vector for transmission of resistance genes between other bacterial populations[27].

Sampling in the monitoring program was done mainly at the slaughterhouse level. Thus, resistant microorganisms should theoretically reflect antimicrobial use patterns and management practices as a whole during the life of the animals. Sample size was sufficient for providing an estimate of the resistant *E. coli* population in each animal species of the entire country and for representativeness, animals were sampled in all months of the year to account for possible seasonal effects[19]. Nonetheless, it should be noted that this sampling is inherently insensitive to detecting resistance at the individual animal level and the resistance measure might not provide as sharp a picture of the situation at individual farms[19,28]. This could be the reason for the drop in prevalence observed in veal calves after 2011, when the sampling scheme changed from farms to slaughterhouses. In the case of dairy cattle, the sharp increase in resistance observed in 2009 was not fully explained but likely attributable to the smaller sample taken in that year (n=136), which made it less representative[19].

Antimicrobial use differed quantitatively and qualitatively by animal sector and this appeared to drive the variation observed in resistance patterns. For instance, quinolone and fluoroquinolone resistance levels were exceptionally high in broilers as a result of the higher use of quinolones as compared to the rest of animal species. Our results reconfirm the potential importance of co-selection (i.e. an antimicrobial selects for resistance to another antimicrobial) in the emergence and perpetuation of this problem. Multi-resistance commonly involved the most widely administered antimicrobials and with several decades of prior usage (penicillins, tetracyclines and trimethoprim/sulphonamides). Multiple resistance can perpetuate for years, even with decreasing or no use of antibiotics, since resistance genes are assembled in complex genetic vectors containing other resistance genes[27,29]. This is the most likely explanation for the observed moderate to high levels of resistance to chloramphenicol and streptomycin, when use of these drugs was virtually zero. Resistance to more recently introduced drugs

(around the 1990s), such as fluoroquinolones and 3rd generation cephalosporins, was much lower but often associated to multi-resistance. Comparable findings have been described[29].

This study suggest that curbing overall resistance rates in animals is possible, to an important extent, by reducing the use of antimicrobials. Specifically, cefotaxime and ciprofloxacin resistance was even more dramatically reduced in relative terms, which we deemed to be a product of the restriction in usage of 3rd/4th generation cephalosporins and fluoroquinolones[30,31]. These findings suggest that, in *E. coli*, resistance to antibiotics with less usage history could be more rapidly reverted. Nonetheless, non-monotonic trends (e.g. reduction of suppression of antimicrobials related to both increased and decreased resistance over different time periods) have been described on several occasions for these drugs[29].

We found positive antimicrobial use-resistance dose-response relationships that varied in size and statistical significance by association and by animal sector. Similar associations have been demonstrated, especially in pigs and veal calves, but rarely based on longitudinal data and at the national level[1,3,6,32,33]. In a recent study, a direct antimicrobial use-resistance correlation at a supranational level was described, but findings were compromised by the cross-sectional nature of the associations and the lack of data per individual animal sectors[2]. We found the strongest usage-resistance relationships in slaughter pigs and veal calves, followed by the broilers (sectors with a relatively high use of antibiotics). In dairy cattle no significant relationships were found. These observations reflect important differences between animal industries in the structure, regimes of drug prescription, and duration of the production cycles (broilers < veal calves < pigs < dairy cattle). In veal production, greater quantities of antimicrobials are applied cycle after cycle, and frequency of animal replacement is lower than in other sectors. Pig production is an age-segregated system with continuous replacement but with more stringent biosecurity conditions. Broilers production is a highly integrated system, where few companies control the supply of animals; broilers are raised highly confined and receive more broad-spectrum drugs such as quinolones (i.e. more multi-resistance). Administration of antimicrobials in dairy cows is usually more limited and on an individual basis, which could explain the lack of significant associations in our models.

We also unravelled differences between types of resistance. Exposure-response slopes were often steeper for newer drugs (i.e. higher ORs for 3th/4th generation cephalosporins in broilers and veal calves and higher ORs for fluoroquinolones in pigs and dairy cattle), but this needs cautious interpretation; baseline resistance levels were lower for these drugs, which might lead to larger estimates, although it also probably explained by a more rapidly reverted resistance to these antimicrobials. Moreover, total antimicrobial use was more significantly related to resistance phenotypes than homologous usage, showing again the importance of co-selection of resistance. This means that if a reduction in resistance to a specific antimicrobial is intended, a reduction in all of the antimicrobials co-selecting for the same resistance is essential. Nonetheless and regardless the significance, direct selection also played a role since some homologous associations had a bigger effect size.

A further decrease in antimicrobial use was predicted to result in more decreased resistance in veal calf and pig sectors. In broilers, resistance trends seemed to level off; a number of hypothesis might explain this. It is clear that use of antimicrobials importantly contributes to resistance emergence, but a reduction in use should not be the only intervention to tackle this problem. Additional forces drive this process, such as movement of carrier animals between premises, transmission from the top of pyramidal production systems, keeping animals in close confinement, biosecurity and hygiene conditions, etc[34-37]. Moreover, illegal use of ceftiofur was noticed up to March 2010 at broiler hatcheries; this use was not recorded and reported, but was deemed to increase levels of resistance to 3rd generation cephalosporins[19].

The data of this study came from publicly available reports, which made it possible to draw big picture conclusions; however, this was also a limiting factor in terms of interpretation and level of detail. Our data were ecological, that is to say, antimicrobial use and resistance were evaluated at country level and not in corresponding and equal epidemiological units (e.g. farm level). This leads to potential misclassification of individual level exposure to antimicrobials, which might be especially important in heterogeneous sectors such as veal calf production. Nonetheless, the temporal and geographical link made the ecological associations more reliable. An extra limitation arising from the level of aggregation of our data was the impossibility to account for other farm-level determinants of resistance and to adjust associations for these[6,38]. Notwithstanding these constraints, we consider the data resolution to have been sufficient for the purpose of evaluating the nationwide program. A methodological limitation was that our models were suited for well-established and long-standing relatively high levels of antimicrobial use and resistance; for obtaining reliable estimates and model convergence, some homologous associations could not be studied when antimicrobials were used in very low quantities.

Conclusion

In conclusion, recent Dutch policies reducing the total veterinary use of antimicrobials, and restricting the use of critically important antimicrobials, appear to have reduced resistance levels in the main livestock industries. Epidemiological evidence highlights the importance of a better understanding on the co-selection of resistance. Phasing out the use of antimicrobials in the coming years was projected to further reduce resistance in veal calves, slaughter pigs and to a lesser extent in dairy cows, but levels in broilers seemed to level off. Additional interventions need to be evaluated in future studies.

Footnotes

Preliminary results from this study were presented at the International Society for Veterinary Epidemiology and Economics (ISVEE XIV), 3-7 November 2015, Mérida, Yucatán, México; oral presentation title: *Establishing dose-response relationships and antimicrobial use thresholds for an active policy against antimicrobial resistance in food-producing animals*.

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Transparency declarations

None to declare.

Supporting material

Supplemental information on antimicrobial use and resistance data used in this study.

In the Netherlands, the veterinary use of antibiotics can be monitored in different but complementary ways. Firstly, overall sales of antimicrobials are reported yearly by the Dutch Veterinary Pharmaceutical Industry Federation (FIDIN). Secondly, annual use of antibiotics is recorded as Defined Daily Dosages per Animal per Year (DDDA/Y) on the basis of drug prescriptions documented at farms. The use of DDDA/Y is a refined way of reporting antibiotic consumption, which has been recommended by ESVAC since it enables the reporting of consumption by animal species and by antibiotic classes.[13,20] From 2004 to 2012, the Agricultural Economic Institute (LEI) of Wageningen University and Research Centre (WUR), calculated DDDA/Y using stratified samples of farms that were weighted to represent each animal sector nationwide.[19,21] In 2011, the large animal production sectors started to implement centralized registration systems for antibiotic prescriptions and the Netherlands Veterinary Medicines Authority (SDa) was installed as an independent institute to reinforce the implementation of the antimicrobial policies (www.autoriteitdiergeneesmiddelen.nl). Since then, antimicrobial use has been reported as DDDA/Y, which is calculated similarly as published previously by the LEI, except that the data from all the farms in a sector is used instead data from a representative sample.[17,22] This study retrieved the total and class-specific DDDA/Y reported by the Netherlands Veterinary Medicines Authority (SDa) during the period 2004-2014 in broilers and pigs, 2007-2014 in veal calves, and 2005-2014 in dairy cattle.[17] Annual data on antimicrobial use in the pig sector were provided separately for fatteners and sows/piglets until 2010. In order to account for exposure to antimicrobials during the entire life of the animals, a joint average antimicrobial use for both animal categories was estimated; their DDDA/Y was weighted in each year with the mean proportion of animals represented by each production type during the period 2000-2010 (0.51 for fatteners and 0.49 for sow/piglets), as obtained from the Netherlands Statistics (http://statline.cbs.nl/Statweb/). The weighting proportions remained stable over the years, which justified this approach.

The antimicrobial resistance monitoring program in the Netherlands is designed to capture changes in resistance at the population level by isolating bacteria from a sample of randomly selected animals at the time of slaughter.[23] Sample size varies by year and animal species, though, as an approximation, a mean number of 245 (±86) animals is sampled throughout each year per animal production sector.[19] The data from the resistance monitoring is available from 1998 onward for broilers and slaughter pigs, and from 2005 onward in veal calves and dairy cattle. In veal calves, sampling was made at farms until 2011, and from 2012 onwards at slaughterhouses. In dairy cattle, pooled samples were taken at farms in all years, except in 2010 and 2011, when individual animals were sampled at slaughterhouses. These results for resistance in *E. coli* are communicated annually in the *Monitoring Antimicrobial Resistance and Antibiotic Use in Animals in the Netherlands* (MARAN) reports in which they identify wild- and non-wild-type isolates through epidemiological cut-off values (www.eucast.org).[19] The use of this non-clinical measure is more objective than clinical breakpoints and allows to detect an early emergence of acquired resistance.[19,24]

Appendix Table S1. Antimicrobial resistance patterns for all *E. coli* isolates obtained from the Dutch antimicrobial resistance monitoring (MARAN) in broilers during the whole study period 2004-2014 and during three consecutive time frames.*

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2004-2014 (whole study period)	Fully susceptible	407 (13)	0	0	0	0	0	0	0	0	0
	Resistant to 1	182 (6)	47	31	15	1	2	0	1	0	4
	Resistant to 2	339 (11)	29	24	23	9	54	54	1	3	3
	Resistant to 3	333 (11)	71	27	64	46	37	37	9	5	5
	Resistant to 4	490 (16)	83	72	86	73	30	31	7	11	6
	Resistant to 5	382 (12)	76	61	91	76	72	72	27	15	10
	Resistant to 6	476 (16)	95	88	98	92	96	96	16	10	9
	Resistant to 7	354 (12)	99	96	100	94	99	100	75	25	10
	Resistant to 8	92 (3)	100	93	100	97	100	100	91	54	64
	Pan-resistant	11 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	3066 (100)	66	55	65	56	54	54	20	11	8
2004-2006	Fully susceptible	79 (10)	0	0	0	0	0	0	0	0	0
	Resistant to 1	48 (6)	33	46	15	0	2	0	2	0	2
	Resistant to 2	73 (10)	30	29	34	15	41	42	1	4	3
	Resistant to 3	98 (13)	62	44	79	57	20	19	11	4	3
	Resistant to 4	133 (18)	82	68	92	80	26	29	5	14	4
	Resistant to 5	103 (14)	62	75	88	83	70	71	22	18	11
	Resistant to 6	116 (15)	93	94	99	95	89	90	20	16	5
	Resistant to 7	88 (12)	100	97	100	100	100	100	80	20	3
	Resistant to 8	15 (2)	100	93	100	87	100	100	93	87	40
	Pan-resistant	2 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	755 (100)	64	61	72	63	48	49	20	13	5
2007-2009	Fully susceptible	97 (13)	0	0	0	0	0	0	0	0	0
	Resistant to 1	38 (5)	47	21	18	0	0	0	0	0	13
	Resistant to 2	66 (9)	33	30	26	11	45	47	2	2	5
	Resistant to 3	67 (9)	60	25	70	40	42	42	6	10	4
	Resistant to 4	107 (14)	81	73	82	66	33	32	12	17	4
	Resistant to 5	101 (13)	69	63	95	79	69	72	20	18	14
	Resistant to 6	119 (15)	93	84	98	95	97	97	14	13	8
	Resistant to 7	135 (17)	100	97	99	93	99	99	69	31	13
	Resistant to 8	36 (5)	100	94	100	97	100	100	86	50	72
	Pan-resistant	8 (1)	100	100	100	100	100	100	100	100	100
	Total of isolates	774 (100)	68	59	71	60	59	59	24	16	12

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2010-2014	Fully susceptible	231 (15)	0	0	0	0	0	0	0	0	0
	Resistant to 1	96 (6)	53	28	14	2	2	0	0	0	1
	Resistant to 2	200 (13)	27	20	18	7	62	61	2	3	3
	Resistant to 3	168 (11)	80	18	53	41	45	45	8	3	5
	Resistant to 4	250 (16)	85	73	85	72	31	32	6	8	8
	Resistant to 5	178 (12)	89	52	90	71	75	72	34	11	7
	Resistant to 6	241 (16)	96	88	97	90	98	98	16	5	12
	Resistant to 7	131 (9)	98	95	100	92	100	100	79	23	11
	Resistant to 8	41 (3)	100	93	100	100	100	100	95	46	66
	Pan-resistant	1 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	1537 (100)	66	49	60	50	54	53	18	7	8

*Resistance patterns during the whole study period do not include streptomycin since it was only tested between 2007-2013.

†Number of resistances (0-9) to the 9 antimicrobial agents tested.

‡Number of isolates resistant to 0-9 antimicrobial agents collected during the whole study period (2004-2014 for broilers).

§Percentage of resistance to each of the 9 antimicrobials by the number of resistances they exhibit (from 0 to 9). Cells are gradually shaded in grey according to percentage (i.e. the larger percentage, the darker the cell and vice versa). AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; GEN, gentamicin.

Appendix Table S2. Antimicrobial resistance patterns for all *E. coli* isolates obtained from the Dutch antimicrobial resistance monitoring (MARAN) in slaughter pigs during the whole study period 2004-2014 and during three consecutive time frames.[a]

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2004-2014 (whole study period)	Fully susceptible	807 (27)	0	0	0	0	0	0	0	0	0
	Resistant to 1	515 (17)	3	79	10	4	0	0	1	0	2
	Resistant to 2	395 (13)	26	72	63	30	1	0	3	1	4
	Resistant to 3	469 (16)	29	80	94	82	1	0	11	0	3
	Resistant to 4	584 (20)	83	96	100	97	1	0	18	2	2
	Resistant to 5	163 (5)	95	99	100	99	5	5	83	5	8
	Resistant to 6	18 (1)	83	100	100	89	78	78	50	11	11
	Resistant to 7	16 (1)	100	100	100	100	100	100	63	31	6
	Resistant to 8	1 (0)	100	100	100	100	100	100	100	0	100
	Pan-resistant	0 (0)	0	0	0	0	0	0	0	0	0
	Total of isolates	2968 (100)	31	62	51	43	2	1	11	1	2
2004-2006	Fully susceptible	177 (26)	0	0	0	0	0	0	0	0	0
	Resistant to 1	125 (19)	2	79	13	4	1	0	0	0	1
	Resistant to 2	95 (14)	25	76	64	28	0	0	2	3	1
	Resistant to 3	110 (16)	25	85	97	83	1	0	8	0	1
	Resistant to 4	129 (19)	81	98	100	99	2	0	19	0	1
	Resistant to 5	32 (5)	94	100	100	100	6	3	88	3	6
	Resistant to 6	2 (0)	50	100	100	100	50	50	100	0	50
	Resistant to 7	4 (1)	100	100	100	100	100	100	75	0	25
	Resistant to 8	0 (0)	0	0	0	0	0	0	0	0	0
	Pan-resistant	0 (0)	0	0	0	0	0	0	0	0	0
	Total of isolates	674 (100)	29	64	52	43	2	1	10	1	1
2007-2009	Fully susceptible	159 (21)	0	0	0	0	0	0	0	0	0
	Resistant to 1	113 (15)	6	81	7	3	0	0	1	0	2
	Resistant to 2	98 (13)	31	72	60	28	2	1	0	1	5
	Resistant to 3	128 (17)	28	82	91	82	1	1	11	0	5
	Resistant to 4	196 (26)	84	96	100	98	1	1	15	3	3
	Resistant to 5	41 (5)	95	100	100	100	5	7	71	10	12
	Resistant to 6	12 (2)	92	100	100	83	92	92	33	8	0
	Resistant to 7	12 (2)	100	100	100	100	100	100	58	42	0
	Resistant to 8	1 (0)	100	100	100	100	100	100	100	0	100
	Pan-resistant	0 (0)	0	0	0	0	0	0	0	0	0
	Total of isolates	760 (100)	39	69	59	51	4	4	11	2	3

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2010-2014	Fully susceptible	471 (31)	0	0	0	0	0	0	0	0	0
	Resistant to 1	277 (18)	1	79	11	5	0	0	2	0	2
	Resistant to 2	202 (13)	23	70	63	33	0	0	5	0	4
	Resistant to 3	231 (15)	31	77	94	82	0	0	12	1	3
	Resistant to 4	259 (17)	84	95	100	96	0	0	20	3	2
	Resistant to 5	90 (6)	96	99	100	99	4	4	88	3	7
	Resistant to 6	4 (0)	75	100	100	100	50	50	75	25	25
	Resistant to 7	0 (0)	0	0	0	0	0	0	0	0	0
	Resistant to 8	0 (0)	0	0	0	0	0	0	0	0	0
	Pan-resistant	0 (0)	0	0	0	0	0	0	0	0	0
	Total of isolates	1534 (100)	28	57	47	40	1	1	12	1	2

*Resistance patterns during the whole study period do not include streptomycin since it was only tested between 2007-2013.

†Number of resistances (0-9) to the 9 antimicrobial agents tested.

‡Number of isolates resistant to 0-9 antimicrobial agents collected during the whole study period (2004-2014 for pigs).

§Percentage of resistance to each of the 9 antimicrobials by the number of resistances they exhibit (from 0 to 9). Cells are gradually shaded in grey according to percentage (i.e. the larger percentage, the darker the cell and vice versa). AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; GEN, gentamicin.

Appendix Table S3. Antimicrobial resistance patterns for all *E. coli* isolates obtained from the Dutch antimicrobial resistance monitoring (MARAN) in veal calves during the whole study period 2007-2014 and during two consecutive time frames.*

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2007-2014 (whole study period)	Fully susceptible	689 (40)	0	0	0	0	0	0	0	0	0
	Resistant to 1	239 (14)	3	94	1	0	0	0	0	0	2
	Resistant to 2	121 (7)	40	88	41	15	4	4	5	1	2
	Resistant to 3	130 (8)	49	90	80	53	8	8	9	0	2
	Resistant to 4	233 (13)	88	98	95	75	5	4	30	3	1
	Resistant to 5	139 (8)	73	99	99	94	25	26	73	1	9
	Resistant to 6	68 (4)	82	99	100	88	78	71	56	6	21
	Resistant to 7	59 (3)	90	98	98	97	97	93	88	12	27
	Resistant to 8	45 (3)	100	100	100	100	100	100	98	7	96
	Pan-resistant	4 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	1727 (100)	34	57	40	32	13	12	19	2	6
2007-2010	Fully susceptible	199 (30)	0	0	0	0	0	0	0	0	0
	Resistant to 1	101 (15)	3	95	1	0	0	0	0	0	1
	Resistant to 2	47 (7)	38	89	38	17	4	4	2	2	4
	Resistant to 3	50 (8)	50	88	78	56	10	10	8	0	0
	Resistant to 4	101 (15)	94	97	96	77	3	3	24	5	1
	Resistant to 5	69 (10)	74	100	99	93	23	25	75	1	10
	Resistant to 6	29 (4)	90	97	100	86	79	72	48	7	21
	Resistant to 7	36 (5)	89	97	100	97	100	94	89	11	22
	Resistant to 8	32 (5)	100	100	100	100	100	100	100	3	97
	Pan-resistant	3 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	667 (100)	43	67	48	41	18	18	24	3	9
2011-2014	Fully susceptible	490 (46)	0	0	0	0	0	0	0	0	0
	Resistant to 1	138 (13)	2	93	1	0	0	0	0	0	3
	Resistant to 2	74 (7)	41	86	43	14	4	4	7	0	1
	Resistant to 3	80 (8)	49	91	81	51	8	8	10	0	3
	Resistant to 4	132 (12)	84	99	95	73	6	5	36	2	1
	Resistant to 5	70 (7)	71	99	100	96	27	27	71	1	7
	Resistant to 6	39 (4)	77	100	100	90	77	69	62	5	21
	Resistant to 7	23 (2)	91	100	96	96	91	91	87	13	35
	Resistant to 8	13 (1)	100	100	100	100	100	100	92	15	92
	Pan-resistant	1 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	1060 (100)	28	51	35	27	10	9	16	1	4

*Resistance patterns during the whole study period do not include streptomycin since it was only tested between 2007-2013.
†Number of resistances (0-9) to the 9 antimicrobial agents tested.
‡Number of isolates resistant to 0-9 antimicrobial agents collected during the whole study period (2007-2014 for veal calves).
§Percentage of resistance to each of the 9 antimicrobials by the number of resistances they exhibit (from 0 to 9). Cells are gradually shaded in grey according to percentage (i.e. the larger percentage, the darker the cell and vice versa).
AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; GEN, gentamicin.

Appendix Table S4. Antimicrobial resistance patterns for all *E. coli* isolates obtained from the Dutch antimicrobial resistance monitoring (MARAN) in dairy cattle during the whole study period 2007-2014 and during two consecutive time frames.*

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2007-2014 (whole study period)	Fully susceptible	1918 (94)	0	0	0	0	0	0	0	0	0
	Resistant to 1	41 (2)	10	54	7	0	0	5	2	2	20
	Resistant to 2	29 (1)	34	79	59	7	3	3	14	0	0
	Resistant to 3	16 (1)	63	81	88	44	0	0	13	0	13
	Resistant to 4	17 (1)	88	88	100	82	0	0	29	12	0
	Resistant to 5	8 (0)	50	100	100	75	38	38	38	25	38
	Resistant to 6	2 (0)	100	100	100	100	100	100	0	0	0
	Resistant to 7	5 (0)	100	80	100	80	100	100	80	40	20
	Resistant to 8	5 (0)	100	100	100	100	100	100	100	20	80
	Pan-resistant	1 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	2042 (100)	3	5	4	2	1	1	1	0	1
2005-2009	Fully susceptible	623 (89)	0	0	0	0	0	0	0	0	0
	Resistant to 1	19 (3)	5	53	11	0	0	11	0	0	21
	Resistant to 2	16 (2)	38	69	50	13	6	6	19	0	0
	Resistant to 3	11 (2)	64	73	82	64	0	0	0	0	18
	Resistant to 4	15 (2)	87	87	100	93	0	0	20	13	0
	Resistant to 5	6 (1)	33	100	100	83	50	50	33	17	33
	Resistant to 6	2 (0)	100	100	100	100	100	100	0	0	0
	Resistant to 7	3 (0)	100	100	100	67	100	100	67	33	33
	Resistant to 8	5 (1)	100	100	100	100	100	100	100	20	80
	Pan-resistant	0 (0)	0	0	0	0	0	0	0	0	0
	Total of isolates	700 (100)	6	8	7	5	2	2	2	1	2

Years	No. of resistance phenotypes†	No. of isolates (%)‡	% of antimicrobial agent resistance§								
			AMP	TET	SMX	TMP	CIP	NAL	CHL	FOT	GEN
2010-2014	Fully susceptible	1295 (97)	0	0	0	0	0	0	0	0	0
	Resistant to 1	22 (2)	14	55	5	0	0	0	5	5	18
	Resistant to 2	13 (1)	31	92	69	0	0	0	8	0	0
	Resistant to 3	5 (0)	60	100	100	0	0	0	40	0	0
	Resistant to 4	2 (0)	100	100	100	0	0	0	100	0	0
	Resistant to 5	2 (0)	100	100	100	50	0	0	50	50	50
	Resistant to 6	0 (0)	0	0	0	0	0	0	0	0	0
	Resistant to 7	2 (0)	100	50	100	100	100	100	100	50	0
	Resistant to 8	0 (0)	0	0	0	0	0	0	0	0	0
	Pan-resistant	1 (0)	100	100	100	100	100	100	100	100	100
	Total of isolates	1342 (100)	1	3	2	0	0	0	1	0	0

*Resistance patterns during the whole study period do not include streptomycin since it was only tested between 2007-2013.

†Number of resistances (0-9) to the 9 antimicrobial agents tested.

‡Number of isolates resistant to 0-9 antimicrobial agents collected during the whole study period (2005-2014 for dairy cattle).

§Percentage of resistance to each of the 9 antimicrobials by the number of resistances they exhibit (from 0 to 9). Cells are gradually shaded in grey according to percentage (i.e. the larger percentage, the darker the cell and vice versa).

AMP, ampicillin; TET, tetracycline; SMX, sulfamethoxazole; TMP, trimethoprim; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; FOT, cefotaxime; GEN, gentamicin.

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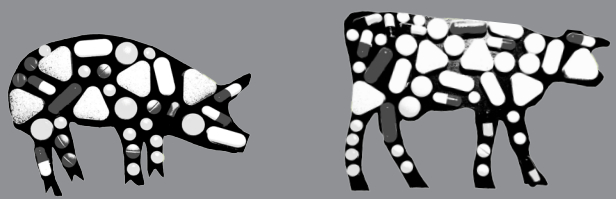
Part IV

Extended-spectrum β-lactamase-producing *Escherichia coli* in humans, animals and their environment



Chapter 7

The epidemiology of ESBL/AmpC genes and plasmids in human, animal and environmental reservoirs: a meta-analysis

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Abstract

In recent years, several animal, food and environmental sources have been suggested to be involved in transmission of extended-spectrum β-lactamase producing *E. coli* (ESBL-*E. coli*) to humans. We did a systematic search of studies containing collections of *E. coli* producing ESBLs or plasmid mediated AmpC beta-lactamases in the Netherlands between 2000-2015. A total of 27 selected isolate collections were used for data extraction. A meta-collection of isolates was constructed with data on ESBL/AmpC genes (n=3646 isolates), plasmid replicons (n=808) and strain types (n=364) across 19 reservoirs representing humans, animals and the environment. Two meta-analyses were done at gene and at plasmid replicon type levels in 4 steps: i) description of relative frequencies for molecular types per reservoir; ii) pairwise quantification of the associations between the previous frequency profiles for all reservoirs through Proportional Similarity Index (PSI) calculation; iii) Principal Component Analyses (PCAs) to visualize clusters and proximities between reservoirs according to their frequency profiles; iv) rarefaction analysis to evaluate diversity of molecular types per reservoir. Results showed a limited (but still existing) molecular proximity between most animal reservoirs and humans in the open population and clinical settings. Plasmid replicon and gene types from wild birds and surface water influenced by treatment plants were closely related to these human populations evidencing that ESBL/AmpC transmission and dissemination occurs beyond humans and domesticated animals. The farming human communities in direct contact with livestock essentially shared same molecular types with their animals revealing transmission between reservoirs. This meta-analysis offers a summary of the accumulated knowledge on ESBL/AmpC by providing a simple snapshot on molecular proximities of isolates collected in different populations over the last years.

Introduction

In the last decade we have witnessed a global expansion of extended-spectrum β-lactamase producing *Escherichia coli* (ESBL-*E. coli*)[1]. This dramatic epidemiological change has led to a rise in incidence and mortality resulting from these bacterial infections in hospitals and the community[2-6]. At the same time, ESBL-*E. coli* has increasingly been reported in all livestock sectors, in the food chain and in companion animals[7-14]. In Europe, particularly in the Netherlands a large amount of data on ESBL-*E. coli* has been collected in recent years. It is hypothesized that animals form a relevant source of at least a proportion of human urinary tract and extraintestinal infections[15-18]. However, the contribution to the public health burden from the animal reservoirs remains an issue of controversy[19].

The epidemiology of ESBL-producing organisms is far from simple. There are multiple direct and indirect exposure routes that can potentially lead to transmission of animal-associated resistance traits to human populations[15]. Moreover, bacterial populations can acquire resistance horizontally through mobile genetic elements in a wide variety of intestinal or extraintestinal environments[15]. Molecular typing techniques have been widely used over the past years to support these epidemiological observations. Parallel occurrence of similar gene subtypes, plasmids and/or bacterial clones between animals and humans have been identified and poultry has been suggested as the most likely source of animal-associated ESBL-*E. coli* infections in humans[15,20-22].

Evidence about the possible biological mechanisms underlying animal-human transmission is not equivocal. Some studies support a major role of whole bacterium or clonal transmission while others support that transmission mediated by plasmids or other mobile genetic elements is more influencial[15]. Recently, a small number of isolates has been investigated by whole genome sequencing (WGS), suggesting the occurrence of clonal transmission of ESBL-producing *E. coli* between pigs and pig farmers[23]. Clonal transmission from poultry through the food chain was not considered to be likely but strong indications for plasmid-mediated transmission between different reservoirs have been found[23].

To our knowledge, narrative reviews on the molecular characteristics of ESBLs are available and only one study has created a meta-collection of ESBL-*E. coli* isolates (n=1,329)[20,24]. They grouped data on *E. coli* ESBL types, phylogroups and antimicrobial susceptibility and found relevant proportions of same subtypes across humans, livestock and companion animals[20]. These results were valuable to identify common clusters at a large scale strengthening evidence of an existing exchange of bacteria or bacterial genes between these populations or a common reservoir[20].

We therefore performed a systematic search for epidemiological studies describing ESBL-*E. coli*-positive isolates from the Netherlands, at least at the ESBL gene subtype level and originated from any human, animal or environmental source. All isolates from selected studies together with isolates from ongoing partnered research projects were compiled for a meta-analysis. Using multivariable analyses, we give a snapshot of the current knowledge

on the molecular epidemiology of ESBL-*E. coli* in terms of proximity of different potential reservoirs to humans by quantifying the similarity and diversity of gene and plasmid profiles between the different sources.

Materials and Methods

Selection of studies

We systematically reviewed published work containing collections of *E. coli* isolates producing ESBL or plasmid mediated AmpC beta-lactamases (ESBL/AmpC) in the Netherlands using PubMed and further explored the references of the selected studies. Our search strategy consisted on the combination of terms "ESBL", "extended-spectrum" or "beta-lactamase" with either terms related to the bacterial specie of interest (i.e. "*Escherichia coli*" or "*E. coli*") or terms referring to the country where isolates were collected ("the Netherlands" or "Dutch"). We only included studies containing isolates with at least gene sequence typing information for all gene groups found; this was our primary outcome. Secondary outcomes were plasmid types identified by PCR-Based Replicon Typing (PBRT) and/or *E. coli* genotypes identified by multilocus sequence types (MLST). If research partners from the "ESBL attribution (ESBLAT)" consortium (http://www.1health4food.nl/nl/show/ESBL-attributie-3.htm) were involved in selected articles, they were contacted to obtain the raw data and possible extra molecular data. Also data from additional ongoing epidemiological studies within the consortium and data from the Dutch antimicrobial national surveillance system (MARAN) of years 2013 to 2015 were obtained. We excluded studies containing only isolates collected prior to 2000, reports or outbreak investigations, studies that focused on the molecular characterization of only specific plasmids and/or ESBL genes, abstracts from scientific conferences and studies written in languages other than English or Dutch. Other reasons for exclusion were the lack of gene typing data for any of the gene groups reported and the impossibility for data extraction at the isolate level when reporting of specific genes was aggregated. The last search was done in February 2016.

Data extraction and synthesis

We extracted the number of ESBL (CTX-M, TEM or SHV families) and AmpC-beta lactamase genes (mainly CMY family) over the total number of isolates with genes sequenced in each study. The numbers of different plasmid replicon types harboring ESBL/AmpCs and/or the strain MLST types over the total number of isolates typed were also extracted together with specific information on the source of origin and reservoir.

Meta-analyses

To make a visual comparison of the isolates' molecular profiles between the different reservoirs, relative frequencies for genes, plasmids and MLST *E. coli* types were plotted in

bar charts using Sigma Plot (version 13, Systat Software Inc, San José, California). These relative frequencies formed the basis of the data to be analyzed in the subsequent stages.

Firstly, we did a pairwise quantification of the associations between the frequency distributions of genes and plasmids in the reservoirs. We used the proportional similarity index (PSI), which is defined as $PSI=1-0.5\sum_k |p_k-q_k|$, where p_k is the relative frequency of the gene or plasmids subtype harboring an ESBL/AmpC-gene k in the first reservoir and q_k is the relative frequency of subtype k in the second reservoir[25]. Confidence intervals (CIs) for PSI were calculated using 500 bootstrap samples.

Secondly, three Principal Component Analyses (PCA) were done in R (version 3.0.2; R Foundation for Statistical Computing, Vienna, Austria) for the meta-collection of genes, plasmids and the combination of plasmids and genes[26]. The dimension of the proportion profiles was reduced to the 2 main components in which each gene and plasmid had a different load according to their contribution for differentiation of reservoirs. Two exclusion thresholds were established: i) for reservoirs with less than 34 isolates (0. 9% over the total collection) to avoid sampling uncertainty; ii) and for genes with relative frequencies below 0.05 in all reservoirs to avoid rare occurrences related with the number of samples. Unlike in most PCAs, relative frequencies were not scaled in relation to each other to keep the effect of relatively large variances of particular genes and/or plasmids between reservoirs. 5000 bootstrap samples of the relative frequencies per reservoir were generated to account for sample uncertainty[27]. Biplots of the space represented by the 2 principal components were constructed and preferences for location of genes, plasmids or their combination among the bootstrapped samples of the different reservoirs' isolates were displayed.

Finally, a rarefaction analysis was done to evaluate the diversity of genes and plasmids in the meta-collection of isolates per reservoir[28].

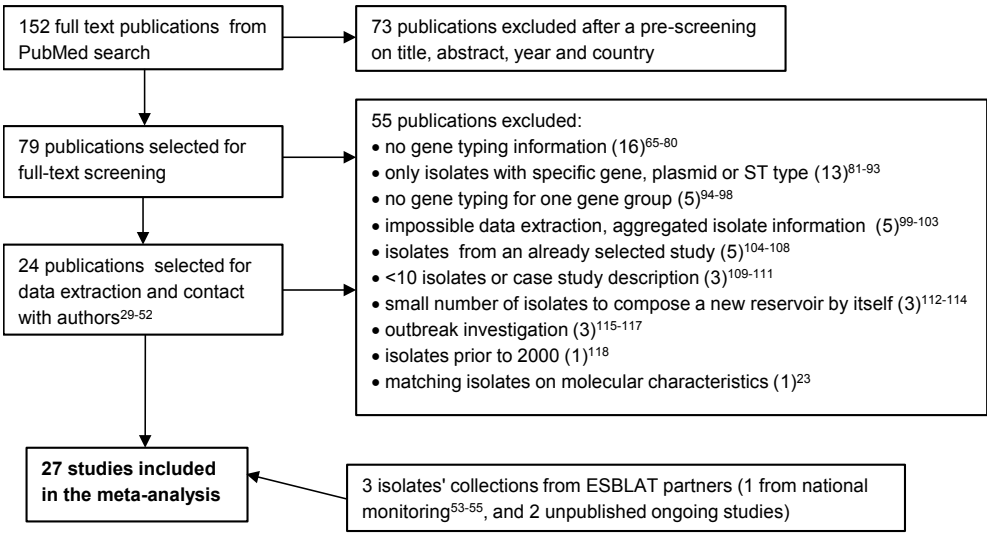
Results

Systematic search and meta-collection of isolates

Figure 1 depicts the selection process for the studies in our meta-analysis. In total, 152 publications were retrieved for inspection. Data were directly extracted from four selected publications[29-32]. For the rest of the 20 selected publications[33-52],raw data were obtained from the partners from the ESBLAT research consortium, that also provided three additional collections of isolates from the Dutch national antimicrobial resistance monitoring system in food-producing animals[53-55].

A total of 3769 isolates from 3193 samples were collated (average 1.2 isolates per sample) from the 27 selected studies[29-55] (Table 1). We excluded the following sources with <0.9% of isolates over the total: clinical samples from cats and horses[37,43], flies and the environment from laying hen farms[35], meat samples other than chicken, turkey or beef[36,53-55]. This resulted in 3646 isolates to be considered for the meta-analyses.

Figure 1. Selection of studies for a meta-analysis of ESBL/AmpC genes and plasmids in the Netherlands.



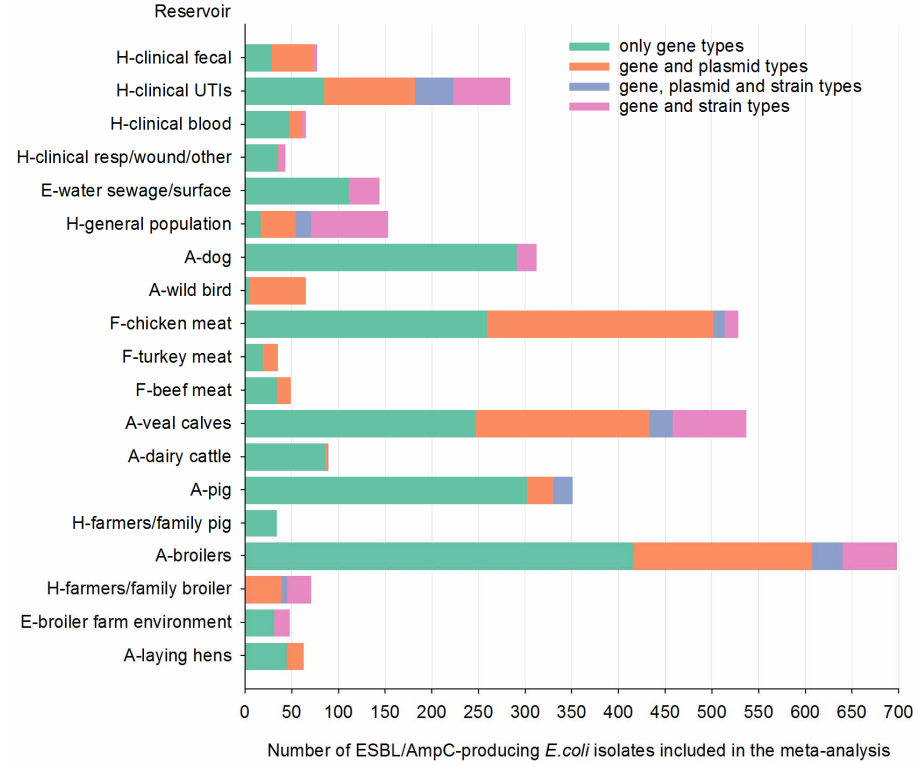
Nineteen reservoirs remained (Table 1) with four of them involving human samples from clinical settings (hospitals, general practitioner of long-term care facility) from different origins, namely fecal samples[30,31,52], urinary tract infections (UTIs)[36,31,52], blood samples[29-31,52] and an aggregation of samples from respiratory infections, wounds and other origins[31,52]. One reservoir represented the human open population[48,50]. Two reservoirs represented the human farming community in contact with pigs[41] and broilers[39,40,46,47]. Animal species constituted independent reservoirs: dogs[33,37,43], wild birds[51], veal calves[42,44,45,53-55], dairy cattle[36,53-55], pigs[36,41,53-55], broilers[32,36,39,46,47,53-55] and laying hens[53-55]. Meat samples were also classified as independent sources as chicken[30,36,53-55],turkey[36,53-55] and beef[53-55]. Finally, two reservoirs represented sewage and surface water[34] and the environment of broiler farms[35,38].

The average number of isolates analyzed per sample was below 1.2 in all reservoirs except for dogs (2.2), the environment of broiler farms (1.7), surface and sewage water (4.4) and farmers and family members in broiler farms (2.0).

The proportion of isolates available over the total final meta-collection greatly differed between the reservoirs (Figure 2). The highest number of isolates was available for broilers, representing 19.1% of the total, followed by isolates from veal calves (14.7%), chicken meat (14.5%), pigs (9.6%), dogs (8.6%), human clinical UTIs (7.8%) and general human population (4.2%). The rest of the reservoirs accounted for between 1 to 4% of the total number of isolates.

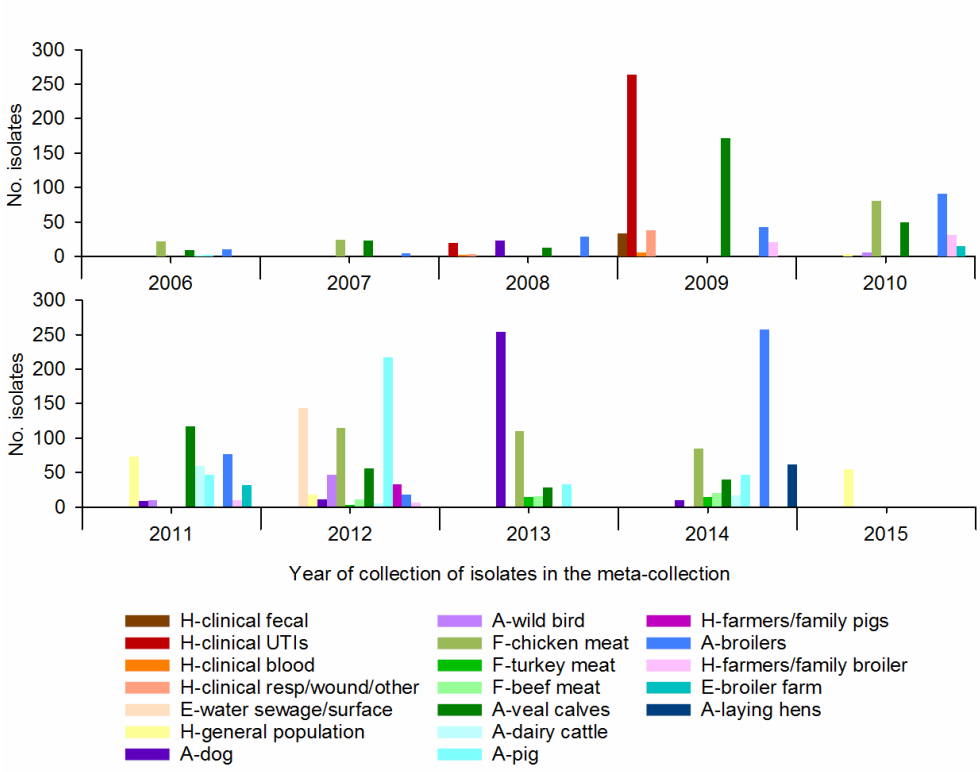
The number of isolates available varied per year of collection (Figure 3); the majority (91.4%) were collected between 2009 and 2015; the remaining 8.6% represented isolates

Figure 2. Number of ESBL/AmpC-producing *E. coli* isolates (n=3646) collated from 27 studies by reservoir for a meta-analysis of ESBL/AmpC genes and plasmids in the Netherlands.
Type of reservoir: H, human; A, animal; F, food; E, environment.



collected from 2000 to 2008. Only in veal calves, 24 isolates (0.7% over the total number in all reservoirs) were collected between 2000-2005, the rest of the isolates from all reservoirs (99,3%) were collected from 2006 to 2015. We observed some shifts in the distribution of number of isolates per reservoir over the years. All human clinical isolates were collected between 2008-2009 while for the community 63.4% corresponded to years 2010-2012 and 36.6% to 2015. Focusing just on the reservoirs with most isolates, we observed that they were mainly collected from 2009 on; 82.8% of chicken meat and 93.6% of broiler samples were evenly distributed between 2009-2014; in veal calves 87% were collected between 2009-2014; in dogs all were collected between 2011-2014 (Figure 3).

Figure 3. Number of ESBL/AmpC-producing *E. coli* isolates (n total=3646) collated from 27 studies by reservoir for a meta-analysis of ESBL/AmpC genes and plasmids in the Netherlands.
Type of reservoir: H, human; A, animal; F, food; E, environment.*



* Isolates from 2000-2005 are not shown in this figure because they represented only 0.7% of the total (n=24). Isolates lacking information on year of collection at isolate level are not represented in this figure (a study of human clinical fecal and blood samples and chicken meat between 2008-200931, a study of broiler samples between 2012-201333 and a study of human clinical blood samples between 2008-200930).

Table 1. Inventory of the 27 selected studies with reservoirs and number of isolates considered for a meta-analysis of ESBL/AmpC genes and plasmids in the Netherlands. Type of reservoir: H, human; A, animal; F, food; E, environment.

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
O. Baede V et al. (2015)[33]	2013-2014	Longitudinal prevalence survey. Samples from 38 dogs providing monthly samples during a 2 year period	A-dog, fecal (266)	A-dogs	266#	0	0
Blaak et al. (2014)[34]	2012	Cross-sectional. Samples from recreational water influenced by wastewater treatment plants.	E-Water (144)	E-surface water/sewage	144#	0	33#
Blaak et al. (2014)[35]	2011	Cross-sectional prevalence survey. Manure and rinse water samples from a broiler and a laying hen farm, pooled samples of flies.	E-broiler farm, fecal (22), other (10) E-Flies in laying hen farm (6) E-manure from laying hen farm (31)	E-broiler farm NA (excluded) NA (excluded)	32#	0	17# E-broiler farm
Day MJ et al. (2016)[36]	2006-2009	Collection of 353 ESBL-*E. coli* isolates from Germany, the UK and the Netherlands. Only the ones of the NL are used.	A-broiler fatteners, fecal (35) A-dairy cattle, fecal (2) H-general practitioner, urine (62) F-chicken, meat surface (47) F-turkey, meat surface (1) F-veal, meat surface (1) A-pig fatteners, fecal (3)	A-broilers A-dairy cattle H-clinical UTIs F-chicken meat F-turkey meat NA (excluded) A-pigs	151#	142# [33 broilers, 2 cattle, 56 human UTIs, 47chicken meat, 1 turkey meat, 3 pigs]	0

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
Dierikx C et al. (2012)[37]	2008	Cross-sectional. A diagnostic center submitted all animal isolates ESBL-suspected from 2000 to 2008.	A-cat clinical, urine (2); wound (2) A-dogs clinical, blood (1); respiratory (2); urine (15); wounds (3); other (3) A-horse clinical Blood (1); wounds (4); other (5)	NA (excluded) A-dogs NA (excluded)	24#	0	21# A-dogs
Dierikx C et al. (2013)[38]	2009-2010	Cross sectional prevalence survey. Samples from broilers at different stages of the production pyramid.	A-broilers fatteners, fecal (3) A-broiler grandparents, fecal (20) A-broiler parents, fecal (7) E-broiler farm, other (16)	A-broilers A-broilers A-broilers E-broiler farm	46#	0	0
Dierikx C et al. (2013)[39]	2009	Cross-sectional prevalence survey. Broiler: samples at 6 weeks of age in 35 farms. Human: samples from farmers.	A-broilers, fecal (31) H-farmers in broiler farm, fecal (21)	A-broilers H-farmers/ family broiler farm	52#	16# [9 A-broilers; 7 H-farmers/ family broiler farm]	23# [16 A-broilers; 7 H-farmers/ family broiler farm]

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
Dierikx C et al. (2010)[40]	2006	Cross-sectional prevalence. Samples from the national monitoring program with non-wild type MICs for cefotaxime.	A-broilers, fecal (10)	A-broilers	10#	10#	0
Dohmen W et al. (2015)[41]	2012	Longitudinal prevalence survey in humans. Cross-sectional prevalence survey in pigs. Samples from pigs and people living and /or working in 40 pig farms. *	H-family members pig farm, fecal (7)* H-farmers pig farm, fecal (27)* A-pig fatteners, fecal (29)* A-gilts, fecal (16)* A-piglets, fecal (34)* A-sows, fecal (38)* A-weaning piglets, fecal (40)*	H-farmers/ family pig farm H-farmers/ family pig farm A-pigs A-pigs A-pigs A-pigs A-pigs	191#	0	0
Hordijk J et al. (2013)[42]	2000-2009	Longitudinal prevalence survey. Samples from 3 veal calf farms upon arrival and after 3,6,8 and 10 weeks.	White veal calves, fecal (143)	A-veal calves	143#	75#	74#
Hordijk J et al. (2013)[43]	2011-2012	Cross-sectional prevalence. Samples collected from healthy dogs and cats.	A-cat clinical, fecal (10) A-dog healthy, fecal (9) A-dog clinical, fecal (13)	NA (excluded) A-dogs A-dogs	22#	0	0
Hordijk J et al. (2013)[44]	2009	Longitudinal prevalence survey assessed with pooled samples.	A-veal calves, fecal (149)	A-veal calves	149#	0	0

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
Hordijk J et al. (2013)[45]	2011	Cross-sectional prevalence survey. Samples from 100 veal calf herds at slaughter.	A-veal calves, fecal (59)	A-veal calves	59#	51#	5#
Huijbers PMC et al. (2014)[46]	2010-2011	Cross-sectional prevalence survey. Broiler: pooled samples at ~31 days of age from 50 farms. Human: people living and/or working at the farms.	A-broiler fatteners, fecal (92) H-family members in broiler farm, fecal (18) H-farmers in broiler farm, fecal (23)	A-broilers H-farmers/ family broiler farm H-farmers/ family broiler farm	133#	70# [43 A-broilers, 27 H-farmers/ family broiler farm]	36# [19 A-broilers; 17 H-farmers/ family broiler farm]
Huijbers PMC et al. (2015)[47]	2011-2012	Longitudinal prevalence survey. Broiler: samples at T1 (~34 days of age) and T2 (~68 days) in 8 farms. Human: people living and/ or working at the farms at T1 and T2.	A-broiler fatteners, fecal (49) H-farmers in broiler farm, fecal (8)	A-broilers H-farmers/ family broiler farm	58#	10# [5 A-broilers, 5 H-farmers/ family broiler farm]	27# [18 A-broilers; 9 H-farmers/ family broiler farm]
Kluytmans JA et al. (2013)[30]	2008-2009	Cross sectional prevalence survey. Isolates from retail chicken meat from supermarkets close to 4 hospitals where human isolates where taken.	H-human clinical, fecal (43), H-human clinical, blood (15) F-chicken, meat surface (87)	H-clinical fecal H-clinical blood F-chicken meat	145*	145**	0

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
Koningstein M et al (2015)[48]	2010-2012	Prospective cohort study in 44 daycare centers attending children. 22-month study period and 852 stool samples	H-childcare facility, fecal (29)	H-general population	29#	0	0
Leverstein-van Hall MA et al. (2011)[49]	2010	Cross-sectional. Human: same samples as in study as Voets CM et al (2012). Meat: retail chicken meat bought at 12 supermarkets.	F-Chicken, meat surface (81)	F-chicken meat	81#	0	14# F-chicken meat
Pacholewicz et al (2015)[32]	2012-2013	Cross, sectional prevalence and quantification of ESBLs in 17 batches of broiler carcasses in 2 slaughterhouses. Isolates from bleeding and chilling phases.	A-broiler slaughterhouse, whole rinsed carcass (165)	A-broilers	165*	0	0
Paltansing S et al (2012)[31]	2008	Cross sectional selection of isolates from a hospital.	H-human clinical, fecal (2) H-human clinical, urine (20) H-human clinical, respiratory (2) H-human clinical, blood (3) H-human clinical, wound/other (2)	H-clinical fecal H-clinical UTIs H-clinical resp inf./wound/ other H-clinical blood H-clinical resp inf./wound/ other	29*	0	29*

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
van der Bij A. et al (2011)[29]	2008-2009	Cross sectional prevalence survey. Isolates of patients with bacteremia from 3 hospitals.	H-human clinical, blood (41)	H-clinical blood	41*	0	0
van Hoek AHAM et al.(2015)[50]	2011	Cross-sectional prevalence survey of 1,033 adults from areas with low and high broiler density.	H-general population, fecal (68)	H-general population	65#	37#	54#
Veldman K et al. (2013)[51]	2010-2012	Cross-sectional prevalence survey. Samples from carcasses of wild birds sent to diagnostic laboratory.	A-wild birds Fecal (65)	A-wild birds	65#	60#	0
Voets GM et al. (2012)[52]	2009	Cross-sectional. Microbiology labs submitted all ESBL-E. coli-positive screened human clinical isolates from hospital, long-term care facilities and GPs during 3 consecutive months.	H-clinical, fecal (36) H-clinical, urine (202) H-clinical, respiratory (14) H-clinical, blood (6) H-clinical, wound/other (25)	H-clinical fecal H-clinical UTIs H-clinical resp inf./wound/ other H-clinical blood H-clinical resp inf./wound/ other	283#	45# [41 H-clinical UTIs; 2 H-clinical fecal; 2 H-clinical blood/resp inf.]	48# [41 H-clinical UTIs; 3 H-clinical fecal; 2 H-clinical blood/ resp inf.; 2 H-clinical wound/ other inf.]

Publication	Years collection of isolates	Study Design	Origin of the isolates (number gene typed)	Aggregated reservoir in the meta-analysis	no. isolates included	no. isolates with plasmid information	no. isolates with MLST type information
MARAN reports 2013, 2014 and 2015[53-55]	2011-2014	Cross-sectional prevalence annual report. Random samples from the national monitoring system collected at slaughter houses and from retail meat at supermarkets.	A-broilers fatteners, fecal (286) A-dairy cattle, fecal (36) A-laying hens, fecal (63) A-pig fatteners, fecal (191) A-rose veal calves, fecal (16) A-white veal calves, fecal (25) A-veal calves, fecal (145) F-chicken, meat surface (313) F-beef , meat surface (49) F-turkey, meat surface (34) F-pig , meat surface (23) F-veal, meat surface (5) F-crocodile, meat surface (4) F-goose, meat surface (1)	A-broilers A-dairy cattle A-laying hens A-pigs A-veal calves A-veal calves A-veal calves F-chicken meat F-beef meat F-turkey meat NA (excluded) NA (excluded) NA (excluded) NA (excluded)	1158#	333# [91 broilers, 18 laying hens, 25 pigs, 60 veal calves, 109 chicken meat, 15 beef meat, 15 turkey meat]	NA
van den Bunt G. (data not published)	2015	Cross sectional prevalence survey in human general population	H-general population Fecal (57)	H-general population	57#	0	29#
Velthuis A. (data not published)	2011	Cross sectional prevalence survey in dairy cattle	A-dairy cattle Fecal (45) Milk (5)	A-dairy cattle	50#	0	0

NA (excluded), sources excluded because they could not constitute a reservoir with more than 0.9% of isolates over the total. # Data obtained directly from ESBLAT project partners. *Data extracted from the publication.**The plasmid typing information from this study was not used in the meta-analyses since the collection also included non-ESBL-carrying plasmids.

Proximity of reservoirs in terms of ESBL/AmpC gene profiles

The number of ESBL/AmpC genes found per isolate ranged from 1 to 1.1. The proportions for $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-52}}$ dominated the distributions in most reservoirs except for poultry, farmers and family members in poultry farms and laying hens with an additional important contribution of $bla_{\text{CMY-2}}$ (Panel 1-A).

The similarity indices applied over the relative frequencies of genes showed that ESBL/AmpC genes from human clinical samples and from the open population were highly comparable (PSIs 0.6 - 0.8) (Panel 1-B). These human reservoirs were also closely related to water samples, wild birds, dogs, veal calves and meat samples (PSIs 0.5 - 0.8). *E. coli* genes from farmers and family members were very similar to those from animals they had direct contact with (PSIs of 0.8 for pig farming and 0.9 for poultry farming). Laying hens, poultry farming community, broilers and environmental samples from broiler farms were least similar to isolates from the human community and the clinical setting. CIs were widest for reservoirs with the smallest sample size such as turkey meat and pig farming community (not shown).

The 2 principal components of the PCA explained 78% of the total variance in our data (Panel 1-C). The first component was dominated by the abundance of $bla_{\text{CTX-M-15}}$ (in the human clinical samples [except fecal], open population and sewage/surface water) and $bla_{\text{CTX-M-1}}$ (in dairy cattle, veal calves, beef and chicken meat, pigs and the pig farming community). The second component represented mainly the distinct abundance of $bla_{\text{CMY-2,}}$ and to a lesser extent $bla_{\text{SHV-12}}$, in laying hens, poultry farming community, broilers and their environment. Isolates from wild birds clustered closed to the human open population and isolates from respiratory or wound infections. Genes from dogs, turkey meat and human clinical fecal samples clustered closed to the center of the 2 component loadings (i.e. PC1 and PC2=0).

Proximity of reservoirs in terms of plasmid replicon types and ESBL/AmpC genes

A total of 808 isolates (22.2% of the meta-collection) contained information on plasmid replicon types harboring ESBL/AmpCs genes. The relative frequencies of genes in this subset of isolates (Appendix Figure 1) was not significantly different from those in the complete meta-collection (Panel 1-A) except for human clinical fecal (increased proportion of $bla_{\text{CTX-M-1}}$ with decreased $bla_{\text{CTX-M-15}}$) and pig isolates (decreased $bla_{\text{CTX-M-1}}$ in favor or increased $bla_{\text{SHV-12}}$, $bla_{\text{TEM-52}}$ and $bla_{\text{CMY-2}}$) (F test< 0.05 in these two reservoirs).

There was information on an average of 1.0-1.1 genes and 1.0-1.7 plasmids harboring an ESBL/AmpC gene per isolate across the different reservoirs (Panel 2-A). The most commonly plasmid replicons found in all reservoirs were IncI1 and multiple IncF replicons. To a lesser extent, ColE (in human fecal), IncN (in general population) and IncK (in veal calves, broilers and farming community and laying hens) were also common (Panel 2 A).

PSIs for plasmid replicon profiles (Panel 2-B) between reservoirs were very close to

those found per genes (Panel 1-B) with the exception of some higher values between chicken meat and clinical human or general population isolates (PSIs~0.6-0.7). CIs were widest for reservoirs with the smallest number of isolates with plasmid replicon typing available such as human clinical blood, and pigs (not shown).

The 2 principal components of the PCA for plasmid replicon types (Panel 2-C) explained 91% of the total variance in the data. Roughly, proximities between reservoirs remained comparable to those seen for gene profiles (Panel 1-C). However, the clusters of broilers and farmers and family members in poultry farms became closer to poultry meat the human open population and wild birds. The first component separated reservoirs with high abundance of IncF (e.g. veal calves) from those with higher abundance of IncI1 (e.g. pigs). The second component defined reservoirs with distinct presence of IncK or IncX1 or IncBO.

The PCA for gene proportions from the complete isolate meta-collection (n=3646) together with the plasmid profiles from the 808 isolates yielded proximities between the reservoirs very similar to those in the analysis for genes with the exception of broilers and poultry farming community getting closer to the center of the 2 component loadings (Panel 2-D). The 2 principal components explained 75% of the observed variance. A sensitivity analysis was done using the proportion of genes from the isolates containing plasmid replicon typing and results were similar to those using the complete meta-collection (Appendix Figures S1 and S2).

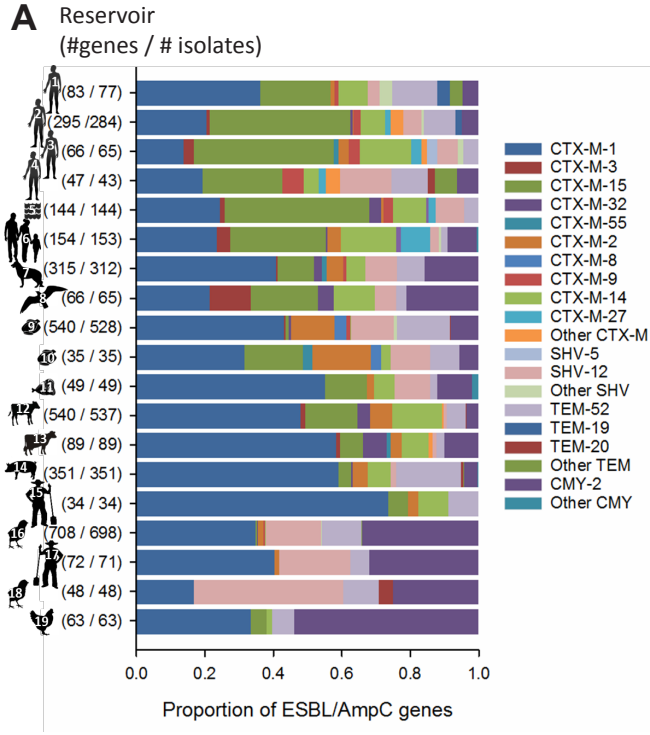
Strain genotypes based on MLST

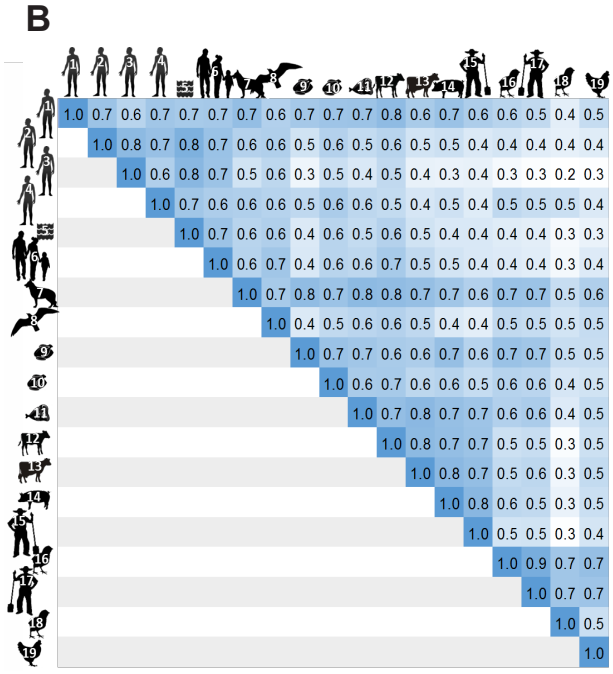
The proportion of *E. coli* isolates containing strain typing (MLST) information was very limited (10% of the total meta-collection) (Figure 4). Thus, we refrained from any analysis at this level further than a mere description of MLST types found in this subset. Differences between reservoirs and diversity of types was very high. ST131 was most commonly found in human UTI followed by isolates from feces from the general population, broilers and poultry farming community; ST10 was present in all reservoirs except for surface and sewage water but it was most common in chicken meat (Figure 4).

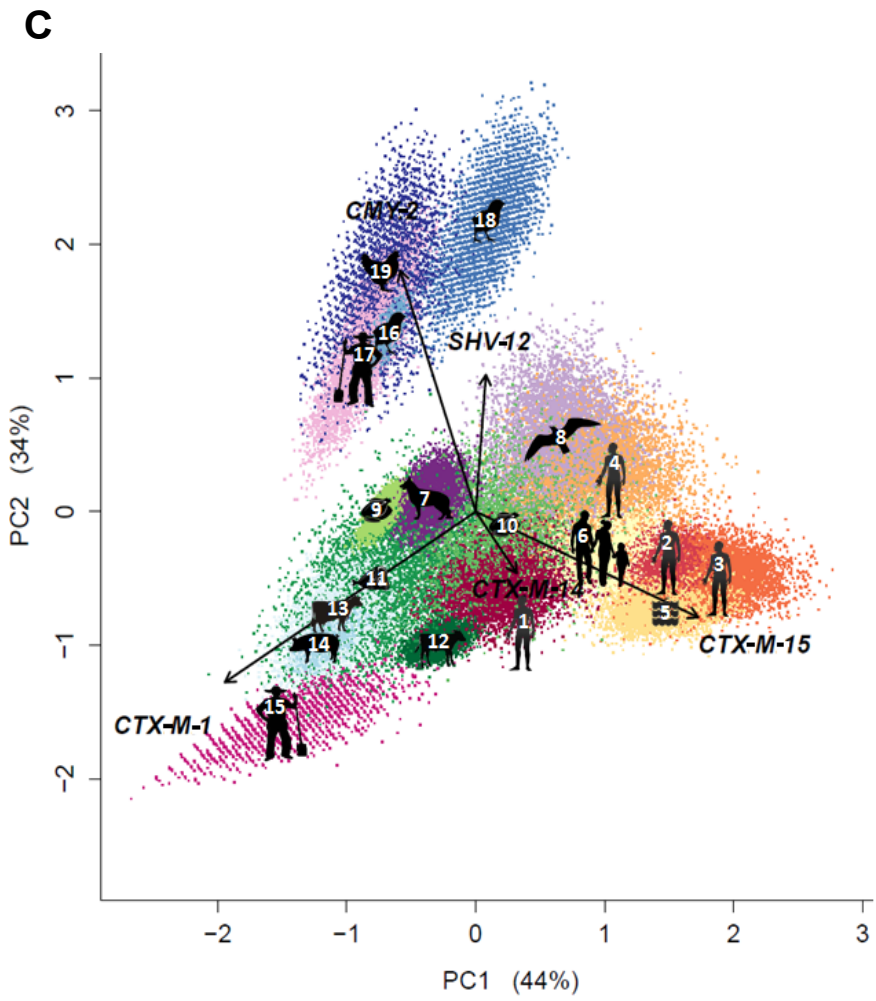
Diversity of gene and plasmid types in the different reservoirs

Rarefaction analysis showed that the human clinical blood and UTI isolates, the human open population, sewage/surface water and dogs had the most diverse pool of ESBL genes (i.e. uppermost rarefaction curves) closely followed by clinical respiratory/ wounds/ other isolates, wild birds veal calves and dairy cattle (~7-9 different genes) (Figure 5). Turkey, chicken and beef meat, pigs and human clinical fecal isolates had in intermediate diversity (~6-7 genes) the lowest diverse gene pool was found in laying hens, broilers and the farming community in direct contact with pigs and poultry (~4 genes).

The diversity in plasmid replicon types across reservoirs with this information available, showed inverse directions in some reservoirs when compared to their ESBL gene richness


A
Reservoir
(#genes / # isolates)
(83 / 77)
(295 /284)
(66 / 65)
(47 / 43)
(144 / 144)
(154 / 153)
(315 / 312)
(66 / 65)
(540 / 528)
(35 / 35)
(49 / 49)
(540 / 537)
(89 / 89)
(351 / 351)
(34 / 34)
(708 / 698)
(72 / 71)
(48 / 48)
(63 / 63)
0.0
0.2
0.4
0.6
0.8
1.0
Proportion of ESBL/AmpC genes
CTX-M-1
CTX-M-3
CTX-M-15
CTX-M-32
CTX-M-55
CTX-M-2
CTX-M-8
CTX-M-9
CTX-M-14
CTX-M-27
Other CTX-M
SHV-5
SHV-12
Other SHV
TEM-52
TEM-19
TEM-20
Other TEM
CMY-2
Other CMY


B
1.0 0.7 0.6 0.7 0.7 0.7 0.7 0.6 0.7 0.7 0.7 0.8 0.6 0.7 0.6 0.6 0.5 0.4 0.5
1.0 0.8 0.7 0.8 0.7 0.6 0.6 0.5 0.6 0.5 0.6 0.5 0.5 0.4 0.4 0.4 0.4 0.4
1.0 0.6 0.8 0.7 0.5 0.6 0.3 0.5 0.4 0.5 0.4 0.3 0.4 0.3 0.3 0.2 0.3
1.0 0.7 0.6 0.6 0.6 0.5 0.6 0.6 0.5 0.4 0.5 0.4 0.5 0.5 0.5 0.4
1.0 0.7 0.6 0.6 0.4 0.6 0.5 0.6 0.5 0.4 0.4 0.4 0.4 0.3 0.3
1.0 0.6 0.7 0.4 0.6 0.6 0.7 0.5 0.5 0.4 0.4 0.4 0.3 0.4
1.0 0.7 0.8 0.7 0.8 0.8 0.7 0.7 0.6 0.7 0.7 0.5 0.6
1.0 0.4 0.5 0.6 0.6 0.5 0.4 0.4 0.5 0.5 0.5 0.5
1.0 0.7 0.7 0.6 0.6 0.7 0.6 0.7 0.7 0.5 0.5
1.0 0.6 0.7 0.6 0.6 0.5 0.6 0.6 0.4 0.5
1.0 0.7 0.8 0.7 0.7 0.6 0.6 0.4 0.5
1.0 0.8 0.7 0.7 0.5 0.5 0.3 0.5
1.0 0.8 0.7 0.5 0.6 0.3 0.5
1.0 0.8 0.6 0.5 0.3 0.5
1.0 0.5 0.5 0.3 0.4
1.0 0.9 0.7 0.7
1.0 0.7 0.7
1.0 0.5
1.0

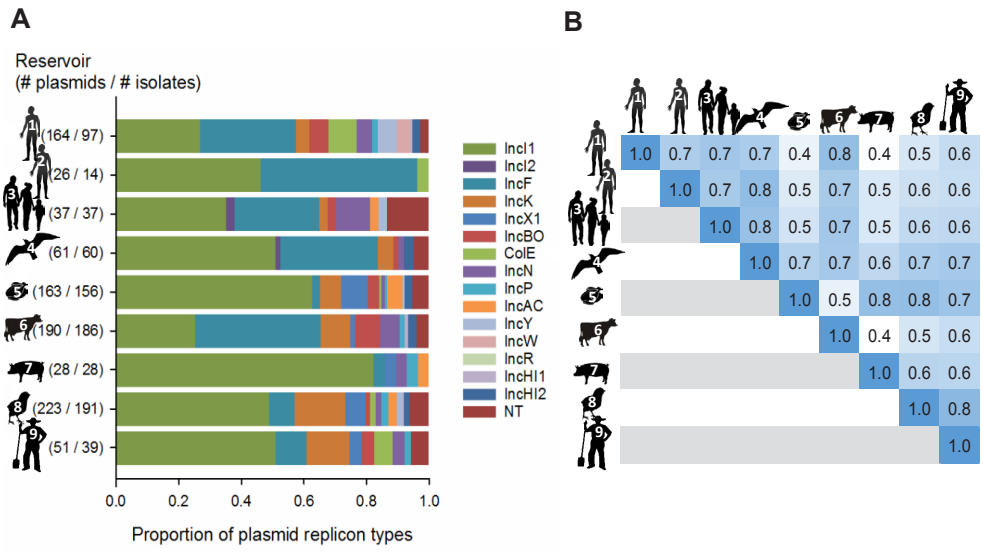


Panel 1. Meta-analysis of ESBL/AmpC gene profiles from 3646 *E. coli* isolates in the Netherlands. Human (H-), animal (A-), food (F-) and the environment (E-) reservoirs represented by silhouettes with the following numbers: 1-H-clinical fecal, 2-H-clinical UTIs, 3-H-clinical blood, 4-H-clinical resp./ wound/other, 5-E-water sewage/surface, 6-H-general population, 7-A-dogs, 8-A-wild bird, 9-F-chicken meat, 10-F-turkey meat, 11-F-beef meat, 12-A-veal calves, 13-A-dairy cattle, 14-A-pig, 15-H-farmers/ family in pig farm, 16-A-broilers, 17-H-farmers/family in broiler farm, 18-E-broiler farm environment, 19-A-laying hens.

A) Proportion of ESBL/AmpC genes over total number of genes found per reservoir.

B) Pairwise PSIs between reservoirs. Cells are shaded gradually according to PSI values (from 0 [no similarity in gene profiles] to 1 [identical profiles]).

C) PCA on the bootstrapped samples of gene relative frequencies per reservoir. Only the most discriminatory genes are plotted (the rest of genes with >0.05% relative frequency in all reservoirs are also included in the analysis). Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.



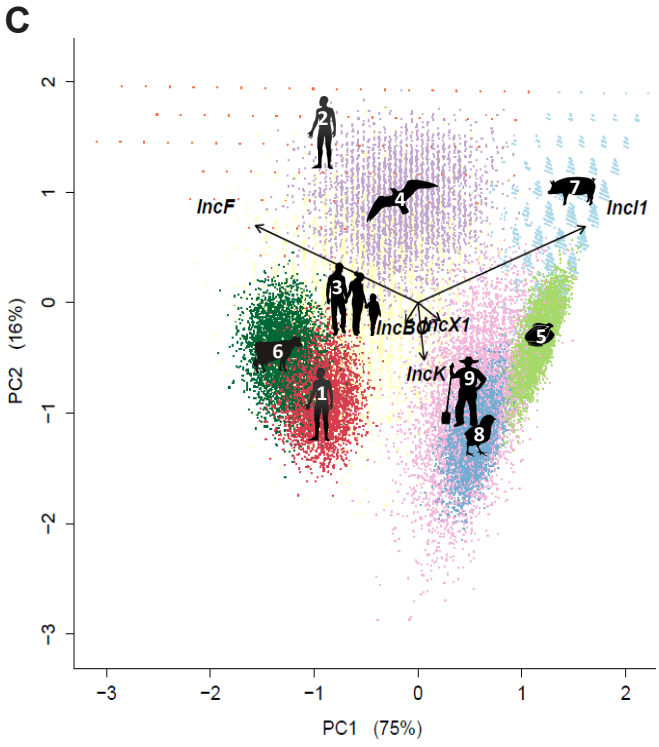
Panel 2. Meta-analysis of plasmid replicon profiles from 808 *E.coli* isolates in the Netherlands. Human (H-), animal (A-), food (F-) and the environment (E-) reservoirs represented by silhouettes with the following numbers: 1-H-clinical UTIs, 2-H-clinical blood, 3-H-general population, 4-A-wild bird, 5-F-chicken meat, 6-A-veal calves, 7-A-pig, 8-A-broilers, 9-H-farmers/family in broiler farm.

A) Proportion of plasmid replicon types over total number of plasmids collated per reservoir.

B) Pairwise PSIs for plasmid replicon types between reservoirs. Cells are shaded gradually according to PSI values (from 0 [no similarity in gene profiles] to 1 [identical profiles])

C) PCA on the bootstrapped samples of plasmid replicon relative frequencies per reservoir. Only the most discriminatory plasmids are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.

D) PCA on the bootstrapped samples of plasmid replicon profiles (from 808 isolates) and of gene profiles (from the complete isolate meta-collection, n=3646) per reservoir. Only the most discriminatory plasmids and genes are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.


C
IncF
IncI1
IncB/orIncX1
IncK
PC2 (16%)
PC1 (75%)

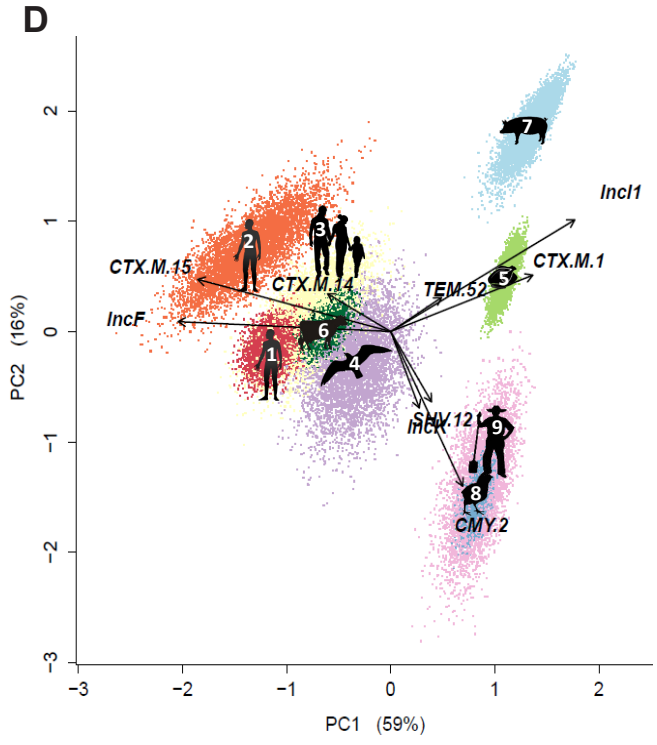
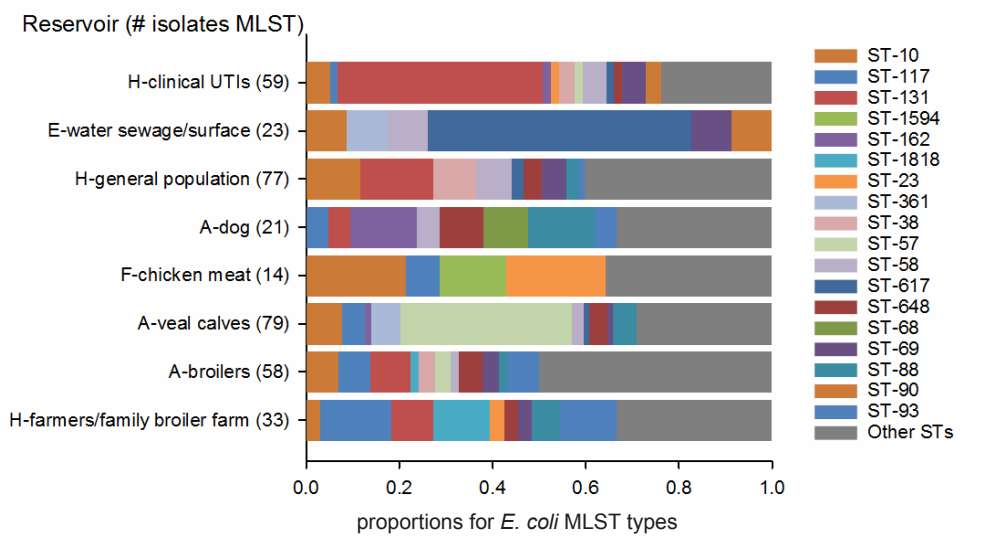

D
IncI1
CTX.M.15
CTX.M.14
CTX.M.1
TEM.52
IncF
SHV.12
IncK
CMY.2
PC2 (16%)
PC1 (59%)

Figure 4. Proportion of ST types per reservoir in the 364 *E. coli* isolates containing strain genotype (MLST) information for a meta-analysis of in the Netherlands.



(Figure 6). The most diverse pool of plasmid replicon types (~6-8 different types) was found in broilers, farming community in contact with poultry, human clinical isolates from UTIs, veal calves, chicken meat and human general population. Turkey meat, pigs, wild birds and clinical fecal isolates had an intermediate diversity (~3-4 types). Finally, beef meat, laying hens and human clinical blood samples showed the lowest diversity (~2-3 types).

Discussion

Our meta-analysis was aimed at providing a visual summary of the knowledge accumulated over the last years on the molecular epidemiology of ESBL-*E. coli* in the Netherlands. By collating information from 27 studies, we gave an overview of similarities between ESBL/AmpC genes and plasmid replicon types harboring these genes in isolates originating from a number of potential human, animal and environmental sources.

In general, we observed a limited similarity in ESBL-*E. coli* gene composition between farm animals and humans in clinical settings or general population. However, this was not the case for farmers and their family members. Broiler and pig reservoirs shared similar gene profiles with people occupationally exposed to these animals[39-41,46,47]. Their distant position in the PCA space from the other human populations gives strong arguments for the notion that directionality of this ESBL transmission mainly occurs from animals to people in close contact with them instead of the other way around.

Interestingly, the centered position of the human general population close to the origin in the PCAs and between the rest of the reservoirs and the human clinical isolates also suggested

Figure 5. Rarefaction analysis for ESBL/AmpC genes in a meta-collection of 3646 *E.coli* isolates from human, animal and environmental reservoirs in the Netherlands.

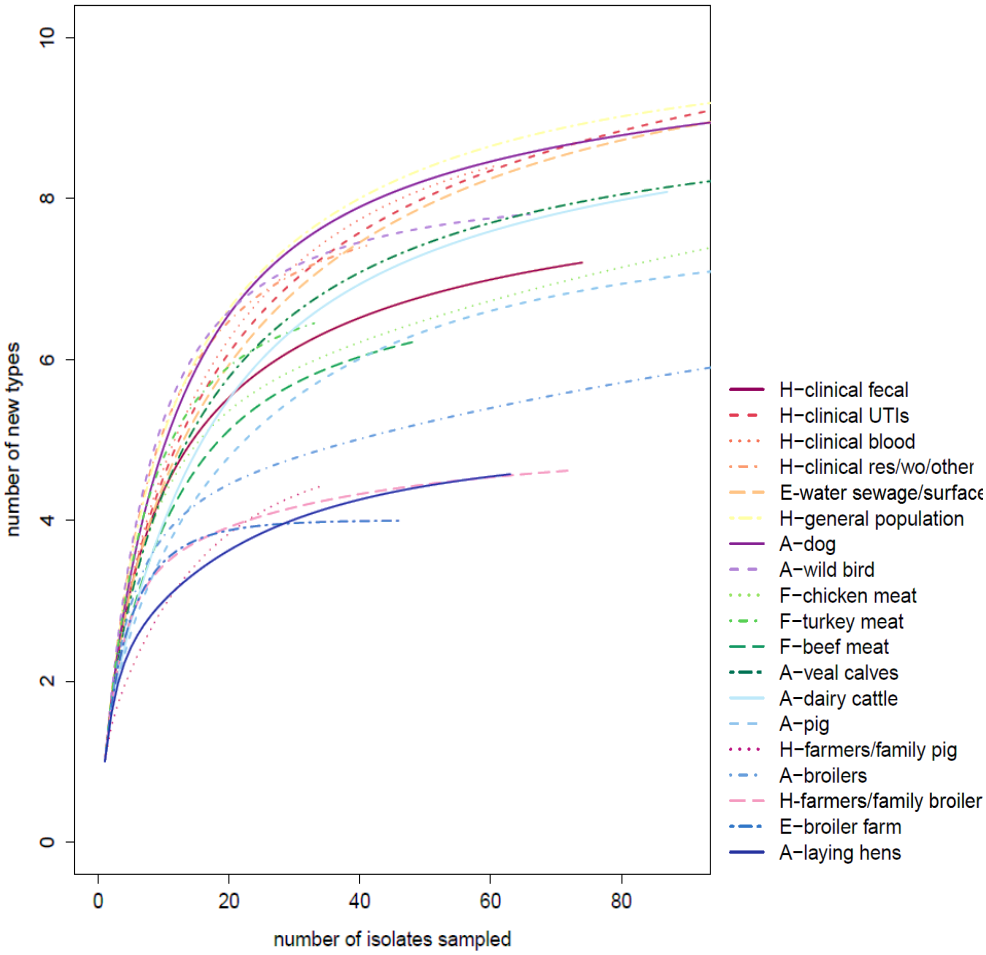
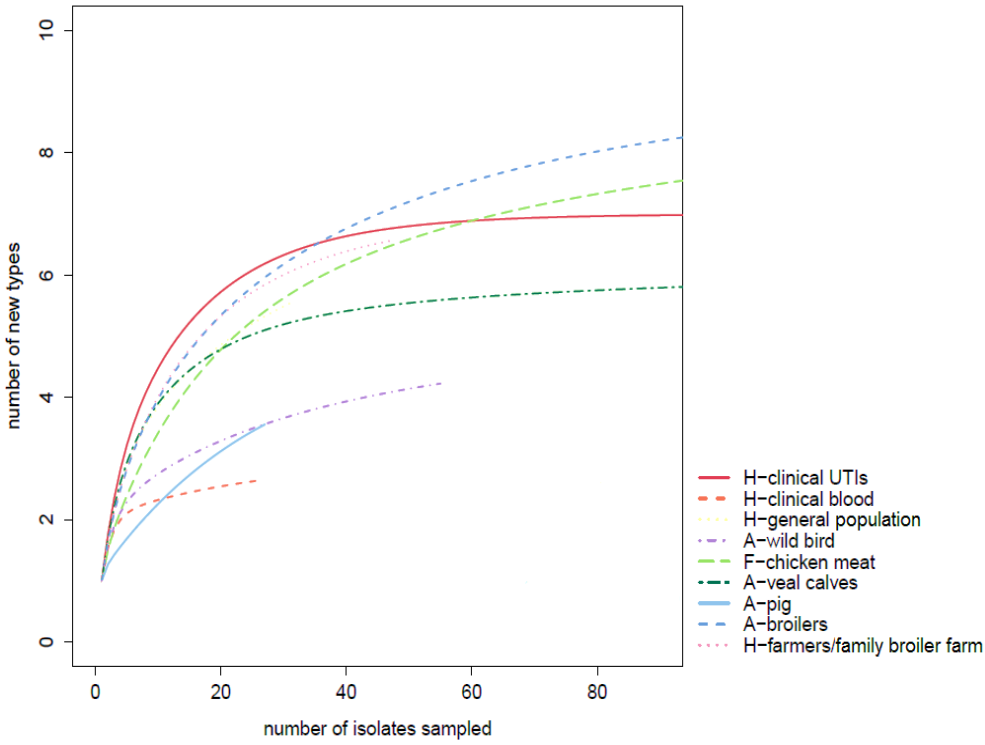


Figure 6. Rarefaction analysis for plasmid replicon types in a meta-collection of 808 *E.coli* isolates from human, animal and environmental reservoirs in the Netherlands.



that most of the transmission events occur in the community, and acquired ESBL resistance is later on introduced in the clinical setting by the human open population.

Given the aforementioned, it is not surprising that additional reservoirs were also sharing many similitudes with human clinical samples. For instance, the surface water samples collected in the study by Blaak et al[34] were influenced by effluent from wastewater treatment plants after human use and thus, ESBL composition was similar to only humans. In contrast, wild birds shared many of the ESBL-*E. coli* molecular characteristics with both animals and humans[51]. Available ESBLs studies in wild birds suggest that they might act as important recipients of human and animal contamination and might contribute to local dissemination[56-60]. These observations are illustrative of the spread of ESBL-*E. coli* to the environment and wildlife beyond the human and domestic animal populations exposed to antimicrobials. Veal calves constituted another reservoir that positioned close to human populations, especially when adding plasmid composition[42,44,45,53-55]. Surprisingly, to our knowledge, no research has been done considering the human community occupationally at risk for this type of animal production.

Many of the studies included in our meta-analysis focused on broilers[32,36,40,46,47,53-55].

However, they constituted a separate cluster far away from human clinical an open populations in terms of ESBL gene and plasmid replicon type profiles. This was due to the distinct abundance of plasmid mediated AmpC genes (mainly bla_{CMY-2}) and IncK replicons but it does not mean that broilers do not constitute a possible reservoir for humans; in fact they showed high proportions of $bla_{CTX-M-1}$ gene and IncI1 replicons that were also very frequent in humans. It was remarkable that chicken meat isolates were always distant from the broiler reservoir; the reason behind this inconsistency is likely found in the different origin of the meat samples imported from different countries such as Brazil or Thailand[53-55]. Future studies should include traceability of meat samples collected at retail level to make more precise epidemiological associations.

Rarefaction analysis allowed us to confirm that the number of isolates collected in most reservoirs was enough to yield correct estimates for their diversity of genes or plasmids (i.e. most rarefaction curves approached a plateau for collected sample sizes)[61,62]. The curves, showed that human clinical and open population samples harbored very rich *E. coli* bacterial populations in terms of ESBL genes and plasmid replicon types. This suggests that human communities are receiving reservoirs from animal populations but it can also be the result of the high antimicrobial selective pressure to which bacteria are exposed in these human communities. Despite the useful information provided by the rarefaction curves we have to note that they should be interpreted cautiously since they do not always give correct estimates of the asymptotic richness[63].

In view of the various study designs and the molecular focus of this work, the general picture we provided did not account for the prevalence levels or frequency of contact between the different reservoirs. These additional aspects are key for future microbial risk assessments. Particularly, 100% of poultry farms and around an 80% of their animals and meat investigated in the Netherlands are ESBL-positive[53-55]. Considering these high prevalences and the frequent human exposure to meat, these reservoirs have received so much attention. Nonetheless, our analysis shows other reservoirs that might be more influential for ESBL transmission to humans and seem to have been underestimated in the last years.

The simple 3–steps methodology we used for the meta-analysis (depiction of proportions, application of PSIs and PCA) has proven to be a powerful tool to visualize a large number of molecular patterns. Moreover, the bootstrapping of observed frequencies, enriched our multivariable analysis and evidenced large uncertainty in some reservoirs with low number of isolates. This is the case for turkey meat, which was positioned in the proximity of human populations but with large uncertainty, thus, requiring careful interpretation.

There are a number of possible limitations to interpret our results and that might affect our conclusions. The most important one is the variation between the sampling periods for the different reservoirs. The complex and dynamic evolution of ESBLs could hamper the temporal link of causation for our associations between some reservoirs. However, the major change in the epidemiology of ESBLs happened with the emergence of the CTX-M group

which happened before the period mainly represented by our meta-collection (2005-2015)[64]. Another limitation of this research arises from the heterogeneity of studies included in terms of designs, objectives and molecular methods; in order to gain statistical power to make comparisons, we did not include more stringent conditions for collating the isolates from selected studies. A small proportion of isolates from some studies were duplicated and we defined the frequencies on base of the total pool of genes and/or plasmids. However, we consider this to have a minor impact on our results since the ratio of isolates per sample and genes/plasmids per isolate were very close to one in most of the reservoirs. Additionally, it is known that the probability of finding more than one gene per isolate is very small[49]. Because of the nature of the collected data, we could not evaluate specific gene-plasmid combinations at the isolate level but we consider the whole pool of genes and plasmid replicons to serve the purposes of representatives for each reservoir. Besides, the number of plasmids per isolate was close to 1 in most reservoirs (except in human clinical isolates from UTI with a ratio of ~1.7), which suggest that most of the plasmid replicon types from our meta-collection harbored ESBL genes.

Conclusions

In view of the epidemiological snapshot on the ESBL-*E. coli* epidemiology given by this work, there is a limited, but still existing, molecular proximity between most of animal reservoirs and humans in the open population and clinical settings. Nonetheless, people in direct contact with livestock shares practically the same pool of ESBL genes and plasmid replicon types with the animals to which they are exposed; this illustrates that transmission between different populations occurs and is highly dependent on exposure intensity. Other potential ESBL sources not directly exposed to antimicrobial selective pressures (e.g. wild birds, water influenced by human activity), were closely related to human populations suggesting that ESBL transmission events and dissemination go beyond humans and domesticated animals to the greater environment and wildlife. The most frequently studied reservoir was poultry and poultry meat, but they shared a more limited similarity with humans in comparison with other reservoirs much less studied (e.g. pigs and veal calves). The methodology used in our meta-analysis was proven to be useful to simplify the complex epidemiological investigations on attributable risks of ESBL transmission to humans. Until higher molecular resolution techniques with more discriminatory power become available at larger scales, these results should encourage to perform similar studies.

Addendum

The work presented here is still in progress and results should not be taken as final. There is still work to do to clean the database and eliminate the possibility of some non-ESBL carrying plasmids in human isolates from UTIs. Moreover, more studies will be incorporated in the coming months and the systematic search and quality assessment of the publications will be done independently by a different person to duplicate the data extraction and accuracy of our database.

Footnotes

* Both authors have equally contributed to this work.

Preliminary results from this study were presented at the International Conference on One Health Antimicrobial Resistance, 30 September - 2 October 2015, Copenhagen, Denmark; poster presentation title: *A meta-collection of Escherichia coli isolates to evaluate distribution patterns of ESBL/AmpC genes and plasmids between different sources.*

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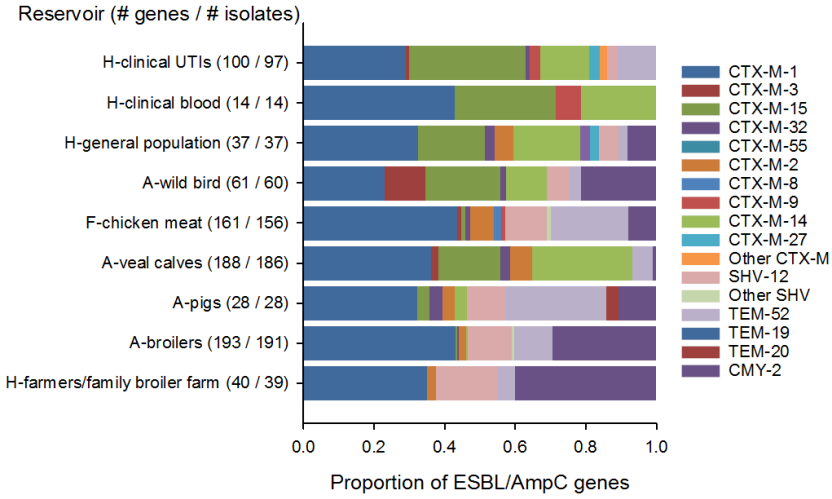
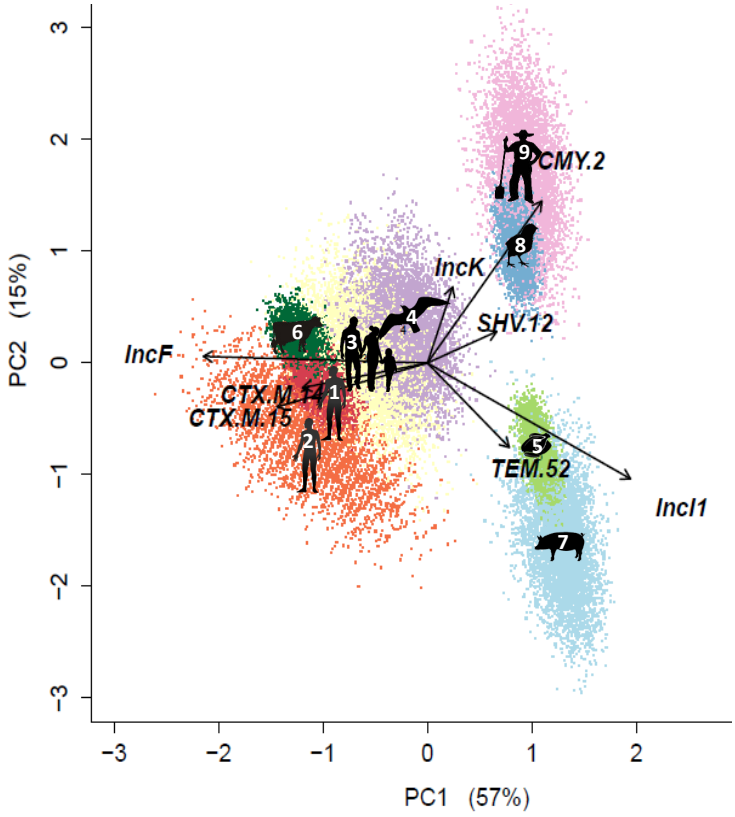
Transparency declarations

None to declare

Supporting material

Appendix figure 1. Proportion of ESBL/AmpC genes per reservoir in the 808 *E. coli* isolates containing plasmid replicon typing information for a meta-analysis of in the Netherlands.





Appendix figure 2. PCA on the bootstrapped samples of plasmid replicon and gene profiles from a meta-collection of 808 *E. coli* isolates in the Netherlands. Human (H-), animal (A-), food (F-) and the environment (E-) reservoirs represented by silhouettes with the following numbers: 1-H-clinical UTIs, 2-H-clinical blood, 3-H-general population, 4-A-wild bird, 5-F-chicken meat, 6-A-veal calves, 7-A-pig, 8-A-broilers, 9-H-farmers/family in broiler farm. Only the most discriminatory plasmids and genes are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.

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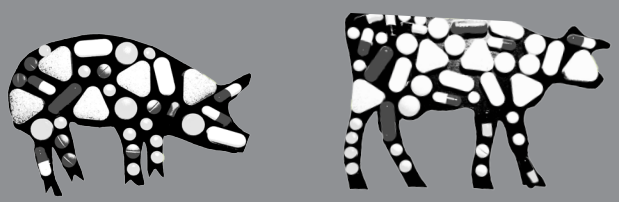
Part V

Concluding remarks



Chapter 8

General discussion

"The future of humanity and microbes likely will unfold as episodes of a suspense thriller that could be titled Our Wits Versus Their Genes"

Joshua Lederber
Infectious History, Science. 14 April 2000

Introduction

The recent emergence of MRSA and ESBLs in livestock represents a complex public health dilemma. There is clear evidence for transfer of antimicrobial resistance (AMR) from animals to humans but the exact burden for human health originated from this transmission is unknown. As a result, current risk management is a balancing act between sensible pre-emptive measures and direct interventions from the perspective of the precautionary principle.

Since the first years of the epidemic expansion of LA-MRSA, many exploratory observational and cross-sectional studies have been performed to identify epidemiological determinants for MRSA in the animal-human interface[1-6]. Control measures in Dutch healthcare settings have kept the cases of human MRSA infections at a minimal level[7]. Livestock-associated clones are perceived to be under control since they are not very virulent and have a considerably lower transmission rate than hospital strains of MRSA[1]. Thus, risks associated with ESBLs in animals and food products have recently received more attention. The epidemiology of these two types of antimicrobial resistance differs in many aspects and approaches to tackle their occurrence and spread likely require different approaches.

The interest in reducing antimicrobial resistance in the Netherlands has led to a series of longitudinal and intervention studies. The studies presented in this thesis are a reflection of these advancements in the field. Moreover, large-scale, sector wide, interventions aimed at reducing the veterinary use of antimicrobials have turned the Dutch livestock production sectors into a natural experiment, which results are starting to be evaluated. Currently, two important developments are observed that will have an important impact on future research: the incorporation of human, animal and environmental components in 'One Health' approaches and the rapid development of new molecular tools for microbiological investigation. These advancements constitute a way forward to strengthen the level of evidence and to inform rational risk management strategies in the future.

In this chapter the relative contribution of animals to the overall AMR public health burden in humans will be discussed. Possible interventions and their limitations will be elaborated on. The crucial role of AMR surveillance programs for evaluation of large-scale interventions will be highlighted. Some future research lines will be proposed addressing the major unanswered questions. Finally, the notable contributions of the Netherlands to this research field are put in a global perspective.

The public health hazard posed by AMR in food-producing animals

Although the origin of most of the resistant bacterial infections in humans might be found, directly or indirectly, in the human use of antimicrobials[8], we should not disregard that bacteria are adapted to live in various ecological niches (skin, lumen of gut, water, soil, etc.) where they are also exposed to antimicrobial selective pressures. In the interface between these niches, transmission of resistance can occur. Based on evidence, many experts argue that veterinary use of antimicrobials is responsible for at least a proportion of infections

caused by resistant organisms in humans[9-16]. Thus, containing increasing AMR threats by just focusing on humans, without the integration of the animal and environmental domains, will likely be a too simplistic approach for a sustainable solution to the resistance problem[17-20].

However, attributing infections in humans with resistant bacteria to the veterinary use of antimicrobials is still a matter of great controversy[11,16,21,22]. Risks emanating from animal reservoirs have been estimated to be from negligible to substantial and even major[20,23]. The reasons for this discrepancy can be found in the complex epidemiology of the emerging antimicrobial resistant threats that complicate the quantification of exact source-attributed risks. Public health problems related with antimicrobial resistance in food-producing animals were classically associated with food-borne pathogens (*Salmonella* and *Campylobacter*). For these pathogens, microbial risk assessments traditionally follow a "simple" unidirectional farm-to-fork pathway[24]. In the last decade, *Staphylococcus* (colonizing skin and nose of humans) and commensal Enterobacteriaceae (e.g. *E. coli)* have come under the resistance spotlight[25-27]. The latter organisms are part of the normal flora in humans and animals and they are among the most important nosocomial pathogens (MRSA and ESBLs-producing bacteria). Consequently, they circulate in open populations and increase the burden of human infections in clinical settings. The epidemiology of these microorganisms has an extra level of complexity and especially for ESBLs, it involves more bidirectional pathways between human, animals and the environment. The current data gaps explain the lack of available quantitative microbial risk assessments (Q-MRAs) for these resistances that would be of great help for informed risk management[19,21,24,28,29].

The case of methicillin-resistant *Staphylococcus aureus* (MRSA) in animals is relatively straightforward and illustrative of the zoonotic capacity of AMR traits. *Staphylococcus* in animals developed resistance in response to the antimicrobial selective pressure and LA-MRSA clones rapidly spread among animal populations, most likely helped by the common trade of animals[30-32]. Since 2005 transmission from livestock to humans started to be documented and occupationally exposed risk groups were identified[33,34]. There is plentiful evidence of a causal relation between MRSA in livestock and in human isolates[5,35-37]. The main reasons for this level of evidence are: i) the presence of well identified animal MRSA clones, ii) the vertical transfer of resistance (located in the bacterial chromosome), iii) the direct animal contact as main route of transmission to humans. Moreover, transmission through the food chain is not considered a public health risk[38]. Consequently, quantification of the impact of LA-MRSA on humans is relatively easy. In the Netherlands, LA-MRSA accounts for approximately 40% of all new MRSA detected mainly through screening of patients and occurrence of serious LA-MRSA infections is very rare[39]. Human-to-human LA-MRSA transmission in hospital settings has been found to be lower than for non-LA-MRSA suggesting that LA-MRSA infection control practices could be less stringent[23,40-43]. Contrarily, recent studies in the Netherlands and Denmark have shown that, in the community, LA-MRSA is maintained and transmitted[44,45]. What is clear is that as long as LA-MRSA is circulating in animals there

will be the chance for transmission events to occur outside farming communities[46]. Moreover, recent findings show the plasticity of MRSA for host adaptation and the emergence of novel resistance genes; a phylogenetic study has indicated that methicillin-susceptible *S. aureus* from humans crossed species and was the origin of the current LA-MRSA clade in farm animals[47]; transfer of mobile genetic elements has been also observed between different LA-MRSA strains[48,49], and recently a novel *mecA* homologue gene, called *mecC* has emerged in MRSA from animals and humans[19,43,50]. All things considered, a close surveillance on LA-MRSA is indispensable for identification of new threats and/or possible epidemiological changes.

For ESBLs it is important to realize that Enterobacteriaceae are present in large numbers in the intestine of animals and humans. In addition to the clonal expansion of resistance, they readily spread their resistance genes horizontally, also between other bacterial species that include pathogenic bacteria (e.g. *Klebsiella* spp.)[24]. ESBL genes are elusive and ubiquitous outlining a risk profile more complex than LA-MRSA. Based on geographical, temporal and genetic associations there is sufficient evidence to establish a link between human isolates and isolates from food animals and animal derived products like meat[16,23,25]. Main transmission routes are direct contact with animals and handling and/or consumption of raw or undercooked meat products, but other routes, including environmental, are also hypothesized as suggested in our meta-analyses on ESBLs. Given this complex epidemiological profile, the level of evidence for quantification of human infections attributed to ESBL-producing bacteria in animals is still quite limited and we are still in the early stages of this process.

In conclusion, despite the difficulties for measuring human risks originated from antimicrobial resistance in animals, there is sufficient scientific evidence to support the interweaving of animal and human bacterial populations[9-16].

Evidence-based interventions to control antimicrobial resistance in livestock

Scope and context

Interventions in the animal domain to contain the transmission of resistant bacteria or genes to humans, seems to be a rational risk-management strategy[30]. Participation of veterinarians and agricultural societies as part of the solution for the global AMR problem, was already highlighted in the WHO's "Global Strategy for Containment of Antimicrobial Resistance"[51-55].

In the process of generating evidence for controlling AMR in livestock it is essential to understand the mechanisms and drivers of resistance. From a theoretical point of view, interventions could act: i) on absolute numbers of resistant bacteria (e.g. reducing selective pressure, host colonization or environmental colonization), ii) on transmission of resistance (e.g. reducing host-to-host and water and food-borne transfer) and iii) on ecology and bio-remediation of resistance (e.g. selecting for susceptible bacterial populations, early

establishment of susceptible microbiota in animal and human newborns, decreasing the horizontal gene transfer)[55]. Additionally, interventions could also be classified by their scope of action, from the individual bacterium or animal to their populations.

Currently, only large-scale interventions directed to the veterinary use of antimicrobials have been applied in Europe (e.g. ban in the use of growth promoters in 2006 and country-specific programs to reduce antimicrobials as a whole or restrict critically important ones)[56-64]. Yet, there are no specific control programs at farms for LA-MRSA or ESBL-producing bacteria. Having in mind the structure and functioning of animal production, it is understandable that AMR control options for individual animals (e.g. decontamination of colonized animals) are very limited and logistically difficult to apply but at farm-level, there is more room for feasible and effective interventions. Farm level interventions such as the ones presented in this thesis should be further explored and included in potential AMR control programs together with the larger scale initiatives for reduction in antimicrobial use.

All the studies included in this thesis, were performed under the context of drastic reductions of antimicrobial use in animal husbandry. The different chapters demonstrate that spread and transmission of AMR in farming sectors can be influenced by changes in use of antimicrobials (i.e. antimicrobial interventions) and/or by changes in other determinants (non-antimicrobial interventions) at the farm level or at a wider sector-level. Options, limitations and considerations for these interventions are discussed in the following sections.

Antimicrobial based interventions

The extensive use of antimicrobials in animal husbandry is well documented as the most important driver for selection and propagation of resistant bacteria from food animals to humans[65]. However, this causal pathway does not always follow a straight line and is influenced by several factors which are yet not fully understood[66]. So far, many countries in Europe have been the testing ground for a series of antimicrobial based large-scale interventions generally successful but with mixed results. For instance, studies after the ban in growth promotor avoparcin showed that level of glycopeptide-resistant enterococci were significantly decreased in Denmark while remained high in Norway[67,68]. Similarly, in the UK, the prevalence of tetracycline-resistant *E. coli* in pigs remained very high several years after the ban in the non-therapeutic use of tetracycline in 1971[69].

The effects of changing the veterinary use of antimicrobials in bacterial resistance have still many data limitations but this thesis significantly contributed to disentangle a true influence of antimicrobials in AMR. Our observations suggest that prevalence of LA-MRSA in veal calves, pigs and farmers and ESBLs in pigs can potentially be reduced at the farm level by a general reduction in the veterinary use of antimicrobials. Reduction of specific antimicrobial classes, such as cephalosporins was shown to be of special importance for reducing LA-MRSA and ESBL-*E. coli* levels. The macro effects of antimicrobial use reductions evaluated at national level showed that the decrease in resistance for commensal

E.coli greatly differed by animal sectors. Underlying all these mixed effects resulting from the reduction in antimicrobial use, there were a set of non-antimicrobial determinants (discussed in the following section) and other antimicrobial resistance interactions (e.g. co- and cross-resistance selection, perpetuation of resistance to long used drugs) that will need special attention in future studies.

Other aspects need to be considered when designing and applying interventions that target antimicrobial use. It is a general belief that antimicrobial resistance implies fitness costs for bacteria (i.e. bacteria are less able to grow or to infect)[51]. Based on this assumption the prevalence of resistance is expected to be easily reverted by removing the selecting pressure of antimicrobials. Unfortunately, some resistant bacteria preserve their wild-type fitness, thus maintaining high levels in the absence of selective pressure[70-72]. A constrain for the evaluation of these interventions is the scarce level of knowledge on the dose-response relationship between antimicrobial use and resistance. Background resistance levels (i.e. levels existing even in absence of antimicrobial selective pressure) should be explored and resistance thresholds (i.e. antimicrobial use volumes from which resistance development starts) should be established[73]. The antimicrobial use-resistance dose-response relationships may vary depending on the bacterial specie as a reflection of its representativeness for other bacterial populations and its epidemiology[74]. This is illustrated in this thesis in which we describe different effect sizes of the antimicrobial use in LA-MRSA and ESBL-*E. coli*.

Although there is limited evidence of sufficient scientific quality, a recent review showed that no clear indications exist that farm technical results, animal health and welfare are being affected by reductions in use of antimicrobials[75]. This has also been shown by Danish data after the ban in growth promoters[76,77] and it is also suggested in the evaluation of technical parameters in our intervention study among veal calves[78]. Nonetheless, undesired effects can be derived from the antimicrobial restrictions, such as the increase in other antimicrobial uses or modest increases in infections or mortality rate[62]. These "side effects" need complementary monitoring but eventually should be overcome by stringent farm health plans improving vaccination coverage, hygiene and biosecurity[27].

Non-antimicrobial based interventions

Results presented in this thesis show that the veterinary use of antimicrobials was not always clearly related to resistance in livestock[78,79]. Other determinants could explain the lack of an association. Risk factor analyses have been previously performed serving as the starting point for conceptualization of some of the non-antimicrobial interventions[11,80-87]. In this thesis several other risk factors for LA-MRSA and ESBL carriage in veal calves, pigs and the farming community were identified. They indicated that some of the current infection control measures, including farm biosecurity, might be inefficient for controlling AMR. Transmission between farms plays a crucial role for LA-MRSA dissemination both in pigs and veal calves; potential measures addressing this point could be the improvement of sanitary

measures during transport or, in countries with low LA-MRSA prevalence, the limitation in purchases of animals from MRSA-positive herds. LA-MRSA and ESBL-*E. coli* within-farm transmission could be prevented by strengthening the level of farm compartmentalization, avoiding circulation of possible vectors (e.g. free-ranging farm cats or vermin, rats and mice), or avoiding movements between different groups of animals[78,79,89].

Hygiene measures such as cleaning and disinfecting of farms are thought to kill bacteria and avoid within-farm transmission but in the studies presented in this thesis they were often related with increasing carriage of resistant bacteria[78,79]. Transmission through air, or possibly transmission by farmers or personnel, are possible routes of bacterial dissemination that have not often been considered in detail[88]. Thorough cleaning and disinfection could favor the LA-MRSA airborne transmission leading to the observed counterproductive effects at least in the short term[78,89].

The presence of other non-antimicrobial selective pressures have to be taken into account. This is the case for substances co-selecting for resistance genes as it has been described with zinc (used as a dietary supplement for livestock) and quaternary ammonium compounds (common ingredients of disinfectants). High levels of these substances have been suggested as important drivers for the selection and persistence of MRSA in pig farming[90-95]. Some observations in this thesis might also be explained by these types of selective pressures[78,79].

As a whole, evidence for some non-antimicrobial interventions is still limited but for LA-MRSA, a control program could in principle be developed and executed. Some sanitary measures such as culling, decontamination, vaccination or regulation of animal trade based on LA-MRSA farm status could be conceptually applied based on other disease eradication programs. However, given the high proportion of LA-MRSA-positive farms in the Netherlands and in many other countries, the economic cost of some of these measures would make them unfeasible. Further evaluation of other farm measures is warranted in future studies.

Antimicrobial resistance and antimicrobial use surveillance systems for assessment of interventions

The establishment of comprehensive, harmonized and effective AMR surveillance programs in the animal and human domains is the cornerstone for assessing the magnitude of the AMR problem. Monitoring the occurrence and development of resistance has been proven to be essential to generate evidence for designing interventions and assessing their effectiveness (chapter 6)[52,54]. Currently different programs have been successfully implemented in many countries (e.g. NARMS in the USA, JARM in Japan, DANMAP in Denmark, MARAN in the Netherlands)[96] but there is an obvious global imbalance in terms of design, quality, objectives and communication of derived information[97]. AMR surveillance should move towards harmonized systems allowing comparison between countries. For such comparison it is necessary to establish common methodological frameworks in terms of microbiological susceptibility testing (e.g. EUCAST in Europe), sampling techniques and harmonized

measures for antimicrobial use (e.g. Defined Daily Dose Animal [DDDA] or similar unified measure that corrects for treatable animal weights)[98].

Currently, most industrialized countries have national AMR systems, but there are many data gaps and biases that could be corrected. The most obvious data gap is the generalized lack of access to antimicrobial prescription data by animal species at the national level. Only a few countries in the world (including Denmark and the Netherlands) have this information, which is essential for evaluation of nationwide antimicrobial interventions. An important limitation is also the lack of contextual information, which makes difficult to disentangle the effects of antimicrobial interventions from the interplay of other determinants. Big data type of approaches could be explored in national databases on AMU when contextual information is available. Collecting extra information longitudinally and at national level about relevant influential factors (e.g. organic/conventional farming, levels of biosecurity or hygiene, animal trade movements) could help to identify shifts in composition or management of livestock sectors (derived or not from non-antimicrobial interventions). Moreover, once the ongoing large-scale antimicrobial interventions in livestock reach their limit for antimicrobial resistance reduction, the effect of additional interventions will need to be evaluated; having readily available systems also tracking sectorial changes in livestock would be of great value in future surveillance programs.

Future research perspectives

Avoiding the emergence, spread and maintenance of antimicrobial resistance in general, and LA-MRSA and ESBL-*E. coli* in particular, in the animal reservoir still presents numerous challenges to overcome. Among some of the most critical points to address in the coming years are: the prompt identification and containment of new emerging resistance risks; the identification, implementation and evaluation of local, national or global interventions; and the quantification of risks for human health attributed to livestock and other potential sources such as the environment. For answering all questions, the field is rapidly changing towards One Health approaches, engaging not only human and veterinary public health experts, but a wide range of actors (e.g. farmers, policy makers, environmental and social scientists)[98].

In addition to the key role of the surveillance systems, epidemiological field studies will always be needed. Observational studies can identify new resistance threats and risk factors for resistance but more experimental designs are required in future research to properly evaluate the effect of local interventions. Furthermore, in the assessment of human risks derived from different animal or environmental reservoirs, there are still great data limitations, especially for ESBLs; new studies could be specially designed to estimate and quantify parameters as input in mathematical modelling such as bacterial populations sizes or horizontal gene transfer rates in different ecosystems and hosts.

At the microbiological level, significant steps are also being taken to study antimicrobial resistance from a different and widened perspective. More and more research projects are

now investigating the whole microbiota and its resistome (i.e. the collection of antibiotic resistance genes and their precursors in bacteria). Apart of the metagenomics approach, new sequenced-based or functional metagenomics are now technically available and envisage expanding our knowledge on the interplay of resistance genes between different bacterial populations[99,100]. New statistical and computational biology tools will need development to decipher what is behind the data generated by metagenomics studies.

Epilogue

The evidence supporting that transmission of antimicrobial resistance from food-animals occurs and has an impact on human health, although still incomplete, is currently sufficient to begin to intervene in this problem. The Netherlands is a successful example of how a substantial reduction in veterinary use of antimicrobials is possible and sustainable. Modern animal production systems could stop depending on routine antimicrobial use by relying in proper diagnostics and well-designed farm health programs. Despite the current knowledge, global antimicrobial use in animals is projected to dramatically increase in the coming years, especially in middle-income countries[101]. Given the way resources are used in different parts of the world and the complexity of the problem it is not strange to compare the current AMR threat with the climate change problematic[102].

In this scenario of constant human and animal movement, increasing veterinary use of antimicrobials and a plummeted number of new antimicrobials in the development pipeline, global actions are needed to tackle the rising AMR. In addition to the significant contributions of individual countries, international and interdisciplinary One Health approaches need to be strengthened. Only this way we will understand the specific barriers and opportunities for intervention and management of risks derived from emerging antimicrobial resistances in food-animals.

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Summary
(in English, Dutch and Spanish)

English Summary

The use and misuse of antimicrobials during the last decades have increasingly generated resistant bacteria. Currently this is one of the greatest human health threats at a global scale. Once-treatable infections are becoming difficult to cure leading to increased economic and social costs. Antibiotics have also been extensively used in animal production and resistant bacteria have emerged in livestock. There are no geographical or biological borders for antimicrobial resistance (AMR) and its transmission from animals to humans can involve different direct, and indirect routes such as the food chain or the environment.

This thesis explores the animal-human interface of the emerging AMR problem. It focuses on two relevant bacterial species which are found in animals and impose a burden for human health: methicillin-resistant *Staphylococcus aureus* (MRSA) and (extended-spectrum beta-lactamase (ESBL)/AmpC-producing) *Escherichia coli*. Over recent years, several exploratory studies have been performed in this field, but due to their observational and cross-sectional nature, causality of the epidemiological associations was often difficult to be established. This work strengthens the level of evidence on the relation between veterinary antimicrobial use (vAMU) and AMR in animals and humans by relying on longitudinal studies and quantitative vAMU data. Two intervention studies in veal calves, pigs, and their farmers and family members are presented together with the evaluation of a country-wide vAMU reduction. Additionally, a meta-analysis on molecular characteristics of ESBL/AmpC-producing *E. coli* in different reservoirs is put in perspective of the current knowledge.

Part I presents a 3-arm intervention study aimed at evaluating strategies to reduce livestock-associated (LA)-MRSA in veal calf farming. Results show that farm environments contaminated with MRSA might enhance the acquisition of LA-MRSA through airborne transmission. Contrary to expectations, farms applying a protocol for cleaning and disinfection of stables had transitory increased levels of LA-MRSA in the air together with higher LA-MRSA carriage in veal calves and farmers. More stringent farm biosecurity conditions (e.g. absence of free-ranging cats, presence of changing room) were associated with less probability of LA-MRSA carriage in animals and humans. Farms at which only vAMU was reduced showed a decreased carriage of LA-MRSA in animals and humans over time, in contrast to farms in the control arm or farms which applied the cleaning and disinfection program.

Part II presents a longitudinal field study in which pragmatic interventions were applied to reduce LA-MRSA and ESBL-producing *E. coli* in pig farming. Most farm management practices did not significantly change during the study period thus, dynamics of resistance attributed to management changes could not be evaluated. However, vAMU decreased 44% during the study period as a result of the Dutch policies in place. LA-MRSA in pigs slightly decreased, while ESBL-*E. coli* carriage showed a more substantial drop. Lower LA-MRSA and ESBL-*E. coli* carriage was positively related to decreasing vAMU. Incidental use of cephalosporins was the most striking risk factor for LA-MRSA and ESBL-*E. coli*

carriage in pigs. vAMU in animals was shown to be a risk factor for LA-MRSA in farmers regardless the level of animal contact, which points at direct exposure to antimicrobials as an occupational hazard. A risk factor analysis provided evidence of higher probability of AMR in farms receiving external supply of animals from other premises, in farms with less stringent biosecurity conditions (e.g. hygiene lock not used as the exclusive entrance in the farm), and in farms with less rigid animal contact structures (e.g. sows not housed in stable groups).

Part III illustrates the importance of having exhaustive antimicrobial resistance monitoring systems in the animal production domain. Through an ecological study, trends in vAMU and AMR in commensal *E. coli* are evaluated in the main animal sectors over a 10 year-period in the Netherlands. The nationwide vAMU reduction was associated to decreasing *E. coli* resistance levels for most antimicrobial drugs, especially in the veal calf and pig sectors. The study highlights that the historical use of antimicrobials and the co-resistance selection are key elements for perpetuation of this AMR problem.

Finally, in Part IV, a meta-collection of 3646 isolates is created to identify reservoirs or sources that may contribute to increase ESBL/AmpC-producing *E. coli* infections and carriage in humans. Gene and plasmid replicon typing data from 27 Dutch studies were collected and the dimensionality of the data was reduced by principal component analyses. Results showed an existing, but limited, *E. coli* molecular similarity between most animal reservoirs, and the open human population and patients in clinical settings. ESBL/AmpC-producing *E. coli* in human farming communities (i.e. in direct contact with livestock) shared basically the same molecular profiles with bacteria isolated from their animals, and this suggested high transmission between these reservoirs. ESBL/AmpC-producing *E. coli* from wild birds, surface water influenced by treatment plants and from human populations were molecularly closely related. Thus, transmission and dissemination of resistance seemed to occur also beyond humans and domesticated animals, without apparent direct selective pressure to antimicrobials.

This thesis calls for future field studies with longer follow up periods and controlled interventions to set strategies containing the animal-human transmission of AMR. Results from the nationwide vAMU reduction should encourage public health authorities and animal sectors to maintain their efforts for prudent vAMU. Considering the multiple reservoirs and sources of possible transmission to humans, the AMR problem needs a holistic and multidisciplinary “One Health” approach.

Nederlandse samenvatting (Summary in Dutch)

Door het gebruik en misbruik van antimicrobiële middelen is de laatste jaren het aantal resistente bacteriën enorm toegenomen. Op dit moment is het een van de grootste wereldwijde humane gezondheidsrisico's. Infecties die voorheen goed te behandelen waren, zijn nu lastiger te genezen met daarbij behorende economische en maatschappelijke gevolgen. Antibiotica worden ook uitgebreid toegepast in de veeteelt en hier zijn meer en meer resistente bacteriën ontstaan. Er zijn geen geografische of biologische grenzen voor antimicrobiële resistentie. Daarnaast kan de overdracht van dier op mens zowel direct als indirect plaatsvinden, bijvoorbeeld via de voedselketen of de omgeving.

Dit proefschrift richt zich op het probleem van antimicrobiële resistentie op het grensvlak van mens en dier. De nadruk wordt gelegd op twee relevante bacteriesoorten die dieren bij zich kunnen dragen en een grote last vormen voor de humane gezondheid: methicillin-resistant *Staphylococcus aureus* (MRSA) en (extended-spectrum beta-lactamase (ESBL)/AmpC-producerende *Escherichia coli*. In de afgelopen jaren zijn er verschillende exploratieve studies gedaan op dit gebied. Doordat dit veelal observationeel onderzoek was, met een cross-sectionele opzet, is het lastig causale verbanden aan te tonen. Het hier beschreven onderzoek beschrijft de relatie tussen het veterinair gebruik van antimicrobiële middelen en antimicrobiële resistentie bij mens en dier door gebruik te maken van longitudinale studies en kwantitatieve antibiotica data. Twee interventies studies zijn uitgevoerd op kalver- en varkensbedrijven, waarin zowel veehouders, werknemers en gezinsleden als dieren zijn betrokken. Deze onderzoeken hebben plaatsgevonden tijdens een landelijke afname van antibioticagebruik in de veehouderij. Tevens is een meta-analyse uitgevoerd naar moleculaire eigenschappen van ESBL/AmpC-producerende *E. coli* afkomstig uit verschillende bronnen.

In deel I wordt een interventie-onderzoek met drie takken in de kalverhouderij gepresenteerd met als doel te achterhalen welke strategieën leiden tot een vermindering van veeteelt gerelateerde MRSA (LA-MRSA). Wanneer MRSA aanwezig is in de omgeving op een kalverbedrijf, zou dit kunnen leiden tot overdracht van LA-MRSA via de lucht. Op kalverbedrijven waar een schoonmaak- en ontsmettingsprotocol voor de stallen werd toegepast werd, tegen de verwachting in, een tijdelijke toename van LA-MRSA in de lucht en LA-MRSA-dragerschap bij kalveren en veehouders waargenomen. Meer strikte biosecurity-maatregelen (zoals bijvoorbeeld geen loslopende katten, aanwezigheid van een omkleedruimte) waren geassocieerd met een lagere kans op LA-MRSA-dragerschap bij mensen en kalveren. Op kalverbedrijven waar uitsluitend minder antibiotica werd gebruikt nam dragerschap van LA-MRSA bij dieren en mensen af in de loop van de tijd, dit in tegenstelling tot controlebedrijven en bedrijven waar tevens een schoonmaak- en desinfectie-programma werd uitgevoerd.

In deel II wordt een longitudinaal veldonderzoek beschreven waarin pragmatische interventies werden toegepast om LA-MRSA en ESBL-producerende *E. coli* in de varkenshouderij te reduceren. Omdat er tijdens het onderzoek geen significante wijzigingen

in de werkwijzen op de veehouderijen hebben plaatsgevonden, konden de veranderingen in resistentie hier ook niet aan worden geassocieerd. Echter, als gevolg van gewijzigd Nederlands beleid nam het gebruik van antimicrobiële middelen in de onderzoeksperiode met 44% af. LA-MRSA in varkens nam enigszins af terwijl ESBL-producerende *E. coli* substantieel afnam. Minder LA-MRSA en ESBL-producerende *E. coli* was te relateren aan een afname in antibioticagebruik. Het incidenteel toepassen van cephalosporines was de grootste risicofactor voor LA-MRSA en ESBL-producerende *E. coli* in varkens. Antibioticagebruik bij dieren blijkt een risicofactor te zijn voor LA-MRSA-dragerschap bij veehouders ongeacht de hoeveelheid diercontact, dit wijst erop dat directe blootstelling aan antimicrobiële middelen een werk gerelateerd risico is. Een risicofactor-analyse toonde dat de kans op het voorkomen van antimicrobiële resistentie groter is op varkensbedrijven waar dieren door andere bedrijven worden aangeleverd, waar minder stringente biosecurity-maatregelen (bijvoorbeeld het niet uitsluitend gebruiken van de hygiënesluis als toegang tot het bedrijf), en waar minder rigide contactroutes worden gehanteerd (bijvoorbeeld zeugen niet gehuisvest in stabiele groepen).

Deel III toont het belang van grondige antibioticaresistentie monitoringsystemen in de veehouderij. In een ecologische studie zijn de trends van antibioticagebruik en antimicrobiële resistentie vergeleken voor de belangrijkste diersectoren over een periode van 10 jaar in Nederlands. Een nationale daling van het gebruik van antimicrobiële middelen is geassocieerd met een afname van resistentie bij *E. coli* voor de meeste antimicrobiële middelen, in het bijzonder in de kalver- en varkenssector. De studie onderschrijft het gebruik van antimicrobiële middelen in het verleden en de selectie van co-resistentie als belangrijke factoren voor het probleem omtrent antibioticaresistentie.

Tot slot wordt in deel IV een meta-collectie van 3646 isolaten gebruikt om de bronnen te identificeren die kunnen leiden tot een toename van ESBL/AmpC-producerende *E. coli* infecties en dragerschap bij mensen. Gegevens met betrekking tot ESBL en AmpC genen en bijbehorende plasmides van 27 Nederlandse studies zijn verzameld en gereduceerd door principale componenten analyses. De uitkomsten wijzen op een beperkte overeenkomst in moleculaire eigenschappen van *E. coli* tussen de meeste dierlijke reservoirs, de open humane populatie en patiënten in een klinische setting. ESBL/AmpC–producerende *E. coli* in mensen woonachtig en/of werkzaam op een veehouderij (d.w.z. in direct contact met de dieren) hebben nagenoeg hetzelfde moleculaire profiel als de bacteriën geïsoleerd uit de dieren. Dit suggereert een aanwezige overdracht tussen deze reservoirs. ESBL/AmpC–producerende *E. coli* van wilde vogels, oppervlaktewater waarin geloosd wordt en humane populatie zijn moleculair nauw gerelateerd. Overdracht en verspreiding van resistentie lijkt dus ook voor te komen buiten mensen en gehouden dieren, zonder directe invloed van antimicrobiële middelen.

Het onderzoek beschreven in dit proefschrift geeft aanleiding tot meer langdurig onderzoek in het veld, waarbij gecontroleerde interventies worden toegepast om de

dier-mens overdracht van antimicrobiële resistentie nader te bestuderen. De resultaten van de nationale reductie in gebruik van antimicrobiële middelen zouden de publieke gezondheidsorganisaties en diersectoren moeten stimuleren om hun inspanningen voor verstandig gebruik van antimicrobiële middelen in de veehouderij voort te zetten. Rekening houdend met de verschillende reservoirs en bronnen voor overdracht naar de mens vraagt het resistentieprobleem om een holistische en multidisciplinaire "One Health" aanpak.

Resumen en español (Summary in Spanish)

El uso excesivo o mal uso de antibióticos durante las últimas décadas ha generado bacterias cada vez más resistentes a estos tratamientos. Actualmente éste es de uno de los mayores problemas para la salud humana a escala mundial. Infecciones que una vez fueron fácilmente tratables se están volviendo difícil de curar con los consecuentes costes económicos y sociales. Los antibióticos también se han utilizado extensivamente en producción animal y las resistencias antimicrobianas no han tardado en aparecer en los animales. No hay fronteras geográficas o biológicas para la resistencia a antibióticos y su transmisión, desde los animales a las personas, puede involucrar diferentes rutas directas e indirectas, tales como la cadena alimentaria o el medio ambiente.

Esta tesis doctoral tiene como objetivo proporcionar una mayor comprensión sobre la epidemiología de resistencia a antibióticos en la interfaz humano-animal, a través de dos especies de bacterias que constituyen un problema para la salud humana, *Staphylococcus aureus* resistente a la meticilina (SARM) y *Escherichia coli* (incluyendo bacterias productoras de betalactamasas de espectro extendido (BLEE) y AmpC). En los últimos años, se han realizados varios estudios exploratorios en este campo, pero debido a su naturaleza observacional y transversal, las relaciones causales de las asociaciones epidemiológicas han sido a menudo difíciles de establecer. El presente trabajo refuerza la evidencia sobre estas relaciones causales apoyándose en asociaciones longitudinales y datos cuantitativos sobre el uso veterinario de antibióticos. También se presentan dos estudios longitudinales de intervención en terneros, cerdos y sus respectivas comunidades de granjeros junto a una evaluación longitudinal de las recientes políticas holandesas para la reducción del uso veterinario de antibióticos. Finalmente, un meta-análisis resume y pone en perspectiva el conocimiento acumulado sobre las características moleculares de *E. coli* productor de BLEE/AmpC en diferentes fuentes alimentarias y ambientales, y en reservorios animales y humanos.

En la Parte I se presenta un estudio de intervención a 3 brazos dirigido a evaluar estrategias para reducir SARM de origen animal (OA) en la producción de terneros. Los resultados indican que en las granjas con ambientes contaminados con SARM-OA, hay una mayor tasa de adquisición de esta bacteria a través de la transmisión aérea. Contrario a lo esperado, en las granjas que aplicaron un protocolo de limpieza y desinfección de los establos, se observó una propagación transitoria de SARM-OA en el aire que resultó en más portadores de SARM-OA entre los terneros, granjeros y miembros de la familia. Condiciones

de bioseguridad más estrictas (p.ej. la ausencia de gatos circulando libremente en las granjas o la presencia de vestuarios) se revelaron como factores de protección para SARM-OA en animales y humanos. En contraste con las granjas en el brazo control o las que aplicaron el programa de limpieza y desinfección, las granjas que sólo redujeron el uso veterinario de antibióticos mostraron tasas disminuidas de portación de SARM-OA en animales y humanos.

En la Parte-II se presenta un estudio de campo longitudinal en el que se aplicaron intervenciones pragmáticas para el control de SARM-OA y *E. coli* productor de BLEE en la producción porcina. La mayoría de las prácticas de manejo en la granja no cambiaron significativamente durante el periodo de estudio, por lo que las dinámicas de resistencia atribuidas a dichos cambios no pudieron ser evaluadas. Sin embargo, el uso veterinario de antibióticos disminuyó un 44% como resultado de la aplicación de las políticas holandesas. Esto se acompañó de una ligera disminución en la prevalencia de cerdos portadores de SARM-OA y una caída más significativa de la prevalencia de BLEEs. El uso ocasional de cefalosporinas fue el factor de riesgo más relevante para la portación de SARM-OA y BLEEs en cerdos. El uso de antibióticos en los animales se mantuvo asociado con más granjeros portadores de SARM-OA, independientemente del nivel de contacto con los animales, por lo que la exposición directa a estos usos de antibióticos puede suponer un riesgo laboral para las personas. Un análisis de factores de riesgo proporcionó más evidencia sobre el aumento de la propagación de resistencia a antibióticos en explotaciones que recibían suministro externo de animales provenientes de otras granjas, en explotaciones con condiciones de bioseguridad menos estrictas (p. ej. cuando el vado sanitario no se utiliza como entrada exclusiva en la granja) y en explotaciones con estructuras de contacto menos rígidas entre los animales (p. ej. cerdas alojadas en grupos no estables).

La Parte III ilustra la importancia de tener sistemas exhaustivos de monitorización de resistencia a antibióticos en el ámbito de la producción animal. A través de un estudio ecológico, se evalúan las tendencias en el uso veterinario de antibióticos y en *E. coli* comensal resistente durante 10 años en los en los principales sectores de producción animal holandeses. La reducción a nivel nacional del uso de antibióticos se asoció a la disminución de niveles de resistencia en *E. coli* para la mayoría de antibióticos, especialmente en los sectores porcino y bovino de terneros. El estudio pone de relieve que la historia de consumo de antibióticos y la selección de resistencias cruzadas son elementos clave para la perpetuación de resistencia.

Por último, en la Parte IV, se crea una meta-colección de 3646 aislados bacterianos de *E. coli* productores de BLEE/AmpC para identificar fuentes o reservorios que puedan contribuir a aumentar infecciones bacterianas en el hombre. Este trabajo recopila los datos sobre genes y replicones plasmídicos provenientes de 27 estudios holandeses y reduce la dimensionalidad de los datos con un análisis de componentes principales. Los resultados mostraron similitudes limitadas, pero aún existentes a nivel molecular entre bacterias *E. coli* provenientes de reservorios animales y de la población general humana y pacientes en entornos clínicos. Se observó que los aislados de *E. coli* en granjeros y sus familias (en

contacto directo con el ganado) compartían básicamente los mismos perfiles moleculares con los aislados de *E. coli* de sus animales, lo que sugiere una alta transmisión entre estos reservorios. Los aislados de *E. coli* de aves silvestres y de agua superficial influenciada por plantas de tratamiento estaban estrechamente relacionados con las poblaciones humanas, lo que indica que la transmisión y difusión de BLEEs y AmpCs ocurre más allá de los seres humanos y los animales domésticos, en entornos sin aparente presión selectiva a antibióticos.

Los resultados de esta tesis doctoral propugnan la realización de futuros estudios de campo con períodos de seguimiento más largos e intervenciones más controladas, para establecer estrategias de control de la transmisión de resistencias antimicrobianas desde los animales al hombre. Los positivos resultados de la reducción del uso veterinario de antibióticos a nivel nacional en los Países Bajos deberían alentar a las autoridades sanitarias y a los sectores de producción animal para mantener sus esfuerzos por un uso prudente de antibióticos. Teniendo en cuenta los múltiples reservorios y fuentes de posible transmisión a los seres humanos, el problema de la resistencia a antibióticos debería abordarse de manera integral a través de enfoques multidisciplinares en el marco del concepto de "Salud Única".

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About the author

Contribution to Scientific Conferences

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Curriculum Vitae

Alejandro Dorado García was born on February 17, 1984 in Madrid, Spain. He graduated as Doctor of Veterinary Medicine in 2008 from Complutense University of Madrid. From 2009 to 2011, he worked in the pharmaceutical industry as Veterinary Pharmacovigilance Technician and as Clinical Research Associate, and obtained his MSc in Monitoring of Clinical Trials from the European School of Pharma Studies and Alcalá de Henares University. In September 2011 he moved to the Netherlands after receiving a grant from "La Caixa" Foundation to study the MSc in Veterinary Epidemiology and Economics at Utrecht University (UU). He joined the Institute for Risk Assessment Sciences (IRAS) at UU as part of the major research project of his MSc. After its completion in March 2013, he continued working at the IRAS to obtain his PhD in epidemiology of antimicrobial resistance in the livestock farming domain. In 2015, he received a fellowship from the SEA-EU-NET to follow a One Health Master class at Montelier University (France) and Kasetsart University (Bangkok, Thailand). During his PhD he has presented the results of his research at several international scientific conferences. Currently, Alejandro lives in Utrecht.