Antimicrobial Resistant *Escherichia coli* in Faeces from Preweaned Dairy Calves

Prevalence, Risk Factors, and Spread

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Abstract

Antimicrobial resistant (AMR) bacteria are increasing threats for human and veterinary medicine. Faecal *Escherichia coli* (*E. coli*) from preweaned dairy calves is often resistant to multiple antimicrobials and calves may therefore serve as reservoirs for these bacteria and their resistance genes. This thesis investigated the prevalence, risk factors, and spread of resistant *E. coli* on Swedish dairy farms, with special emphasis on quinolone resistant *E. coli* (QREC). Faecal samples from preweaned calves and post-partum cows were analysed for resistant *E. coli* and set in relation to potential risk factors. The farm environment was sampled to study the occurrence and spread of QREC.

The occurrence of faecal resistant *E. coli* in calves was strongly age-dependent, but was also associated with herd size, milking system, calf housing, and geographic location of the farm. Treatment with some broad-spectrum antimicrobials in cows or calves increased the occurrence of resistant *E. coli* in calves. Feeding waste milk from cows treated with antimicrobials during lactation to calves increased the proportion of streptomycin and quinolone resistant *E. coli* in calves, but feeding waste colostrum from cows treated with antimicrobials at drying off had no effect on AMR *E. coli*. Feeding such colostrum or milk to calves was a common practice on Swedish dairy farms, in particular on farms in southern Sweden, on non-organic farms, and on farms with tie stall housing. On farms where QREC is common in faeces of calves, these bacteria were also widespread in the farm environment. In particular, the calf feed and water trough contained QREC. The same QREC genotype was found throughout the same and on different farms, suggesting contagious spread of QREC within and between farms. Fluoroquinolone treatment, WM feeding, group calving, poor farm hygiene, purchasing cattle or shared animal transports were some risk factors for increasing the occurrence of QREC on the farm.

Altogether, the results indicate that proper biosecurity and improved hygiene, less exposure to broad-spectrum antimicrobials, and restrictive waste milk feeding may be important factors to reduce the burden of AMR *E. coli* on dairy farms.

Keywords: Calf, *Escherichia coli*, antimicrobial resistance, waste milk, antimicrobials, quinolone resistance, genetic diversity, risk factor, spread

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Dedication

To my grandmother Ingrid, who passed away during the making of this book and never got to see me finish. I hope you get to watch the final while wearing the prettiest angel wings ever!

“Whatever you do in life, surround yourself with smart people who'll argue with you.”

John Wooden

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Duse A., Waller K.P., Emanuelson U., Unnerstad H.E., Persson Y., Bengtsson B. (2013). Farming practices in Sweden related to feeding milk and colostrum from cows treated with antimicrobials to dairy calves. *Acta Veterinaria Scandinavica* 55, 49.

II Duse A., Waller K.P., Emanuelson U., Unnerstad H.E., Persson Y., Bengtsson B. (2015). Risk factors for antimicrobial resistance in fecal *Escherichia coli* from preweaned dairy calves. *Journal of Dairy Science* 98(1), 500–516.

III Duse A., Waller K.P., Emanuelson U., Unnerstad H.E., Persson Y., Bengtsson B. Risk factors for quinolone resistant *Escherichia coli* in faeces from preweaned dairy calves and post-partum dairy cows. (Submitted manuscript).

IV Duse A., Waller K.P., Emanuelson U., Unnerstad H.E., Persson Y., Bengtsson B. Occurrence and spread of quinolone resistant *Escherichia coli* on Swedish dairy farms. (Manuscript).

The contribution of Anna Duse to the papers included in this thesis was as follows:

I Was involved in the planning of the study. Developed the questionnaire with input from the co-authors and was responsible for the survey logistics. Analysed the results in collaboration with the supervisors. Performed the statistical analyses under supervision and wrote the manuscript with regular input from the co-authors.

II Was involved in the planning of the practical study. Was responsible for the recruitment of herds and for the sampling logistics. Performed most laboratory work and analysed the results under supervision. Was responsible for writing and completing the manuscript with regular input from the co-authors.

III Was involved in the research idea and planning of the study. Was responsible for the recruitment of herds and visited the herds. Contributed to laboratory work. Performed the statistical analyses under supervision and wrote the manuscript with regular input from the co-authors.

IV Was involved in the research idea and planning of the study. Was responsible for the recruitment of herds and visited the herds. Contributed to laboratory work and performed all genotyping analyses. Performed the statistical analyses under supervision and wrote the manuscript with regular input from the co-authors.

Abbreviations

AMR	Antimicrobial resistant
Am^r	Ampicillin resistant
CFU	Colony Forming Unit
Ci^r	Ciprofloxacin resistant
Cm^r	Chloramphenicol resistant
Ctx^r	Cefotaxime resistant
DCT	Dry cow therapy
DHS	Dihydrostreptomycin
DI	Diversity Index
ESBL	Extended Spectrum Betalactamases
GI	Gastrointestinal
H-farm	High farm (where QREC is common in faeces from calves)
Km^r	Kanamycin resistant
L-farm	Low farm (where QREC is rare in faeces from calves)
MIC	Minimum Inhibitory Concentration
MLVA	Multiple Locus Variable-number Tandem Repeat Analysis
NADRS	National Animal Disease Recording Scheme
Nal^r	Nalidixic acid resistant
PCR	Polymerase Chain Reaction
QREC	Quinolone resistant *Escherichia coli*
SEC	Select *E. coli* Count
Sm^r	Streptomycin resistant
SOMRS	Swedish Official Milk Recording Scheme
Su^r	Sulphametoxazole resistant
Tc^r	Tetracycline resistant
TWM	Waste milk produced during ongoing treatment
WC	Waste colostrum
WT	Waste transition milk
WWM	Waste milk produced during the withdrawal period

1 Introduction

Antimicrobial resistance is an increasing threat for human and animal health. Antimicrobial resistant (AMR) *Escherichia coli* from the gut of calves is normally harmless for the calf itself, but may cause intractable infections in the animal or its herd-mates. There is also a risk that AMR *E. coli* strains in the cattle population are transferred to humans by direct contact or via contaminated milk or meat products. Preweaned dairy calves often shed AMR *E. coli* in faeces. Many of these *E. coli* strains are multi-drug resistant, even when the calf has never been exposed to antimicrobials. Hence, calves may act as reservoirs for AMR *E. coli* causing intractable infections in animals and humans. The epidemiology of faecal AMR *E. coli* in calves is not yet fully understood and more knowledge is needed to define measures that could reduce the burden of AMR *E. coli* on dairy farms.

This thesis investigates the prevalence, risk factors, and spread of faecal AMR *E. coli* in preweaned dairy calves in relation to farm and calf characteristics, usage of antimicrobials in the herd, management factors such as the feeding of milk from antimicrobial-treated cows to calves, and factors associated with within- and between- farm biosecurity.

1.1 General aspects of antimicrobial resistance

The global emergence of antimicrobial resistance is a rising concern for human health (WHO, 2012b). The consequences of antimicrobial resistance extend beyond treatment failures in individual cases. Without effective antimicrobials, important procedures such as major surgery, organ transplantation, and cancer chemotherapy will be hazardous (Cars *et al.*, 2008; Laxminarayan *et al.*, 2013). It has been estimated that by 2050, approximately ten million deaths per year will be due to infections with resistant bacteria. This means that the number of

deaths due to resistant bacteria will exceed the number of deaths due to cancer in 2050 (O'Neill, 2014).

The consequences of antimicrobial resistance in animals are similar to those for humans, leading to increased suffering and mortality (Bengtsson & Greko, 2014). However, future veterinary medicine has to rely mainly on the efficacy of already existing antimicrobials (Schwarz *et al.*, 2001). Moreover, the World Health Organization has stated that some antimicrobials (fluoroquinolones, third and fourth generation cephalosporins and macrolides) should be reserved only for treating human infections (WHO, 2012a). It is also likely that any new antimicrobial will be reserved for human medicine (Schwarz *et al.*, 2001; Bengtsson & Greko, 2014). Loss of effective treatment options for animals may not only lead to therapy failures, but also to decreased welfare and reduced productivity for food-producing animals, resulting in major setbacks for the animal and global food production (Bengtsson & Greko, 2014).

1.1.1 Emergence and spread of antimicrobial resistance

Emergence refers to the conversion from wild-type to resistance phenotypes, whereas spread refers to the dissemination of resistance between hosts and the environment, or spread of resistance determinants between bacteria. Often emergence and spread may overlap.

Emergence

Emergence of antimicrobial resistance is a normal step in bacterial evolution, as the survival of bacteria with the phenotypical traits best adapted to the current environment (Sykes, 2010). Exposure to antimicrobials imposes a selective pressure on the bacterial population, allowing only resistant subpopulations of bacteria to survive.

Antimicrobial resistance can be intrinsic or acquired (Alekshun & Levy, 2007). Intrinsic resistance is conferred by naturally occurring genes in the bacterium's genome or by inherent characteristics of the bacterium, which allow tolerance to specific antimicrobials (Alekshun & Levy, 2007; Cox & Wright, 2013). Intrinsic resistance is common for all members of a bacterial species and is independent of the selective pressure from antimicrobials (Cox & Wright, 2013). Acquired resistance is when a particular bacterium obtains the ability to resist a specific antimicrobial agent to which it was previously susceptible (Alekshun & Levy, 2007). Unlike intrinsic resistance, acquired resistance traits are found only in some strains or subpopulations of a bacterial species (Alekshun & Levy, 2007).

There are two mechanisms by which bacteria acquire resistance - by spontaneous mutations in chromosomal genes or through acquisition of

naturally occurring resistance genes from other bacteria (Schwarz *et al.*, 2001; Alekshun & Levy, 2007; Sykes, 2010). Horizontal transfer of genes can occur within a bacterial species or over species boundaries either by uptake of naked DNA or through the integration of DNA in plasmids, bacteriophages, transposons, or other mobile genetic elements (Alekshun & Levy, 2007; Sykes, 2010). Many resistance genes are clustered together on mobile genetic elements, meaning that a single transfer can result in the acquisition of resistance to multiple antimicrobials (Guardabassi & Kruse, 2008).

The use of antimicrobials creates optimal conditions for resistance to emerge (Guardabassi & Kruse, 2008). Exposure to antimicrobials allows AMR strains to multiply in the absence of susceptible competitors (Schwarz *et al.*, 2001). Exposure to some bactericidal antimicrobials, such as betalactams, fluoroquinolones, and aminoglycosides, may also stimulate bacteria to produce reactive oxygen species (Kohanski *et al.*, 2007). Reactive oxygen species may damage bacterial DNA, which results in the accumulation of mutations (Kohanski *et al.*, 2010). Thus, exposure to low concentrations of bactericidal antimicrobials results in formation of multidrug-resistant mutants (Kohanski *et al.*, 2010). Exposure to betalactam antimicrobials (Miller *et al.*, 2004) or reactive oxygen species (Carlsson & Carpenter, 1980) may also activate the SOS-response. The SOS-response is evoked by DNA-damage which arrests cell division and induces mutagenesis and DNA repair (Janion, 2008). This response also promotes the transfer of resistance genes by increasing the expression of genes needed for gene transfer (Beaber *et al.*, 2004).

Exposure to one antimicrobial may select for resistance to other antimicrobials, because of cross- or co-resistance. Cross-resistance refers to single resistance genes or mutations conferring resistance to more than one antimicrobial class (Schwarz *et al.*, 2001; Guardabassi & Kruse, 2008). Co-resistance is the co-existence of several genes conferring resistance to different antimicrobials (Schwarz *et al.*, 2001; Guardabassi & Kruse, 2008).

Spread

Resistant bacteria or their genes do not respect ecological, phylogenetic or geographical borders and thus, the epidemiology of resistance must be seen from a holistic and global point of view (Guardabassi & Kruse, 2008). Antimicrobial resistance spreads through bacteria populations both vertically, when new generations inherit resistance determinants, and horizontally, when bacteria share or exchange resistance genes with other bacteria (Witte, 2004). Horizontal transfer of resistance genes can occur within and between bacterial species (Schwarz *et al.*, 2001; Witte, 2004). Bacteria that have acquired resistance may then spread between hosts by skin to skin contact, via excreta or

saliva containing the resistant bacteria, or by exposure to contaminated food, feed, air, or water (Schwarz *et al.*, 2001). Human or animal excreta that contain resistant bacteria may contaminate the environment directly, or via the application of sludge or manure/slurry on lands (Marshall *et al.*, 2009; Wellington *et al.*, 2013). Spread to humans and animals then occurs through contact with soil, irrigation of crops, water, or wildlife (Wellington *et al.*, 2013). Finally, the movement of animals, food, and humans is a factor in the global dissemination of antimicrobial resistance (Laxminarayan *et al.*, 2013). When resistant bacteria have reached the new host, they can either colonize, infect, or reside only transiently (Schwarz *et al.*, 2001). In the new host, the resistant bacteria can spread their resistance genes to other bacteria, and also acquire other resistance genes from them (Schwarz *et al.*, 2001).

Use of antimicrobials by some individuals may enhance the spread of resistant bacteria to other individuals sharing the same environment. First, antimicrobial treatment decreases the ratio of susceptible to resistant organisms in the bacteria population that may colonize other animals or humans (Lipsitch & Samore, 2002). Second, antimicrobial treatment reduces the competition from the residing microbiota in the treated individual, and thus, increases the treated individual's risk of being colonized with a resistant strain from the environment (Lipsitch & Samore, 2002).

1.1.2 The role of commensal *E. coli*

Commensalism is a relationship between two organisms in which one benefits from the other without affecting it (Hogan, 2012). Commensalism exists between bacteria and animal/human hosts in various sites of their bodies, e.g., the skin and the gastrointestinal (GI) tract (Andremont, 2003). Although commensal bacteria are per definition harmless to their host, under certain conditions or in certain individuals, they can become pathogenic (Lupp & Finlay, 2005; Marshall *et al.*, 2009). The commensal microbiota are large populations of bacteria (Andremont, 2003; Marshall *et al.*, 2009), that are often in transient, but intimate, contact with bacteria from outside the body (Courvalin, 2008). During antimicrobial therapy for an infectious agent, the commensals are also exposed to selective pressure from the antimicrobial (Andremont, 2003; Courvalin, 2008; Marshall *et al.*, 2009). Given the versatile pool of bacteria and genes in the commensal microbiota, there are ample opportunities for antimicrobial resistance to emerge (Andremont, 2003; Courvalin, 2008), both through emergence and selection of resistant strains (Andremont, 2003) and by transfer of resistance genes from indigenous bacteria to bacteria from outside the host and vice versa (Courvalin, 2008). The general belief is that resistance first emerges in the commensal microbiota and

then spreads by horizontal transfer to pathogens (Andremont, 2003). Hence, the level of AMR in commensals is considered a good indicator of the selection pressure by antimicrobials, but it is also predictive of the emergence of resistance in pathogens (van den Bogaard & Stobberingh, 2000).

The GI microbiota represents by far the largest commensal population in the body (Andremont, 2003). It is therefore an important reservoir for multidrug-resistant bacteria (van den Bogaard & Stobberingh, 2000; Wellington *et al.*, 2013). *E. coli* is a species that normally colonizes the GI tract of warm-blooded animals and humans (Anderson *et al.*, 2006). This species is also found among some cold-blooded animals and in the environment, such as sediments and water reservoirs (Anderson *et al.*, 2006; Marshall *et al.*, 2009). Due to its ubiquity, its relevance to human medicine, and the ease with which it acquires conjugative plasmids, *E. coli* from animal faeces is often used in resistance monitoring programmes as an indicator for acquired resistance in Gram-negative bacteria (Swedres-Svarm 2013; EFSA, 2014).

1.1.3 Antimicrobials and resistance of special concern

Fluoroquinolones are classified as critically important antimicrobials for humans (WHO, 2012b) and emerging resistance to them is therefore of utmost concern. Unfortunately, quinolone resistance emerges rapidly as a consequence of exposure to it. After the introduction of enrofloxacin as a therapeutic agent on a US dairy farm in 2008, quinolone resistant *E. coli* (QREC) from faeces of calves increased from 1.3% in 2006 to 47.9% in 2011 (Jones *et al.*, 2013). Due to the importance of this antimicrobial class for human medicine, this thesis focuses especially on risk factors and spread of QREC.

Quinolone antimicrobials inhibit the activity of the DNA gyrase and topoisomerase II and IV, enzymes that relax the supercoiling of bacterial DNA and complete cell division (Ruiz, 2003). Quinolone resistance can be due to chromosomal mutations, acquisition of plasmid-mediated genes, or decreased uptake of the antimicrobial (Ruiz, 2003). In *E. coli*, mutations occur in the gyrase (*gyrA* or *gyrB*) or topoisomerase genes (*parC* or *parE*) (Ruiz, 2003). Accumulation of mutations in these genes results in stepwise increases in the minimum inhibitory concentration (MIC) (Ruiz, 2003; Cavaco & Aarestrup, 2009) and cross-resistance to all members of the quinolone class is common (Ruiz, 2003). Plasmid-mediated resistance is conferred by the *qnr* genes, which have a protective effect on DNA gyrase, or by the aac(6)Ib-cr gene, which modifies fluoroquinolones enzymatically (Robicsek *et al.*, 2006). Plasmid-mediated resistance is often expressed as low-level resistance to fluoroquinolones (Cavaco & Aarestrup, 2009). Resistance due to decreased

uptake occurs either by increased impermeability or by overexpression of efflux pumps (Ruiz, 2003). Decreased uptake may also be associated with decreased susceptibility to other antimicrobials (Ruiz, 2003).

1.2 Antimicrobial resistant faecal *E. coli* from dairy calves

1.2.1 Occurrence of AMR *E. coli* in faeces of dairy cattle

Antimicrobial resistance in faecal *E. coli* is less common in cattle than in other food-producing animals (Jong *et al.*, 2009; EFSA, 2014). In Sweden, AMR *E. coli* is rarely isolated from slaughtered cattle (Swedres-Svarm 2013). The vast majority of *E. coli* isolated from calves aged 6 to 11 months 2013 (92%) and mature cows 2006 (97%) were susceptible to all tested antimicrobials (Svarm 2006; Swedres-Svarm 2013). The occurrence of resistance in faecal *E. coli* from cattle is however, age-dependent. Faecal *E. coli* from calves is significantly more resistant, and often multidrug-resistant, compared to that in older cattle (DeFrancesco *et al.*, 2004; Khachatryan *et al.*, 2004; Sato *et al.*, 2005; Dolejská *et al.*, 2008; Berge *et al.*, 2010; Yamamoto *et al.*, 2013; EFSA, 2014). Wierup (1975) observed that *E. coli* isolates from 5-day old calves were more resistant and carried more transferrable resistance than *E. coli* isolates from 30-day old calves. Moreover, resistant strains from the younger calves transferred significantly more en bloc resistance, i.e. resistance to multiple antimicrobials (Wierup, 1975).

Calves and cows are often housed in proximity to each other, often looked after by the same personnel, and exposed to the same microorganisms. The high level of resistance in faecal *E. coli* from calves is therefore a peculiar phenomenon. The following text offers potential explanations.

1.2.2 Factors affecting faecal AMR *E. coli* in preweaned dairy calves

Factors that may have an impact on the occurrence of faecal AMR *E. coli* in calves can be related to the calf itself, to external influences such as the diet or exposure to antimicrobials, to characteristics of the farm, and to management-associated factors.

Use of antimicrobials

High levels of faecal AMR *E. coli* in calves may be due to frequent exposure to antimicrobials. Likewise, antimicrobial use has been associated with increased occurrence of resistance among faecal *E. coli* from calves (Berge *et al.*, 2005a; b, 2006; Di Labio *et al.*, 2007; Jones *et al.*, 2013; Yamamoto *et al.*, 2013; Pereira *et al.*, 2014b). Berge *et al.* (2005b, 2006) and Singer *et al.* (2008) observed that the effect of individual antimicrobial treatment was transient,

with resistance levels returning to pre-treatment levels already within a few weeks post-treatment. On the other hand, some of the studies showed an association between farm-level usage of antimicrobials and AMR *E. coli* in calves (Di Labio *et al.*, 2007; Jones *et al.*, 2013; Yamamoto *et al.*, 2013). This means that frequent antimicrobial use by individual animals may maintain a high level of AMR bacteria in the farm environment (Berge *et al.*, 2005a). These AMR bacteria could then colonize the GI tract of the calves. However, levels of resistance in *E. coli* from young individuals are often high even without previous exposure to antimicrobials (Khachatryan *et al.*, 2004; Berge *et al.*, 2005a; Karami *et al.*, 2006; de Verdier *et al.*, 2012). Hence, frequent usage of antimicrobials is an insufficient explanation as to why calves carry more AMR *E. coli* than older animals do.

1.2.3 The age of the calf

Independent of resistance phenotype, colonization with AMR *E. coli* occurs shortly after birth (Hinton *et al.*, 1985a; b; Hoyle *et al.*, 2004b; Donaldson *et al.*, 2006), and reaches a maximum within a few weeks (Hinton *et al.*, 1985b; Hoyle *et al.*, 2004a; Berge *et al.*, 2005a; Donaldson *et al.*, 2006). Thereafter it decreases with calf age to negligible levels at four to six months (ampicillin and non-specific resistance), four months (apramycin), and two months (nalidixic acid and extended spectrum betalactamase; ESBL) (Hinton *et al.*, 1984, 1985b; Hoyle *et al.*, 2004a; b; Watson *et al.*, 2012; Brunton *et al.*, 2014). Hinton *et al.* (1985b) suggested that the age-related occurrence of resistance is due to the frequent turnover of *E. coli* strains in the developing gut of calves—moving from mainly susceptible strains, to resistant ones, and then back to susceptible ones again. Susceptible *E. coli* serotypes from older calves were different from those isolated from the younger ones, indicating that the shift was due to acquisition of new susceptible strains rather than to re-establishment of the same susceptible strains (Hinton *et al.*, 1985a; b).

Khachatryan *et al.* (2004) observed that resistant strains outcompeted susceptible strains in calves but not in older cattle. However, the resistance genes were not by themselves associated with the higher fitness of AMR *E. coli* in the calves' GI tract (Khachatryan *et al.*, 2006b). Berge *et al.* (2005a) and Khachatryan *et al.* (2006b) suggested therefore that resistance is linked to traits that increase the colonization capacity in the young calf's GI tract. Such traits could be the production of virulence or adhesion factors that promote colonization in the gut (Karami *et al.*, 2006).

Diet factors

A factor in common for most of the calf studies is that resistance is highest in the milk-feeding period (Hinton *et al.*, 1984; Hoyle *et al.*, 2004a; b; Khachatryan *et al.*, 2004; Edrington *et al.*, 2012b; Watson *et al.*, 2012). Therefore, the milk diet has been proposed to be a risk factor. Given that ingestion of milk by itself would create optimal conditions for resistant strains in the calf gut, resistance levels should drop after weaning, but this was not the case in the studies of Edrington *et al.* (2012b) and Hinton *et al.* (1984). Likewise, AMR and susceptible *E. coli* grow equally well on agar plates supplemented with milk powder, indicating that milk by itself does not favour AMR strains over susceptible strains (Khachatryan *et al.*, 2006a).

If milk instead were a vehicle for the transfer of AMR bacteria to calves, pasteurizing the milk before feeding would decrease colonization with resistant strains. However, feeding pasteurized milk did not result in fewer faecal AMR *E. coli* (Aust *et al.*, 2012; Edrington *et al.*, 2012b).

Supplementation of calf milk with antimicrobials is, in some countries, a common practice for disease prevention and growth promotion. This practice increased faecal AMR *E. coli* in the studies by Pereira *et al.* (2011) and Berge *et al.* (2006), but not in the study by Khachatryan *et al.* (2006a). However, Khachatryan *et al.* (2006a) added only a tetracycline component to the milk whereas Pereira *et al.* (2011) and Berge *et al.* (2006) added both tetracycline and sulfamethazine or neomycin. Thus, the lack of an effect from the tetracycline in Khachatryan *et al.* (2006a) may have been due to chelation of the antimicrobial by calcium and magnesium ions in the milk.

Antimicrobials in milk fed to calves may also come from cows treated with antimicrobials. When a cow is given antimicrobials, the antimicrobial itself or degradation products thereof are usually excreted in the milk (Langford *et al.*, 2003; Randall *et al.*, 2013; Pereira *et al.*, 2014a). Colostrum or milk from cows treated with antimicrobials (here defined as waste colostrum; WC and waste milk; WM) may not be sold for human consumption and must be disposed elsewhere. One way of using WC and WM is to feed it to calves, which is a cost-effective solution for the farmer. Likewise, surveyed farmers in the UK stated that saving money was the major reason for feeding WM to calves, followed by avoiding problems with the disposal (Brunton *et al.*, 2012). Although WC and WM can be of good nutritional quality, if they contain antimicrobials, this may put a selection pressure on the calves' GI microbiota, possibly favouring resistant bacteria. This was investigated already in 1980 by Yndestad *et al.* (1980) and although they did not find a higher prevalence of faecal AMR *E. coli* among calves that were exposed repeatedly to antimicrobial-containing milk, their study material was small. Ten years later,

Wray *et al.* (1990), observed that the faecal *E. coli* from calves given WM had significantly higher MIC for streptomycin, but not for ampicillin than *E. coli* from calves fed milk substitute. Langford *et al.* (2003) revealed a dose-related relationship between the penicillin concentration in milk and the degree of penicillin inhibition of unspecific bacteria from calves fed such milk. However, the results of that study must be interpreted cautiously due to the inaccuracy of the methods used. Researchers in Denmark did not find a difference in resistance levels of faecal *E. coli* on farms that gave milk from antimicrobial-treated cows to calves compared to farms that did not (Sörensen *et al.*, 2008), but again, the study material was small. Finally, Würgler-Aebi (2004) advised against the feeding of milk from cows treated with antimicrobials after observing that the relative number of AMR *Enterococci* increased markedly in calves fed such milk. Taken together, these results are inconclusive and thus, more and better studies are needed. Moreover, to our knowledge, no one has investigated the importance of feeding colostrum from cows treated with dry-cow antimicrobials. It is therefore uncertain to what extent feeding WC selects for AMR *E. coli* in the GI tracts of calves.

Other factors

Many other factors affect the carriage of faecal AMR *E. coli* in calves, such as the incidence of diarrhoea (Gunn *et al.*, 2003; de Verdier *et al.*, 2012), herd size (de Verdier *et al.*, 2012), farm type (beef, calf ranch, or dairy) (Berge *et al.*, 2010), production type (organic or non-organic) (Sato *et al.*, 2005), infrequent disinfection of calf feeding equipment, storage of slurry in a pit, keeping purchased cattle in quarantine (Snow *et al.*, 2012), purchase of calves (particularly from several suppliers) (Di Labio *et al.*, 2007), and vitamin supplements (Khachatryan *et al.*, 2006a). There may also be other, yet unidentified factors.

1.3 Dissemination of AMR *E. coli* on dairy farms

When studying factors that affect the occurrence of faecal AMR *E. coli* in calves, it is crucial to investigate the spread of AMR *E. coli* between calves, in other cattle, and in the farm environment.—these may be potential sources and dissemination routes. However, one of the major problems when studying the epidemiology of antimicrobial resistance is that different resistance traits may behave in different ways. What complicates the picture is that spread by clonal dissemination, horizontal plasmid transfer, horizontal transfer of genes, or a combination of these events may occur (Liebana *et al.*, 2006). Overall, little is known about the spread of AMR *E. coli* within and between dairy farms.

Watson *et al.* (2012) made a longitudinal analysis of the epidemiology of ESBL-resistance in *E. coli* on a dairy farm in the UK. In that study, ESBL-producing *E. coli* was found in calf faecal samples and persistently in water troughs in the calving pen, but only occasionally in those in the dry cow pen. Moreover, such strains were persistently recovered from pen walls in the calving- and calf pens. Cows were also more likely to shed ESBL-producing *E. coli* after compared to before calving. These authors therefore suggested that transition via the calving area may be a crucial pathway for the dissemination of ESBL-producing *E. coli* between different cattle categories (Watson *et al.*, 2012). These results indicate that ESBL resistance disseminates within the farm via both clonal spread and via transfer of genes on conjugative plasmids (Watson *et al.*, 2012), similar to the earlier study by Liebana *et al.* (2006). Liebana *et al.* (2006) also showed that the same ESBL-producing *E. coli* clone persisted on the farm over time.

Since horizontal transfer of genes conferring quinolone resistance is still a rare event in *E. coli* from cattle (Jurado et al., 2008; Kirchner et al., 2011; Hordijk *et al.*, 2012; Marchese *et al.*, 2012), quinolone resistance is assumed to spread mainly by clonal expansion. Likewise, the same clone of QREC disseminated throughout the herd in Hoyle *et al.* (2005). Clonal spread of faecal AMR *E. coli* between different age categories was also observed on dairy farms in Japan (Yamamoto *et al.*, 2013).

It has been suggested that young animals are colonized with AMR *E. coli* from faeces of their mothers at birth (Bettelheim *et al.*, 1974; Watson *et al.*, 2012; Callens *et al.*, 2014). However, neither Gow *et al.* (2008) nor Watson *et al.* (2012) could find evidence that the mother's faecal microbiota was the only source of AMR *E. coli*. Instead, the farm environment may be a more important source for colonizing strains, as suggested by Hinton *et al.* (1985b) and Yamamoto *et al.* (2013).

More knowledge about the dissemination of AMR *E. coli* within and between farms and related risk factors is needed before making best-practice recommendations to reduce the dissemination of AMR *E. coli*.

1.4 Zoonotic aspects

A zoonosis is defined by WHO as any infection that is naturally transmissible between vertebrate animals and humans (WHO, 2015) and resistant bacteria is thus also covered by this definition. The interface between human and animals is complex; numerous pathways exist for the spread of AMR bacteria. Van den Bogaard *et al.* (2001) suggest that AMR *E. coli* reaches humans by direct contact or via contaminated food. Resistant *E. coli* from cattle may

contaminate milk (Straley *et al.*, 2006) and meat products (Alexander *et al.*, 2010) and subsequently spread to humans via the food-chain. Normally, proper cooking and pasteurization is sufficient to inactivate bacteria in meat and milk (Alexander et al., 2010). However, improper handling of the faeces-contaminated food may pose a risk for humans to be colonized with AMR *E. coli* from food (Alexander *et al.*, 2010). Corpet (1988) showed that the amount of contamination with AMR bacteria on food was correlated to the levels of AMR bacteria in the faecal microbiota of humans ingesting the food. Smith (1969) observed that *E. coli* of animal origin persisted poorly in the GI tract of humans, whereas both Linton *et al.* (1977) and Trobos *et al.* (2009) came to the opposite conclusion. Although *E. coli* in itself may cause infections in humans, the zoonotic potential of AMR *E. coli* in the food-chain comes mainly from the transfer of resistance genes from animal origin commensals to human pathogenic bacteria (van den Bogaard & Stobberingh, 2000). Although colonisation of the human gut by animal-derived strains is occasionally transient, there may be sufficient time for the transfer of resistance genes (Trobos *et al.*, 2009). Levy *et al.* (1976) observed that tetracycline resistance genes could be transferred between chicken and human *E. coli*. Winokur *et al.* (2001) showed that an AmpC-type betalactamase gene was transmitted from animal *E. coli* to human pathogenic *Salmonella*.

1.5 The current dairy cow sector in Sweden

Sweden currently has 344,000 dairy cattle, distributed on 4,400 farms with an average herd size of 78.4 cows (Swedish Board of Agriculture, 2014). Eighty-four percent of Swedish dairy cows are enrolled in the Swedish Official Milk Recording Scheme (SOMRS) (Växa Sverige, 2014). The average milk production for cows enrolled in SOMRS is 9,535 kg energy-corrected milk and the average geometric somatic cell count is 192,000 cells per mL of delivered bulk milk (Växa Sverige, 2014). Since 1990, the number of farms has decreased by 70%, the herd size has increased by 178%, and the milk yield has increased by 30% (Swedish Board of Agriculture, 2014). Hence, the Swedish dairy sector has undergone dramatic changes over a short period. In 2013, approximately 13% of all milk produced was certified by one or another organisation for organic farming (Swedish Board of Agriculture, 2014). Swedish cows are housed in either insulated or cold barns, a majority of them in free stall housing (60%)[1] for most of the year. In contrast to other countries, all Swedish cows must be released on pasture for stipulated periods each year.

[1]. Eva Stormwall, Växa Sverige, personal communication

Dairy farming in Sweden is strictly regulated under the Animal Welfare Act[2] and the Animal Protection Ordinance[3]. Sweden also has a long tradition of preventive health measures; these have been successful in the eradication of bovine viral diarrhoea, brucellosis, infectious bovine rhinotracheitis, tuberculosis, and bovine enzootic leucosis (Anonymous, 2013). Sweden is also presently considered free from paratuberculosis (Anonymous, 2013). Control programmes have been initiated for diseases such as *Salmonella*; the incidence in slaughtered Swedish cattle is therefore low (Anonymous, 2013). Consistent work with preventive measures has led to a low disease incidence among Swedish dairy cows (Växa Sverige, 2015) which minimizes the need for antimicrobials.

1.5.1 Usage of and prescription of antimicrobials

In Sweden, usage of antimicrobials in dairy cattle requires a prescription and such drugs cannot be dispensed without veterinary examination of the animal (Swedish Board of Agriculture, 2013). Moreover, veterinarians are not allowed to make profit by selling drugs (Veterinärutredningen, 2007). Follow-up treatment of the same animal or a group of animals can be administered by the farmer for a limited time (Swedish Board of Agriculture, 2013). As of 2016, *conditional delegated treatment* (Villkorad läkemedelsanvändning) may become a possibility on dairy farms. This means that farmers are allowed to diagnose and treat certain conditions on their own, given close collaboration with a herd-veterinarian (including regular visits). For the farmer, this requires strict recording of disease events and treatments as well as the completion of a course on legal requirements, risks associated with antimicrobials including resistance, handling and storage of medicines, injection techniques and safety aspects. The aim is to promote preventive measures to increase animal health and welfare (Swedish Board of Agriculture, 2013).

Antimicrobials for growth promotion were banned in 1986 (as the first country ever) (Cogliani *et al.*, 2011) and the use of antimicrobials for prophylaxis is not recommended. For domestic animals in Sweden, 90% of the antimicrobials consumed are for treatment of individual animals and only 10% for treatment of flocks or groups (Swedres-Svarm 2013). This is in contrast to many other European countries (ESVAC 2012). Furthermore—unlike in other European countries—few drug classes are used and the by far most sold drug is betalactamase-sensitive penicillin (ESVAC 2012). Recently, the veterinarian's right to prescribe fluoroquinolones and third and fourth generation cephalosporins is restricted to situations where culture and susceptibility

[2]. SFS no: 2003:1077, issued by the Ministry for Rural Affairs
[3]. SFS no: 1988:539, issued by the Ministry for Rural Affairs

testing have proven that no other available antimicrobial is expected to be effective. However, acute, life-threatening cases do not have to await culturing/susceptibility testing results. Moreover, fluoroquinolones can be prescribed in cases where culturing and susceptibility testing of the same infectious agent in the previous six months showed that no effective alternatives were available (Swedish Board of Agriculture, 2013).

Benzylpenicillin is the drug of choice against many bacterial infections in dairy cattle (Sveriges Veterinärmedicinska Sällskap, 2013). It is by far the most commonly prescribed antimicrobial (84% of total amount prescribed to cows in SOMRS), followed by tetracycline (7%), trimethoprim-sulfonamides (5%), enrofloxacin (3%), and ceftiofur (0.5%). The majority of systemic antimicrobials used in dairy cattle in SOMRS are prescribed for clinical mastitis (69%) (Växa Sverige, 2015). These are most often administered systemically; only a few veterinarians use the local route (Hårdemark, 2014). In contrast to many other countries, selective and not blanket dry cow therapy (DCT) is applied, meaning that antimicrobial treatment at drying off is used only for selected cows. Therefore, only approximately 26% of Swedish dairy cows were treated at drying off in 2013 (Swedres-Svarm 2013).

Data on the use of antimicrobials for calves is scarce due to incomplete registration in the National Animal Disease Recording Scheme (NADRS) (Mörk *et al.*, 2009). Treatment statistics are therefore limited to single surveys. Ortman & Svensson (2004) observed that 669 of the 3081 dairy heifers from birth to 90 days acquired an infectious disease. Of those, 38% were treated with antimicrobials (Ortman & Svensson, 2004). Respiratory illness and diarrhoea were the most common causes for treatment (Ortman & Svensson, 2004). In more than half of the cases, the drug was administered without consulting a veterinarian, probably with left-over drugs from a previous veterinary prescription. Despite recommendations, broad-spectrum antimicrobials were often used (Ortman & Svensson, 2004). For calves between birth and 90 days, the most commonly used drug and administration route was systemic penicillin in combination with dihydrostreptomycin (DHS; 55% of total amounts used), followed by systemic procaine penicillin (14%), DHS tablets for per oral administration (11%), systemic tetracycline (10%), and systemic trimethoprim-sulfonamide combinations (6%) (Ortman & Svensson, 2004). Benzylpenicillin is the drug of choice for most calf diseases requiring antimicrobials, except for severe diarrhoea or septicaemia for which trimethoprim-sulfonamide is a more appropriate alternative (Sveriges Veterinärmedicinska Sällskap, 2013).

1.5.2 Feeding milk to calves from cows treated with antimicrobials

Milk from cows treated with antimicrobials may not be sold for human consumption until stipulated time periods have elapsed since the last administration (withdrawal period). In Sweden, these withdrawal periods are determined by the Medical Products Agency as the time periods required for drugs in milk to decrease to below their maximum residue limit (Medical Products Agency, 2014). The withdrawal period is typically between zero and twelve days, depending on the antimicrobial, and up to fourteen days for some trimethoprim-sulfonamide combinations when administered twice daily (FASS Vet, 2015). There are two licensed, long-acting products for dry cow treatment. For one of them, the milk must be withheld for four days after calving if used at the latest one month before calving. If the administration was less than 30 days before calving, the withdrawal period has to be extended substantially. For the other product, milk may not be sent to the dairy plant for 36 hours after calving if it was administered at latest 35 days prior to calving. If administered later than 35 days before calving, the total withdrawal period is 37 days from the treatment day (FASS vet, 2015). On organic farms, withdrawal periods are in general the double and two days if the statutory period is zero days (KRAV, 2015).

Instead of discarding withdrawn milk, it is sometimes fed to calves. It is likely that Swedish dairy calves are fed milk from cows treated with antimicrobials, but to what extent is unknown. Healthy animals should not be exposed to antimicrobials, and the feeding of such milk may therefore be controversial from a resistance point-of-view. For non-organic farms, there are no formal regulations because the Animal–By-Products regulation[4] does not cover animal waste disposed on the farm. The disposal of such milk is probably done according to the farmer's own experiences and traditions.

Feeding milk from antimicrobial-treated cows is prohibited on organic farms during the statutory withdrawal period, except to the cow's own offspring (KRAV, 2015). Växa Sverige (one of three livestock associations in Sweden) has issued some recommendations related to the use of such milk as calf feed, but these are not scientifically based. They recommend that milk from cows treated with antimicrobials during lactation should be discarded until the second day after treatment and for colostrum from cows given DCT, from the third complete milking after calving[5]. However, it is unknown to what extent these recommendations are followed in the field.

As stated previously, there are unique prerequisites in Sweden for use of antimicrobials. The majority of discarded milk from antimicrobial-treated cows

[4]. Animal-by-products regulation (EC) No 1069/2009 issued by the European Union

[5]. Håkan Landin, Växa Sverige, personal communication

comes from cows treated systemically with benzylpenicillin. Since *E. coli* is intrinsically resistant to that drug (Giguré *et al.*, 2006), feeding milk with penicillin residues is not assumed to have an effect on the AMR levels in faecal *E. coli* from calves, but studies are still needed to confirm or dismiss such an assumption.

2 Aims of the thesis

The overall aim of the thesis was to obtain knowledge about the prevalence, risk factors, and spread of faecal AMR *E. coli* in preweaned dairy calves. This information will be used to define measures to reduce the overall burden of AMR *E. coli* on dairy farms. Specific objectives were to describe:

- The occurrence of AMR *E. coli* (with special focus on QREC) in relation to calf and farm characteristics, use of antimicrobials on the farm, feeding milk from antimicrobial-treated cows to calves, management factors and biosecurity factors.

- Practices regarding the feeding of colostrum and milk from cows given antimicrobials to calves in relation to different farm characteristics.

- The within-farm occurrence of QREC on dairy farms in relation to the prevalence of QREC in calves to identify potential dissemination routes for these bacteria.

- The genetic diversity of QREC within and between dairy farms and relate it to risk factors for the spread of QREC.

3 Materials and Methods

This section gives an overview over the materials and methods that forms the basis of the studies in this thesis. Detailed information is given in papers I-IV.

3.1 Summary of the study designs

- Paper I was a questionnaire survey investigating management routines related to the feeding of colostrum and milk from cows treated with antimicrobials to calves.
- Paper II was a cross-sectional study for risk factors related to the shedding of AMR *E. coli* by preweaned dairy calves.
- Paper III was a case-control study, investigating risk factors related to the shedding of QREC by preweaned dairy calves and post-partum dairy cows.
- Paper IV was a case-control study on the occurrence and genetic diversity of QREC on dairy farms.

3.2 Study populations

The study populations in papers I-IV were all derived from a group of farmers that took part in the questionnaire survey in paper I. Figure 1 summarizes the selection process of study farms.

The study population in paper I was recruited using a list of farms with e-mail addresses obtained from Växa Sverige. The use of a web-based survey tool limited the study population to farms that could be contacted electronically. The group of farms responding to the questionnaire was a representative sample of the general dairy-farm population with regard to geographic location and herd size.

The study population in paper II was recruited via the questionnaire in paper I. As a final question, respondents were asked to take part in the study in

paper II which involved submission of faecal samples from calves. Of those that voluntarily signed up for the study in paper II, only farms with ≥ 30 cows were enrolled. The farms enrolled were also representative of Swedish dairy farms with ≥ 30 cows regarding geographic location and herd size.

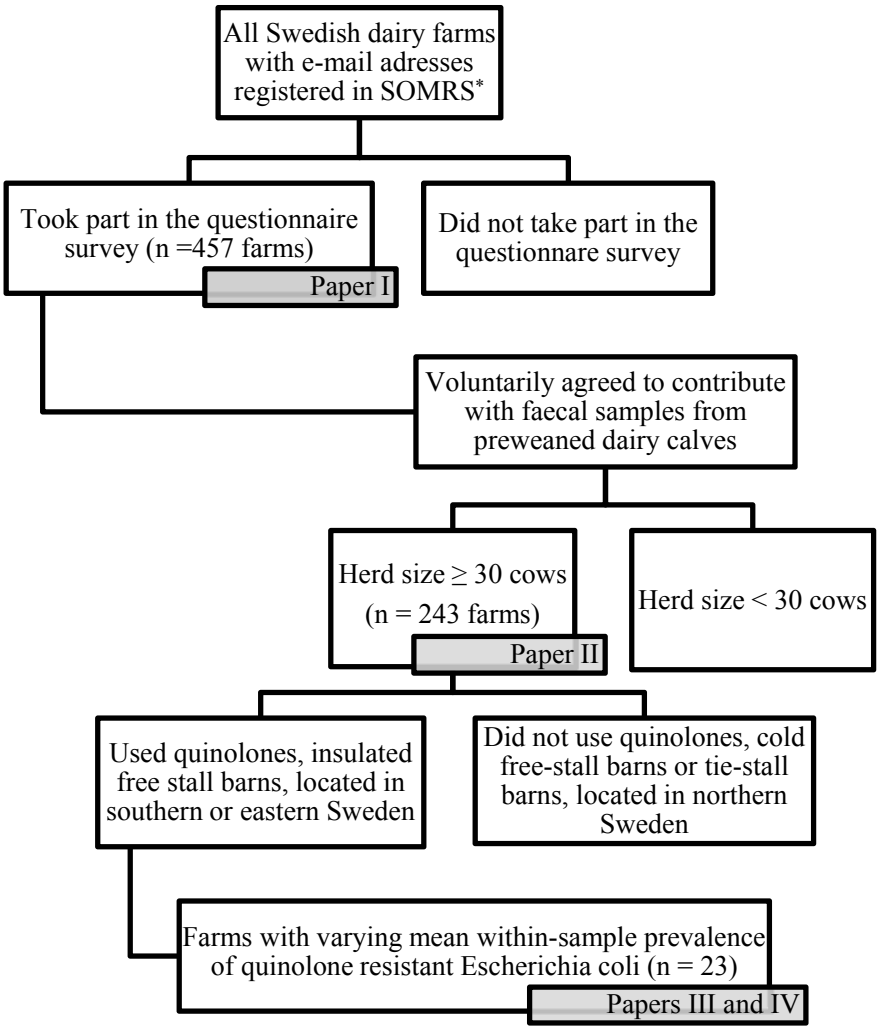

Figure 1. A flow-diagram of the selection process of farms enrolled in the studies in papers I-IV. *SOMRS is the Swedish Official Milk Recording Scheme

In papers III and IV, the study population consisted of 23 farms from the study in paper II, all which used fluoroquinolones, had insulated free-stall barns and were located in southern or eastern Sweden, but differed regarding their prevalence of QREC in faecal samples from calves. The farms were selected based on the mean within-sample prevalence of faecal QREC for three calves per farm (paper II). Potential case farms had a mean within-prevalence of QREC > 10% (n = 14 farms) and potential control farms had no QREC (n = 20 farms). Case farms were asked in a descending order of prevalence to take part in the study, and a total of ten farms were recruited. A total of eleven control farms were, in a random order, asked to participate, before ten farms could be enrolled. Finally, three farms with moderate mean within-sample prevalence of QREC (0.02 - 0.8%) were enrolled. These three farms did neither meet the criteria for cases nor controls, but were selected due to the presence of *E. coli* carrying genes encoding ESBL which were of interest for a parallel study where the shedding of *E. coli* resistant to third generations' cephalosporins was studied.

3.3 Sampling procedures

In papers II-IV, different types of samples were collected to investigate the occurrence of AMR *E. coli* in faecal, environmental and milk samples. The same faecal samples from cows and calves were used in papers III and IV.

3.3.1 Faecal samples

In papers II-IV, faecal samples from individual animals were collected by rectal swabs using Amie's charcoal culture swabs (Copan Diagnostics Inc., Murrieta, California, USA). The faecal samples in paper II were from three calves (7 to 28 days old) per farm, collected by the farmers and sent by postal transport to the National Veterinary Institute (SVA). The faecal samples in papers III and IV were from 15 calves (0 to 30 days), and five cows (0 to 3 days post-partum). Samples in papers III and IV were collected by the author of the thesis with some additional sampling by the farmers and sent by post or brought by car to the laboratory.

3.3.2 Environmental samples

The environments within the dairy farms in paper IV were sampled using a commercial kit called "Sterile cloth" (Sodibox, Névez, France). This product was developed for surface sampling on farms and food industries for monitoring of *Salmonella* and other bacteria. The cloths were used to sample calf feed troughs, calf water troughs, milk buckets, automatic milk feeders, and

walls in calf pens and the calving area. Up to three samples per sampling site were collected.

Overshoe sampling (Aho, 1992) was conducted to obtain pen floor samples from all cattle categories on the farms (except calves below 30 days) (paper IV). For this purpose, another commercial kit called "Sterisocks humid" (Sodibox) was used. Samples were collected from the floors in the lactating cow area, dry cow area, calving area, and in pens with young stock aged 1 to 6 months, and 7 to 24 months. Up to three samples per sampling site were collected. After collection, all samples were sent by post or brought by car to the laboratory.

3.3.3 Milk samples

In paper IV, five milk/milk substitute and five colostrum samples were collected in sterile plastic tubes by each farmer. The milk, milk substitute, or colostrum designated as calf feed was sampled just before feeding by dipping the plastic tube into the milk in the feeding equipment using a gloved hand. All milk samples were sent by post at ambient temperature (Nov – Feb) to the laboratory and stored frozen until analysis.

3.3.4 Determination of farm hygiene

While sampling for papers III and IV, the general hygiene of the farms was determined as the degree of faecal contamination in all environmental sampling sites described above. The hygiene was scored by the primary investigator (AD) on user-defined scales (0 to 3), where 0 was defined as clean surfaces and 3 as heavily faeces-contaminated areas.

3.4 Antimicrobial susceptibility testing

3.4.1 Preparation of samples

Rectal swabs were transferred to tubes with isotonic saline, 1 mL in paper II and 3 mL in papers III-IV, and vortex mixed to release faecal content. Isotonic saline, in aliquots of 100 mL, was added to the environmental samples (socks and cloths) before they were treated in a stomacher for 30 seconds. Environmental samples from the same sampling site within each farm were pooled by mixing aliquots (2 mL) of each sample suspension. Tenfold dilutions down to 10^{-5} (paper II), or 10^{-6} (papers III and IV) were prepared from the mixed suspension using isotonic saline.

3.4.2 Selective media

In papers II-IV, the proportion of resistant *E. coli* to total *E. coli* in each sample (within-sample prevalence) was determined for all sample suspensions by parallel plating on selective and non-selective media.

In paper II, samples were plated and *E. coli* counted on non-supplemented MacConkey agar plates and MacConkey agar plates supplemented with either streptomycin (32 mg/L), nalidixic acid (32 mg/L), or cefotaxime (1 mg/L).

In papers III and IV, sample suspensions were cultured and *E. coli* counted on Petrifilm Select *E. coli* Count Plate (SEC plate; 3M Microbiology Products, St. Paul., MN, USA). Sample suspensions were cultured on SEC plates directly and after addition of a nalidixic acid solution. The final concentration of nalidixic acid on the SEC plate was 32 mg/L.

3.4.3 Broth microdilution

In paper II, the MIC (for each of 12 antimicrobials) of a random *E. coli* from the non-supplemented MacConkey (confirmed as *E. coli* using the spot indole test) was determined using broth microdilution according to the CLSI (2013) standards. Tests were performed in VetMIC GN-mo panels (ver. 4, National Veterinary Institute, Uppsala, Sweden).

3.5 Molecular typing methods

3.5.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was used in paper II to investigate the presence of genes encoding transferrable ESBL or plasmid-mediated AmpC betalactamases (pAmpC). For this purpose, all cefotaxime resistant (Ctx^r) isolates confirmed as *E. coli* (using the spot indole test) were screened by two multiplex-PCRs for detection of the following phylogenetic gene groups: pAmpC (Pérez-Pérez & Hanson, 2002) and $bla_{CTX\text{-}M}$ (Woodford *et al.*, 2006), respectively.

PCR was also used in paper IV for preparation of amplicons for Multiple Locus Variable-number Tandem Repeat Analysis (MLVA), see details in 3.5.2. In the MLVA-assay, ten loci were amplified in four multiplex-PCRs and one singleplex-PCR (Lindstedt *et al.*, 2007; Løbersli *et al.*, 2012). The protocol was modified according to Lindstedt *et al.* (2007) and Løbersli *et al.* (2012).

3.5.2 Multiple-Locus Variable-number tandem repeat Analysis

In paper IV, MLVA was used to determine the genetic relatedness of QREC. Preparation of amplicons is described under section 3.5.1. In the final step of the MLVA-assay, the length of PCR amplicons (the number of tandem repeats

in each of ten loci) was determined by capillary electrophoresis. On the basis of the number of repeats, each locus was designated an allele number, resulting in a unique MLVA allelic profile for each strain.

3.5.3 Sequencing of specific variants of ESBL and pAmpC genes

For genes encoding ESBL or pAmpC found in the studies in paper II, the specific gene variants of the phylogenetic groups identified by PCR were determined by sequencing, using group-specific primer-pairs according to Sundsfjord *et al.* (2004).

3.6 Data from questionnaires and interviews

A major part of the data used to test the effect of various factors on resistance in faecal *E. coli* form calves was obtained from the farmers via questionnaires.

3.6.1 Paper I

Paper I was based mainly on questionnaire data regarding farm practices related to the feeding of calves with waste colostrum (WC: from the first milking after calving), waste transition milk (WT: from the second milking until the fourth day after calving) and waste milk (WM) from cows treated with antimicrobials during lactation (during ongoing treatment; TWM and during the statutory withdrawal period; WWM). The questionnaire was developed by the author in the online survey platform Easyresearch[6] and a survey URL link was made available to farmers via e-mails, on Växa Sverige's webpage, and in farmer-managed groups in social media. The questionnaire was developed with conditional branching, which directed questions only to those respondents that they were applicable for.

3.6.2 Paper II

Data on the use of colostrum and milk from cows treated with antimicrobials from the survey in paper I was used in the risk factor analysis for AMR in faecal *E. coli* from calves (paper II). A second web-based survey was sent by e-mail to farms (paper II) to obtain data on use of antimicrobials to cows and preweaned dairy calves. The questions were designed to obtain a semi-quantitative estimate of the frequency of antimicrobial treatments; more precisely as how often antimicrobials were used in a defined time interval (year, month, or week). Semi-quantitative responses were transformed to quantitative estimates, using the "best-guess" interpretation of the number of

[6] www.easyresearch.se

antimicrobial treatments included in each response option and adjusted for herd size to allow between-herd comparisons.

3.6.3 Paper III

In paper III, data on biosecurity factors, management and feeding of calves, and routines around calving were obtained through a structured (face-to-face) interview with the farmer during the farm visits. Data on recent use of antimicrobial drugs (last four months) were obtained from on-farm records and veterinary invoices.

3.7 Data from other sources

In paper I, data on herd size, geographic location (postal codes), and predominant milking and housing system were retrieved from the SOMRS. In paper II, data on prescription of antimicrobials on each farms from NADRS, data on predominant breed, mean herd-size, and geographic location were obtained from the SOMRS database. In paper IV, data on animal trade patterns were obtained from the central register of bovine animals (Swedish Board of Agriculture, Jönköping, Sweden), whereas data on distances between farm-pairs were recorded by manually measuring distances on the map in Google maps (https://maps.google.com) using the "distance measuring" application.

3.8 Statistical analysis

Statistical analyses were used in all four papers to investigate significant differences between groups of farms or to assess the association between various factors on the occurrence of AMR *E. coli*.

In paper IV, farms were categorised as high (H) and low (L) farms, based on the mean within-sample prevalence of QREC in faeces from preweaned calves ($H \geq 0.5\% > L$).

Fisher's exact test was used to investigate the association between farm characteristics and management routines (paper I), and to compare the occurrence of QREC in different age categories or samples (paper IV), between farms where QREC was common in faeces from calves (H-farms) and farms where QREC was rare (L-farms) in calves (paper IV). Moreover, this test was used to assess the representativeness (geography) of studied farms in papers I and II compared with the general population of dairy farms in Sweden.

The Wilcoxon signed rank test was used to assess the representativeness of farms (herd size, papers I and II), and to investigate significant differences in

the within-sample prevalence of QREC in various samples and on H- and L-farms (paper IV).

Analysis of correlation, using the Spearman rank-order correlation coefficient, was used in paper IV to investigate significant associations between the genetic diversity of QREC and the number of purchased cattle. A two sided t-test was used to compare the between-farm distance for farm-pairs with and without at least one shared MLVA type.

Multivariable regression analyses was conducted in papers II and III to investigate the importance of various risk factors for the occurrence of AMR *E. coli*. If the outcome was the proportion of animals shedding resistant *E. coli*, logistic regression was used. If the outcome variable was the within-sample prevalence of resistant *E. coli*, zero-inflated negative binomial regression was used. The individual animal (cow or calf) was the study unit and clustering within a farm was accounted for using robust standard errors. Potential risk factors were first tested in univariable analyses. All variables (given no collinearity) in paper III, and variables with $p \leq 0.2$ in paper II, were eligible for the multivariable analyses. In paper II, a manual stepwise-backwards elimination was applied, whereas in paper III, a manual stepwise-forwards selection procedure was used.

4 Results

A summary of the results presented in papers I to IV is given below. For a more detailed description of the studies, the reader is referred to each paper.

4.1 Prevalence of AMR *E. coli*

4.1.1 Selective plates

Streptomycin resistance (paper II)

Streptomycin resistant (Sm^r) *E. coli* was isolated from 90% of the calves (aged 7 to 28 days), representing 96% of the 243 farms. The within-sample prevalence of Sm^r *E. coli* for individual calves ranged from 0 to 100%, where the median, 25th, and 75th percentiles were 4, 0.07, and 32%, respectively.

Quinolone resistance (papers II and III)

In paper II, QREC was isolated from 49% of the calves (aged 7 to 28 days), representing 60% of the farms. The within-sample prevalence of QREC for individual calves ranged from 0 to 100% and the median, 25th, and 75th percentiles were 0, 0, and 0.5%, respectively.

In paper III, QREC was found on 22 of the 23 farms. Quinolone resistant *E. coli* was isolated from calves (aged 0 to 30 days) on all but one farm and from post-partum cows on 13 of the 23 farms. Significantly more calves (60%) than cows (27%) carried QREC. Likewise, a significantly higher within-sample prevalence of QREC (median 0.0003%) was found in faeces from calves than cows (median 0%).

Cefotaxime resistance and ESBL (paper II)

Cefotaxime resistant *E. coli* was isolated from 11% of the 729 calves (aged 7 to 28 day), representing 18% of the 243 farms. The within-sample prevalence of

Ctx^r *E. coli* for individual calves ranged from 0 to 78%, and the median, 25th, and 75th percentiles were all 0%.

Genes conferring ESBL or pAmpC-resistance were found in 9 of 81 tested isolates of Ctx^r *E. coli*. Four of these isolates carried *bla*$_{CTX\text{-}M\text{-}15}$, one carried *bla*$_{CTX\text{-}M\text{-}1}$, and four carried *bla*$_{CMY\text{-}2}$. The remaining 72 isolates did not carry any of these transferable genes.

4.1.2 Resistance in randomly selected *E. coli* isolates (paper II)

The proportion of the 729 *E. coli* isolates (one isolate each from calves aged 7 to 28 days) resistant to each of 12 antimicrobials is described in Figure 2. Resistance to at least one antimicrobial was seen in 48% of the isolates, and multidrug resistance (resistance to three or more antimicrobials) was observed in 27% of the isolates. The most common multiresistant phenotype was the AMR combination Sm^r-Sulfamethoxazole (Su^r)-Tetracycline (Tc^r).

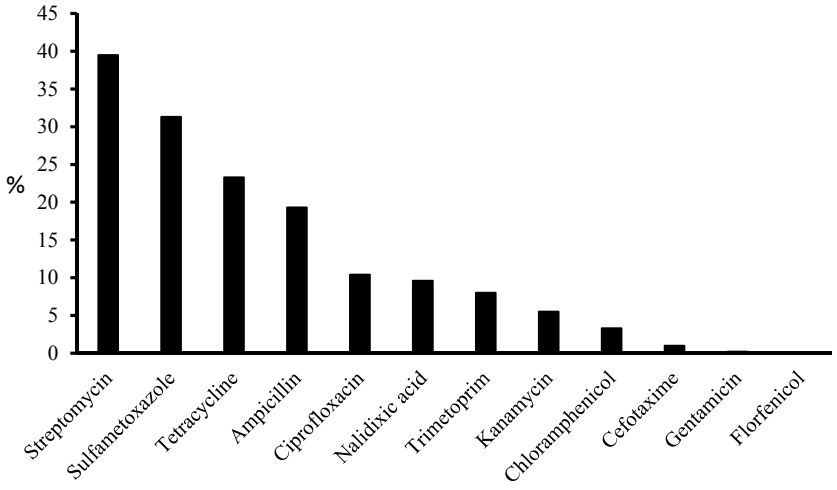

Figure 2. The proportion of randomly selected *E. coli* isolates (n = 729) from preweaned dairy calves aged 7 to 28 days resistant to each tested antimicrobial (paper II).

4.2 Risk factors for AMR *E. coli* (paper II)

Significant risk factors for the shedding of AMR *E. coli* were related to calf factors, farm characteristics and seasonal effects, farm-level antimicrobial usage, and farm-level use of WM as calf feed. A simplified summary of significant risk factors that affected the shedding of AMR *E. coli* in faeces of

preweaned calves is found in Table 1, followed by a more detailed explanation in later sections.

Table 1. *Summary of factors in paper II associated with the shedding of resistant E. coli in faeces of preweaned dairy calves. Lower occurrence of resistance is indicated by an arrow pointing down, and higher occurrence by an arrow pointing up. The symbol – indicates no significant association between a factor and resistance.*

Factor	Selective plates			Random *E. coli*							
	Ctx	QREC[1]	Sm	Am	Cm	Ci	Km	Nal	Sm	Su	Tc
Ageing calf	-	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Group vs. single calf housing[2]	↑	-	-	-	-	-	-	-	-	-	↓
Large vs. small herds	↑	↑	↑	-	-	↑	↑	↑	↑	-	↑
South/East[3] vs. North Sweden	-	↑	-	-	↓	-	-	-	-	-	-
Swedish Holstein vs. Swedish Red or mixed breed	-	↑	-	-	↓	↑	-	↑	-	-	-
Tie stall /Automatic milking system vs. parlour milking	-	-	-	↓	-	-	-	-	-	-	↓
Feeding waste milk to calves at least occasionally	-	↑	↑	-	-	-	-	↑	↑	-	-
Oct – Dec vs. Jan – Sep	-	-	↓	-	-	-	-	-	-	-	-
Ceftiofur to cows	-	-	-	↑	-	-	-	-	-	-	-
Ceftiofur to calves	↑	-	-	-	-	-	-	-	-	-	-
Oral DHS[4] calves	-	↑	↑	-	-	↑	-	↑	↑	-	-
Penicillin-DHS to calves	-	-	↑	-	-	-	-	-	-	-	-
More cows treated with long-acting DCT[5]	-	-	↑	-	-	-	-	-	-	-	-
Short-acting DCT	-	-	↑	-	-	-	-	-	-	-	-
Fluoroquinolones to cows	-	↑	-	-	-	-	-	-	-	-	-
Trimethoprim-Sulfonamide to calves	-	↑	-	-	-	-	-	-	-	-	-
Tetracycline to cows	-	-	-	-	-	↑	-	↑	-	-	↑
Tetracycline to calves	-	-	-	-	↑	-	-	-	-	-	↑

[1]Quinolone resistant *E. coli*, [2]For Tc^r group housing with at least one calf > 30 days, [3]For Cmr, South vs. North/East Sweden, [4]Dihydrostreptomycin, [5]Dry cow therapy with antimicrobials

Calf factors

Increasing calf age led to a decreased within-sample prevalence of Sm^r and QREC and to decreased odds of all resistance traits except Ctx^r. The occurrence of AMR *E. coli* was at its maximum at one week of age and declined gradually thereafter.

Group housing of the calf led to higher odds of Ctx^r *E. coli*, but lower odds of Tc^r *E. coli* (if housed with calves older than one month). There were no significant differences in AMR *E. coli* shedding between bull and heifer calves.

Farm characteristics

In general, shedding of AMR *E. coli* was more common in large than small herds. Calves on farms in some regions were more likely to shed QREC (South and East > North Sweden) and chloramphenicol resistant (Cm^r) *E. coli* (North and East > South Sweden) than calves in other regions. Higher odds of ciprofloxacin (Ci^r) and nalidixic acid resistant (Nal^r), but lower odds of Cm^r *E. coli,* were observed on farms with predominantly the Swedish Holstein breed than on farms with either Swedish Red or mixed breeds. For QREC, a significant interaction was found between predominant herd breed and geographic location. Calves on farms with tie-stall milking or automatic milking systems (AMS) had lower odds of ampicillin resistant (Am^r) or Tc^r *E. coli* than farms with parlour milking. No differences in the occurrence of AMR *E. coli* were found between non-organic and organic farms.

Feeding waste milk from antimicrobial-treated cows to calves

Neither feeding WC nor WT to calves affected the faecal shedding of AMR *E. coli* among calves.

In the univariable analyses in paper II, feeding WM to calves increased faecal shedding of AMR *E. coli*, but no difference was found between calves on farms that fed TWM and those on farms that only fed WWM. Thus, these two categories were amalgamated to one category (WM) in the multivariable analysis. Higher within-sample prevalence of Sm^r *E. coli* and QREC and higher odds of Nal^r and Sm^r *E. coli* were observed for calves on farms that fed WM to calves compared to calves on farms where WM always was discarded. A significant interaction between using fluoroquinolones in cows and feeding WM to calves was also found for QREC.

Using antimicrobial-treated cows for nursing was not associated with the occurrence of AMR *E. coli*.

Use of antimicrobials

Systemic fluoroquinolone treatment of cows was associated with more faecal QREC among calves, but the shedding of QREC by calves varied on farms that used fluoroquinolones regularly (Figure 3). This made us look more in detail (papers III and IV) to determine which other factors were associated with the shedding of QREC.

Systemic tetracycline treatment of cows was associated with increased odds of Nal^r, Ci^r, or Tc^r *E. coli* and systemic tetracycline treatment of calves led to higher odds of Tc^r or Cm^r *E. coli*. Systemic ceftiofur treatment of cows increased the odds of Am^r *E. coli*, and using ceftiofur in calves increased the odds of Ctx^r *E. coli*. Systemic trimethoprim-sulfonamide treatment of calves was associated with increased within-sample prevalence of QREC. Short-acting DCT and treating a higher proportion of cows in the herd with long-acting DCT resulted in more calves with Sm^r *E. coli*. Orally administered DHS

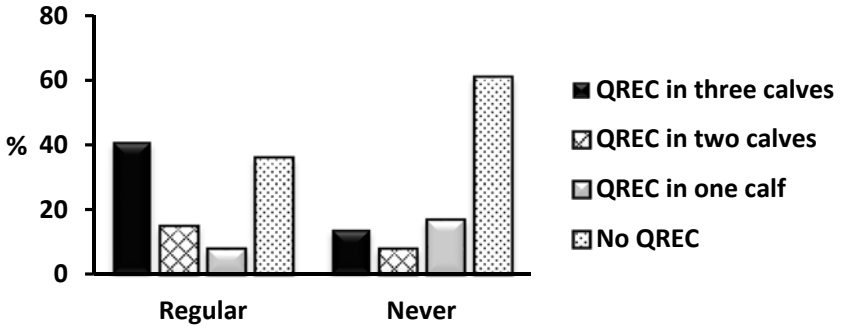

Figure 3. Proportion of calves with at least one quinolone resistant *E. coli* (QREC) relative to calves with only quinolone susceptible *E. coli* on farms that used fluoroquinolones (FQ) regularly for cows and those that never used FQ for cows (paper II).

to calves was associated with higher within-sample prevalence of QREC and Sm^r *E. coli*, and with higher odds of Nal^r, Ci^r, or Sm^r *E. coli*. Use of systemic benzylpenicillin in combination with DHS in calves also gave higher within-sample prevalence of Sm^r *E. coli*. However, the use of systemic benzylpenicillin in cows and calves, intrauterine tetracycline treatment of cows, and intramammary antimicrobials (penicillin only or penicillin-DHS combination) for the treatment of cows during lactation was not associated with any of the resistance traits.

4.3 Farming practices related to feeding waste milk

Of the 457 Swedish dairy farmers that completed the questionnaire in paper I, 89% fed WC and 85% fed WT at least occasionally to calves. The most important reason not to feed WC was if it was of insufficient quality (as determined by the farmer) or if alternative colostrum was available. If WT was not always fed, it was only fed to certain calves (in particular bull calves). Waste colostrum and WT were fed to calves more often on non-organic than organic farms and on more farms with tie-stall barns than free-stalls.

Waste milk from cows treated during lactation was at least occasionally fed to calves 56% of the farms (TWM) and on 79% of the farms (WWM). All farms that fed TWM also fed WWM to calves. The most important reason for not feeding TWM to calves was to avoid feeding them milk from cows with mastitis. For WWM, a common practice was to only give such milk to certain calves (in particular bull calves). Seven percent of the farmers reported that antimicrobial-treated cows occasionally were used as nursing cows. Feeding TWM and WWM was more common on non-organic than organic farms, on farms with cows housed in tie stall compared to free stall barns, and on farms located in South Sweden compared to other regions.

4.4 Risk factors for QREC (paper III)

Risk factors for QREC in calves 0 to 30 days old

Calves between 18 and 30 days of age had a significantly higher within-sample prevalence of QREC than calves 0 to 17 days old. The use of fluoroquinolones in the herd during the previous four months also resulted in a higher within-sample prevalence of QREC. Waste milk feeding on the farm-level, as well as on the individual calf-level, resulted in a higher within-sample prevalence of QREC. Individual calves— independent of whether it was fed WM or not— all shed QREC at negligible levels at approximately three weeks of age.

There were two significant risk factors for carriage of at least one QREC in preweaned calves. The first was carriage of QREC by at least one of the sampled post-partum cows on the farm. The second was use of the calving pen as a sick pen rarely rather than often.

Risk factors for QREC in post-partum cows 0 to 3 days after calving

Since the carriage of QREC in preweaned calves was associated with carriage of QREC in post-partum cows, risk factors for QREC shedding by post-partum cows were also investigated. Calving in a group (free stall or group calving pen) compared to calving in single pens or tie stalls led to higher odds of QREC in the cow faeces. Other risk factors for QREC shedding in cows were poorer than average farm hygiene, purchase of cattle, and sharing animal transports with other livestock farmers.

4.5 Dissemination and genetic diversity of QREC (paper IV)

4.5.1 QREC contamination of the farm environment and milk

The proportion of samples positive for QREC for different sampling sites was 48% for calf feed troughs, 55% for calf water troughs, 32% for milk buckets, 55% for automatic milk feeders, 52% for calf pen walls (single and group pens), 50% for calving pen walls, 52% for calving pen floors, 39% for lactating cow pen floors, 26% for dry cow pen floors, and 35% for young stock pen floors (all ages). In addition, 3% of all colostrum and milk samples contained QREC.

Quinolone resistant *E. coli* was significantly more common on H- than L-farms in feed and water troughs in the calf area, in milk buckets, on the walls of calf single and group pens, on the calving pen floors and on the floors of pens for young stock aged 1 to 6 months. There were no differences between H- and L-farms for samples from the automatic milk feeders, the calving pen walls, or from the floors in the dry and lactating cow areas and in pens with young stock aged 7 to 24 months. The proportion of milk and colostrum samples with QREC was similar on H- and L-farms. The proportion of positive samples, the median and max within-sample prevalence in samples from the farm environment is described in Table 2.

Table 2. *Proportion of positive samples, the median and max within-sample prevalence of quinolone resistant E. coli (QREC) in environmental samples from farms where QREC is common in faecal samples from preweaned dairy calves (H) compared to farms QREC is rare in faecal samples from such calves (L).*

Sample type (number of samples)	H-farms			L-farms		
	% positive	Median (%)	Max (%)	% positive	Median (%)	Max (%)
Calf feed troughs (23)	82	10	100	17	0	0.2
Calf water troughs (20)	100	5	100	10	0	0.05
Calf milk buckets (22)	70	4	33	0	0	0
Calf automatic milk feeders (11)	83	3	26	20	0	0.005
Calf single pen walls (22)	82	4	17	27	0	45
Calf group pen walls (23)	82	1	49	25	0	0.33
Calving pen walls (22)	60	0.8	42	42	0	40
Calving pen floors (23)	82	0.08	30	25	0	0.36
Lactating cow pen floors (23)	55	0.02	5	25	0	4
Dry cow pen floors (19)	33	0	20	20	0	13
Young stock 1 to 6 months pen floors (23)	64	0.04	2	8	0	0.006
Young stock 7 to 24 months pen floors (23)	45	0	0.1	25	0	2

4.5.2 Genetic diversity and dissemination of QREC

A total of 136 isolates from H-farms were genotyped by MLVA. Twenty-three unique MLVA types were identified. The within-farm diversity index (DI) was calculated as the proportion of unique MLVA types of the tested isolates on each farm. Two to nine unique MLVA types were found on each farm, resulting in a DI between 0.1 and 0.6. Six MLVA types were identified on more than one farm, whereas the remaining types were found only on a single farm. One MLVA type was identified on six farms, another on five, and four different types were found on two farms each.

Farm-pairs with shared MLVA type were located significantly closer to each other than farm-pairs without shared MLVA types (196 km vs. 276 km). There was a strong positive correlation ($\rho = 0.81$) between the number of purchased cattle since 1998 and the DI. A group of cattle had been traded once between two farms in the study and on these two farms, 90% of the total number of isolates tested had the same genotype.

5 Discussion

The papers in this thesis demonstrate that AMR *E. coli* is commonly isolated from faeces of preweaned dairy calves. A number of risk factors at the farm and calf level have been identified and the dissemination of QREC has been assessed. Here, the most important results are discussed in a general manner across papers and across aims. The focus of the discussion was to find measures to reduce the overall burden of AMR *E. coli* on dairy farms.

5.1 Factors related to the occurrence of AMR *E. coli*

One specific aim of this thesis was to describe the occurrence of AMR *E. coli* in relation to various risk factors. Several factors were shown in this thesis to have an impact on the occurrence of AMR *E. coli*, but special focus was on the age of the calf, use of antimicrobials in the herd and the use of WC, WT and WM as calf feed.

5.1.1 Age-related dynamics of AMR *E. coli*

The age of the calf was an important factor for the shedding of AMR *E. coli*. Not only did calves carry more QREC than post-partum cows (paper III), but most resistance traits also declined with increasing age of the calf (papers II and III). The more frequent isolation of resistant *E. coli* from young compared to old animals is consistent with earlier studies on cattle as well as humans and pigs (Reves *et al.*, 1990; Moro *et al.*, 1998; DeFrancesco *et al.*, 2004; Sato *et al.*, 2005; Dolejská *et al.*, 2008; Gow *et al.*, 2008; Berge *et al.*, 2010; Hansen *et al.*, 2013; Yamamoto *et al.*, 2013). Given that calves and cows are exposed more or less to the same microbiota on the farm, the difference probably relies on factors related to colonization ability of AMR *E. coli* in the gut at various ages (Edrington *et al.*, 2008).

The ability for a strain to colonize the gut depends on the colonization resistance in the GI tract, i.e. competition from the indigenous microbiota, but also the host immune system (Buffie & Pamer, 2013). The GI microbiota of newborn calves is non-versatile and low in bacterial counts and therefore offers a less developed colonization resistance than the GI microbiota of older cattle (Lukáš *et al.*, 2007; Edrington *et al.*, 2012a; Mayer *et al.*, 2012; Oikonomou *et al.*, 2013; Klein-Jöbstl *et al.*, 2014b). Moreover, although all essential components of the immune system are present at birth, they may not be functional until two to four weeks later (Reber *et al.*, 2006). Young calves are therefore more vulnerable than older, healthy cattle to the establishment of unwanted strains, such as pathogenic bacteria or AMR *E. coli*, in the GI tract (Edrington *et al.*, 2008). Two other examples are the establishment of diarrhoeagenic *E. coli*, which is most common during the first postnatal week (Wieler *et al.*, 2007) and the more frequent occurrence of *Salmonella* infection in young calves than older cattle (Edrington *et al.*, 2008).

However, underdeveloped colonization resistance and immature mucosal immune system do not in and of themselves explain why AMR *E. coli* strains are favoured over susceptible ones in the young calf gut. A possible explanation is that AMR *E. coli* strains sometimes carry more virulence factors, e.g. adhesion factors, than susceptible *E. coli* strains (Nowrouzian *et al.*, 2005; Karami *et al.*, 2006; de Verdier *et al.*, 2012; Lastours *et al.*, 2014). Human QREC strains are also significantly more resistant to acid and are better at using the main nutrient source in the gut than quinolone susceptible strains (Lastours *et al.*, 2014). Eberhart *et al.* (2012; 2014) observed that a bacteriocin produced by some AMR *E. coli* strains from calves can inhibit growth of susceptible *E. coli*. Hence, AMR *E. coli* strains seem to possess colonization factors or inhibiting substances that balance the cost of producing resistance elements. All these factors may facilitate their survival and establishment in the GI tract and explain their selective advantage over susceptible strains, even in the absence of a selective pressure from exposure to antimicrobials.

A decline in the prevalence of resistant *E. coli* with increasing age has also been observed by others (Hinton *et al.*, 1985a; b; Hoyle *et al.*, 2004a; b; Berge *et al.*, 2005a; Watson *et al.*, 2012; Brunton *et al.*, 2014). In paper II, resistance in *E. coli* was most common at around one week of age, which coincides with the peak occurrence of *E. coli* in general in the GI tract of calves (Lukáš *et al.*, 2007; Mayer *et al.*, 2012). The displacement of resistant *E. coli* strains by susceptible ones (Hinton *et al.*, 1985a; b) may be a consequence of increasing activity of the immune system and competition from the indigenous microbiota as the latter expands in number and diversity. The species mainly responsible for the colonization resistance are the anaerobic ones (Andremont, 2003),

which increase rapidly in numbers during the second and third week of the calf's life (Lukáš *et al.*, 2007; Oikonomou *et al.*, 2013; Klein-Jöbstl et al., 2014b). In general, the GI microbiota increases in diversity and species richness at this time point (Edrington et al., 2012a; Oikonomou *et al.*, 2013; Klein-Jöbstl *et al.*, 2014b). Hence, colonization resistance is probably enhanced after two to three weeks of age. Runnels *et al.* (1980) observed that some *E. coli* strains colonize less and less efficiently with increasing calf age. These authors propose that host-resistance to specific strains is mediated by the innate immune system (Runnels *et al.*, 1980). Because AMR *E. coli* strains carry more virulence factors (Karami *et al.*, 2006; de Verdier *et al.*, 2012; Lastours *et al.*, 2014), it can be assumed that AMR strains also stimulate the immune system more than susceptible ones. Thus, increased host resistance to AMR *E. coli* with increasing age may to some extent explain the age-related decline in AMR *E. coli*. Since neither colonization resistance (Lukáš et al., 2007; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014b) nor the innate immune system is very active before two weeks of age (Reber *et al.*, 2006), inhibition of most resistant strains cannot occur until after that. All the above changes coincide with the sudden decline in the within-sample prevalence of QREC observed in paper III.

5.1.2 Antimicrobial use and its implications for AMR *E. coli*

In papers II and III, sampled calves were supposedly healthy and untreated with antimicrobials. The aim was therefore not to assess the impact of individual treatments on the calf gut microbiota, but to assess the impact of farm-level use of antimicrobials on the overall occurrence of faecal resistant *E. coli* in preweaned calves. The relative importance of farm-level as opposed to individual-level antimicrobial use in the occurrence of AMR *E. coli* is confirmed by Tragesser *et al.* (2006). In papers II and III, we observed that farm-level use of antimicrobials influenced the occurrence of AMR *E. coli* in faeces of calves. Not only antimicrobial treatment of preweaned calves, but also treatment of cows increased faecal shedding of AMR *E. coli* by the calves. Although antimicrobial treatments of individuals only transiently select for AMR *E. coli* in the GI microbiota (Berge *et al.*, 2006; Singer *et al.*, 2008), resistant strains will concentrate in faeces and subsequently, the farm environment. Resistant *E. coli* from these cows may then enter the GI tract of other individual animals. As stated by Lipsitch & Samore (2002), treatment of one individual in the herd may increase the ratio of resistant to susceptible *E. coli* in the surroundings, thereby facilitating colonization with AMR *E. coli* in the animals sharing the same environment. Since AMR *E. coli* may outcompete susceptible ones in the calf gut (Khachatryan *et al.*, 2004), it is therefore likely

that AMR *E. coli* strains from other animals in the herd may enrich in the calf gut.

Streptomycin resistance was a common finding in the *E. coli* from calves (paper II). Such resistance emerges rapidly as a consequence of treatment with streptomycin or DHS, for which cross-resistance to streptomycin is almost complete (Giguré *et al.*, 2006). Oral DHS-treatment of calves, systemic treatment of calves with benzylpenicillin/DHS combinations, as well as DCT with long- and short-acting intramammary tubes (often containing DHS) increased shedding of Sm^r *E. coli* (paper II). Oral DHS-treatment for calf diarrhoea is not recommended in Sweden (Sveriges Veterinärmedicinska Sällskap, 2013); it is not likely to be effective given the high Sm^r levels obtained in this study and in clinical isolates from the GI tract of calves (Swedres-Svarm 2013). Oral administration of DHS to calves also increased the shedding of Nal^r and Ci^r *E. coli*. Previous studies have shown that oral DHS may select for *E. coli* resistant to multiple types of antimicrobials, probably because of limited absorption from the gastrointestinal tract (Gaines et al., 1978). Only 19% and 18% of the farms in paper II used oral DHS or systemic benzylpenicillin/DHS, respectively, which indicates that these drugs can be avoided without severe consequences for calf health. A synergy between penicillin and DHS has been proposed, but has not been proven in vivo for treatment of mastitis (Whittem & Hanlon, 1997). Long-acting intramammary antimicrobials without an aminoglycoside component are, however, not available in Sweden. Overall, the results indicate that the inclusion of the DHS components in products for dairy cows and calves scarcely can be justified from a resistance point of view.

In paper II, we observed that the use of certain antimicrobials was associated with resistance to unrelated drugs, in line with the results of Bosman *et al.* (2014). Systemic treatment with tetracycline not only increased the odds of Tc^r *E. coli*, but also the odds of *E. coli* with unrelated resistance traits, such as Nal^r, Ci^r and Cm^r. This is probably due to co-resistance between Tc^r and unrelated antimicrobials as 55%, 51%, and 91% of the Nal^r, Ci^r and Cm^r isolates, respectively, were also Tc^r (results not shown). Likewise, tetracycline and chloramphenicol resistance is very common in QREC isolates from cattle in other countries (Hordijk et al., 2012; Marchese et al., 2012). Another explanation is that so called Mar (multiple antibiotic resistant) mutants may emerge following tetracycline exposure (George & Levy, 1983). Isolates with the Mar locus exhibit decreased expression of the OmpF porin, which results in less accumulation of the drug in bacterial cells (Cohen *et al.*, 1988). This mechanism has been associated with resistance to tetracycline,

chloramphenicol, quinolones, betalactams, and rifampicin (George & Levy, 1983).

Use of fluoroquinolones in cows was a risk factor for shedding of QREC in both papers II and III. The use of these drugs has also been associated with a higher prevalence of QREC in other studies, either after treatment of the individual calf or on the farm-level (Pereira *et al.*, 2011; Cummings *et al.*, 2013; Jones *et al.*, 2013; Yamamoto *et al.*, 2013; Bosman *et al.*, 2014). Until 2012, fluoroquinolones were the second most prescribed antimicrobial drug class for systemic use in dairy cows enrolled in SOMRS (Växa Sverige, 2015), and they were used regularly for the treatment of coliform mastitis. In January 2013, prescription of this drug was restricted due to its importance in human medicine (Swedish Board of Agriculture, 2013). The prescription of fluoroquinolones has since then decreased (Växa Sverige, 2015), but it is uncertain whether the decrease is due to these restrictions or other factors.

The within-sample prevalence of QREC in calves was also higher on farms that treated calves with systemic trimethoprim-sulfonamides products (paper II). This association is probably due to co-resistance, as 38% of the Su^r isolates were also Nal^r (results not shown). Likewise, sulfonamide and trimethoprim resistance is very common in QREC isolates from cattle in other countries (Hordijk et al., 2012; Marchese et al., 2012).

Farms that reported treatment with systemic ceftiofur (a third-generation cephalosporin) of calves were more likely to have calves with Ctx^r *E. coli*, which is in line with the findings of Tragesser *et al.* (2006). Farms that had used third- and fourth-generation cephalosporins were also nearly four times more likely to have ESBL-producing *E. coli* than farms that had not used such drugs (Snow *et al.*, 2012). In our study, farms that used ceftiofur in cows had more calves with Am^r *E. coli* than farms that did not. Many third- and fourth-generation cephalosporins alter the composition of the GI microbiota, in particular the anaerobic part which is important for colonization resistance (Edlund & Nord, 1993). The higher prevalence of Am^r *E. coli* on farms that use ceftiofur in cows may be explained by enrichment of Am^r strains in the cow gut facilitated by ceftiofur-mediated decrease in gut colonization resistance. Ceftiofur is the only licensed third-generation cephalosporin, while no fourth-generation cephalosporin is available for use in dairy cattle in Sweden (FASS vet, 2015). Ceftiofur is used restrictively on Swedish dairy farms (Växa Sverige, 2015), which probably explains the low prevalence of ESBL-producing *E. coli* in calves in this study (1%).

Benzylpenicillin is by far the most used drug for cattle in Sweden (Växa Sverige, 2015) and this was also the case in paper II (results not shown). However, we did not find a significant association between the use of systemic

benzylpenicillin and faecal AMR *E. coli* in calves (paper II). This is contrary to the results of Grønvold *et al.* (2011) who isolated significantly more resistant *E. coli* in faeces from calves after systemic penicillin treatment. However, their conclusion is based on susceptibility testing of single *E. coli* isolates before and after treatment (Grønvold *et al.*, 2011), a method associated with a high degree of uncertainty. Benzylpenicillin administered parenterally is excreted quickly, almost entirely via the kidneys, whereas other antimicrobials are excreted, to some extent, via the GI tract (Giguré *et al.*, 2006). Thus, parenterally administered benzylpenicillin is likely to have minimal impact on the GI tract microbiota, as shown in humans (Edlund & Nord, 1993).

Overall, the results indicate that the use of broad-spectrum antimicrobials should be limited both in the cow and calf populations. In line with current guidelines on antimicrobial use in cattle in Sweden, the use of benzylpenicillin should instead be promoted.

5.1.3 Waste milk feeding and its implications for AMR *E. coli*

Feeding colostrum and transition milk

To our knowledge, paper II is the first to investigate the effects of feeding WC or WT to calves on faecal AMR *E. coli* from preweaned calves. Blanket DCT is common in most other countries, whereas in Sweden, sales records show that 26% of the dairy cows are treated with antimicrobials at drying off (selective DCT) (Växa Sverige, 2015). Hence, farmers in Sweden have the option of feeding colostrum and transition milk from untreated cows, which is not the case in blanket DCT. Still, the vast majority of the farms in the survey in paper I reported that WC or WT was fed to calves at least occasionally. Feeding only colostrum from non-treated cows to calves would require storing large volumes of colostrum from non-treated cows, which is not feasible on some dairy farms.

In paper II, we did not find a significant difference in the occurrence of AMR *E. coli* between farms that fed WC or WT at least occasionally and farms that did not. If the cow is dried off 60 days before calving, and the withdrawal period is between 35 and 37 days, the antimicrobial may already be eliminated at calving. This is confirmed by Olivier *et al.* (1984) who were unable to detect antimicrobial residues in colostrum from the first milking, even after dry periods of varying length. If the antimicrobials are given according to the manufacturer's recommendations, WC or WT feeding is safe from a resistance point of view. However, care must be taken when extrapolating these results to other countries where broader-spectrum antimicrobials are used or where most or all calves are given WC or WT.

The current recommendations state that WC, as defined in this thesis, should not be fed to calves at all, whereas WT can be given to calves from the third milking after calving[7]. Based on our results, we suggest that these recommendations be abolished. Instead, WC and WT can be given to calves without restrictions.

Feeding waste milk from cows treated during lactation

Waste milk was at least occasionally fed to calves on 79% of the farms in paper I, which is similar to a survey in the UK and Wales (83%) (Brunton *et al.*, 2012). Interestingly, over one-fourth of the organic farmers (paper I) stated that they fed WM during on-going treatment although this is prohibited on organic farms (KRAV, 2015). If this result is not due to a misinterpretation of the question, it indicates a lack of compliance with current regulations for organic certification.

More farms with cows housed in tie stall than free stall barns fed WM to calves. It can be hypothesized that there is a generation shift between tie stall and free stall farms, and that younger farmers are more aware of risks of feeding WM to calves. Finally, there were regional differences in feeding of WM to calves. It is possible that farmers in proximity to each other shared similar strategies, eventually as a result of using the same advisory services.

In our studies, we observed that feeding WM to calves increased the shedding of Sm^r *E. coli* (paper II) and QREC (papers II and III) by preweaned calves, but did not affect the shedding of *E. coli* with other resistance traits (paper II). Since the initiation of this project work, several studies have investigated the effect of WM feeding on the occurrence of resistant bacteria in faeces of calves. The conclusions drawn by these studies all point in the same direction—WM feeding favours faecal *E. coli* with certain resistance traits (Aust *et al.*, 2012; Brunton *et al.*, 2014; Pereira *et al.*, 2014d; Rebelo, 2014). Brunton *et al.* (2014) also observed that the shedding of ESBL-producing *E. coli* persists longer in WM-fed than non-WM-fed calves.

Twenty-three percent of the farms in paper I reported that they fed WM during the withdrawal period only, possibly to reduce the exposure of calves to antimicrobial residues. Nonetheless, we did not find a significant difference in the occurrence of AMR *E. coli* on farms that chose to discard TWM and to only feed WWM to calves compared to those that fed both TWM and WWM. Both these practices increased the occurrence of AMR *E. coli* among calves. This suggests that even the lower concentrations of residues in WWM are sufficient to exert a selection pressure on the GI microbiota. Hence, our results are in agreement with those by Pereira *et al.* (2014d) who show that even very

[7]. Håkan Landin, Växa Sverige, personal communication

low concentrations of antimicrobials in milk favour AMR *E. coli* in faeces of calves.

Since benzylpenicillin is by far the most common antimicrobial used in dairy cows in Sweden (Växa Sverige, 2015), this is probably the drug that most Swedish calves are exposed to if fed WM. We therefore assume that the higher levels of AMR *E. coli* on farms feeding WM to calves at least occasionally, compared to those that discarded all WM was mainly due to exposure to benzylpenicillin residues. Although benzylpenicillin is to some extent degraded in the GI tract, the bioavailability of benzylpenicillin administered in milk is 10% in one-week old calves (Musser & Anderson, 2001). Since penicillin is absorbed mainly from the intestines (McDermott *et al.*, 1946), this means that at least a portion of the drug reaches there. Langford *et al.* (2003) also show that milk containing penicillin affects the composition of the faecal flora, further indicating that the penicillin is reaching the intestines. All members of the species *E. coli* are intrinsically resistant to benzylpenicillin (Giguré *et al.*, 2006), but benzylpenicillin in milk may inhibit benzylpenicillin-susceptible species in the gut, which decreases the colonization resistance and facilitates colonization with AMR *E. coli*. Feeding WM from cows treated with broad-spectrum antimicrobials probably exerts a similar effect on the colonization resistance in the gut of calves, but with a direct selection of resistant *E. coli* superimposed on it. The significant interaction effect between WM feeding and use of fluoroquinolones in cows on the shedding of faecal QREC (paper II) further suggests that this is the case.

The calf GI microbiota is very simple until two to three weeks of age (Edrington et al, 2012a; Oikonomou *et al.*, 2013; Klein-Jöbstl *et al.*, 2014b) and thus, probably more vulnerable to inhibition by antimicrobials than in older animals. Based on the results of paper III, we therefore hypothesized that calves fed WM before two to three weeks of age were at higher risk of acquiring AMR *E. coli* than older calves, but this requires further study for confirmation.

British farmers reported that saving money was the most important reason for feeding WM to calves (Brunton *et al.*, 2012). The economics in Swedish dairy farming are currently very strained and every effort to save money is important. Thus, feeding WM to calves is an attractive way to reduce costs also for Swedish farmers. Godden *et al.* (2005) observed that calves fed conventional milk replacer have higher morbidity and mortality rates, and lower growth rates than calves fed pasteurized WM. They estimate that net savings from feeding pasteurized waste milk is $0.69 per calf and day compared to milk replacer (Godden *et al.*, 2005). Hence, the farm economy

may benefit from WM feeding, but at the cost of an increased burden of AMR *E. coli* to the society as a whole.

One way of minimizing the burden of AMR *E. coli* on dairy farms without compromising the farm economy could be to inactivate antimicrobials in WM before feeding it to calves. Pasteurizing or acidifying WM as well as adding inactivating enzymes to WM have been proposed for this purpose. Pasteurisation decreases the bacterial load of milk fed to calves (Godden *et al.*, 2005), but betalactams, aminoglycosides and quinolones are very heat-stabile and normal pasteurisation is insufficient to inactivate them in WM (Zorraquino *et al.*, 2008, 2009; Roca *et al.*, 2010, 2011). Benzylpenicillin is inactivated by acid (Giguré *et al.*, 2006) and hence, acidifying WM by fermentation or adding acid may reduce the benzylpenicillin concentration. However, WM fermentation may require up to 100 hours due to penicillin-inhibition of fermenting bacteria (Keys *et al.*, 1976, 1979). Fermentation of WM is therefore only a good alternative for inhibition of benzylpenicillin when the concentration in WM is low. Likewise, adding formic acid to WM requires one to seven days to inactivate benzylpenicillin (Raustein, 2003). A more promising method is to add a betalactamase enzyme (Antipen, Finnzymes, Esbo, Finland). This is reported to inactivate the drug residues within three hours (Raustein, 2003). This is a quick approach, but the effect of feeding betalactamase-treated WM on the occurrence of faecal AMR *E. coli* still needs to be evaluated.

The current Swedish recommendation is that WM should not be fed until the second post-treatment day[8]. Based on our results, we suggest instead that WM should not be fed to calves, at least not during the first few weeks of life when they may be more susceptible for colonization with AMR *E. coli*. However, if penicillin residues can be inactivated, WM from benzylpenicillin-treated cows can probably be fed to calves. Waste milk from cows treated with broad-spectrum antimicrobials should, on the other hand, always be discarded.

5.2 Factors related to the dissemination of AMR *E. coli*, with special reference to QREC

Another aim of this thesis was to describe the within-farm dissemination of QREC and factors of relevance for its spread. The results may also apply to other resistant bacteria that behave in a similar manner. The following is a discussion on the dissemination of AMR *E. coli* that focuses on measures to reduce the burden of those bacteria on dairy farms.

[8]. Håkan Landin, Växa Sverige, personal communication

5.2.1 Acquisition of QREC by calves

Shedding of AMR *E. coli* occurs shortly after birth as shown in paper III and by others (Donaldson *et al.*, 2006; Watson *et al.*, 2012; Brunton *et al*., 2014). One-fifth and one-third of the calves shed QREC already on the day of birth and day one, respectively (paper III). The normal retention rate in the GI tract for milk-fed calves is nine hours (Cannon *et al.*, 2010), indicating that calves acquired AMR *E. coli* strains relatively early after birth. Colonizing bacteria may originate from the vaginal or GI tract of the mother, from her skin or colostrum during ingestion of the first meal, or from the surroundings to which the calf is exposed (Mackie *et al.*, 1999). In paper IV, we observed that QREC were widespread in the surroundings on H-farms, in particular in the calf- and calving pens, resulting in ample oppurtinities for the newborn calf to pick up a resistant strain from the surroundings.

The maternal faecal microbiota may affect the composition of the offspring's GI tract microbiota (Bettelheim *et al.*, 1974; Callens *et al.*, 2014), but this may not be the only source of AMR *E. coli* for calves (Gow *et al.*, 2008; Watson *et al.*, 2012). Gow *et al.* (2008) found no association between the resistance patterns of *E. coli* from the newborn beef calf and its dam. Watson *et al.* (2012) observed that, in most cases, dairy calves and their dams shed different clones of ESBL-producing *E. coli*. Both Gow *et al.* (2008) and Watson *et al.* (2012) conclude that the farm environment microbiota is more important than the dam's microbiota for the occurrence of AMR *E. coli* in calves. We observed that QREC was more common on the calving pen floors of H- than L-farms, and the calving pen environment is probably a source of colonizing QREC for newborn calves. Bacteria in the calving area may either be derived from cows or calves that has been kept previously in the calving area. In this study, many post-partum cows shed QREC in faeces and the prevalence among these cows seemed slightly higher than in lactating and dry cows, indicating that the shedding of QREC increases around calving. In a small-scale study, the shedding of faecal Tc^r *E. coli* was compared between peri-partum and mid-lactation cows from the same farm (Gustafsson, 2014). The results showed that none of the mid-lactation cows shed Tc^r *E. coli*, whereas 18% of the post-partum cows shed Tc^r *E. coli* approximately one week after calving (Gustafsson, 2014). An increase in the shedding of AMR *E. coli* after calving is also reported by Watson *et al.* (2012). However, both in that study and in paper III, calves shed significantly more AMR *E. coli* than post-partum cows, suggesting that calves are the main sources for AMR *E. coli* in the calving area. Rather than being the actual source of QREC, it can be speculated that the post-partum cows become colonized with QREC in the calving area. Peri-partum cows compared to mid-lactation cows may be at

higher risk of colonization with AMR *E. coli*, as a consequence of disturbances in the GI flora induced by changes in diet, stress by movement to the calving area etc. (Anderson *et al.*, 1984). Moro *et al.* (1998, 2000) also observed that the occurrence of AMR *E. coli* in faeces from pigs increases when they are cold- or heat-stressed, and one can assume that stress has a similar effect on the GI microbiota of cows.

We also hypothesized that calves become colonized with QREC by ingestion of contaminated milk, but the lack of QREC in most milk samples in paper IV suggests that this is not the case. Rather, it seems that the composition of the farm environment microbiota is the most important source for AMR *E. coli* in calves. To reduce the risk of acquisition of AMR *E. coli* strains a clean calving environment should be provided.

5.2.2 Dissemination of AMR *E. coli* within farms

Within-farm dissemination of QREC correlated with the shedding of QREC by calves. We also found that QREC shedding by at least one post-partum cow was a risk factor for QREC shedding by calves, indicating that the presence of QREC in one category affects its occurrence in other categories on the farm. The overall genetically homogenous population of QREC in different samples from the same farm suggests that QREC is disseminated clonally throughout farms, in line with the findings by Hoyle *et al.* (2005). Clonal dissemination of QREC strains within-farms is probably facilitated by poor farm hygiene, which was also a significant risk factor for QREC shedding by post-partum cows. Poor farm hygiene is also a risk factor for shedding of quinolone resistant *Campylobacter* in pigs (Taylor *et al.*, 2009). Bosman *et al.* (2014) also found that poor farmworker hygiene, i.e. changing clothes infrequently, increases fluoroquinolone resistant *E.coli* in veal calves.

The calf environment—in particular feed troughs, water troughs and milk buckets—were more often contaminated with QREC on H- than L-farms, which indicates that QREC circulates on a faecal-oral route in the calf area. Watson *et al.* (2012) likewise observed that water troughs are heavily contaminated with ESBL-producing *E. coli* on a farm where ESBL-producing *E. coli* are commonly isolated from individual cattle. Snow *et al.* (2012) also concluded that infrequent disinfection of milk feeding equipment is a risk factor for the occurrence of ESBL-producing *E. coli* on dairy farms. Although not elucidated in this study, cleaning and disinfection of feed troughs, water troughs and milk buckets could reduce the burden of QREC in the farm environment and among calves. Furthermore, reducing the overall contamination of *E. coli* in the surroundings of the calf may be beneficial also

in terms of reducing the load of intestinal pathogens (Klein-Jöbstl *et al.*, 2014a).

As stated in the previous section, the calving area could be important in the acquisition of QREC for cows and calves. Exposure to a QREC-contaminated calving area by peri-partum cows may therefore spread QREC to the lactating cow pen. As Watson *et al.* (2012) suggested, transition via the calving area may be crucial for the dissemination of AMR *E. coli* between cattle categories on the farm. In paper III, we observed that cows that had calved in groups were more likely to shed QREC than cows that calved in tie stall barns or single pens. It seems that group calving increased the risk of between-individual sharing of faecal flora and thus, colonization with QREC. Moreover, group calving may be stressful for the cow as she cannot isolate from the herd, which could result in stress-induced changes in the GI microbiota like those observed in other species (Moro *et al.*, 1998, 2000; Bailey *et al.*, 2004). Further, new cows may enter the group calving pen on a continuous basis instead of using an all-in–all-out practice, making it more difficult to keep the pens clean. Using the calving pen as a sick pen rarely compared to often was a risk factor for the shedding of faecal QREC by calves. This is paradoxical since sick cows are sometimes treated with antimicrobials and therefore would be expected to shed more AMR *E. coli* than healthy cows. However, farms that often used the calving pen as a sick pen reported that they cleaned it more frequently than farms that rarely housed sick cows there. It is likely, therefore, that frequent cleaning of the calving area decreases the burden of QREC on the farm, as for other faecal-oral transmitted bacteria, such as *Mycobacterium avium* subspecies *paratuberculosis* (Pithua *et al.*, 2013).

The movement and inter-mingling of animals and people on the farm may also be associated with a higher occurrence of AMR *E. coli*. Not only do such movement disseminate one strain of *E. coli* to another place on the farm but they also increase the contact between potential donors and recipients for horizontal transfer of resistance genes. We can assume that movement of animals and people is greater in larger herds. We observed in paper II that large herds had more AMR *E. coli* than smaller ones. Furthermore, large herds have a higher incidence of disease (Hill *et al.*, 2009; Klein-Jöbstl *et al.*, 2014a), probably resulting in higher incidence of antimicrobial treatments. The higher occurrence of AMR *E. coli* in large herds is in line with the results from other studies (de Verdier *et al.*, 2012; Rebelo, 2014). Given the rapid increase in herd size in Sweden, this finding is a concern.

Transmission of AMR *E. coli* may also be facilitated by certain milking systems, as calves on farms with milking parlour had more AMR *E. coli* than those with tie stall milking or AMS. Parlour milking may be associated with a

greater movement and inter-mingling of cattle and people than tie stall or AMS milking where cows are confined to certain cubicles or pens. The type of milking system may, however, be a proxy for other, unidentified factors affecting the occurrence of AMR *E. coli*.

Group housing of the calf also increased Ctx^r *E. coli* shedding, whereas group housing with calves older than one month decreased Tc^r shedding compared to single calf housing. Such inconsistent findings were also observed by Pereira *et al.* (2014c), who conclude that no housing system is preferable. Nonetheless, it is likely that inter-mingling with calves in a group pen is important for the exchange of *E. coli* strains and that mixing younger and older calves dilutes the resistant *E. coli* population in the surroundings with susceptible strains.

Overall, the results suggest that the burden and dissemination of AMR *E. coli* may be reduced by improving farm hygiene, with special focus on the calf and calving areas, and by using only single pens at calving.

5.2.3 Dissemination of AMR *E. coli* between farms

In paper II, regional differences were observed in the occurrence of QREC and Cm^r *E. coli*. Likewise, Yamamoto *et al.* (2013) found that *E. coli* with the same resistance patterns are genetically similar on farms located in the same geographic regions. Clustering of the occurrence of AMR *E. coli* in different regions may result from clonal dissemination of resistant strains between closely located farms. Moreover, the same QREC genotype was found on more than one farm in paper IV, suggesting clonal dissemination of QREC between farms. In line with these findings, Marchese *et al.* (2012) observed that the same clone of QREC caused septicaemic colibacillosis on several farms. In addition, Lim *et al.* (2010) found the same clone on two nearby farms. In paper IV, farm-pairs that shared a QREC genotype were also more closely located than farm-pairs with no shared QREC genotypes.

The shorter the distance between farms, the more likely there is to be an epidemiologic link between them. Clonal spread of AMR *E. coli* between farms requires an epidemiological link and movement of animals, equipment and people between farms is the most straightforward one. In paper III, we observed that purchasing cattle and sharing animal transports with other livestock farmers increased the odds of QREC-shedding by post-partum cows. Interestingly, there was also a strong correlation between the number of purchased cattle and the genetic diversity of QREC on the farm, indicating that new genotypes were introduced with purchased cattle. These results are in line with the findings by DiLabio *et al.* (2007) who isolated more AMR *E. coli* from calves on farms that purchased calves, in particular from those that

purchase from multiple suppliers. In contrast, Snow et al. (2012) showed that farms were equally likely to have ESBL-producing *E. coli* independent of whether they had received cattle from a specific farm with ESBL-producing *E. coli* or not. However, if not from that farm, they could have received cattle from other farms with ESBL-producing *E. coli*. These authors found, nonetheless, that operating a closed farm policy is protective against the occurrence of ESBL-producing E. coli on the farm (Snow et al., 2012), which is in line with our findings regarding QREC.

Two QREC clones were more widespread than others, suggesting that these have characteristics that enhance their survival and spread in the farm environment. We also observed that, on some farms, many calves shed QREC but to a lesser extent. On these farms, QREC was seldomly found in the environment. Overall, this probably indicates that some QREC strains are more successful than others. In humans, some QREC isolates are more adapted to the habitat of the GI tract (Lastours *et al.*, 2014); such differences might also exist in the QREC population from cattle. Acquisition of resistance by bacteria is mostly associated with a fitness cost, which decreases the growth rate and survival of the bacteria (Andersson & Hughes, 2010). The lack-of-fitness cost is often necessary for the survival of a mutant in the absence of a selective pressure (Andersson & Hughes, 2010). On the other hand, some mutations may actually lead to increased fitness. Gullberg *et al.* (2011) found that the fitness cost of strains with a mutation in the *gyrA*(S83L) is lower than those with the *gyrA(*D87N) mutation. This might explain the widespread occurrence of some strains. However, the genetic basis for quinolone resistance was not investigated in any of our studies.

Once established on the farm, QREC may be difficult to eliminate due to its adaptability to a commensal habitat (Lastours et al., 2014). Marshall et al. (1990) also observed that QREC strains—in contrast to rifampicin resistant *E. coli*— persist in the GI tract of the inoculated heifer and the surroundings for at least 70 days post-inoculation, indicating that the survival of bovine QREC strains is better than for other *E. coli*.

The results emphasise the importance of proper between-farm biosecurity, such as minimizing cattle trade and the sharing of equipment between farms. Not only does good biosecurity protect against the transmission of infectious diseases, but it may also decrease the spread of AMR *E. coli* in the dairy farm population.

5.3 Clinical importance of AMR *E. coli* on dairy farms

The *E. coli* isolates in studies in papers II-IV were obtained from commensal microbiota and are probably not harmful for the calf itself under normal conditions. Nonetheless, a high prevalence of faecal AMR *E. coli* in the farm environment puts the animal at a higher risk of being infected with a resistant strain. This is a concern since the proportion of multi-drug resistant isolates of *E. coli* from calf intestinal infections has increased substantially in Sweden (Swedres-Svarm 2013).

Resistant *E. coli* from calves can also cause infection in older cattle. The contamination of AMR *E. coli* in the calving area may pose a risk for cows to acquire an intramammary or intrauterine infection with AMR *E. coli*. However, the occurrence of resistance in *E. coli* isolates causing intramammary or uterine infections in Sweden is low (Swedres-Svarm 2013), suggesting that this is currently not a problem on Swedish dairy farms.

The spread of AMR *E. coli* to humans may occur through contamination of the meat at slaughter; therefore AMR *E. coli* in faeces of slaughter-ready animals is a concern. In Sweden, the majority (80%) of slaughtered cattle are above one year of age (Swedish Board of Agriculture, 2014). Hence, a high prevalence of AMR *E. coli* in preweaned calves does not necessarily pose a risk for colonization of humans via the food chain, but it may pose a risk for humans handling calves (Marshall *et al.*, 1990). On the other hand, bacteria are not confined to one cattle category and AMR *E. coli* may be spread to slaughter-ready animals or cows producing milk for humans. It can be assumed that the higher proportion of AMR *E. coli* on the farm, the higher is the risk of spreading such bacteria to humans.

The occurrence of AMR *E. coli* in faeces of calves may also have implications for the spread to the general environment. Cattle faeces (including that of calves) is often applied on land as a fertilizer and AMR *E. coli* from calves may therefore end up in soil, water, crops and in wild animals. A high proportion of AMR *E. coli* in calf faeces may therefore increase the ratio of resistant to susceptible *E. coli* in the general environment.

5.4 Methodological considerations

The choice of study designs, study populations, and methods for data collection affects the validity of the results. Biases are systematic errors that may deviate the results more or less from the true situation as a consequence of methodological flaws (Dohoo *et al.*, 2010). This section covers methodological considerations that could have introduced bias in the results of this thesis.

5.4.1 Study populations and study designs

Selection bias is a concern when the study population deviates too much from the target population (Dohoo *et al.*, 2010). Approximating the study population to the target is therefore critical in cross-sectional studies where the aim is to assess the prevalence of certain traits in the target populations, such as the prevalence of AMR *E. coli* (paper II) or management routines (paper I). The target populations in papers I and II were all Swedish dairy farms, and all Swedish dairy farms with ≥ 30 cows, respectively. Farms with smaller herd sizes were excluded in paper II to make the results of the study more valid in the longer term, as farms with < 30 cows would not be representative of future Swedish dairy farms. The study populations in all papers were based on farms that participated in SOMRS, had registered their e-mail address in the SOMRS database, and that responded to the questionnaire in paper I. Thus, this approach relied both on the quality of e-mail addresses and the willingness of farmers to respond to the questionnaire. The response rate was only 30%, but an analysis of respondents and non-respondents proved that they were similar in terms of geographic location and herd size. Likewise, there were no differences between farms included in papers I and II and their respective target populations with regard to herd size and geographic location. Although there may be differences in other farm-level characteristics that could distort the validity of the study, we believe that the study populations in papers I and II were reasonably similar to their targets.

The study in papers III (and IV) was designed as a case-control study. The advantages of case-control studies are that results can be obtained with less funding and that multiple exposure factors can be studied using fewer study objects (Lewallen & Courtright, 1998). Disadvantages are that incidence data cannot be generated and that selection of controls can be challenging (Lewallen & Courtright, 1998). For this study, only a small number of farms could be included due to time constraints, and since there was only a small number of appropriate case farms available, the case-control design was more appropriate than a cross-sectional design. The study design (case-control) was therefore chosen to ensure that the variation in the prevalence of QREC was large enough to identify differences in management routines. This was done at the expense of the external validity of the results since the farms were neither chosen by random nor to be representative of a larger population. Care should therefore be taken when extrapolating the results of papers III and IV to other populations.

The inclusion of farms with a high prevalence of Ctx^r in calves and with moderate prevalence of QREC in the studies in papers III and IV can be questioned. These farms did not meet the criteria for cases or controls, but were

visited for a parallel study on the within-farm dissemination of ESBL-producing *E. coli*. However, we do not believe that the inclusion of these farms markedly affected the results since all farms were recategorized as H- or L-farms based on samples from 15 calves. Instead, they served to increase the sample size.

5.4.2 Collection of data on antimicrobial usage

Papers II and III rely on data on the use of antimicrobial drugs; obtaining such data with good quality from dairy farms is extremely challenging. Redding *et al.* (2014) reviewed different types of methods for data collection on antimicrobial use, such as surveillance at the national level, drug sales records, questionnaires or interviews, on-farm treatment records, collection of empty drug vials, and detection of tissue residues. Each of these may be more or less accessible. Collecting empty vials requires the study to be conducted prospectively rather than retrospectively, as in papers II and III. This approach also requires that the empty vials are physically collected from the farms which may not be feasible when there are many farms.

In Sweden, antimicrobials for use in animals must be prescribed by a veterinarian and registration in the NADRS is compulsory. Thus, in an ideal situation, complete data on antimicrobial usage could be obtained from NADRS. However, NADRS suffers from under-reporting, in particular for antimicrobial treatments of preweaned calves (Mörk *et al.*, 2009). Using NADRS records as the only source of antimicrobial consumption data may therefore lead to severe underestimation. Questionnaires or interviews on antimicrobial use may introduce recall bias (Dohoo *et al.*, 2010), i.e. when the farmer does not recall what types and what quantities of antimicrobials that was used on the farm. In paper II, two approaches were used: a questionnaire and the prescription data from NADRS. The highest estimate from either the questionnaire data or the NADRS was used to avoid underestimation of treatment incidence. However, it is difficult to assess the precision of this method as the true treatment incidence is unknown.

In paper III, a combination of interviews and on-farm treatment records was used to obtain information about antimicrobial usage. The time limit was set to four months before the visit, which was considered a reasonable time to remember events. Unfortunately, a four-month limit could also lead to over- or underestimation due to seasonal effects or disease outbreaks affecting the treatment incidence. All farms were visited during the winter season and none of the farms mentioned outbreaks during the previous four months. Therefore we believe that the measure gave a good estimate of the recent use of antimicrobials.

5.4.3 Limitations with questionnaire data

Information (or misclassification) bias may occur in data collected in questionnaires, or by using imprecise methods (Dohoo *et al.*, 2010). In this thesis, papers I-III included questionnaire or interview data, which may have introduced misclassification bias in the results. Several challenges are involved in the design of a questionnaire. First, the questions can be misunderstood; second, the questionnaire may not include the appropriate options, and third, it may be too long and tedious to be completed. To reduce these risks, the questionnaires in papers I and III were each pilot-tested on six dairy farmers. However, the questionnaire on antimicrobial usage (paper II) consisted of more straightforward questions and options that were less likely to be misunderstood. This questionnaire was therefore only tested on colleagues at SVA.

As for all questionnaires, there is also a risk that the respondents may answer what they believe is the expected or appropriate answer instead of the true one. Such errors are more common the more controversial the true answer is. It is difficult to assess the magnitude of such errors in the studies in this thesis or to what extent they may have changed the final result. However, some of the answers of the questionnaires in papers I and II could be regarded as controversial, i.e. the use of ceftiofur or feeding of TWM on organic farms, indicating that some of the respondents at least to some extent were giving the true answer.

5.4.4 Sampling considerations

When designing studies, budget and time constraints may limit the total number of animals or farms that can be sampled. Thus, either many animals on few farms or few animals on many farms can be sampled (Dunlop *et al.*, 1999). For monitoring the prevalence of resistant bacteria, the "single sample per farm" is the approach recommended by the European Food Safety Authority (EFSA, 2014). This approach assumes that isolates from the same farm are expected to show a similar resistance pattern due to a common environmental microbiota. However, inter-individual differences may be large (Dunlop *et al.*, 1999). Use of a single sample from each farm in risk factor analyses may lead to misclassification of the farm-level resistance status and thus, bias the estimates of associations. Therefore, we sampled three calves per farm in paper II to increase the precision of the farm-level estimate on the prevalence of AMR *E. coli*. Three to five calves per farm was also the sampling routine applied by Di Labio *et al.* (2007). The results of paper III showed, however, that three calves were probably insufficient to determine the farm-level status of QREC. Two of the control farms were recategorized as H-farms and one

case farm turned into an L-farm when 15 calves per farm were sampled. A larger number of sampled calves per farm in paper II could have increased the precision of the farm-level resistance status, but would also have reduced the number of farms that could be sampled due to increased costs and the willingness of farmers to participate.

5.4.5 Methods for susceptibility testing

The *E. coli* population in the GI tract is very numerous, while only a small proportion of the total *E. coli* population is present in the faecal sample (Hinton *et al.*, 1985a). This is a major problem when drawing conclusions based on the susceptibility of a single random *E. coli* sample from a calf. Such methods may underestimate the true prevalence of AMR *E. coli* (Scott *et al.*, 2011). A better approach is to use selective plates for which the susceptibility of multiple isolates can be tested simultaneously (Scott *et al.*, 2011). Nonetheless, selective plates require that a breakpoint (concentration of antimicrobial in the plate) must be decided ahead of culturing. For some resistance traits there is a clear-cut difference between the MIC for wild-type and resistant subpopulations, whereas they overlap for others. In paper II, > 32 mg/L was the cut-off chosen to select for Sm^r isolates, which is one $\log_2$ step higher than the epidemiological cut-off issued by EUCAST (EUCAST, 2015). Nonetheless, Garcia-Migura *et al.* (2012) observed that the MIC span of 4 to 32 mg/L may represent an overlapping area for the wild-type and resistant subpopulation. Thus, a cut-off at > 32 mg/L minimized false-positives at the expense of misclassifying some resistant isolates as susceptible.

Nalidixic acid is the preferred quinolone drug to identify chromosomal mutations (Cavaco & Aarestrup, 2009). In papers II-IV, > 32 mg/L was the cut-off chosen to select for QREC, one $\log_2$ step higher than the epidemiological cut-off (EUCAST, 2015). With this approach, some low-level resistant isolates may have been misclassified as susceptible and plasmid-mediated quinolone resistance may have gone undetected (Cavaco & Aarestrup, 2009). However, only 0.6% of the 729 isolates in paper II (results not shown) had MICs of 32 mg/L, indicating that the proportion of misclassified isolates was negligible. Further, plasmid-mediated quinolone resistance is very rare in *E. coli* from cattle (Jurado *et al.*, 2008; Kirchner *et al.*, 2011; Hordijk *et al.*, 2012; Marchese *et al.*, 2012), so we did not expect this to be an issue.

In paper II, we used selective agars supplemented with 1 mg/L cefotaxime without preceding pre-enrichment to select for third- and fourth-generation cephalosporin resistance. However, the preferred method for isolation of ESBL- or AmpC-producing *E. coli* is to use selective (containing

antimicrobials) pre-enrichment in a broth followed by culture on selective agar supplemented with antimicrobials (EFSA, 2011). Excluding the pre-enrichment step may have led to an underestimation of the prevalence of ESBL-producing *E. coli* in the calf population in Sweden. However, since third- and fourth-generation cephalosporins are rarely used in Swedish cattle (Växa Sverige, 2015), we do not expect the prevalence of ESBL-producing *E. coli* to be much higher than what was observed. In addition, it was shown that the sensitivity of screening methods for ESBL-producing *E. coli* does not increase significantly by adding a pre-enrichment step (Diederen *et al.*, 2012).

6 Conclusions

The results from this thesis improve knowledge about the prevalence, risk factors, and spread of faecal AMR *E. coli* in preweaned dairy calves. Important conclusions are that:

- Antimicrobial resistant *E. coli* strains are widespread among preweaned dairy calves in Sweden. The occurrence was strongly age-dependent, but was also associated with herd size, milking system, calf housing, and geographic location of the farm. Farm-level treatment with broad-spectrum antimicrobials increased the occurrence of faecal AMR *E. coli* in calves. Feeding WM to calves increased the proportion of faecal streptomycin and quinolone resistant *E. coli* in calves, but feeding WC had no effect on faecal AMR *E. coli*.
- Waste milk and WC was fed to calves at least occasionally on a majority of surveyed farms. Farmers were more reluctant to feed TWM than WWM. Feeding WM to calves was in general more common on farms in southern than northern/eastern Sweden, on non-organic compared to organic farms, and on farms with tie stall barns compared to free stall barns.
- Within-farm dissemination of QREC was correlated to the shedding of QREC by calves and post-partum cows. These calves and cows may have been colonized with QREC in the calving pen. The same QREC genotype was found in different sample types within the farm and on different farms, suggesting contagious spread of QREC within and between farms.
- Fluoroquinolone treatment, WM feeding, and infrequent compared to frequent use of the calving pen as sick pen increased faecal QREC in calves, while group calving, poor farm hygiene, purchasing cattle or sharing animal transporter with other farmers increased faecal QREC in post-partum cows. Moreover, the more purchased cattle, the higher was the QREC genetic diversity within the farm. Thus, proper within-and between-farm biosecurity may be valuable to reduce the spread of QREC.

7 Practical recommendations

The knowledge generated in this thesis can be used to define measures to reduce the burden of AMR *E. coli* on dairy farms. Here, some practical recommendations of the generated results are highlighted:

- The use of broad-spectrum antimicrobials in the herd should be limited. In particular, we advise against the use of orally administered DHS in calves since the efficacy of these products in *E. coli* diarrhoea may be questionable, given the high prevalence of streptomycin resistance. Moreover, other broad-spectrum antimicrobials, such as fluoroquinolones should be avoided because the use of these drugs increases the prevalence of resistance.
- Colostrum from cows treated with antimicrobials at drying off can be given to calves without increasing the prevalence of AMR *E. coli*, at least under Swedish conditions. However, milk from cows treated with antimicrobials during lactation should not be given to calves, not even during the withdrawal period.
- Calving in single pen should be preferred over group-pen calving.
- Improved farm hygiene, at least in the calving pens and in the feed and water troughs as well as milk feeding equipment in the calf pens, could potentially reduce the occurrence of AMR *E. coli* on the farm.
- Reducing the number of epidemiological links between farms, such as purchase of cattle, may be important to reduce the spread of AMR *E. coli*. We therefore recommend that contacts that involve the spread of faecal material between farms should be minimized.

8 Perspectives for the future

During this thesis work, some new questions that could be of interest in future studies were raised:

Can the use of a commercial competitive exclusion product reduce the burden of AMR E. coli on dairy farms?

If insufficient colonization resistance is a key factor in the establishment of AMR *E. coli* in the gut of calves, treatment with competitive exclusion products may be a way to reduce the burden of AMR *E. coli*. Competitive exclusion is when harmless bacteria, such as lactic acid bacteria, outcompetes harmful bacteria by competing for the same nutrients. The use of commercial competitive exclusion products has been associated with a reduction in ESBL-producing *E. coli* in the broiler industry (Anderson *et al.*, 1984; Nuotio *et al.*, 2013), reduction of diarrhoea cases in calves (von Buenau *et al.*, 2005), and enterohaemorrhagic *E. coli* in weaned calves (Tkalcic *et al.*, 2003). However, the ability of competitive exclusion products to reduce the shedding of AMR *E. coli* in calves has not been investigated and deserves attention in future research. If proven effective against colonization with AMR *E. coli*, such products could be given to calves and peri-partum cows after cleaning and disinfection of the calving pens. An example of such a product is the probiotic *E. coli* strain Nissle 1917, which is commercially available in Germany (von Buenau *et al.*, 2005).

Can prior betalactamase-treatment or fermentation of milk from penicillin-treated cows lower the prevalence of faecal AMR E. coli in calves fed such milk?

Because benzylpenicillin is used in the majority of systemic antimicrobial treatments of cows in Sweden, the bulk of WM that is fed to Swedish calves is likely to contain benzylpenicillin residues. If benzylpenicillin residues in WM can be inactivated, such milk can be a good nutrition source for calves.

Treatment of WM with a commercially available betalactamase enzyme (Antipen, Finnzymes, Oy, Finland) and fermentation of milk inactivate penicillin residues in WM. Three hours was sufficient for the betalactamase enzyme to inactivate all the penicillin in milk (Raustein, 2003), whereas fermentation of penicillin-milk took more time for inavtivation, at least when the concentration of penicillin residues in milk was high (Keys *et al.*, 1976, 1979). These tools may therefore be valuable to obtain penicillin-free WM that could be fed to calves. However, tudies on how the feeding of fermented or betalactamase-treated WM from penicillin-treated cows influences the shedding of AMR *E. coli* by calves are not available. Hence, a study that compares the occurrence of AMR *E. coli* in calves fed betalactamase-treated penicillin-milk, fermented penicillin-milk and untreated penicillin-milk would be valuable to find measures that reduce the risk of WM feeding.

What is the farm-level clinical importance of having high prevalence of AMR E. coli in healthy dairy calves?

Although the benefits of reducing the burden of antimicrobial resistance is relevant to the society as a whole, direct costs of measures to reduce the occurrence of AMR *E. coli* may be on the farm. Such measures may be difficult to implement on the farm if the farmer does not see any direct benefits of applying them. Therefore, it is necessary to evaluate the benefits of having low prevalence of AMR *E. coli* on the farm as compared to a high prevalence. Resistance in clinical *E. coli* isolates from the GI tract of calves seems to be on the rise in Sweden, with resistance to streptomycin or tetracycline occurring in 76% of the isolates in 2013 (Swedres-Svarm 2013). It is logical to assume that a higher proportion of multidrug-resistant *E. coli* will be found in healthy calves on farms that have experienced GI infections with multi-drug resistant isolates than on farms with mostly susceptible clinical isolates, but this has so far not been investigated. Also, since resistant isolates more often carry virulence genes (de Verdier *et al.*, 2012) than susceptible ones, it could be valuable to assess the association between the susceptibility of clinical isolates and the presence of virulence genes. Such a study could show potential farm-level benefits of reducing the occurrence of AMR *E. coli* in healthy calves.

Do widespread QREC genotypes possess characteristics that enhance their survival and spread compared to rare genotypes?

In paper IV, we observed that some QREC genotypes seem to be more widespread within and between farms than other, rarer genotypes. This may be due to expression of factors that enhance their survival and spread. Studies in healthy humans have shown that QREC strains more often express virulence

traits, grow better in the main nutrient medium in the gut, and are more resistant to oxidative and acid stress than susceptible strains (Lastours *et al.*, 2014). Also, the fitness cost of resistance was dependent on the type of mutation conferring quinolone resistance (Gullberg *et al.*, 2011). Hence, it could be of value to assess the genetic basis of resistance, the fitness, competitiveness in the gut, and survival in the farm environment of widespread compared to rare genotypes of QREC.

9 Populärvetenskaplig sammanfattning

Bakgrund

Antibiotika används för att behandla bakterieinfektioner hos människor och djur. Dock kan bakterier bli motståndskraftiga (resistenta) mot antibiotika, vilket kan leda till mycket svårbehandlade infektioner. Behandling med antibiotika kan leda till att resistens uppstår. Dessutom kan resistenta bakterier gynnas i förhållande till känsliga bakterier. Antibiotikaresistenta bakterier hos djur innebär inte bara en risk för djuren själva utan de kan också överföras till människor genom direktkontakt eller via livsmedel. Ett exempel på bakterier som kan överföras mellan djur och människor är *Escherichia coli* (kolibakterier) som finns i tarmen hos alla djur och människor. I normala fall vållar inte dessa kolibakterier några problem, men de kan ge sjukdom hos svaga individer. De här bakterierna kan även överföra resistensarvsanlag till andra bakterier som orsakar sjukdom, till exempel salmonella.

I mjölkbesättningar bär unga kalvar oftare på resistenta kolibakterier i tarmen än äldre nötkreatur, trots att många av kalvarna inte har behandlats med antibiotika samt att kalvar och kor vistas i samma miljö. En teori till varför resistenta bakterier är så vanliga hos unga kalvar är att användningen av specifika antibiotika är hög i besättningen. Det kan till exempel vara en skillnad om man använder så kallade bredspektrumantibiotika, som verkar mot många sorters bakterier, jämfört med så kallade smalspektrumantibiotika, som endast verkar mot ett fåtal bakterietyper. Ett exempel på det sistnämnda är penicillin. Att ge kalvarna mjölk från antibiotikabehandlade kor är en annan teori till varför kalvar har så många resistenta kolibakterier. Sådan mjölk kan innehålla rester av antibiotika och får inte säljas till mejeriet varför den ibland ges till kalvarna istället. Få studier har dock gjorts om detta och man vet därför inte så mycket om varför resistenta kolibakterier är så vanliga hos kalvar. Eftersom kalvarna kan vara en viktig källa för resistenta bakterier för djur och människor är det viktigt att ta reda på vilka faktorer som har betydelse för

förekomsten samt spridningen av resistenta kolibakterier. Dessa kunskaper kan sedan användas för att ge rekommendationer om åtgärder för att minska förekomsten av resistenta bakterier i mjölkbesättningar. Av speciellt intresse i avhandlingen är resistens mot kinoloner som anses vara mycket viktiga för behandling av människor.

Metoder och resultat

Totalt fyra studier ligger till grund för avhandlingen. I den första studien var syftet att undersöka hur vanligt det är att råmjölk och mjölk från antibiotikabehandlade kor ges till kalvar i svenska mjölkbesättningar och om rutiner för detta skiljer sig åt mellan olika typer av gårdar. Detta undersöktes i en enkätstudie bland 457 besättningar. Resultatet visade att på 89 procent av gårdarna utfodrades kalvar någon gång med råmjölk (från första mjölkningen efter kalvning) från kor som antibiotikabehandlats vid sinläggning. Övergångsmjölk (från andra mjölkningen till och med fjärde dagen efter kalvning) från kor som antibiotikabehandlats vid sinläggning användes på 85 procent av gårdarna. Mjölk från kor som behandlats med antibiotika under laktationen (mjölkgivningsperioden) gavs till kalvar på 56 procent (under både behandling- och karenstid) och 79 procent (enbart under karenstiden) av gårdarna. Överlag var det vanligare att man gav mjölk från behandlade kor på icke-ekologiska än ekologiska gårdar samt på gårdar med uppbundna kor jämfört med lösdrift eller gårdar som var belägna i södra Sverige jämfört med andra delar av landet.

I den andra studien var syftet att undersöka hur förekomsten av resistenta bakterier skiljer sig åt mellan olika typer av gårdar och olika kalvar, samt hur användning av antibiotika och utfodring med mjölk från antibiotikabehandlade kor påverkar förekomsten av sådana bakterier. Avföringsprover från kalvar på 243 gårdar runt om i Sverige samlades in och undersöktes för andelen streptomycin-, kinolon- och cefalosporinresistenta kolibakterier samt resistens mot olika antibiotika hos en slumpvist utvald kolibakterie. Resistens mot streptomycin var mycket vanlig, resistens mot kinoloner förekom på vissa gårdar, men resistens mot cefalosporiner var relativt ovanlig. Generellt sett var resistens vanligast vid en veckas ålder för att sedan gradvis minska hos kalvarna. Detta tros bero på att nyfödda kalvars tarmflora och immunförsvar är under utveckling och att resistenta bakterier därför lättare etablerar sig i tarmkanalen hos kalvar än hos äldre djur. Många resistenta kolibakterier har speciella egenskaper som ökar deras konkurrenskraft gentemot känsliga kolibakterier. Minskningen av resistenta kolibakterier med stigande ålder kan bero på en kombination av immunitet mot vissa kolibakterier samt ökad konkurrens från en mer utvecklad tarmflora. Att behandla kor eller kalvar med

bredspektrumantibiotika ökade förekomsten av resistenta kolibakterier hos kalvarna. De hade till exempel fler kinolonresistenta bakterier på gårdar där man behandlar med kinolonantibiotika jämfört med gårdar där man inte gör det. Däremot påverkade inte behandling med penicillin förekomsten av resistenta kolibakterier. Utfodring med råmjölk från kor som antibiotikabehandlats vid sinläggning gav inte upphov till fler resistenta kolibakterier hos kalvarna. Utfodring med mjölk från kor som behandlats med antibiotika under laktationen ökade däremot förekomsten av streptomycin- och kinolonresistenta kolibakterier. Antibiotika i mjölk hämmar antagligen känsliga mikroorganismer i tarmfloran och stör därmed balansen i tarmfloran, vilket i sin tur innebär att resistenta kolibakterier lättare etablerar sig i tarmen. Utfodring av kalvar med mjölk från kor som behandlats under laktationen kan därför inte rekommenderas ur resistenssynpunkt.

Resistenta kolibakterier var vanligare på stora jämfört med små gårdar. Det fanns också skillnader i förekomsten av resistenta kolibakterier hos kalvar på gårdar med olika mjölkningssystem (vanligare på gårdar med mjölkgrop än robot eller uppbundna kor) samt på gårdar belägna i olika delar av landet. Exempelvis var kinolonresistens vanligare i södra och östra Sverige än i norra Sverige.

I den tredje och fjärde studien studerades kinolonresistens lite närmare. Syftet med dessa studier var att undersöka förekomst och spridning av kinolonresistenta kolibakterier i gårdsmiljön och mellan gårdar samt att ta reda på vilka faktorer som har betydelse för förekomst och spridning av dessa bakterier. Dessa studier genomfördes på 23 gårdar som alla hade använt kinolonantibiotika, men som hade olika mycket kinolonresistenta kolibakterier hos kalvarna. På gårdar med en stor andel kinolonresistenta kolibakterier hos kalvarna hittades dessa bakterier ofta på väggar i kalvboxar, i kalvarnas fodertråg, vattentråg, och mjölkhinkar samt i kalvningsboxen, hos yngre ungdjur och i avföringsprov från nykalvade kor. Det kan därför antas att kinolonresistenta bakterier cirkulerar mellan individer på gården genom träckförorening av inredningen. Däremot verkar inte mjölk eller råmjölk vara en källa för kinolonresistenta kolibakterier för kalvarna. Resultaten tyder också på att kalvningsboxen kan utgöra en källa för kinolonresistenta kolibakterier för både kor och kalvar.

På de flesta av de undersökta gårdarna var det samma typ av kinolonresistenta kolibakterier på olika ställen i gårdsmiljön, vilket tyder på dessa bakterier sprids inom gården. Faktorer som gynnade kinolonresistenta kolibakterier hos kalvarna var om man hade använt kinolonantibiotika i besättningen de senaste fyra månaderna, om man generellt sett utfodrar med mjölk från antibiotikabehandlade kor, och om kalven i fråga fått sådan mjölk

samt att sällan jämfört med ofta använda kalvningsboxen till sjuka kor. Det sistnämnda fyndet beror troligen på att kalvningsboxen tvättas oftare om man ofta ställer in sjuka kor i boxen. Fler kalvar bar dessutom på kinolonresistenta kolibakterier om minst en nykalvad ko av dem som undersöktes bar på dessa bakterier, vilket tyder på att förekomsten hos kalvar påverkas av eller påverkar förekomsten hos nykalvade kor. Kinolonresistenta kolibakterier var vanligare hos nykalvade kor om stallhygienen på gården bedömdes som sämre än genomsnittet i studien och om de hade kalvat i grupp. Detta indikerar att förekomsten borde kunna minskas genom ordentlig rengöring i kalv- (speciellt foder- och vattentråg) och kalvningsboxarna samt genom att låta kor kalva enskilt.

Samma typ av kinolonresistenta kolibakterier hittades på mer än en gård, vilket tyder på att dessa bakterier kan spridas mellan gårdar. Gårdar som hade samma typ av resistent kolibakterie var belägna närmare varandra än gårdar som bara hade sina egna typer. Kinolonresistenta kolibakterier var vanligare hos nykalvade kor på de gårdar som brukar köpa in nötkreatur från andra gårdar eller som delar djurtransport med andra djurbönder. Dessutom var det fler olika typer av kinolonresistenta kolibakterier ju fler nötkreatur som hade köpts in under årens lopp, vilket tyder på att nya resistenta typer införs på gården via inköp av djur. Dessa resultat tyder på att kinolonresistenta kolibakterier sprids mellan besättningar, eventuellt genom inköp av nötkreatur och delad utrustning.

Slutsatser och rekommendationer

Sammanfattningsvis understryker resultaten vikten av ansvarsfull antibiotikaanvändning samt god hygien och gott smittskydd då dessa faktorer verkar ha betydelse för förekomsten och spridningen av resistenta kolibakterier inom och mellan gårdar. Följande rekommendationer kan ges utifrån studierna i denna avhandling:

- Användningen av bredspektrumantibiotika bör begränsas och i första hand ska penicillin övervägas.
- Råmjölk från kor som antibiotikabehandlats vid sinläggning kan ges till kalvar.
- Mjölk från kor som behandlats med antibiotika under laktationen bör däremot inte ges till kalvar.
- Kor bör i möjligaste mån kalva i enskilda boxar istället för gruppboxar.
- Rengöring av kalvningsboxar samt inredningen i kalvboxar (speciellt foder- och vattentråg) kan troligtvis ge mindre resistens.

- Kontakter mellan gårdar som innebär att gödsel sprids från en gård till en annan, exempelvis inköp av djur eller att dela djurtransport med andra bönder, bör också i möjligaste mån undvikas.

References

Aho, M. (1992). Problems of Salmonella sampling. *International Journal of Food Microbiology*, 15(3-4), pp 225–235.

Alekshun, M. N. & Levy, S. B. (2007). Molecular Mechanisms of Antibacterial Multidrug Resistance. *Cell*, 128(6), pp 1037–1050.

Alexander, T. W., Inglis, G. D., Yanke, L. J., Topp, E., Read, R. R., Reuter, T. & McAllister, T. A. (2010). Farm-to-fork characterization of *Escherichia coli* associated with feedlot cattle with a known history of antimicrobial use. *International Journal of Food Microbiology*, 137(1), pp 40–48.

Anderson, M. A., Whitlock, J. E. & Harwood, V. J. (2006). Diversity and Distribution of *Escherichia coli* Genotypes and Antibiotic Resistance Phenotypes in Feces of Humans, Cattle, and Horses. *Applied and Environmental Microbiology*, 72(11), pp 6914–6922.

Anderson, W. R., Mitchell, W. R., Barnum, D. A. & Julian, R. J. (1984). Practical Aspects of Competitive Exclusion for the Control of Salmonella in Turkeys. *Avian Diseases*, 28(4), pp 1071–1078.

Andersson, D. I. & Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*, 8(4), pp 260–271.

Andremont, A. (2003). Commensal flora may play key role in spreading antibiotic resistance. *ASM news*, 69(12), pp 601–607.

Anonymous (2013). *Surveillance of infectious diseases in animals and humans in Sweden 2013*. Uppsala, Sweden: National Veterinary Institute (SVA). (SVA:s rapportserie 2 8; ISSN 1654-7098).

Aust, V., Knappstein, K., Kunz, H.-J., Kaspar, H., Wallmann, J. & Kaske, M. (2012). Feeding untreated and pasteurized waste milk and bulk milk to calves: effects on calf performance, health status and antibiotic resistance of faecal bacteria. *Journal of animal physiology and animal nutrition*, 97(6), pp 1091-1103.

Bailey, M. T., Lubach, G. R. & Coe, C. L. (2004). Prenatal stress alters bacterial colonization of the gut in infant monkeys. *Journal of Pediatric Gastroenterology and Nutrition*, 38(4), pp 414–421.

Beaber, J. W., Hochhut, B. & Waldor, M. K. (2004). SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature*, 427(6969), pp 72–74.

Bengtsson, B. & Greko, C. (2014). Antibiotic resistance—consequences for animal health, welfare, and food production. *Upsala Journal of Medical Sciences*, 119(2), pp 96–102.

Berge, A. C. B., Atwill, E. R. & Sischo, W. M. (2005a). Animal and farm influences on the dynamics of antibiotic resistance in faecal *Escherichia coli* in young dairy calves. *Preventive Veterinary Medicine*, 69(1–2), pp 25–38.

Berge, A. C. B., Epperson, W. B. & Pritchard, R. H. (2005b). Assessing the effect of a single dose florfenicol treatment in feedlot cattle on the antimicrobial resistance patterns in faecal *Escherichia coli*. *Veterinary Research*, 36(5-6), pp 723–734.

Berge, A. C. B., Moore, D. A. & Sischo, W. M. (2006). Field Trial Evaluating the Influence of Prophylactic and Therapeutic Antimicrobial Administration on Antimicrobial Resistance of Fecal *Escherichia coli* in Dairy Calves. *Applied and Environmental Microbiology*, 72(6), pp 3872–3878.

Berge, A. C., Hancock, D. D., Sischo, W. M. & Besser, T. E. (2010). Geographic, farm, and animal factors associated with multiple antimicrobial resistance in fecal *Escherichia coli* isolates from cattle in the western United States. *Journal of the American Veterinary Medical Association*, 236(12), pp 1338–1344.

Bettelheim, K. A., Breadon, A., Faiers, M. C., O'Farrell, S. M. & Shooter, R. A. (1974). The Origin of O Serotypes of *Escherichia coli* in Babies after Normal Delivery. *The Journal of Hygiene*, 72(1), pp 67–70.

Bogaard, A. E. van den, London, N., Driessen, C. & Stobberingh, E. E. (2001). Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry slaughterers. *Journal of Antimicrobial Chemotherapy*, 47(6), pp 763–771.

Van den Bogaard, A. E. & Stobberingh, E. E. (2000). Epidemiology of resistance to antibiotics: Links between animals and humans. *International Journal of Antimicrobial Agents*, 14(4), pp 327–335.

Bosman, A. B., Wagenaar, J. A., Stegeman, J. A., Vernooij, J. C. M. & Mevius, D. J. (2014). Antimicrobial resistance in commensal *Escherichia coli* in veal calves is associated with antimicrobial drug use. *Epidemiology & Infection*, 142(09), pp 1893–1904.

Brunton, L. A., Duncan, D., Coldham, N. G., Snow, L. C. & Jones, J. R. (2012). A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. *The Veterinary record*, 171(12), pp 296–302.

Brunton, L. A., Reeves, H. E., Snow, L. C. & Jones, J. R. (2014). A longitudinal field trial assesing the impact of feeding waste milk containing antibiotic residues on the prevalence of ESBL-producing *Escherichia coli* in calves. *Preventive Veterinary Medicine*, 117(2), pp 403-12.

Von Buenau, R., Jaekel, L., Schubotz, E., Schwarz, S., Stroff, T. & Krueger, M. (2005). *Escherichia coli* Strain Nissle 1917: Significant Reduction of Neonatal Calf Diarrhea. *Journal of Dairy Science*, 88(1), pp 317–323.

Buffie, C. G. & Pamer, E. G. (2013). Microbiota-mediated colonization resistance against intestinal pathogens. *Nature reviews. Immunology*, 13(11), pp 790–801.

Callens, B., Faes, C., Maes, D., Catry, B., Boyen, F., Francoys, D., de Jong, E., Haesebrouck, F. & Dewulf, J. (2014). Presence of antimicrobial resistance and antimicrobial se in sows are risk factors for antimicrobial resistance in their offspring. *Microbial Drug Resistance* [online],

Available from: http://online.liebertpub.com/doi/abs/10.1089/mdr.2014.0037. [Accessed 2014-08-18].

Cannon, S. J., Fahey Jr., G. C., Murphy, M. R., Dikeman, C. L., Miller, B. L. & Drackley, J. K. (2010). Inclusion of psyllium in milk replacer for neonatal calves. 1. Effects on growth, digesta viscosity, rate of passage, nutrient digestibilities, and metabolites in blood1. *Journal of Dairy Science*, 93(8), pp 3652–3660.

Carlsson, J. & Carpenter, V. S. (1980). The recA+ gene product is more important than catalase and superoxide dismutase in protecting *Escherichia coli* against hydrogen peroxide toxicity. *Journal of Bacteriology*, 142(1), pp 319–321.

Cars, O., Hogberg, L. D., Murray, M., Nordberg, O., Sivaraman, S., Lundborg, C. S., So, A. D. & Tomson, G. (2008). Meeting the challenge of antibiotic resistance. *BMJ*, 337(sep18 3), pp a1438–a1438.

Cavaco, L. M. & Aarestrup, F. M. (2009). Evaluation of Quinolones for Use in Detection of Determinants of Acquired Quinolone Resistance, Including the New Transmissible Resistance Mechanisms qnrA, qnrB, qnrS, and aac(6′)Ib-cr, in *Escherichia coli* and Salmonella enterica and Determinations of Wild-Type Distributions. *Journal of Clinical Microbiology*, 47(9), pp 2751–2758.

CLSI (2013). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved Standard – Fourth Edition VET01-A4.* Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Cogliani, C., Goossens, H. & Greko, C. (2011). Restricting antimicrobial use in food animals: lessons from Europe. *Microbe Magazine*, 6(6), p 274.

Cohen, S. P., McMurry, L. M. & Levy, S. B. (1988). MarA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. *Journal of Bacteriology*, 170(12), pp 5416–5422.

Corpet, D. E. (1988). Antibiotic resistance from food. *The New England Journal of Medicine*, 318(18), pp 1206–1207.

Courvalin, P. (2008). Predictable and unpredictable evolution of antibiotic resistance. *Journal of Internal Medicine*, 264(1), pp 4–16.

Cox, G. & Wright, G. D. (2013). Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *International Journal of Medical Microbiology*, 303(6–7), pp 287–292 (Special Issue Antibiotic Resistance).

Cummings, K. J., Aprea, V. A. & Altier, C. (2013). Antimicrobial resistance trends among *Escherichia coli* isolates obtained from dairy cattle in the northeastern United States, 2004–2011. *Foodborne Pathogens and Disease*, 11(1), pp 61–67.

DeFrancesco, K. A., Cobbold, R. N., Rice, D. H., Besser, T. E. & Hancock, D. D. (2004). Antimicrobial resistance of commensal *Escherichia coli* from dairy cattle associated with recent multi-resistant salmonellosis outbreaks. *Veterinary Microbiology*, 98(1), pp 55–61.

Diederen, B., Chang, C., Euser, S. & Stuart, J. C. (2012). Evaluation of four screening protocols for detection of extended-spectrum β-lactamase-producing members of the Enterobacteriaceae. *Journal of Medical Microbiology*, 61(3), pp 452–452.

Dohoo, I., Martin, W. & Stryhn, H. (2010). *Veterinary epidemiologic research.* 2. ed Charlottetown, Prince Edward Islands, Canada: VER Inc. ISBN 978-0-919013-60-5.

Dolejská, M., Senk, D., Cízek, A., Rybaríková, J., Sychra, O. & Literák, I. (2008). Antimicrobial resistant *Escherichia coli* isolates in cattle and house sparrows on two Czech dairy farms. *Research in Veterinary Science*, 85(3), pp 491–494.

Donaldson, S. C., Straley, B. A., Hegde, N. V., Sawant, A. A., DebRoy, C. & Jayarao, B. M. (2006). Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. *Applied and environmental microbiology*, 72(6), pp 3940–3948.

Dunlop, R. H., McEwen, S. A., Meek, A. H., Friendship, R. M., Black, W. D. & Clarke, R. C. (1999). Sampling considerations for herd-level measurement of faecal *Escherichia coli* antimicrobial resistance in finisher pigs. *Epidemiology and Infection*, 122(3), pp 485–496.

Eberhart, L. J., Deringer, J. R., Brayton, K. A., Sawant, A. A., Besser, T. E. & Call, D. R. (2012). Characterization of a novel microcin that kills enterohemorrhagic Escherichia coli O157:H7 and O26. *Applied and Environmental Microbiology*, 78(18), pp 6592–6599.

Eberhart, L. j., Ochoa, J. n., Besser, T. e. & Call, D. r. (2014). Microcin MccPDI reduces the prevalence of susceptible *Escherichia coli* in neonatal calves. *Journal of Applied Microbiology*, 117(2), pp 340–346.

Edlund, C. & Nord, C.-E. (1993). Ecological impact of antimicrobial agents on human intestinal microflora. *Alpe Adria Microbiology Journal*, 2(3), pp 137–164.

Edrington, T. S., Callaway, T. R., Anderson, R. C. & Nisbet, D. J. (2008). Prevalence of Multidrug-Resistant Salmonella on Commercial Dairies Utilizing a Single Heifer Raising Facility. *Journal of Food Protection*, 71(1), pp 27–34.

Edrington, T. S., Dowd, S. E., Farrow, R. F., Hagevoort, G. R., Callaway, T. R., Anderson, R. C. & Nisbet, D. J. (2012a). Development of colonic microflora as assessed by pyrosequencing in dairy calves fed waste milk. *Journal of Dairy Science*, 95(8), pp 4519–4525.

Edrington, T. S., Farrow, R. L., Carter, B. H., Islas, A., Hagevoort, G. R., Callaway, T. R., Andersson, R. C. & Nisbet, D. J. (2012b). Age and diet effects on fecal populations and antibiotic resistance of a multi-drug resistant *Escherichia coli* in dairy calves. *Agriculture, Food and Analytical Bacteriology*, 2(3), pp 162–174.

EFSA (2011). Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β-lactamases and/or AmpC β-lactamases in food and food-producing animals. *EFSA Journal*, 9(8), p 2322.

EFSA (2014). Scientific Report of EFSA: EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2012. *The EFSA Journal*, 12(3), p 336.

EUCAST. *Antimicrobial wild type distributions of microrganisms*. [online] (2015). Available from: http://mic.eucast.org/Eucast2/. [Accessed 2015-03-22].

ESVAC 2012 (2014). *Sales of veterinary antimicrobial agents in 26 EU/EEA countries in 2012 - Fourth ESVAC report* [online]. European Medicines Agency. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Report/2014/10/WC500175671.pdf [Accessed 2015-04-13], (EMA/333921/2014).

FASS vet. *FASS Djurläkemedel - Startsida*. [online] (2015). Available from: http://www.fass.se/LIF/startpage;jsessionid=3rsTSn-8Sauf6gxsIC465-6YmwnQc0tKdFcvDcdciS9LNNIdGf-Q!870332124?userType=1. [Accessed 2015-03-13].

Gaines S.A., Rollins L.D., Silver R.P., Washington M. (1978). Effect of low concentrations of dihydrostreptomycin on drug resistance in enteric bacteria. *Antimicrobial Agents Chemotherapy*, 14, pp 252–256

Garcia-Migura, L., Sunde, M., Karlsmose, S., Veldman, K., Schroeter, A., Guerra, B., Granier, S. A., Perrin-Guyomard, A., Gicquel-Bruneau, M., Franco, A., Englund, S., Teale, C., Heiska, H., Clemente, L., Boerlin, P., Moreno, M. A., Daignault, D., Mevius, D., Hendriksen, R. S. & Aarestrup, F. M. (2012). Establishing streptomycin epidemiological cut-off values for Salmonella and *Escherichia coli*. *Microbial Drug Resistance (Larchmont, N.Y.)*, 18(1), pp 88–93.

George, A. M. & Levy, S. B. (1983). Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. *Journal of Bacteriology*, 155(2), pp 531–540.

Giguré, S., Prescott, J. F., Baggot, J. D., Walker, R. D. & Dowling, P. M. (2006). *Animicrobial Therapy in Veterinary Medicine*. Ames, Iowa, USA: Blackwell Publishing.

Godden, S. M., Fetrow, J. P., Feirtag, J. M., Green, L. R. & Wells, S. J. (2005). Economic analysis of feeding pasteurized nonsaleable milk versus conventional milk replacer to dairy calves. *Journal of the American Veterinary Medical Association*, 226(9), pp 1547–1554.

Gow, S. P., Waldner, C. L., Rajíc, A., McFall, M. E. & Reid-Smith, R. (2008). Prevalence of antimicrobial resistance in fecal generic *Escherichia coli* isolated in western Canadian beef herds. Part II — Cows and cow-calf pairs. *Canadian Journal of Veterinary Research*, 72(2), pp 91–100.

Grønvold, A.-M. R., Mao, Y., L'Abée-Lund, T. M., Sørum, H., Sivertsen, T., Yannarell, A. C. & Mackie, R. I. (2011). Fecal microbiota of calves in the clinical setting: Effect of penicillin treatment. *Veterinary Microbiology*, 153(3–4), pp 354–360.

Guardabassi, L. & Kruse, H. (2008). Principles of Prudent and Rational Use of Antimicrobials in Animals. In: Guardabassi, L., Jensen, L. B., & Kruse, H. (Eds) *Guide to Antimicrobial Use in Animals*. pp 1–12. Blackwell Publishing, Ltd. ISBN 9781444302639.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D. & I., A. D. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *Plos pathogens*, 7(7), p pg:e1002158.

Gunn, G. J., Hall, M. & Low, J. C. (2003). Comparison of antibiotic resistance for *Escherichia coli* populations isolated from groups of diarrhoeic and control calves. *Veterinary journal (London, England: 1997)*, 165(2), pp 172–174.

Gustafsson, L. (2014). *High prevalence of antibiotic resistance in Escherichia coli from the gut flora of young calves – caused by increased shedding of resistant E. coli from the cow around parturition? [in Swedish]*. Master Thesis. Uppsala, Sweden: Swedish University of Agricultural Sciences. Available from: http://stud.epsilon.slu.se/6515/7/gustafsson_l_140320.pdf. [Accessed 2015-03-24].

Hansen, K. H., Damborg, P., Andreasen, M., Nielsen, S. S. & Guardabassi, L. (2013). Carriage and fecal counts of cefotaxime M-producing *Escherichia coli* in pigs: a longitudinal study. *Applied and Environmental Microbiology*, 79(3), pp 794–798.

Hill, A. E., Green, A. L., Wagner, B. A. & Dargatz, D. A. (2009). Relationship between herd size and annual prevalence of and primary antimicrobial treatments for common diseases on dairy operations in the United States. *Preventive veterinary medicine*, 88(4), pp 264–277.

Hinton, M., Hedges, A. j. & Linton, A. h. (1985a). The ecology of *Escherichia coli* in market calves fed a milk-substitute diet. *Journal of Applied Bacteriology*, 58(1), pp 27–35.

Hinton, M., Linton, A. m. & Hedges, A. j. (1985b). The ecology of *Escherichia coli* in calves reared as dairy-cow replacements. *Journal of Applied Bacteriology*, 58(2), pp 131–138.

Hinton, M., Rixson, P. D., Allen, V. & Linton, A. H. (1984). The persistence of drug resistant *Escherichia coli* strains in the majority faecal flora of calves. *The Journal of Hygiene*, 93(3), pp 547–557.

Hogan, C. *Commensalism*. [online] (2012). Available from: http://www.eoearth.org/view/article/171918/. [Accessed 2015-02-10].

Hordijk, J., Veldman, K., Dierikx, C., van Essen-Zandbergen, A., Wagenaar, J. A. & Mevius, D. (2012). Prevalence and characteristics of quinolone resistance in *Escherichia coli* in veal calves. *Veterinary Microbiology*, 156(1–2), pp 136–142.

Hoyle, D. V., Knight, H. I., Shaw, D. J., Hillman, K., Pearce, M. C., Low, J. C., Gunn, G. J. & Woolhouse, M. E. J. (2004a). Acquisition and epidemiology of antibiotic-resistant *Escherichia coli* in a cohort of newborn calves. *Journal of Antimicrobial Chemotherapy*, 53(5), pp 867–871.

Hoyle, D. V., Shaw, D. J., Knight, H. I., Davison, H. C., Pearce, M. C., Low, J. C., Gunn, G. J. & Woolhouse, M. E. J. (2004b). Age-Related Decline in Carriage of Ampicillin-Resistant *Escherichia coli* in Young Calves. *Applied and Environmental Microbiology*, 70(11), pp 6927–6930.

Hoyle, D. V., Yates, C. M., Chase-Topping, M. E., Turner, E. J., Davies, S. E., Low, J. C., Gunn, G. J., Woolhouse, M. E. J. & Amyes, S. G. B. (2005). Molecular epidemiology of antimicrobial-resistant commensal *Escherichia coli* strains in a cohort of newborn calves. *Applied and Environmental Microbiology*, 71(11), pp 6680–6688.

Hårdemark, V. (2014). *Enkätundersökning bland svenska veterinärer angående behandling av klinisk mastit hos mjölkkor* [in Swedish]. Master Thesis. Uppsala Sweden: Swedish University of Agricultural Sciences. Available from: http://stud.epsilon.slu.se/6527/7/hardemark_v_140320.pdf. [Accessed 2015-02-23].

Janion, C. (2008). Inducible SOS Response System of DNA Repair and Mutagenesis in *Escherichia coli*. *International Journal of Biological Sciences*, pp 338–344.

Jones, L. P., Sischo, W., Besser, T. E. & Davis, M. A. (2013). Correlation between quinolone-resistant commensal *E. coli* in dairy calves and enrofloxacin use. *Proceedings of American Society of Microbiology - 113th general meeting*, Denver, CO, USA, 2013. Denver, CO, USA.

Jong, A. de, Bywater, R., Butty, P., Deroover, E., Godinho, K., Klein, U., Marion, H., Simjee, S., Smets, K., Thomas, V., Vallé, M. & Wheadon, A. (2009). A pan-European survey of antimicrobial susceptibility towards human-use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food-producing animals. *Journal of Antimicrobial Chemotherapy*, 63(4), pp 733–744.

Jurado, S., Orden, J. A., Horcajo, P., De La Fuente, R., Ruiz-Santa-Quiteria, J. A., Martínez-Pulgarín, S. & Domínguez-Bernal, G. (2008). Characterization of fluoroquinolone resistance

in *Escherichia coli* strains from ruminants. *Journal of Veterinary Diagnostic Investigation: Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc*, 20(3), pp 342–345.

Karami, N., Nowrouzian, F., Adlerberth, I. & Wold, A. E. (2006). Tetracycline Resistance in *Escherichia coli* and Persistence in the Infantile Colonic Microbiota. *Antimicrobial Agents and Chemotherapy*, 50(1), pp 156–161.

Keys, J. E., Pearson, R. E. & Fulton, L. A. (1976). Fermentation of Mastitic Milk from Antibiotic Treated Cows. *Journal of Dairy Science*, 59(10), pp 1746–1751.

Keys, J. E., Pearson, R. E. & Weinland, B. T. (1979). Starter Culture, Temperature, and Antibiotic Residue in Fermentation of Mastitic Milk to Feed Dairy Calves1. *Journal of Dairy Science*, 62(9), pp 1408–1414.

Khachatryan, A. R., Besser, T. E., Hancock, D. D. & Call, D. R. (2006a). Use of a nonmedicated dietary supplement correlates with increased prevalence of streptomycin-sulfa-tetracycline-resistant *Escherichia coli* on a dairy farm. *Applied and environmental microbiology*, 72(7), pp 4583–4588.

Khachatryan, A. R., Hancock, D. D., Besser, T. E. & Call, D. R. (2004). Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. *Applied and environmental microbiology*, 70(2), pp 752–757.

Khachatryan, A. R., Hancock, D. D., Besser, T. E. & Call, D. R. (2006b). Antimicrobial drug resistance genes do not convey a secondary fitness advantage to calf-adapted *Escherichia coli*. *Applied and environmental microbiology*, 72(1), pp 443–448.

Kirchner, M., Wearing, H. & Teale, C. (2011). Plasmid-mediated quinolone resistance gene detected in *Escherichia coli* from cattle. *Veterinary Microbiology*, 148(2-4), pp 434–435.

Klein-Jöbstl, D., Iwersen, M. & Drillich, M. (2014a). Farm characteristics and calf management practices on dairy farms with and without diarrhea: a case-control study to investigate risk factors for calf diarrhea. *Journal of Dairy Science*, 97(8), pp 5110–5119.

Klein-Jöbstl, D., Schornsteiner, E., Mann, E., Wagner, M., Drillich, M. & Schmitz-Esser, S. (2014b). Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. *Frontiers in Microbiology*, 5, pp 662

Kohanski, M. A., DePristo, M. A. & Collins, J. J. (2010). Sub-lethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular cell*, 37(3), pp 311–320.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A. & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, 130(5), pp 797–810.

KRAV (2015). *Standards for KRAV-certified production 2015 [in Swedish]*. [online]. Available from: http://www.krav.se/kravs-regler [Accessed 2015-02-23].

Di Labio, E., Regula, G., Steiner, A., Miserez, R., Thomann, A. & Ledergerber, U. (2007). Antimicrobial Resistance in Bacteria from Swiss Veal Calves at Slaughter. *Zoonoses and Public Health*, 54(9-10), pp 344–352.

Langford, F. M., Weary, D. M. & Fisher, L. (2003). Antibiotic resistance in gut bacteria from dairy calves: A dose response to the level of antibiotics fed in milk. *Journal of Dairy Science*, 86(12), pp 3963–3966.

Lastours, V. de, Bleibtreu, A., Chau, F., Burdet, C., Duval, X., Denamur, E. & Fantin, B. (2014). Quinolone-resistant *Escherichia coli* from the faecal microbiota of healthy volunteers after

ciprofloxacin exposure are highly adapted to a commensal lifestyle. *Journal of Antimicrobial Chemotherapy*, 69(3), pp 761–768.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D. & Cars, O. (2013). Antibiotic resistance—the need for global solutions. *The Lancet Infectious Diseases*, 13(12), pp 1057–1098.

Lewallen, S. & Courtright, P. (1998). Epidemiology in Practice: Case-Control Studies. *Community Eye Health*, 11(28), pp 57–58.

Levy, S. B., Fitzgerald, G. B. & Macone, A. B. (1976). Spread of antibiotic-resistant plasmids from chicken to chicken and from chicken to man. *Nature*, 260(5546), pp 40–42.

Liebana, E., Batchelor, M., Hopkins, K. L., Clifton-Hadley, F. A., Teale, C. J., Foster, A., Barker, L., Threlfall, E. J. & Davies, R. H. (2006). Longitudinal Farm Study of Extended-Spectrum β-Lactamase-Mediated Resistance. *Journal of Clinical Microbiology*, 44(5), pp 1630–1634.

Lim, S.-K., Lim, K.-G., Lee, H.-S., Jung, S.-C., Kang, M.-I. & Nam, H.-M. (2010). Prevalence and molecular characterization of fluoroquinolone-resistant *Escherichia coli* isolated from diarrheic cattle in Korea. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science*, 72(5), pp 611–614.

Lindstedt, B.-A., Brandal, L. T., Aas, L., Vardund, T. & Kapperud, G. (2007). Study of polymorphic variable-number of tandem repeats loci in the ECOR collection and in a set of pathogenic *Escherichia coli* and Shigella isolates for use in a genotyping assay. *Journal of Microbiological Methods*, 69(1), pp 197–205.

Linton, A. H., Howe, K., Bennett, P. M., Richmond, M. H. & Whiteside, E. J. (1977). The colonization of the human gut by antibiotic resistant *Escherichia coli* from chickens. *The Journal of Applied Bacteriology*, 43(3), pp 465–469.

Lipsitch, M. & Samore, M. H. (2002). Antimicrobial Use and Antimicrobial Resistance: A Population Perspective. *Emerging Infectious Diseases*, 8(4), pp 347–354.

Lukáš, F., Koppová, I., Kudrna, V. & Kopečný, J. (2007). Postnatal development of bacterial population in the gastrointestinal tract of calves. *Folia microbiologica*, 52(1), pp 99–104.

Lupp, C. & Finlay, B. B. (2005). Intestinal microbiota. *Current Biology*, 15(7), pp R235–R236.

Løbersli, I., Haugum, K. & Lindstedt, B.-A. (2012). Rapid and high resolution genotyping of all *Escherichia coli* serotypes using 10 genomic repeat-containing loci. *Journal of Microbiological Methods*, 88(1), pp 134–139.

Mackie, R. I., Sghir, A. & Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *The American Journal of Clinical Nutrition*, 69(5), p 1035s–1045s.

Marchese, A., Coppo, E., Barbieri, R., Zoppi, S., Pruzzo, C., Rossi, F., Bergagna, S., Dondo, A. & Debbia, E. (2012). Characterization of fluoroquinolone-resistant *Escherichia coli* causing septicemic colibacillosis in calves in Italy: emergence of a multiresistant O78 clonal group. *Microbial drug resistance (Larchmont, N.Y.)*, 18(1), pp 94–99.

Marshall, B. M., Ochieng, D. J. & Levy, S. B. (2009). Commensals: underappreciated reservoir of antibiotic resistance. *Microbe*, 4(5), pp 231–238.

Marshall, B., Petrowski, D. & Levy, S. B. (1990). Inter- and intraspecies spread of *Escherichia coli* in a farm environment in the absence of antibiotic usage. *Proceedings of the National Academy of Sciences of the United States of America*, 87(17), pp 6609–6613.

Mayer, M., Abenthum, A., Matthes, J. M., Kleeberger, D., Ege, M. J., Hölzel, C., Bauer, J. & Schwaiger, K. (2012). Development and genetic influence of the rectal bacterial flora of newborn calves. *Veterinary microbiology*, 161(1-2), pp 179–185.

McDermott, W., Bunn, P. A., Benoit, M., DuBois, R. & Reynolds, M. E. (1946). THE ABSORPTION, EXCRETION, AND DESTRUCTION OF ORALLY ADMINISTERED PENICILLIN 1. *Journal of Clinical Investigation*, 25(2), pp 190–210.

Medical Products Agency (2014). Drug residues in food [in Swedish]. *Läkemedelsrester i livsmedel* [online], Available from: http://www.lakemedelsverket.se/malgrupp/Halso---sjukvard/Forskrivning/Veterinarmedicinska-lakemedel/Lakemedelsrester-i-livsmedel/. [Accessed 2015-02-23].

Miller, C., Thomsen, L. E., Gaggero, C., Mosseri, R., Ingmer, H. & Cohen, S. N. (2004). SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science*, 305(5690), pp 1629–31.

Moro, M. h., Beran, G. w., Griffith, R. w. & Hoffman, L. j. (2000). Effects of heat stress on the antimicrobial drug resistance of *Escherichia coli* of the intestinal flora of swine. *Journal of Applied Microbiology*, 88(5), pp 836–844.

Moro, M. H., Beran, G. W., Hoffman, L. J. & Griffith, R. W. (1998). Effects of cold stress on the antimicrobial drug resistance of *Escherichia coli* of the intestinal flora of swine. *Letters in Applied Microbiology*, 27(5), pp 251–254.

Musser, J. M. B. & Anderson, K. L. (2001). Bioavailability and disposition of sodium and procaine penicillin G (benzylpenicillin) administered orally with milk to calves. *Journal of Veterinary Pharmacology and Therapeutics*, 24(3), pp 161–169.

Mörk, M., Lindberg, A., Alenius, S., Vågsholm, I. & Egenvall, A. (2009). Comparison between dairy cow disease incidence in data registered by farmers and in data from a disease-recording system based on veterinary reporting. *Preventive veterinary medicine*, 88(4), pp 298–307.

Nowrouzian, F. L., Wold, A. E. & Adlerberth, I. (2005). *Escherichia coli* Strains Belonging to Phylogenetic Group B2 Have Superior Capacity to Persist in the Intestinal Microflora of Infants. *Journal of Infectious Diseases*, 191(7), pp 1078–1083.

Nuotio, L., Schneitz, C. & Nilsson, O. (2013). Effect of competitive exclusion in reducing the occurrence of *Escherichia coli* producing extended-spectrum β-lactamases in the ceca of broiler chicks. *Poultry Science*, 92(1), pp 250–254.

O'Neill, J. (2014). *Review on Antimicrobial Resistance - Tackling a crisis for the health and wealth of nations* [online]. Available from: http://www.jpiamr.eu/wp-content/uploads/2014/12/AMR-Review-Paper-Tackling-a-crisis-for-the-health-and-wealth-of-nations_1-2.pdf. [Accessed 2015-02-23].

Oikonomou, G., Teixeira, A. G. V., Foditsch, C., Bicalho, M. L., Machado, V. S. & Bicalho, R. C. (2013). Fecal Microbial Diversity in Pre-Weaned Dairy Calves as Described by Pyrosequencing of Metagenomic 16S rDNA. Associations of Faecalibacterium Species with Health and Growth. *PLoS ONE* [online], 8(4), pp e63157

Oliver, S. P., Duby, R. T., Prange, R. W. & Tritschler, J. P. (1984). Residues in Colostrum Following Antibiotic Dry Cow Therapy. *Journal of Dairy Science*, 67(12), pp 3081–3084.

Ortman, K. & Svensson, C. (2004). Use of antimicrobial drugs in Swedish dairy calves and replacement heifers. *Veterinary Record*, 154(5), pp 136–140.

Pereira, R. V., Siler, J. D., Bicalho, R. C. & Warnick, L. D. (2014a). Multiresidue screening of milk withheld for sale at dairy farms in central New York State. *Journal of Dairy Science*, 97(3), pp 1513-9

Pereira, R. V., Siler, J. D., Ng, J. C., Davis, M. A., Grohn, Y. T. & Warnick, L. D. (2014b). Effect of on-farm use of antimicrobial drugs on resistance in fecal *Escherichia coli* of preweaned dairy calves. *Journal of Dairy Science*, 97(12), pp 7644–7654.

Pereira, R. V., Siler, J. D., Ng, J. C., Davis, M. A. & Warnick, L. D. (2014c). Effect of preweaned dairy calf housing system on antimicrobial resistance in commensal *Escherichia coli*. *Journal of Dairy Science*, 97(12), pp 7633–7643.

Pereira, R. V. V., Santos, T. M. A., Bicalho, M. L., Caixeta, L. S., Machado, V. S. & Bicalho, R. C. (2011). Antimicrobial resistance and prevalence of virulence factor genes in fecal *Escherichia coli* of Holstein calves fed milk with and without antimicrobials. *Journal of Dairy Science*, 94(9), pp 4556–4565.

Pereira, R. V. V., Siler, J. D., Bicalho, R. C. & Warnick, L. D. (2014d). In vivo selection of resistant *E. coli* after ingestion of milk with added drug residues. *PloS One*, 9(12), p e115223.

Pérez-Pérez, F. J. & Hanson, N. D. (2002). Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. *Journal of clinical microbiology*, 40(6), pp 2153–2162.

Pithua, P., Espejo, L. A., Godden, S. M. & Wells, S. J. (2013). Is an individual calving pen better than a group calving pen for preventing transmission of *Mycobacterium avium* subsp *paratuberculosis* in calves? Results from a field trial. *Research in Veterinary Science*, 95(2), pp 398–404.

Randall, L., Heinrich, K., Horton, R., Brunton, L., Sharman, M., Bailey-Horne, V., Sharma, M., McLaren, I., Coldham, N., Teale, C. & Jones, J. (2013). Detection of antibiotic residues and association of cefquinome residues with the occurrence of Extended-Spectrum β-Lactamase (ESBL)-producing bacteria in waste milk samples from dairy farms in England and Wales in 2011. *Research in Veterinary Science,* 96(1), pp 15-24.

Raustein, T. (2003) *Effekt av myrsyra och beta-laktamas på penicillin- och S. aureus-halt i mjölk till kalvar [in Swedish]*. Master Thesis. Uppsala Sweden: Swedish University of Agricultural Sciences. Available from: http://ex-epsilon.slu.se:8080/archive/00000019/. [Accessed 2013-06-12].

Rebelo, I. B. (2014). *Risk of feeding calves with waste milk unfit for human consumption [in Portugees]*. Master Thesis. Lisboa, Portugal: University of Lisboa. Available from: http://www.repository.utl.pt/handle/10400.5/7578. [Accessed 2015-03-06].

Reber, A. J., Lockwood, A., Hippen, A. R. & Hurley, D. J. (2006). Colostrum induced phenotypic and trafficking changes in maternal mononuclear cells in a peripheral blood leukocyte model for study of leukocyte transfer to the neonatal calf. *Veterinary Immunology and Immunopathology*, 109(1–2), pp 139–150.

Redding, L. E., Cubas-Delgado, F., Sammel, M. D., Smith, G., Galligan, D. T., Levy, M. Z. & Hennessy, S. (2014). Comparison of two methods for collecting antibiotic use data on small dairy farms. *Preventive Veterinary Medicine*, 114(3–4), pp 213–222.

Reves, R. R., Fong, M., Pickering, L. K., Bartlett, A., Alvarez, M. & Murray, B. E. (1990). Risk factors for fecal colonization with trimethoprim-resistant and multiresistant *Escherichia coli* among children in day-care centers in Houston, Texas. *Antimicrobial Agents and Chemotherapy*, 34(7), pp 1429–1434.

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Hye Park, C., Bush, K. & Hooper, D. C. (2006). Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nature Medicine*, 12(1), pp 83–88.

Roca, M., Castillo, M., Marti, P., Althaus, R. L. & Molina, M. P. (2010). Effect of heating on the stability of quinolones in milk. *Journal of Agricultural and Food Chemistry*, 58(9), pp 5427–5431.

Roca, M., Villegas, L., Kortabitarte, M. L., Althaus, R. L. & Molina, M. P. (2011). Effect of heat treatments on stability of β-lactams in milk. *Journal of Dairy Science*, 94(3), pp 1155–1164.

Ruiz, J. (2003). Mechanisms of resistance to quinolones: targ*et al.*terations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy*, 51(5), pp 1109–1117.

Sato, K., Bartlett, P. C. & Saeed, M. A. (2005). Antimicrobial susceptibility of *Escherichia coli* isolates from dairy farms using organic versus conventional production methods. *Journal of the American Veterinary Medical Association*, 226(4), pp 589–594.

Schwarz, S., Kehrenberg, C. & Walsh, T. R. (2001). Use of antimicrobial agents in veterinary medicine and food animal production. *International Journal of Antimicrobial Agents*, 17(6), pp 431–437.

Scott, H. M., Norby, B. & Loneragan, G. H. (2011). Antimicrobial resistance surveillance: bacterial prevalence estimates are not enough. *Proceedings of International Conference on Animal Health Surveillance (ICAHS)*, Lyon, France, May 17th 2011. pp 182–184. Lyon, France: Association pour l'Étude de l'Épidémiologie des Maladies Animales.

Singer, R. S., Patterson, S. K. & Wallace, R. L. (2008). Effects of Therapeutic Ceftiofur Administration to Dairy Cattle on *Escherichia coli* Dynamics in the Intestinal Tract. *Applied and Environmental Microbiology*, 74(22), pp 6956–6962.

Snow, L. C., Warner, R. G., Cheney, T., Wearing, H., Stokes, M., Harris, K., Teale, C. J. & Coldham, N. G. (2012). Risk factors associated with extended spectrum beta-lactamase *Escherichia coli* (CTX-M) on dairy farms in North West England and North Wales. *Preventive Veterinary Medicine*, 106(3–4), pp 225–234.

Straley, B. A., Donaldson, S. C., Hedge, N. V., Sawant, A. A., Srinivasan, V., Oliver, S. P. & Jayarao, B. M. (2006). Public health significance of antimicrobial-resistant gram-negative bacteria in raw bulk tank milk. *Foodbourne Pathogens & Disease*, 3(3), pp 222–233.

Sundsfjord, A., Simonsen, G. S., Haldorsen, B. C., Haaheim, H., Hjelmevoll, S.-O., Littauer, P. & Dahl, K. H. (2004). Genetic methods for detection of antimicrobial resistance. *APMIS*, 112(11-12), pp 815–837.

Svarm (2006). *Swedish Veterinary Antimicrobial Resistance Monitoring (Svarm) 2006.* Uppsala, Sweden: National Veterinary Institute (SVA). (ISSN 1650-6332).

Swedish Board of Agriculture (2013). *Regulations amending the State Board of Agriculture Regulation (SJVFS 2009: 84) on medicines and medication* [online]. Available from: http://www.jordbruksverket.se/download/18.6c157f5413b5fe03aa780002586/2012-032.pdf. [Accessed 2015-03-11].

Swedish Board of Agriculture (2014). *Yearbook of agricultural statistics 2014 [in Swedish]*. [online]. Örebro: Statistics Sweden. Available from: http://www.jordbruksverket.se/webdav/files/SJV/Amnesomraden/Statistik%2C%20fakta/Hus Husd/JO20/JO20SM1403/JO20SM1403.pdf. [Accessed 2015-03-11].

SWEDRES-Svarm (2013). *Use of antimicrobials and occurrence of antimicrobial resistance in Sweden.* Solna/Uppsala, Sweden: Public Health Agency of Sweden and National Veterinary Institute. (ISSN 1650-6332).

Sveriges Veterinärmedicinska Sällskap (2013). *Guidelines for the use of antimicrobials for food-producing animals [in Swedish]* [online]. Available from: http://www.svf.se/Documents/S%C3%A4llskapet/Husdjurssektionen/SVS%20Riktlinjer%20f%C3%B6r%20anv%C3%A4ndning%20av%20antibiotika%20till%20produktionsdjur%202013.pdf [Accessed 2013-08-09].

Sykes, R. (2010). The 2009 Garrod Lecture: the evolution of antimicrobial resistance: a Darwinian perspective. *Journal of Antimicrobial Chemotherapy*, 65(9), pp 1842-52.

Sörensen, J. T., Hegelund, L. & Aarestrup, F. M. *Antimicrobial resistance in milk fed calves (in Danish).* [online]. Available from: https://www.landbrugsinfo.dk/Kvaeg/Malkekoeer-og-opdraet/Smaakalve/Sider/Antibiotikaresistens_hos_maelkefodrede_k.aspx. [Accessed 2013-08-09].

Taylor, N. M., Clifton-Hadley, F. A., Wales, A. D., Ridley, A. & Davies, R. H. (2009). Farm-level risk factors for fluoroquinolone resistance in *E. coli* and thermophilic *Campylobacter* spp. on finisher pig farms. *Epidemiology and infection*, 137(8), pp 1121–1134.

Tkalcic, S., Zhao, T., Harmon, B. G., Doyle, M. P., Brown, C. A. & Zhao, P. (2003). Fecal Shedding of Enterohemorrhagic *Escherichia coli* in Weaned Calves following Treatment with Probiotic *Escherichia coli*. *Journal of Food Protection*, 66(7), pp 1184–1189.

Tragesser, L. A., Wittum, T. E., Funk, J. A., Winokur, P. L. & Rajala-Schultz, P. J. (2006). Association between ceftiofur use and isolation of *Escherichia coli* with reduced susceptibility to ceftriaxone from fecal samples of dairy cows. *American Journal of Veterinary Research*, 67(10), pp 1696–1700.

Trobos, M., Lester, C. H., Olsen, J. E., Frimodt-Møller, N. & Hammerum, A. M. (2009). Natural transfer of sulfonamide and ampicillin resistance between *Escherichia coli* residing in the human intestine. *Journal of Antimicrobial Chemotherapy*, 63(1), pp 80–86.

Watson, E., Jeckel, S., Snow, L., Stubbs, R., Teale, C., Wearing, H., Horton, R., Toszeghy, M., Tearne, O., Ellis-Iversen, J. & Coldham, N. (2012). Epidemiology of extended spectrum beta-lactamase *E. coli* (CTX-M-15) on a commercial dairy farm. *Veterinary microbiology*, 154(3-4), pp 339–346.

Wellington, E. M., Boxall, A. B., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M., Otten, W., Thomas, C. M. & Williams, A. P. (2013). The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases*, 13(2), pp 155–165.

De Verdier, K. de, Nyman, A., Greko, C. & Bengtsson, B. (2012). Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Veterinaria Scandinavica*, 54(1), p 2.

Veterinärutredningen (2007). *SOU 2007:024 Veterinär fältverksamhet i nya former*. Norstedts Juridik AB. ISBN 9789138227299.

Whittem, T. & Hanlon, D. (1997). Dihydrostreptomycin or streptomycin in combination with penicillin G in dairy cattle therapeutics: A review and re-analysis of published data Part 1: Clinical pharmacology. *New Zealand Veterinary Journal*, 45(5), pp 178–184.

WHO (2012a). Antimicrobial resistance. [online]. Available from: http://www.who.int/mediacentre/factsheets/fs194/en/. [Accessed 2015-01-27].

WHO (2012b). *Critically important antimicrobials for human medicine – 3 rd revision* [online]. Geneva, Switzerland: World Health Organization. (Critically important antimicrobials for human medicine – third revision; 978 92 4 150448 5).

WHO (2015). *WHO - Zoonoses and the Human-Animal-Ecosystems Interface*. [online]. Available from: http://www.who.int/zoonoses/en/. [Accessed 2015-01-27].

Wieler, L. H., Sobjinski, G., Schlapp, T., Failing, K., Weiss, R., Menge, C. & Baljer, G. (2007). Longitudinal prevalence study of diarrheagenic *Escherichia coli* in dairy calves. *Berliner Und Münchener Tierärztliche Wochenschrift*, 120(7-8), pp 296–306.

Wierup, M. (1975). [Antibiotic resistance and transferable antibiotic resistance of *Escherichia coli* isolated from Swedish calves 5 and 30 days old]. *Nordisk Veterinaermedicin*, 27(2), pp 77–84.

Williams Smith, H. (1969). Transfer of antibiotic resistance from animal and human strains of *Escherichia coli* to resident *E. coli* in the alimentary tract of man. *The Lancet*, 293(7607), pp 1174–1176 (Originally published as Volume 1, Issue 7607).

Winokur, P. L., Vonstein, D. L., Hoffman, L. J., Uhlenhopp, E. K. & Doern, G. V. (2001). Evidence for Transfer of CMY-2 AmpC β-Lactamase Plasmids between *Escherichia coli* andSalmonella Isolates from Food Animals and Humans. *Antimicrobial Agents and Chemotherapy*, 45(10), pp 2716–2722.

Witte, W. (2004). International dissemination of antibiotic resistant strains of bacterial pathogens. *Infection, Genetics and Evolution*, 4(3), pp 187–191 (6th International Meeting on Microbial Epidemiological Markers).

Woodford, N., Fagan, E. J. & Ellington, M. J. (2006). Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *Journal of Antimicrobial Chemotherapy*, 57(1), pp 154–155.

Wray, C., Furniss, S. & Benham, C. L. (1990). Feeding antibiotic-contaminated waste milk to calves--effects on physical performance and antibiotic sensitivity of gut flora. *Brittish Veterinary Journal*, 146(1), pp 80–7.

Würgler-Aebi, I. C. (2004). *Entwicklung von Resistenzen gegen Makrolid-Antibiotika bei Enterokokken im Kot von Kälbern, gefüttert mit Antibiotika-haltiger Milch*. Doctoral Thesis. University of Bern, Switzerland. Availiable from: http://www.anresis.ch/files/pdf/Project_Schaellibaum.pdf. [Accessed 2015-01-27].

Växa Sverige (2014). *Cattle Statistics 2014 [in Swedish]*. [online]. Stockholm, Sweden. Available from: http://www.vxa.se/Documents/Husdjursstatistik2015_ver2015-02-11.pdf [Accessed 2015-03-21].

Växa Sverige (2015). *Animal Health 2013/2014: Annual report from the animal health section [in Swedish]*. [online]. Stockholm, Sweden. Available from: http://www.vxa.se/Global/Bildbank/Redog%C3%B6relse%20f%C3%B6r%20husdjursorganisationens%20djurh%C3%A4lsov%C3%A5rd%202013_14.pdf. [Accessed 2015-03-21].

Yamamoto, S., Iwabuchi, E., Hasegawa, M., Esaki, H., Muramatsu, M., Hirayama, N. & Hirai, K. (2013). Prevalence and molecular epidemiological characterization of antimicrobial-resistant *Escherichia coli* isolates from Japanese black beef cattle. *Journal of Food Protection*, 76(3), pp 394–404.

Yndestad, M. (1980). Milk containing antibiotics used as food for young calves--an investigation concerning the health aspect of periodically supplying penicillin and dihydrostreptomycin (in Norwegian). *Nor veterinaertidsskr*, 92(7/8), pp 435–441.

Zorraquino, M. A., Althaus, R. L., Roca, M. & Molina, M. P. (2009). Effect of heat treatments on aminoglycosides in milk. *Journal of Food Protection*, 72(6), pp 1338–1341.

Zorraquino, M. A., Roca, M., Fernandez, N., Molina, M. P. & Althaus, R. (2008). Heat inactivation of beta-lactam antibiotics in milk. *Journal of Food Protection*, 71(6), pp 1193–1198.

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