Eesti Maaülikool
Estonian University of Life Sciences

ANTIMICROBIAL RESISTANCE OF *ESCHERICHIA COLI* AND ENTEROCOCCI ISOLATED FROM SWINE, CATTLE AND DOGS AND MASTITIS PATHOGENS ISOLATED IN ESTONIA IN 2006–2015

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BIRGIT AASMÄE

A Thesis
for applying for the degree of Doctor of Philosophy in
Veterinary Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks loomaarstiteaduse erialal

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To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following research papers, which are referred to by their Roman numerals (I-V in the text).

I **Aasmäe, B.**, Kalmus, P. (2012). Antimicrobial resistance of animal pathogens 2006-2009 in Estonia. Research for Rural Development 1:181-187.

II **Aasmäe, B.**, Häkkinen, L., Kaart, T., Kalmus, P. (2019). Antimicrobial resistance of *Escherichia coli* and enterococci isolated from cattle and swine from 2010 to 2015 in Estonia. Acta Vet Scand. 61:5.

III **Aasmäe, B.**, Volkova, J., Häkkinen, L., Orro, T., Tenson, T., Kalmus, P. (2015). *In vitro* antimicrobial resistance of intestinal *Escherichia coli* and enterococci in clinically healthy dogs in Estonia. Vet Med Zoot. 72:3-8.

IV Kalmus, P., **Aasmäe, B.**, Kärssin, A., Orro, T., Kask, K. (2011). Udder Pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. Acta Vet Scand. 53:4.

The contribution of authors to the research paper

Article	Original idea, study design	Data collection, sample analysis	Data analysis	Manuscript writing
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ABBREVIATIONS

AMR	Antimicrobial resistance
BHI	Brain heart infusion
CI	Confidence intervals
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase negative staphylococci
DTU	Technical University of Denmark
EARC	Estonian Animal Recording Centre
EC	European Commission
ECDC	European Centre for Disease Control
ECOFF	Epidemiological cut-off values
EFSA	European Food Safety Authority
EMB	Eosin methylene blue
EPEC	Enteropathogenic E. coli
ESBL	Extended spectrum beta-lactamases
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EURL-AREU	Reference Laboratory for antimicrobial resistance
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
OR	Odds ratio
PCR	Polymerase chain reaction
VFL	Veterinary and Food Laboratory
WHO	World Health Organization
VRE	Vancomycin-resistant enterococci

1. INTRODUCTION

Antimicrobial resistance (AMR) has been a global emerging threat in both human and veterinary medicine during last two decades. However, Alexander Fleming, the scientist who first discovered the antibiotic properties of penicillin in 1928, reported in 1945 that within one year of the widespread use of penicillin, a significant number of *Staphylococcus (S.) aureus* strains had become resistant to penicillin (Levy, 2002; Capita and Alonso-Calleja, 2013). Fleming warned about the inappropriate use of penicillin that could lead to selection of resistant "mutant forms" of bacteria. Several years later, already more than 50% of *S. aureus* had become resistant to penicillin (Levy, 2002). Thus, from the discovery of antibiotics, AMR became an unavoidable result of antimicrobial therapy because of the metabolic and protective mechanisms of bacteria against antibiotics (Alanis, 2005).

According to data already from 2009 reported by the European Centre for Disease Control (ECDC), in the Europa AMR causes 25,000 deaths together with 2.5 million extra hospital days each year (ECDC, 2009). In the review by O'Neill (2014), a group of scientists estimated that without rapid changes in the use of antimicrobials, antimicrobial resistance could cause 10 million deaths a year by 2050. Approximately 300 million people are expected to die prematurely because of drug resistance over the next 35 years and the world's gross domestic product will be 2 to 3.5% lower than it otherwise would be in 2050.

Regarding veterinary medicine, already over a decade ago it was generally acknowledged that the use (especially irresponsible and excessive use) of antimicrobials in animals has contributed to the development of resistance of pathogens that can be transmitted to humans (Shea, 2004; Carnevale, 2005). However, some authors do not consider the impact of veterinary antimicrobials being high in spread of resistant bacteria to humans (Capita and Alonso-Calleja, 2013; Horigan *et al.*, 2016), but we need to keep in mind that large amounts of antibiotics used in food animals definitely contribute to the development of resistant bacteria that may be transmitted to the environment and humans. It is of utmost importance to monitor the amount of antibiotics consumed by food-producing animals each year and analyse possible relation between development of antimicrobial resistance of bacteria and use

of antibiotics. Furthermore, also resistance of bacteria isolated from pet animals should be monitored as pets are in very close contact and share the same environment with humans (Carvalho *et al.*, 2016).

Several documents and guidelines have been issued by the European Commission and its institutions confirming the importance of continuous monitoring of AMR in member states and taking appropriate measures to minimise the risk for development of resistant microbes both in human and veterinary medicine. In June 2017, the European Commission adopted the EU One Health Action Plan against AMR to summarise the ongoing and forthcoming plans and activities regarding AMR (European Commission, 2017). Setting out the current situation at a certain timepoint enables future researches to monitor changes and take appropriate measures to diminish the development of antimicrobial resistance. Similar data from other countries enable researches to compare resistance patterns in different countries and regions and to consider possible transmission of resistant strains. In order to reduce development of resistant bacteria and provide appropriate suggestions for the use of antibacterials, the adequate survey of the current situation in each country is necessary.

The main goal of the present work was to describe antimicrobial resistance of different bacterial species originating from healthy or diseased animals in Estonia. In studies I and II, antimicrobial resistance of *Escherichia (E.) coli* and *Enterococcus* spp. in healthy and diseased swine and dairy cattle was investigated. In study III, antimicrobial resistance of *E. coli* and enterococci originating from healthy dogs was analysed and associated risk factors were identified. In study IV, the distribution and antimicrobial resistance of mastitis pathogens in dairy cattle was evaluated.

2. REVIEW OF THE LITERATURE

2.1. Antimicrobial resistance – a global emerging threat to human and animal health, as well as a challenge to the food industry

Bacterial infections are one of the most prevalent disease groups in animals and are commonly treated with antimicrobial drugs (Horigan *et al*., 2016). Antibacterial treatment is essential to treat sick animals, however, one of the negative impacts is expansion of antimicrobial resistance (AMR). The antimicrobial resistance of bacterial species originating from production animals also influence human health through the transfer of resistant microbes or genes via the food chain (Greig *et al.*, 2015; Händel *et al*., 2016). Antimicrobial resistance is one of the fastest developing problems in human medicine, and the World Health Organisation (WHO) has named antibiotic resistance as one of the most important public health threats of the 21st century (WHO, 2015).

The emergence of AMR was not an unexpected phenomenon, but today it has turned into a global public health threat. It affects animals and humans and involves both current and future generations. Antibiotic-resistant microbes are responsible for reduced quality of life of the patients, for metastatic bacterial infections, recurrent and chronical infections and future opportunistic infections (Capita and Alonso-Calleja, 2013). AMR is a serious threat to the modern health-care system and could set back the modern medicine (Ferri *et al.,* 2017). The impact and probability of AMR to the public health is considered as high as terrorism or climate change (http://www3.weforum.org/docs/WEF_GRR18_Report.pdf). AMR as a global problem needs to be handled on national, regional and international levels as a "One Health" approach with involvement of professionals in human and veterinary medicine.

Studies have demonstrated that the antimicrobial-resistant bacteria and their resistance genes are circulating in the soil, plants, food-producing animals and the food-chain (Heuer *et al.,* 2011; Ferri *et al.,* 2017; McKinney *et al.,* 2018). Animal trade has been identified to be a driver of bacteriae (Espinoso-Gongora *et al.*, 2012). Pathogens from food-producing animals can infect humans through the consumption of contaminated or cross-contaminated foods of animal

origin (Aarestrup *et al.*, 2007; Overdevest *et al.*, 2011), by water or environmental contamination (Weese *et al.*, 2006), as well as through direct animal contact (Price *et al.*, 2007; Soavi *et al.*, 2010). The latter issue is especially relevant for farm workers or veterinarians that can be colonised (or infected) with resistant bacteria from animals. Although direct transmission lacks relevant public health significance, the infected workers or their families might constitute a port-of-entry of resistant genes in the community and hospital environments (Lyons *et al.*, 1980; Voss *et al.*, 2005). There is also evidence that commensal bacteriae, such as *E. coli* and *Enterococcus* spp., originated not only from the intestine of farm animals, but also bacteria living in human intestine can form a potential reservoir of resistance genes that may be transferred between bacterial species, including organisms that can cause disease in both humans and animals (Van den Bogaard and Stobberingh, 2000; Winokur *et al.*, 2001; Penders *et al.*, 2013).

2.2. An impact of antimicrobial use on the development of resistance

The development and spread of resistance is an outcome of natural selection, survival of the fittest organisms (microbes that can survive in the presence of antibacterials). As a result of long-term use of antimicrobials, susceptible organisms are eliminated, while resistant populations remain and becomes predominant (Levy and Marshall, 2004). Exposure to one antibiotic may co-select for bacteria resistant to another antimicrobials (Canton and Ruiz-Garbajosa, 2011; Tadesse *et al.*, 2012).

The intensive use of antimicrobials in food producing animals may lead to dissemination of resistant bacteria in humans, especially *E. coli* strains with several antibiotic-resistant phenotypes, including co-resistance to other, unrelated groups of antibiotics (Saenz *et al.*, 2004). Differently from human medicine, in production animals, mass medication is used quite often (Ferry *et al.*, 2017). The massive use of antimicrobials, particularly critically important antimicrobials (e.g., quinolones and 3rd and 4th generation cephalosporins) in animals is a threat for transfer of resistant genes and might corroborate the significance of animals, especially food animals, as reservoirs of resistance for human infection (Ferri *et al.*, 2017). One severe consequence of antimicrobial treatment in animals is that approximately 75-90% of antibiotics administered to

food animals are excreted (mostly unmetabolised) into the environment and may also create resistance outside the animal and human bacterial population (Rosenblatt-Farrell, 2009; Subbiah *et al.*, 2016). Gullberg *et al.* (2011) showed that even sub-minimum inhibitory concentration (MIC) levels of antibiotics in the environment are relevant in the presence and maintenance of resistance.

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 and eventually the formation of multidrug-resistant mutants (Kohanski *et al.,* 2010). 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. Co-resistance is the co-existence of several genes conferring resistance to different antimicrobials (Schwarz *et al.,* 2001; Guardabassi and Kruse, 2008). Moreover, there is an interesting concept that development of resistance is not a simple selective pressure induced by antibacterials. It includes physiological changes in the bacteria: if the new environment (antimicrobials) is not lethal to the microbe, it may uniformly induce changes in the physiological state of the cell, and it can make bacterial cells more receptive to foreign DNA and influence frequency of mutation (Heinemann, 1999). There might be a link also between the use of biocides and disinfectants and the development of antimicrobial resistance (Hegestad *et al.,* 2010). Some studies show that when not under the selective pressure of antimicrobials, bacterial population may slowly revert to mainly susceptible (Aarestrup *et al.,* 2001; Butler *et al.,* 2007). Other studies have shown that resistance can also remain without the presence of antimicrobials (Borgen *et al.,* 2000; Heuer *et al.,* 2002; Johnsen *et al.,* 2005). Some authors have suggested that in the original host microbe, the resistance genes are involved in detoxification of non-antibiotic agents and play a role in metabolic functions (Piddock, 2006; Martinez, 2008). A reservoir of such genes is present in the microbes in natural environments and can be transferred to other bacteria, including pathogens, via different gene exchange mechanisms (Wright, 2010). Thus, the issue of resistance must be considered beyond the human medicine and veterinary profession and specific pathogen, it is the „One Health" issue. The effects of administration of antimicrobials on the

development and spread of resistance at the individual and global level are complex and require further investigations (MacLean, 2010).

It is likely that any new antimicrobial will be reserved for human medicine. Future veterinary medicine has to rely mainly on the efficacy of already existing antimicrobials (Schwarz *et al.*, 2001). The WHO has stated that some antimicrobials (fluoroquinolones and 3rd and 4th generation cephalosporins and macrolides) should be reserved only for treating human infections (WHO, 2012). As the antimicrobial resistance is a „One Health" issue, it is necessary to find more effective preventive measures at the farm level to reduce the use of antimicrobials, to improve prudent use of antibiotics in clinical area and strengthen AMR surveillance system in animal and human population (Ferri *et al.*, 2017).

2.2.1. The use of antimicrobials in Estonia

General rules for the use of antibiotics in veterinary medicine in Estonia are similar to those in Nordic countries. This also reflects the total amounts of antibiotics sold out for veterinary purposes. According to the data from the Estonian State Agency of Medicines, the most often used group of antibiotics in Estonia is betalactams. However, the use of 3rd and 4th generation cephalosporins is extremely high, with Estonia having the highest use in Europe (ESVAC, 2017). Also, the use of quinolones is intensive. It is necessary to increase the awareness of veterinarians and farmers and make necessary decisions on the political and legislative levels.

There are approximately 84,800 dairy cows (549 farms in total) and 306,200 pigs (217 farms in total) in Estonia (Eesti Põllumajanduse Registrite ja Informatsiooni Amet (PRIA) 2019). The number of both animal species has continuously decreased from 2010 to 2019. In 2010 there were about 96,000 dairy cows and 374,000 pigs as compared to about 84,000 cows and 306,000 pigs in 2019. Poultry population has remained quite stable, at around 2,100,000 over the past 15 years (Eesti Statistika Andmebaas (ESA) 2019). In Estonia the poultry sector is relatively small and well covered by the state control plans, therefore the investigations in poultry are not included in this thesis.

Table 1. The use of antimicrobials (active ingredients in grams) in animals in Estonia in 2012-2017[1].

Antimicrobial	2012	2013	2014	2015	2016	2017
Aminoglycosides	780,004	813,162	899,478	549,235	408,401	438,665
Amphenicols	20,640	41,030	52,275	39,195	40,750	55,490
Fluoroquinolons	128,401	202,448	201,013	225,059	148,513	150,700
Lincosamides	146,677	208,437	301,812	97,867	33,608	53,982
Macrolides	463,463	465,627	584,247	348,656	266,753	239,927
Penicillins and penicillin combinations	2,710,632	3,543,896	3,153,837	3,613,840	3,683,293	2,485,167
Pleuromutilins	560,985	512,223	1,827,408	931,330	728,050	691,654
Polymyxins	540,080	697,311	397,520	136,601	82,860	109,438
Sulfonamide and trimetoprim combinations	140,082	296,622	207,090	118,315	200,942	405,311
Tetracyclines	1,806,153	1,765,621	2,223,540	2,032,185	1,832,813	1,675,350
Cephalosporins 1st and 2nd generation	119,070	94,157	85,994	80,076	77,977	68,986
Cephalosporins 3rd and 4th generation	70,970	80,444	80,086	75,512	82,211	91,503
Other antibacterials	20,909	21,422	25,104	33,536	52,345	65,397
TOTAL	**7,508,067**	**8,742,399**	**10,039,405**	**8,281,408**	**7,638,516**	**6,531,570**

[1] Data from Estonian State Agency of Medicine, unpublished.

In 2012–2017, the average amount of tetracycline used for the treatment of swine was approximately 1,500 kg of pure active substance per year, for the treatment of cattle approximately 210 kg/year (data from the Estonian State Agency of Medicines, unpublished). The same figures for ampicillin/amoxicillin were 2,200/500 kg and for sulpha/trimethoprim 110/50 kg, respectively. In Estonia, tetracyclines (including doxycycline), ampicillin/amoxicillin and sulpha/trimethoprim are authorised for oral treatment in swine and poultry, but not in cattle (data from the Estonian State Agency of Medicines). Enrofloxacin and other quinolones are still used quite extensively for the treatment of swine and cattle in Estonia (amounts of active ingredients 85/55 kg per year respectively). This is not in line with the local rules of prudent use of antimicrobials. The increasing use of 3rd and 4th generation cephalosporins and amphenicols is also worrying (Table 1). As a positive trend, we can see the decreased use of aminoglycosides, lincosamides and macrolides. The use of polymyxins has decreased, but considerable amount of these antibiotics is still used. Pleuromutilins are used in relatively high amount which is against the rules of prudent use of antibiotics.

2.3. Antimicrobial resistance monitoring programs

The problem of antimicrobial resistance is not eradicable, but has to be managed; thus, additional control measures regarding antibiotic treatment are necessary. Continuous monitoring of the antimicrobial resistance in indicator (commensal) bacteria is of utmost importance, as commensal bacteria, such as *E. coli* and enterococci are considered good indicators of the antimicrobial resistance development on intestinal populations of bacteria in food animals following the use of antimicrobials (EFSA, 2008).

According to the Directive 2003/99/EC EU and Commission decision 2013/652/EL, member states are obliged to monitor and report data on the resistance of *Salmonella* spp, *Campylobacter (C.) jejuni*, *C. coli*, indicator commensal *E. coli*, *E. faecalis* and *E. faecium* isolates from animals and animal products. There is an AMR monitoring programme of the abovementioned bacteria in place in Estonia. Additional national resistance monitoring programmes are in place in many countries. In Estonia, there is no annual AMR monitoring programme regarding other bacteria, which are not covered by EU legislation, e.g., bacteria from diseased animals and commensal bacteria from pet animals.

2.4. Antimicrobial resistance of *E. coli* in animals

E. coli is a commensal bacteria of the gastrointestinal tract of humans and animals, but pathogenic strains can cause infections in different organ systems (Ramos *et al.,* 2013).

E. coli is considered an important indicator bacteria characterising the level of resistance in intestinal bacteria of healthy animals (Hammerum and Heuer, 2009), as well as a species that acts as an early warning of the development of resistance by related pathogens (van den Bogaard and Stobberingh, 2000).

2.4.1. Antimicrobial resistance of *E. coli* isolated from dairy cattle and swine

Antimicrobial resistance of both pathogenic and commensal *E. coli* isolated from food animals has been investigated all over the world for decades. *E. coli* resistance is probably the most frequently emerging issue regarding isolates originating from swine. Many isolates are multi-resistant, and resistance genes are frequently on plasmids (Barton, 2014). It has also been reported that transmission via foods is the most important method of transmission of resistant bacteria and resistance genes from farm to the food consumer (van den Bogaard and Stobberingh, 2000); thus, the meat (from swine as well as from other food animals and poultry) contaminated with resistant bacteria can be the potential threat to human health.

Isolates from clinical submission can be more frequently resistant than isolates from healthy animals because of the more frequent exposure to antimicrobials, and use of antimicrobial agents may select for bacteria carrying virulence genes (Boerlin *et al*., 2005). Regarding clinical submission from swine, many authors report that *E. coli* has the highest resistance against streptomycin, tetracycline, trimethoprim-sulphametoxazole and ampicillin (SWEDRES/SVARM, 2014; FINRES-Vet, 2010-2012; Boireau *et al.,* 2018). The same authors also report that unless the level of *E. coli* resistance to enrofloxacin and ceftiofur is low, it is nevertheless concerning because these antibiotics are „last-generation antibacterials", both in human and veterinary medicine. Studies have also reported that in cattle, *E. coli* clinical isolates exhibit

the highest resistance to ampicillin, tetracycline, trimethopim and sulphamethoxazole (MARAN, 2015; Boireau *et al*., 2018).

Several studies have confirmed that *E. coli* strains isolated from healthy swine are mainly resistant to tetracycline, trimethoprim-sulphametoxazole, streptomycin and ampicillin (MARAN, 2015; Österberg *et al.,* 2016; Valiakos *et al.,* 2016). *E. coli* from healthy cattle has been reported to show the highest resistance against ampicillin, tetracycline, streptomycin and sulphamethoxazole (Sawant *et al.,* 2007; SWEDRES/SVARM, 2017), as well as to ceftazidime and neomycin, but resistance patterns are related to the country, farm and use of antimicrobials (Bok *et al.,* 2015). In many European countries, the resistance level of *E. coli* to fluoroquinolones is generally low and has been decreasing during recent years. Ciprofloxacin resistance in chickens increased, but clear tendencies were absent (de Jong *et al.,* 2012). The amount of multi-resistant *E. coli* isolates depends on farm and use of antimicrobials (Mazurek *et al.,* 2013; Ramos *et al.,* 2013).

Regarding human health, the enteropathogenic *E. coli* (EPEC) found in animals is a concern (Moura *et al.,* 2009). There is limited data currently available on virulence genes and antibiotic resistance in this pathogenic *E. coli* group in animals, but some authors have confirmed that cattle are a potential source of EPEC, and therefore, further studies are required to investigate cattle and other animals as a source of human infection and to examine the role in EPEC in the dissemination of antibiotic resistance (Trabulsi *et al.,* 2002; Moura *et al.,* 2009; Bolton *et al.,* 2014).

2.4.2 Antimicrobial resistance of *E. coli* isolated from dogs

Some studies have reported that *E. coli* isolates from dogs are mostly resistant to ampicillin and tetracycline, but there are differences between countries and geographical areas (Wedley *et al.,* 2011; Davis *et al.,* 2011). National resistance surveillance programmes (DANMAP, MARAN, SWEDRES-SVARM, Finres-Vet) focus mainly on food animals, but data on pet animals are clearly needed because healthy pet animals can also harbour resistant *E. coli* on body sites that come in contact with humans – animal owners (Davis *et al.,* 2011). Thus, both food and pet animals should be the focus of antimicrobial resistance studies.

2.4.3. Extended spectrum beta-lactamases (ESBL) and AmpC producing *E. coli*

Extended spectrum beta-lactamases (ESBL) are enzymes produced by bacteria, which are capable of conferring bacterial resistance to the penicillins, first-, second- and third-generation cephalosporins and aztreonam. ESBLs are able to hydrolyse those antibiotics but are still sensitive to clavulanic acid (Paterson and Bonomo, 2005; Rawat and Nair, 2010). Many bacteria can be ESBL producers, but special attention has been paid to *E. coli, Salmonella* spp. and *Klebsiella* spp. as these are the bacteria with high clinical importance (Falagas and Karageorgopoulos, 2009). The total number of ESBLs characterised is over 200 (Rawat and Nair, 2010). ESBLs are often encoded by genes located on large plasmids, which can also carry genes for resistance to other antimicrobials, such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (Paterson, 2000), as well as fluoroquinolones (Mammeri *et al.*, 2005). Clinically, ESBLs limit the efficacy of beta-lactam antibiotics (Rawat and Nair, 2010). AmpC beta-lactamases are bacterial enzymes encoded on the chromosomes of many of the *Enterobacteriaceae* and a few other organisms. They mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins and beta-lactamase inhibitor - beta-lactam combinations. AmpC enzymes can be both plasmid and chromosomally determined. In most parts of the world, resistance due to plasmid-mediated AmpC enzymes is less common than ESBL production, but it may be both harder to detect and broader in spectrum. For instance, AmpC enzymes can hydrolyse broad-spectrum cephalosporins more efficiently. Additionally, regarding AmpC β-lactamase production by mutation, the development of resistance is a concern (Jacoby, 2009).

The presence of ESBL/AmpC-producing *E. coli* in food animals is a public health concern (Dohmen *et al.*, 2017). Severals studies have reported a presence of ESBLs in swine farms (Mesa *et al*., 2006; SWEDRES/SWARM, 2014; Dahms *et al.*, 2015; MARAN, 2015; DANMAP, 2015) and in foods of animal origin (SWEDRES/SWARM, 2014). Transmission of ESBL genes from animals to humans can occur through food or direct contact (Dohmen *et al.*, 2015). However, one recent study showed a different standpoint. Dorado-García *et al.* (2018) reported that in their study, *E. coli* isolates from most livestock- or food-associated reservoirs did not show a high level of similarity in their gene profiles compared with humans from the general and clinical

populations. This indicates that livestock reservoirs, including poultry and poultry meat, are probably not major contributors to ESBL/AmpC occurrence in humans.

2.5 Antimicrobial resistance of *Enterococcus* spp.

Enterococci are commensal bacteria in the gastrointestinal tract of humans and domestic animals. Enterococci can also be found in the environment, from soil, water, plants, wild animals, birds, and insects. In humans, some enterococcal species, e.g., *E. faecalis* and *E. faecium*, can cause infections in the urinary tract and wounds, as well as bacteraemia and endocarditis (Hammerum, 2012). Thus, monitoring of enterococci, especially vancomycin-resistant enterococci (VRE) of animal origin and their resistance to antimicrobials has a great significance for public health. Special attention should be payed to VRE and streptogramin-resistant *E. faecium* strains isolated outside of hospitals (Hammerum, 2012). The role of nonhuman hosts as reservoirs of highly transmissible VRE and the consequent risk of gene transfer to human bacteria are issues still under discussion and need to be further investigated (Freitas *at al.,* 2011). There is evidence that vancomycin resistance encoding genes can be detected in food-producing animals more than a decade after the ban of glycopeptides as growth promoters (Haenni *et al.,* 2009). This indicates that resistant enterococci in the intestines may act as donors of resistance genes (Hammerum, 2012).

Enterococci are intrinsically resistant to a number of first-line antimicrobial agents, such as β-lactams and cephalosporins, and also show low-level resistance to aminoglycosides and can acquire resistance to other antimicrobial agents, including quinolones, macrolides, tetracyclines, streptogramins and glycopeptides (Iweriebor *et al.,* 2016). According to Boerlin *et al.,* 2001, the use of avoparcin and tylosin has been associated with a high level of vancomycin-resistant and erythromycin-resistant enterococci in farm animals.

In several studies, both swine and cattle enterococci isolates showed the highest resistance against tetracyclines and macrolides/lincosamides (erythromycin, lincomycin) (Jackson *et al.,* 2011; Finres-Vet, 2010-2012; DANMAP, 2015; MARAN, 2015). Resistance to other antibiotics depends on the region and use of antibiotics, but co-selecting factors

may play a role in development of resistance to different antibiotics, including glycopeptides (Haenni *et al.,* 2009).

The prevalence of resistant enterococci isolated from dogs is different in different countries. For example, it is low in Finland and Canada (Rantala *et al.,* 2004; Murphy *et al.,* 2009) but high in Portugal (Leite-Martins *et al.,* 2014).

2.6 Antimicrobial resistance of mastitis pathogens

Mastitis is an inflammation of the mammary gland caused by different pathogens entering into the udder via the teat canal. The list of mastitis pathogens contains many different bacteria, and several antibiotics are used for the treatment of mastitis. Despite the variation of antibiotics that have been used in the dairy industry for treatment and prevention of mastitis for several decades, emerging resistance to antibacterial drugs has not been found, and most mastitis pathogens are generally susceptible to antibiotics (Oliver and Murinda, 2012; de Jong *et al.,* 2018). *S. aureus* has shown increased resistance, particularly to penicillin and ampicillin, by producing β-lactamase (Persson *et al.,* 2011; Oliver and Murinda, 2012; Thomas *et al.,* 2015; de Jong *et al.,* 2018). Bengtsson *et al.,* 2009 reported that the prevalence of penicillin resistance among *S. aureus* has changed very little over 25 years. Also, coagulase-negative staphylococci (CNS) remain mostly susceptible and resistance has been developed mainly against penicillin (Persson Waller *et al.,* 2011; Oliver and Murinda, 2012; de Jong *et al.,* 2018). *Streptococcus (Str.) uberis* and *Str. dysgalactiae* strains are also susceptible to the β-lactam antibiotics, and some resistance can be detected to erythromycin and tetracycline.

Methicillin-resistant *S. aureus* (MRSA) is an important human pathogen, but it can cause infection in dairy cows as well as in other animal species. Emergence of MRSA in dairy cattle may be associated with contact with other host species of animals (e.g. pigs or humans) or bacterial host species, such as CNS, that often carry antimicrobial resistance determinants that could be transferred to coagulase-positive *S. aureus* associated with mastitis (Holmes and Zadoks, 2011).

According to broad based overviews, mastitis causing *E. coli* and *Klebsiella* spp. resistance is in most cases moderate to tetracycline, sometimes higher to lincomycin and high to cephapirin, whereas

resistance to other β-lactam antibiotics is low (Oliver and Murinda, 2012; Thomas *et al.*, 2015; de Jong *et al.*, 2018). The same authors concluded that antibiotic resistance is diminished when antibiotic use is decreased or discontinued. Although some individual bacterial strains may retain resistance genes, they are often replaced by susceptible strains when antimicrobial treatment is stopped (Butler, 2007).

3. AIMS OF THE STUDY

Aim of the study: estimate the antimicrobial resistance of *E. coli*, *Enterococcus* spp. and mastitis pathogens in Estonia.

Tasks of the study:

1. Estimate the occurrence of antimicrobial resistance of *E. coli* and *Enterococcus* spp. isolated from healthy and diseased swine in Estonia from 2006 to 2009 (I).

2. Estimate the occurrence of antimicrobial resistance of *E. coli* and *Enterococcus* spp. isolated from healthy and diseased swine and cattle in 2010-2015 and study whether antimicrobial resistance differs between swine and cattle isolates (II).

3. Estimate the occurrence of antimicrobial resistance of intestinal *E. coli* and enterococci in clinically healthy dogs in Estonia in 2012 and identify the risk factors associated with resistance (III).

4. Estimate the distribution of mastitis pathogens and their antimicrobial resistance in Estonia from 2007 to 2009 (IV).

4. MATERIALS AND METHODS

4.1 Collection of study material

4.1.1. Faecal samples and clinical submission (organ material) from swine and dairy cattle (I, II)

Faecal samples from healthy and diseased swine in 2006-2009, (I) and from healthy and diseased cattle and swine in 2010-2015 (II), were collected in the course of the annual national salmonella surveillance programme carried out in Estonia in these years. According to the number of faecal samples sent to the laboratory from one herd, one to three randomly chosen samples were cultivated for the isolation of *E. coli*, *E. faecium* and *E. faecalis* as follows: one sample was selected when the total number of samples from one farm was up to 15, two samples when the sample numbers ranged from 15-30 samples from one farm and three samples when the number of samples from one farm varied from 31-50. *E. coli* isolates from clinical material (post mortem samples, organ material) originated from diseased cattle and swine. These samples were sent to the National Veterinary and Food Laboratory (VFL; Tartu, Estonia) by veterinarians during the study years and all isolates were included in the study (Table 2).

Table 2. The number of E*scherichia coli*, *Enterococcus faecium* and *Enterococcus faecalis* isolates from swine and cattle.

	Number of isolates during study period	
Bacteria	**Years 2006-2009**	**Years 2010-2015**
E. coli from healthy swine	139	120
E. coli from diseased swine	94	143
E. coli from healthy cattle	not collected	171
E. coli from diseased cattle	not collected	63
Enterococci from swine	63	60
Enterococcus faecalis	24	20
Enterococcus faecium	39	40
Enterococci from cattle	not collected	51
Enterococcus faecalis		21
Enterococcus faecium		30

4.1.2. Faecal samples from healthy dogs (III)

Faecal samples were collected from clinically healthy dogs in Estonia. The dogs were selected randomly, with permission and interest of dog owners. The dogs were selected from those brought to veterinary clinics for vaccination or veterinary consultation. Only one (the oldest) dog from the same household was selected. The first inclusion criterion was that dogs were not treated with antimicrobials during the last three months. The data about the dogs' health, living environment and travelling history was collected from the dog owners. The information about the previous (last two years) antibiotic treatments was collected from the databases of veterinary clinics.

All dogs were examined clinically before collection of the faecal samples. Only clinically healthy dogs were included in the study: body temperature <39.0ºC, heart rate <120/min, respiratory rate <30/min, and no visible enlargement of the main lymph nodes. Five-gram faecal samples were collected immediately after defecation using a sterile spoon and collection tube. Faecal samples were placed in the refrigerator (+2…4ºC) initially and thereafter stored at -80ºC. All collected faecal samples were sent to the Estonian Veterinary and Food Laboratory for the bacteriological analysis. A total of 86 dogs (53 females and 33 males) of 39 different breeds were included in the study. One dog was excluded due to fever (39.8ºC).

4.1.3. Collection of milk samples from dairy cows for isolation of mastitis pathogens (IV)

Milk samples were submitted to the Estonian Veterinary and Food Laboratory during the period from 2007 to 2009. Quarter milk samples were collected from cows on Estonian dairy farms by local veterinarians or farmers. The samples were sent to the laboratory either for isolation of the clinical mastitis pathogen and determination of its antimicrobial susceptibility or to determine the reason for an increased somatic cell count. Clinical mastitis was diagnosed when visible abnormalities of udder (swelling) were detected or milk from a quarter had abnormal viscosity (watery, thicker than normal), colour (yellow, blood-tinged) or consistency (flakes or clots) were identified (IDF 1999).

4.2. Isolation and identification of bacterial species

4.2.1. Identification of *E. coli*, *E. faecium* and *E. faecalis* isolated from swine, cattle and dogs (I- III)

The isolation and identification of *E. coli* and enterococci were performed according to accredited methods at the VFL.
For the identification of *E. coli,* the colonies were inoculated to eosin methylene blue (EMB) agar. Based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies after incubation at 37°C overnight, *E. coli* was confirmed by biochemical tests (IMViC – indole, methyl red, Voges-Proskauer, Simmons citrate). All strains isolated from clinical submissions were serotyped using *E. coli* OK O antisera for live culture produced in rabbits and F4, F5 antisera according to the manufacturer's protocol (SSI Diagnostica A/S, Denmark).

For the isolation of enterococci, one gram of faeces was incubated at 37°C overnight in enrichment broth agar (6.5% NaCl brain heart infusion (BHI)), and ten µl of enrichment suspension was spread on Slanetz-Bartley agar and incubated for 48 h at 42°C. Up to four colonies with morphology typical of *E. faecalis*/*E. faecium* were sub-cultivated on blood agar. Colonies were identified by the following criteria: haemolysis on blood agar, aesculin hydrolysis on Edward's medium, growth in presence of tellurite and the ability to ferment mannitol, sorbitol, arabinose and raffinose. All pure isolates of *E. coli*, *E. faecium* and *E. faecalis* were stored at -80ºC for the antimicrobial susceptibility testing.

4.2.2 Identification of mastitis pathogens (IV)

Bacterial species were cultured and identified using accredited methodology based on the National Mastitis Council standards (2004) in the Estonian Veterinary and Food Laboratory. From each sample, 0.01 ml of milk was cultured on blood-esculin agar and incubated for 48 h at 37°C. The plates were examined after 24 and 48 h of incubation. A minimum of five colonies of the same type of bacterium was recorded as bacteriologically positive, and growth of more than two types of bacterial colonies was categorised as mixed growth. No bacterial growth was recorded when fewer than five colony-forming units were detected during 48 h of incubation.

4.3. Determination of *in vitro* antimicrobial susceptibility

4.3.1. Microdilution method (I, II)

In study I and II, the *in vitro* antimicrobial susceptibility of *E. coli* was determined using the microdilution method (VetMIC®, Sweden). The antimicrobial susceptibility of *E. coli* isolates was tested for ampicillin, cephotaxime, nalidixic acid, chloramphenicol, florfenicol, tetracycline, ceftiofur, gentamycin, kanamycin, streptomycin, ciprofloxacin, trimethoprim and sulphamethoxazole. In study II, resistance to colisitin was also determined.

The susceptibility of *E. faecalis* and *E. faecium* was tested for ampicillin, erythromycin, virginiamycin, gentamycin, streptomycin, kanamycin, tetracycline chloramphenicol, vancomycin, narasin, bacitracin and linezolid. Ampicillin was used as a test substance, whereas ampicillin covers both antimicrobial resistance ampicillin and amoxicillin. Phenotypic vancomycin resistance of enterococci was confirmed by microdilution. Enterococci were considered resistant when MIC was over 4mg/ml.

For the interpretation of minimum inhibitory concentration from the susceptibility testing of *Escherichia coli, E. faecalis* and *E. faecium* cut-off values available in SVARM, 2007 (I) and the Clinical and Laboratory Standards Institute (CLSI) document M100-S21, European Committee on Antimicrobial Susceptibility Testing (EUCAST) and SWEDRES-SVARM 2015 report Table 7.12 (II) were used (Table 3). In study I the *E. coli* isolate was classified as multidrug-resistant (MDR) when it was resistant to three or more antibiotics; in study II, when it was resistant to three or more of the following antimicrobials: ampicillin, tetracycline, chloramphenicol, colistin and florfenicol or to the following antimicrobial classes: trimethoprim/sulfamethoxazole, fluoroquinolones (ciprofloxacin or nalidixic acid), aminoglycosides (gentamicin, streptomycin or kanamycin), extended-spectrum cephalosporins (cephotaxime or cephtazidime). *E. faecium* or *E. faecalis* was classified as MDR if the resistance was detected to any antibiotic in three or more of the following antimicrobials/antimicrobial classes: ampicillin, tetracycline, erytromycin, vancomycin, virginiamycin, aminoglycosides (gentamicin, streptomycin or kanamycin), narasin, bacitracin and linezolid.

Table 3. Minimum inhibitory concentration (MIC) breakpoints for *E. coli* and *Enterococcus* spp., isolated from clinically healthy and diseased swine and cattle.

Antimicrobial	MIC Breakpoint (mg/l)	
	Escherichia coli	***Enterococcus faecalis, Enterococcus faecium***
Ampicillin	≥8	≥4
Ciprofloxacin	≥0.06	
Nalidixic acid	≥16	
Gentamycin	≥2	≥32
Ceftiofur	≥1	
Streptomycin	≥16	≥512[1] / ≥128[2]
Tetracyclin	≥8	≥4
Florfenicol	≥16	
Kanamycin	≥8	≥1024
Sulfamethoxasol	≥256	
Trimethoprim	≥2	
Chloramphenicol	≥16	≥32
Cefotaxim	≥0.25	
Erythromycin		≥4
Virginiamycin		≥32[1] / ≥4[2]
Vancomycin		≥4
Narasin		≥2
Bacitracin		≥32
Linesolid		≥4

[1] Epidemiologial cut-off for *Enterococcus faecalis*

[2] Epidemiological cut-off for *Enterococcus faecium*

4.3.2. Disc diffusion assay (III; IV)

In studies III and IV the *in vitro* antibacterial susceptibility was determined with the disc diffusion assay on Mueller–Hinton agar. In study III, the antimicrobial susceptibility of *E. coli* was tested with ampicillin, gentamycin, streptomycin, kanamycin, trimethoprim, sulfamethoxasole, tetracycline, nalidixic acid, ciprofloxacin, cefotaxime and ceftazidine. The antimicrobial susceptibility of enterococci was tested with ampicillin, erythromycin, gentamycin, tetracycline, chloramphenicol, vancomycin, ciprofloxacin and linezolid.

The testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) M31-A3 in 2008.

Quality control strains *E. coli* ATCC® 25922, *E. faecalis* ATCC 29212 and *E. faecium* VLA 58 were included with each batch of isolates tested. For interpretation of results for susceptibility testing of indicator bacteria (*E. coli* and enterococci), epidemiological cut-off values (ECOFF) issued by the EUCAST (http:// www.escmid.org) were used. When no ECOFFs were issued by EUCAST, the clinical breakpoints recommended for animal pathogens by CLSI (Clinical and Laboratory Standard Institute, 2008) were taken into consideration (Table 4).

In Study IV, the antimicrobial susceptibility of Gram-positive bacteria was tested with penicillin, ampicillin, cephalothin, clindamycin, erythromycin, gentamycin, trimethoprim/sulfa and tetracycline. The antimicrobial susceptibility of Gram-negative bacteria was tested with ampicillin, gentamycin, trimethoprim/sulfamethoxasole, tetracycline, enrofloxacin, streptomycin, neomycin and cefaperazone. The recommendation of the CLSI document M31-A2 in 2002 and M31-A3 in 2008 was used (Table 5). Quality control strains, *S. aureus* ATCC® 25923, *E. coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Streptococcus pneumoniae* ATCC® 49619, were included with each batch of isolates tested.

Table 4. Zone diameter (mm) interpretive criteria for *E. coli* and *Enterococcus* spp., isolated from clinically healthy dogs.

Antimicrobial,	**Zone diameter (mm)**	
concentracion in disc (µg)	***E. coli***	***Enterococcus* spp.**
Ampicillin 2		≥10
Ampicillin 10	≥14	
Erythromycin 15		≥13
Gentamycin 10	≥16	
Gentamycin 30		≥8
Kanamycin 30	≥13	
Chloramphenicol 30	≥17	≥12
Linezolid 10		≥19
Nalidixic acid 30	≥19	
Streptomycin 10	≥11	
Sulfamethoxazole 250	≥12	
Tetracycline 30	≥11	≥14
Trimethoprim 5	≥20	
Cefotaxime 10	≥23	
Ciprofloxacin 5	≥25	≥15
Vancomycin 5		≥12

Table 5. Zone diameter (mm) interpretive criteria of susceptible (S), intermediate (I) and resistant (R) mastitis pathogens.

Disc content (µg)	*Staphylococcus* spp.			*Streptococcus* spp.			*Enterococcus* spp.			*Enterobacteriaceae* spp.		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin 10	≥29	-	≤28	≥26	19-25	≤18	≥17	-	≤16	≥17	15-16	≤14
Penicillin 10	≥29	-	≤29	≥24	-	-	≥15	-	≤14	-	-	-
Cephalothin 30				≥		≤	-	-	-			
Cefaperazone 75	-	-	-	-	-	-	-	-	-	≥21	16-20	≤15
Clindamycin 2	≥21	15-20	≤14	≥19	16-18	≤15	-	-	-	-	-	-
Erythromycin 15	≥23	14-22	≤14	≥21	16-20	≤15	-	-	-	-	-	-
Gentamycin 10	≥12	13-14	≤15	≥12	13-14	≤15	≥10	7-9	≤6	≥12	13-14	≤15
Tetracycline 30	≥19	15-18	≤14	≥23	19-22	≤18	≥19	15-18	≤14	≥19	15-18	≤14
Enrofloxacin 5										≥20	15-19	≤14
Trimethoprim/sulfa 1.25/23.75	≥16	11-15	≤10	≥16	11-15	≤10	≥16	11-15	≤10	≥16	11-15	≤10

4.3.3. Detection of ESBL and AmpC producing *E. coli* (II)

For *E. coli* isolates resistant to either cefotaxime or ceftazidime, the phenotypic confirmatory test (National Veterinary Institute, Technical University of Denmark (DTU) scheme) for the production of ESBLs and AmpC was performed (CLSI M100-S21) (CLSI 2011). Genotypic confirmation of ESBL and AmpC-positive *E. coli* (n = 16) was performed in the EU Reference Laboratory for antimicrobial resistance (EURL-AR) at DTU, where the presence of genes encoding *bla*TEM, *bla*CTX and *bla*SHV were examined. Polymerase chain reaction (PCR) assay and sequence analysis was performed at DTU as described by Xia *et al.* (2011).

4.4 Statistical analysis

In study I, the proportion of *E. coli* and *Enterococcus* spp. resistant isolates with 95% confidence intervals (95% CI) in swine were calculated.

In study II, association between occurrence of antibiotic resistance (0-susceptible; 1-resistant) of *E. coli* and animal species (dairy cattle vs swine) was studied. A multiple logistic regression mixed model with random herd effect to control for clustering was used in all analyses. Year of sampling as a fixed factor was included in all models for controlling confounding effects. Due to a small number of samples, the resistance of *E. faecium* and *E. faecalis* originated from healthy animals were analysed together. Odds ratios (OR) with 95% CI were calculated. Statistical significance was assumed at $p \leq 0.05$. An association between multi-resistance (simultaneous resistance to more than three antimicrobials) of *E. coli* from clinically healthy animals or diagnostic submission, and animal species were analysed with logistic regression models with random herd effect. In addition, according to *E. coli* serotyping results, an impact of pathogenic *E. coli* (O-serotype positive vs. no confirmation of serotype) on antimicrobial resistance was analysed.

In study III, associations between resistance of *E. coli* and enterococci originated in healthy dogs and different risk factors were analysed. All estimated risk factors were categorised before statistical analysis. At first, a univariate logistic regression model was used to evaluate every single variable. All variables with $p < 0.2$ were included in the final logistic regression model. The full models included age of dog as 4-level

categorical variable (less than 1 years, 1-5 years, 5-10 years, more than 10 years); dog bodyweight as a 4-level categorical variable (less than 10 kg, 10-25 kg, 25-40 kg, 40-60 kg); living environment as a two level variable (living inside, but going out; living only outside); visit to abroad last year (yes, no); visit to veterinary clinic (yes, no). Odds ratios with 95% CI were calculated. Statistical significance was set at $p \leq 0.05$.

In study IV, the farm, herd size and year were recorded and categorised before statistical analysis. A logistic regression model with a random herd effect for the control clustering was used for all the analyses in this study. Odds ratios with 95% CI were calculated. The influence of milk samples with mixed growth or no bacterial growth on the occurrence of clinical or subclinical mastitis was assessed. Potential interactions (no growth or mixed growth x year) were assessed in the logistic regression model. The effects of herd size and year on the pathogens that caused clinical and subclinical mastitis were analysed. Statistical significance was set at $p \leq 0.05$.

Stata 10.2 (III and IV) and 11.0 (II; StataCorp, Texas, USA) was used for statistical analysis. Online calculator was used for resistant isolates proportions confidence intervals calculations. (https://www.allto.co.uk/tools/statistic-calculators/confidence-interval-for-proportions-calculator/).

5. RESULTS

5.1. Resistance profile of *E. coli* isolated from swine and cattle (I; II)

5.1.1 Resistance profile of *E. coli* isolated from healthy and diseased swine from 2006-2009 (I)

Study I shows that *E. coli* from healthy swine (n = 139) has developed resistance against several antibiotics (Table 6). No resistance was detected to florfenicol and ceftiofur. The highest resistance (proportion of resistant isolates) was developed against streptomycin (23.7%), tetracycline (15.8%), sulfamethoxazole (12.9%) and ampicillin (12.2%).

E. coli isolates from diseased swine (n = 94) showed the highest resistance to sulphamethoxazole (71.3%), trimethoprim (57.4%), tetracycline (57.4%), streptomycin (51.5%) and ampicillin (48.9%). Considerable resistance to ciprofloxacin (31.9%), nalidixic acid (31.9%) and chloramphenicol (20.2%) was also detected.

Multiresistance has been detected between 60-73% among all isolates (n = 139) during all study years. The contemporaneous resistance to ampicillin, streptomycin and trimethoprim-sulphonamides was the most common trait, occurring in 84% of the multiresistant isolates. During the study period, one ESBL producing isolate was found.

Table 6. Resistance of *E. coli* isolates originating from faecal samples of healthy and diseased swine, collected from 2006 to 2009 in Estonia (I).

Antimicrobial	Breakpoints for resistance (mg/l)	Healthy (n = 139)		**Diagnostic submission (n = 94)**	
		%	**(95% CI)**	**%**	**(95% CI)**
Ampicillin	>8	12.2	(6.7-17.6)	48.9	(40.6-57.2)
Cephotaxime	>0.5	0.7	(-0.7-2.1)	1.1	(-0.6-2.8)
Ceftiofur	>0.5	6.5	(2.4-10.5)	0.0	NA[1]
Streptomycin	>16	23.7	(16.6-30.7)	51.1	(42.8-59.4)
Gentamycin	>4	5.6	(1.8-9.4)	6.4	(2.3-10.5)
Kanamycin	>16	7.2	(2.9-11.5)	13.8	(8.1-19.5)
Ciprofloxacin	>0.06	0.7	(-0.7-2.1)	31.9	(24.1-39.6)
Nalidixic acid	>16	3.6	(0.5-6.7)	31.9	(24.1-39.6)
Tetracycline	>8	15.8	(9.7-21.8)	57.4	(49.2-65.6)
Chloramphenicol	>16	4.3	(0.9-7.7)	20.2	(0.1-4.7)
Florfenicol	>16	0.0	NA[1]	2.1	(13.5-26.9)
Trimethoprim	>2	7.9	(3.4-12.4)	57.4	(49.2-65.6)
Sulfamethoxazole	>64	12.9	(7.3-18.5)	71.3	(63.8-78.8)

[1] Not assessed (NA).

5.1.2. Resistance profile of *E. coli* isolated from dairy cattle and swine from 2010 to 2015 in Estonia (II)

5.1.2.1 Resistance profile of *E. coli* from healthy animals

Among the *E. coli* isolates from swine (n = 120), we found high occurrence of resistance to streptomycin (39.2%), tetracycline (32.5%) and sulfamethoxazole (30.0%). In clinically healthy cattle (n = 171), the most prevalent resistance was observed against aminoglycosides (7.0-8.8%) and tetracycline (7.0%) (Table 7).

The resistance of *E. coli* originated from faecal samples from clinically healthy swine compared to cattle was significantly higher to ampicillin (OR = 6.5; 95% CI 2.70-15.56; $p < 0.001$), streptomycin (OR = 8.5, 95% CI 4.27-17.03; $p < 0.001$), ciprofloxacin (OR = 10.5; 95% CI 1.27-86.76; $p = 0.029$), tetracycline (OR = 6.4; 95% CI 3.16-12.89; $p < 0.001$) colistin (OR = 5.5; 95% CI 1.7-17.3; $p = 0.004$), sulfamethoxazole (OR = 8.7; 95% CI 3.87-19.70; $p < 0.001$) and trimethoprim (OR = 8.4; 95% CI 3.33-21.04; $p < 0.001$).

5.1.2.2. Resistance profile of *E. coli* from diagnostic submissions

In the 143 *E. coli* isolates from swine, 136 originated from post-mortem organ material and seven isolates from animals with diarrhea. Among the 83 *E. coli* isolates, 15 different serotypes were determined. Serotyping did not show results among the other 60 *E. coli* isolates. The most common serotype was K88 (n = 38), followed by O138 (n = 14) and O149 (n = 12).

Out of the 63 *E. coli* isolates from dairy cattle, 18 originated from calves with signs of diarrhea, and 45 were post-mortem samples. Among the 63 *E. coli* isolates from cattle, serotypes were confirmed in 22 isolates, where the most frequent serotype was O26.

E. coli isolates from clinical submission showed the most prevalent resistance against sulfamethoxazole (68.6%), tetracycline (60.2%), streptomycin (54.6%), ampicillin (53.9%) and trimethoprim (53.9%). *E. coli* isolates from cattle clinical submissions were also mainly resistant to streptomycin (63.5%), sulfamethoxazole (60.3%), tetracycline (58.8%), ampicillin (58.7%) and trimethoprim (55.6%) (Table 7).

The resistance against gentamycin was significantly lower (OR = 0.17; 95% CI 0.06-0.47; $p < 0.001$) and resistance against nalidixic acid significantly higher (OR = 2.24; 95% CI 1.07-4.72; $p = 0.034$) in swine *E. coli* isolates compared to cattle isolates.

Table 7. Resistance of *E. coli* isolates originating from faecal samples of healthy swine and cattle and clinical submissions collected from 2010 to 2015 in Estonia (II).

Antimicrobial	Breakpoints for resistance (mg/l)[1]	Healthy animals				Diagnostic submissions			
		Dairy cattle (n = 171)		Swine (n = 120)		Dairy cattle (n = 63)		Swine (n = 143)	
		%	(95% CI)	%	(95% CI)	%	(95% CI)	%	(95% CI)
Ampicillin *H	>8	**3.5**	**(0.8-6.3)**	**21.5**	**(14.3-29.1)**	58.7	(46.5-70.9)	53.9	(45.7-62.1)
Cephotaxime	>0.5	1.2	(-0.4-2.8)	2.5	(-0.3-5.3)	7.9	(1.2-14.6)	4.2	(0.9-7.5)
Cephazidime	>0.5	2.9	(0.4-5.4)	3.3	(0.1-6.5)	7.9	(1.2-14.6)	7.7	(3.3-12.1)
Streptomycin *H	>16	**7.0**	**(3.2-10.8)**	**39.2**	**(30.5-40.8)**	63.5	(51.6-6.4)	54.6	(46.4-62.8)
Gentamycin *D	>4	7.0	(3.2-10.8)	12.5	(6.6-18.4)	**20.6**	**(10.6-30.6)**	**5.6**	**(1.8-9.4)**
Kanamycin	>16	8.8	(4.6-13.1)	10.0	(4.6-15.4)	0.0	NA[2]	0.0	NA[2]
Ciprofloxacin *H	>0.06	**0.6**	**(-0.6-1.8)**	**5.8**	**(1.6-10.0)**	38.1	(26.1-50.1)	32.2	(24.5-39.9)
Nalidixic acid *D	>16	0.6	(-0.6-1.8)	3.3	(0.1-6.5)	**17.5**	**(8.1-26.9)**	**32.2**	**(24.5-39.9)**
Tetracycline *H	>8	**7.0**	**(3.2-10.8)**	**32.5**	**(24.1-40.9)**	58.5	(46.3-70.7)	60.2	(52.2-68.3)
Colistin *H	>2	**2.4**	**(0.1-4.7)**	**11.6**	**(5.9-17.3)**	3.2	(-1.6-7.6)	5.6	(1.8-9.4)
Chloramphenicol	>16	2.4	(0.1-4.7)	5.8	(1.6-10.0)	9.5	(2.3-16.7)	18.2	(11.9-24.5)
Florfenicol	>16	0.0	NA[2]	0.8	(-0.8-2.4)	0.0	NA[2]	0.7	(-0.7-2.1)
Trimethoprim *H	>2	**3.5**	**(0.8-6.3)**	**22.4**	**(14.9-29.9)**	55.6	(43.3-67.9)	53.9	(45.7-62.1)
Sulfamethoxazole *H	>64	**4.7**	**(1.5-7.9)**	**30.0**	**(21.8-38.2)**	60.3	(48.2-70.4)	68.5	(60.1-76.1)

[1] SWEDRES/SVARM 2015. Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. Solna/Uppsala ISSN 1650-6332, 117, Table 2.17.

*H and *D Statistically significant difference ($p < 0.05$) between healthy dairy cattle and swine, and between dairy cattle's and swine's clinical submissions. Corresponding percentages are also presented in bold face.

[2] Not assessed (NA).

5.1.2.3. Multidrug-resistance of *E. coli* isolates (II)

The distribution of susceptible and multi-drug resistant *E. coli* isolates from swine and cattle is shown in Table 8. The *E. coli* isolates from clinically healthy swine (n = 35; 29.2%) showed significantly higher multidrug resistance (OR = 11.2; 95% CI 4.23-29.22; p < 0.001) than the isolates from cattle (n = 6; 3.5%). The proportion of MDR isolates from clinical submission was very high both in cattle (n = 42; 66.7%) and swine (n = 93; 65.0%), without statistical differences.

Table 8. Distribution of susceptible and multi-drug resistant *E. coli* isolates in dairy cattle and swine.

Number of antimicrobials	Clinically healthy animals		Diagnostic submissions	
	Dairy cattle (n = 171)	Swine (n = 120)	Dairy cattle (n = 63)	Swine (n = 143)
Number and proportion (%) of susceptible isolates				
Susceptible to all tested antimicrobials/antimicrobials classes[1]	135 (78.9)	40 (33.3)	12 (19.0)	19 (13.3)
Resistant to 1-2 antimicrobials/ antimicrobial classes	29 (16.9)	46 (38.3)	9 (14.3)	31 (21.7)
Number and proportion (%) of multi-drug resistant isolates				
Resistant to 3-5 antimicrobials	6 (3.5)	33 (27.5)	40 (63.5)	85 (59.4)
Resistant to 6-8 antimicrobials	0	2 (1.7)	2 (3.2)	8 (5.6)

[1] Antimicrobial classes: Quinolones (ciprofloxacin and nalidix acid); Aminoglycosides (streptomycin, kanamycin, gentamycin); 3th-4th generation cephalosporines (cephotaxime+cefazidime), sulfamethoxazole+trimethoprim.

5.1.2.4. Determination of ESBL- and AmpC-producing *E. coli* (II)

All 16 *E. coli* isolates with cefotaxime and/or ceftazidime MIC above cut-off level were analysed for confirmation of ESBL and AmpC production. ESBL phenotype was confirmed in one *E. coli* isolate from clinically healthy cattle and in eight isolates from organ materials both from cattle and swine. Three *E. coli* strains out of nine exhibiting an ESBL phenotype were found to be the same genotype $bla_{\text{TEM-52C}}$. All these strains originated from swine organ material that was collected post-mortem.

In total, four strains representing AmpC phenotypes were found. One plasmid-encoded AmpC type β-lactamases producing *E. coli* from clinically healthy cattle was found to harbour the bla_{CMY-1} gene, and another from clinically healthy swine carried the bla_{CMY-2} gene.

5.2. Resistance profile of enterococci isolated from swine and cattle (I, II)

5.2.1. Resistance profile of enterococci isolated from healthy swine from 2006 to 2009 in Estonia (I)

In study I, 63 *Enterococcus* spp. isolates from healthy swine were analysed. For both, *Enterococcus faecalis* (n=24) and *Enterococcus faecium* (n=39), resistance was most frequently detected against tetracycline (38.1%), erythromycin (38%), streptomycin (25.4%) and kanamycin (22.2%) (Table 9). Multiresistance was detected mainly against kanamycin, streptomycin and tetracycline.

Table 9. Proportion of resistance of *Enterococcus* spp. isolates originating from faecal samples of healthy swine (n = 63) in 2006-2009 in Estonia (I).

Antimicrobial	Breakpoints for resistance (mg/l)	%	(95% CI)
Ampicillin	>4	0	NA[1]
Erythromycin	>4	38.0	(15.5-37.9)
Virginiamycin			
E. faecalis	>32	3.1	(-0.5-10.5)
E. faecium	>4		
Gentamycin	>32	4.8	(-1.6-5.0)
Streptomycin			
E. faecalis	>512	25.4	(22.9-47.1)
E. faecium	>128		
Kanamycin	>1024	22.2	(1.5-37.9)
Tetracycline	>4	38.1	(27.6-52.4)
Chloramphenicol	>32	6.3	(0.8-13.3)
Vancomycin	>4	7.9	(2.4-17.6)
Narasin	>2	6.3	(-1.2-7.8)
Bacitracin	>32	6.3	(0.4-13.3)
Linezolid	>4	1.6	(-1.6-5.0)

[1] Not assessed (NA).

5.2.2. Resistance profile of enterococci isolated from healthy cattle and swine from 2010-2015 in Estonia (II)

Resistance of *E. faecalis* and *E. faecium* is presented in Table 10. Altogether, 51 isolates from healthy cattle (21 isolates of *E. faecalis* and 30 isolates of *E. faecium*) and 60 isolates from healthy swine (20 isolates of *E. faecalis* and 40 isolates of *E. faecium*) were analysed.

Enterococci from both animal species were mainly resistant to tetracycline (33.3% in cattle, 40.4% in swine) and erythromycin (21.6% in cattle, 26.7% in swine). Enterococci from swine were also resistant to streptomycin (30.0%) and kanamycin (26.7%). Enterococci isolated from swine had a significantly higher resistance against streptomycin (OR = 4.0; 95% CI 1.46-11.14; p = 0.008) and kanamycin (OR = 8.9; 95% CI 1.91-41.66; p = 0.006) compared to isolates from cattle. The proportion of fully susceptible *Enterococcus* spp. isolates was 49% (n = 25) in cattle and 35% (n = 21) in swine. Multidrug resistance was significantly higher (OR = 4.4; 95% CI 1.17-16.78; p = 0.029) in swine isolates (n = 13) than in isolates that originated from cattle (n = 3).

Table 10. Proportion of resistance of *Enterococcus* spp. isolates originating from faecal samples of healthy cattle and swine in 2010-2015 in Estonia (II).

Antimicrobial	**Break-points for resistance (mg/l)[1]**	**Dairy cattle (n = 51)**		**Swine (n = 60)**	
		%	**(95% CI)**	**%**	**(95% CI)**
Ampicillin	>4	0.0	NA[2]	1.7	(-1.6-5.0)
Erythromycin Virginiamycin	>4	21.6	(10.3-21.9)	26.7	(15.5-37.9)
E. faecalis	>32	1.9	(-1.9-5.7)	5.0	(-0.5-10.5)
E. faecium	>4				
Gentamycin Streptomycin*	>32	1.9	(-1.9-5.7)	1.7	(-1.6- 5.0)
E. faecalis	**>512**	**11.7**	**(2.9-20.5)**	**35.0**	**(22.9-47.1)**
E. faecium	>128				
Kanamycin*	**>1024**	**3.9**	**(-1.4-9.2)**	**26.7**	**(1.5-37.9)**
Tetracycline	>4	33.3	(20.4-46.2)	40.4	(27.6-52.4)
Chloramphenicol	>32	1.9	(-1.9-5.7)	6.7	(0.8-13.3)
Vancomycin	>4	5.9	(-0.63-9.4)	10.0	(2.4-17.6)
Narasin	>2	3.9	(-1.4-9.2)	3.3	(-1.2-7.8)
Bacitracin	>32	3.9	(-1.4-9.2)	6.6	(0.4-13.3)
Linezolid	>4	0.0	NA[2]	1.7	(-1.6-5.0)

[1] Swedres-Svarm 2015. Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. Solna/Uppsala ISSN 1650-6332, 117, Table 2.17.

[2] Not assessed (NA).

[*] Statistically significant difference ($p < 0.05$) between resistant *Enterococcus* spp. isolates from healthy dairy cattle and swine. Corresponding percentages are also presented in bold face.

5.3. Resistance profile of *E. coli* and *Enterococcus* spp. in healthy dogs (III)

In study III, *E. coli* was isolated in 68 of the 86 (79.1%) faecal samples, and *Enterococcus* spp. was isolated in 66 (76.7%) cases (40 isolates of *E. faecalis* and 26 isolates of *E. faecium*). Resistance to at least one antimicrobial agent was found among 10.3% (n = 7) of *E. coli* and 60.6% (n = 40) of *Enterococcus* spp. isolates. Two *E. coli* and two *Enterococcus* spp. isolates were multiresistant.

All *E. coli* isolates were susceptible to cefotaxime and ceftazidime. Three (4.4%) *E. coli* isolates were resistant to ampicillin and streptomycin, and two (2.9 %) of the isolates showed resistance against tetracycline, ciprofloxacin and sulfamethoxazole. In total, 45.5 % (n = 30) of enterococci were resistant to tetracycline, 21.2% (n = 14) to ciprofloxacin and 10.6% (n = 7) to erythromycin.

5.3.1. Risk factors of antimicrobial resistance

Sixteen of the 86 dogs (18.6%) lived only outdoors, and 70 (81.4%) lived indoors but walked outside regularly. Out of the 86 dog owners, 28% (n = 24) had visited other countries during the last year. The main regions visited were Scandinavia and western parts of Europe. During the last three years, 76.7% (n = 66) of dogs had visited veterinary clinics, and 66.7% (n = 44) of these dogs were treated with antibiotics. Health records and information on antibiotic treatment were available for 36 (87.8%) dogs. The main purposes for antimicrobial treatment were trauma and urogenital tract infections (19.4%), followed by an equal proportion (13.9%) of ear and skin infections and respiratory infection. The most frequently used antibiotics were amoxicillin in combination with clavulanic acid (83.3%) and cephalosporins (19.4%).

We did not find any significant associations between resistance of enterococci and *E. coli* and estimated risk factors, such as previous

antibiotic treatment, dog age, bodyweight, travelling and living environment.

5.4. Mastitis pathogens and their resistance to antimicrobials (IV)

5.4.1. Isolation of mastitis pathogens from milk samples submitted to the Veterinary and Food Laboratory from 2007-2009

Over the study period, 3,058 clinical mastitis samples from 190 farms and 5,146 subclinical mastitis samples from 274 farms were investigated. Mastitis pathogens were isolated from 4,680 out of 8,204 (57% of total amount) samples. The proportion of bacteriologically negative samples was 22.3%, and that of mixed growth was 20.6%. There was a significantly higher chance (OR = 1,15; 95% CI = 1.01-1.33; p = 0.042) of finding bacteriologically negative samples in the presence of subclinical mastitis (n = 1317; 25.6%) in comparison with clinical mastitis (n = 554; 16.8%). The probability of obtaining mixed growth from milk samples was also significantly higher (OR = 2.2; 95% CI=1.9-2.6; p < 0.001) if subclinical mastitis was found. Among the bacteriologically positive (n = 2016) clinical mastitis samples, *Streptococcus (Str.) uberis* was the bacterium isolated most frequently (n = 371; 18.4% of the positive samples), flollowed by *E. coli* (n = 321; 15.9%) and *Str. agalactiae* (n = 239; 11.9%). *Staphylococcus (S.) aureus* (n = 532; 20%) and CNS (n = 411; 15.4%) were the bacteriae isolated most commonly from milk in cases of subclinical mastitis, followed by *Corynebacterium* spp. (n = 395; 14.8%).

5.4.2. Antimicrobial resistance of clinical mastitis pathogens

The percentage of *S. aureus* isolates resistant to penicillin and ampicillin was 61.4% and 59.5% respectively. In addition, CNS showed resistance to penicillin and ampicillin (38.5% and 34.4%, respectively), while resistance to erythromycin and lincomycin was also common (14.9% and 17.6%, respectively). Six isolates (3.8%) of *S. aureus* and three isolates (3.6%) of CNS were resistant to cephalothin. All streptococci (Table 11) were susceptible to penicillin, ampicillin and cephalothin, except for one isolate of *Str. uberis*. Of the 90 isolates of *Str. dysgalactiae*, 32.2% were classified as resistant to tetracycline. Of the 151 isolates of *Str. uberis,* 14.3% of isolates were resistant to tetracycline.

Table 11. Propotion of resistant isolates of staphylococci and streptococci isolated from bovine clinical mastitis cases (IV).

Disc content (µg)	*Saphylococcus aureus*		CNS[1]		*Streptococcus agalactiae*		*Streptococcus dysgalactiae*		*Streptococcus uberis*	
	n	R[2] (%)	**n**	**R (%)**	**n**	**R (%)**	**n**	**R (%)**	**n**	**R (%)**
Ampicillin 10	173	59.5	91	38.5	162	0	111	0	265	0.4
Penicillin 10	174	61.4	93	34.4	168	0	111	0	267	0.4
Cephalothin 30	160	3.8	84	3.6	143	0	101	0	254	0.4
Clindamycin 2	169	18.1	91	17.6	161	6.2	115	7.8	273	6.6
Erythromycin 15	83	4.8	47	14.9	77	1.3	60	6.7	134	8.2
Tetracycline 30	147	4.1	86	11.6	151	14.6	90	32.2	234	19.7
Trimethoprim/sulfa 1.25/23.75	162	3.4	76	2.6	140	6.4	103	1	223	3.2
Gentamycin 10	146	6.8	69	1.4	143	24.5	88	11.4	210	18.6

[1] Coagulase negative staphylococci (CNS).

[2] Proportion of resistant (R) isolates.

Table 12. Propotion of resistant isolates of *Escherichia coli* and *Klebsiella* spp. isolated from bovine clinical mastitis cases (IV).

Disc content (µg)	*E. coli*				*Klebsiella* spp.			
	n	S[1] (%)	I (%)	R (%)	n	S (%)	I (%)	R (%)
Ampicillin 10	201	68.7	7.0	24.3	39	15.4	7.7	76.9
Cefaperazone 75	137	100	0	0	32	100	0	0
Tetracycline 30	184	77.8	8.7	13.5	39	79.6	10.2	10.2
Trimethoprim/ sulfa 1.25/23.75	191	84.3	3.7	12.0	40	97.5	0	2.5
Gentamycin 10	161	94.3	2.5	2.2	40	95.0	0	5.0
Streptomycin 30	154	78.6	5.8	15.6	37	73.0	8.1	18.9
Neomycin 30	155	72.9	20.6	6.5	37	83.8	13.5	2.7
Enrofloxacin 5	185	98.4	0	1.6	37	100	0	0

[1] Propotion of susceptible (S), intermediate (I) and resistant (R) isolates.

Among the *E. coli* isolates (Table 12), the highest percentage showing intermediate susceptibility and resistance was observed for ampicillin, neomycin, streptomycin and tetracycline. *E. coli* was 98.4% susceptible to enrofloxacin and 100% susceptible to cefaperazone.

6. DISCUSSION

6.1. Resistance of *E. coli* and enterococci in dairy cattle and swine

6.1.1 Antimicrobial resistance of *E. coli* and enterococci isolated from swine from 2006-2009 (I)

Study I shows that *E. coli* and enterococci isolated from healthy swine have developed resistance against several antibiotics. No resistance was detected to ceftiofur and florfenicol. The highest resistance can be detected against streptomycin, tetracycline, sulfametoxazole and ampicillin. During the study years, resistance to steptomycin and tetracycline decreased slightly. For *E. faecalis* and E. *faecium,* resistance was most frequently detected against erythromycin, tetracycline, streptomycin and kanamycin. Multiresistance occurred mainly against kanamycin, streptomycin and tetracycline.

The resistance level of enteric microflora in healthy swine was higher in Estonia compared to that in Sweden and Norway (NORM/NORM-VET, 2003; SVARM, 2009), but at a similar level with Denmark (DANMAP, 2009) and the Netherlands (MARAN, 2008). Both *E. coli* and enterococci showed highest resistance to tetracycline which can be explained with the wide use of doxycycline for oral treatment of pigs. Also, tylosin and sulfonamides with trimetoprim are commonly used in Estonia which explains the high level of macrolide and sulfa/trimetoprim resistance in normal enteric microflora. As enterococci are intrinsically resistant to many antimicrobial agents (Huycke *et al.,* 1998; Garrido *et al.,* 2014), antimicrobials used for treatment of enterococcal infection are limited. Although food-producing animals are rarely sources of enterococcal infection in humans (Higuita and Huycke, 2014), antimicrobial-resistant strains of animal origins may cause transmission of their resistance genes from animal to human bacteria. Therefore, prevalence of antimicrobial resistant enterococci, including vancomycin- resistant enterococci (VRE) in food-producing animals, has become a serious problem in several countries (Garrido *et al.,* 2014).

Higher resistance was shown in bacteria from diagnostic submissions compared to bacteria originating from healthy animals sampled at the farm level. For instance, *E. coli* from pathological material is more resistant than isolates from healthy animals. However, there is a high probability of bias toward animals with recurrent infections, previously treated with antimicrobials that could explain the high levels of resistance. On the other hand, the number of isolates of animal pathogens are quite low for making generalisations. Veterinarians do not often send samples to the laboratory for isolation and identification of bacteria. According to VFL annual reports, every year only 70-80 samples from diseased swine were sent to microbiological analysis. Therefore, we can say that antibacterial treatment is often initiated without bacterial diagnosis, which can lead to multidrug resistance.

6.1.2. Antimicrobial resistance of *E. coli* and enterococci isolated from swine and cattle in 2010-2015 (II)

This study is the latest broad-based overview of antimicrobial resistance of these animal pathogens in Estonia. Currently, there is an extensive movement of live animals and food of animal origin between countries and continents. Regarding the possible transfer of resistant microbes, overview of the situation in each region cannot be underestimated (European Medicines Agency, 2017).

The proportion of resistant *E. coli* isolates and MDR *E. coli* isolates originating from healthy swine was higher than that of *E. coli* isolates that originated from healthy cattle. The resistance against tetracycline, ampicillin, streptomycin, sulfamethoxazole, trimethoprim, ciprofloxacin and colistin differed significantly. Monitoring programmes in Finland, the Netherlands and Denmark have also described higher resistance among the swine isolates (FINRES-Vet, 2010-2012; MARAN, 2015; DANMAP, 2015). The overall higher resistance of swine isolates is stated also in the European Union Summary Report on AMR (EFSA 2019).

Isolates originating from swine were more resistant to mainly orally administered antibiotics. For instance, doxycycline, ampicillin/amoxicillin and sulpha/trimethoprim have been used for the treatment of swine diseases in a large volume and over a long time period in Estonia (European Medicines Agency, 2017; Estonian State Agency of

Medicines, unpublished data). From 2012-2016, the average amount of tetracycline used for the treatment of swine was approximately 1,500 kg of pure active substance per year, and approximately 210 kg/year for the treatment of cattle (Estonian State Agency of Medicines, unpublished data). The same figures for ampicillin/amoxicillin were 2,200/500 and for sulpha/trimethoprim 110/50, respectively. In Estonia, tetracyclines (including doxycycline), ampicillin/amoxicillin and sulpha/trimethoprim are authorised for oral treatment in swine and poultry, but not in cattle (Estonian State Agency of Medicines, unpublished data). Considering this we can say that in Estonia, there might be a link between the use of antibiotics and the level of resistance, and enteric bacteria in swine are more often exposed to antibiotics than those in cattle. There is a higher probability for commensal *E. coli* to become a reservoir of resistance when oral antibiotics are widely used in the swine farms. Several authors have confirmed that oral administration of antibiotics to swine increases the level of antimicrobial resistance (Burow *et al.*, 2014; Hanon *et al.*, 2015) and there is a strong correlation between the use of antimicrobials and the extent of antimicrobial resistance in *E. coli* isolated from livestock (Chantziaras *et al.*, 2014; Gibbons *et al.*, 2014). This could explain the high resistance of commensal *E. coli* strains isolated from healthy swine in our study, as oral antibiotics are not commonly used for the treatment of cattle in Estonia.

We found high resistance to ciprofloxacin and nalidixic acid in bacteria originating from diseased animals in both animal species. It is an alarming issue as the European Union Summary Report on AMR (EFSA 2019) stated that the resistance to ciprofloxacin was moderate and the resistance to nalidixic acid was low at the EU level. Enrofloxacin and other quinolones are still used quite extensively for the treatment of swine and cattle in Estonia (amounts of active ingredients 85/55 kg per year respectively (Estonian State Agency of Medicines, unpublished data). This is not in line with the local rules of prudent use of antimicrobials (Aasmäe and Kalmus, 2012). That could explain the high resistance to quinolones as there can be a link between the presence of antibiotics in the body and the number of resistant bacteria (Nguyen *et al.*, 2012). Monitoring of resistance to fluoroquinolones should be continued in future studies as well as resistance to virginiamycin and chloramphenicol – compounds which are not used in veterinary practice in Estonia and which resistance can be associated with the use of tetracyclines at low concentrations (Mirzaagha *et al.*, 2011).

We found considerable phenotypic resistance to colistin in *E. coli* isolates from healthy swine, whilst the Summary Report asserts that at the EU level the colistin resistance both in swine and cattle is low (EFSA 2019). We did not investigate colistin genotypic resistance in this study. However, future studies should focuse on colistin resistance of swine *E. coli*, as a plasmid carrying the colistin resistance gene *mcr*-1 was isolated from a pig slurry sample in Estonia (Brauer *et al.*, 2016). Some enterococci isolated from healthy swine showed vanomycin resistance, but confirmatory tests were negative. In study II, we did not analyse the difference in the resistance of *E. coli* isolates from healthy animals and diagnostic submissions because the origin and collection of that kind of material is different, and comparison may lead to biased conclusion, although higher number of resistant isolates among the clinical submissions were observed, which is in line with the results of other authors (MARAN, 2015; DANMAP, 2015). Isolates from clinical submission can be more frequently resistant than isolates from healthy animals because of the more frequent exposure to antimicrobials, and in veterinary practice we need to keep in mind that the use of antimicrobial agents may select bacteria carrying virulence genes (Boerlin *et al.*, 2005).

When comparing resistance data of *E. coli* and enterococci isolated from healthy swine during different time periods (I and II), a continuous increase in resistance against ampicillin, streptomycin, tetracycline and sulfamethoxazole can be identified. At the EU level, the resistance to tetracycline in swine and cattle indicator *E. coli* is the most common trait and overall resistance to sulfamethoxazole, ampicillin and trimethoprim is high in both animal species (EFSA 2019). It indicates that because of long-term use of antimicrobials, susceptible organisms will be eliminated, while resistant populations remain and become predominant (Levy and Marshall, 2004). Data about overall sales of antimicrobials in Estonia (Estonian State Agency of Medicines, unpublished data) confirm that there has been no decrease in the use of veterinary antimicrobials during the last decade. According to the ESVAC report 2017, in Estonia, overall sales of antimicrobials for food-producing species, in mg/PCU among 30 European countries is rather low, but sales numbers for fluoroquinolones, polymyxins, pleuromutilins and 3rd and 4th generation cephalosporins is relatively high. In Estonia, the experience of other countries in decreasing the use of antimicrobials and regulating the use of different classes of antimicrobials should be considered, as it may be rapidly reflected in marked decline in resistance levels (Levy, 2014).

For example, in the Netherlands, several compulsory and voluntary actions on the government and farm levels resulted in reduction of use of antimicrobials up to 56% in farm animals between 2007 and 2012 (Speksnijder *et al.,* 2014). Also, in Demnark, after change of antibiotic policy, antibiotic use per kilogram of swine raised in Denmark dropped by more than 50% from 1992 to 2008, whereby overall productivity increased in the swine industry (Levy, 2014).

In Estonia, a central database for the collection of reports on the use of antibiotics in animals should be created, followed by the establishment of strict regulations regarding the use of antimicrobial classes (e.g., restricted use of fluoroquinolones and cephalosporins in farm animals). The joint visit report of the Euroeapean Centre for Disease Prevention and Control and the Directorate General for Health and Food Safety of the European Commission (2019) also concludes that there are no benchmarking systems concerning prescription and use of antimicrobials and their use by veterinarians at farm level. The introduction of such benchmarking systems would require obtaining data on the use of antimicrobials by veterinarians at farm and clinic levels.

Resistance of enterococci, as well as development of multidrug resistance was lower in cattle isolates compared to swine isolates, which has also been reported in other investigations (FINRES-Vet 2010-2012, 2015; MARAN, 2015; DANMAP, 2015).

This was the first time in Estonia when the ESBL-producing *E. coli* harbouring the $bla_{\text{TEM-52C}}$ genotype was found in swine post-mortem tissue samples. TEM-52 and CTX-M are often the most dominant types of enzymes in swine isolates in other countries (Geser *et al.,* 2012, Rodrigues *et al.,* 2013; Brolund, 2014). Several studies (Paterson and Bonomo, 2005; Jacoby, 2009; Sunde *et al.,* 2009; Schmid *et al.,* 2013) have reported that strains producing AmpC and ESBL are often resistant to multiple agents. As faecal carriage of plasmid-mediated AmpC β-lactamases was found in healthy swine and cattle, the possible development and transmission methods of antimicrobial resistance in cattle and swine must be investigated in future studies.

6.2. Antimicrobial resistance of *E. coli* and enterococci isolated from dogs (III)

In this study, the antimicrobial resistance of normal enteric microflora in clinically healthy dogs was investigated. The number of microbial isolates is quite small, and the results of that investigation do not represent the antimicrobial resistance situation in the Estonian dog population, but it gives a preliminary standpoint for future discussion and investigations. The antimicrobial resistance of *E. coli* was generally low in our study, but resistance among enterococci was prevalent. No cefotaxime-resistant *E. coli* or VRE were found. The prevalence of cephalosporin-resistant *E. coli* varies between countries: 12% in Canada (Murphy *et al.*, 2009), 6% in the USA (Shaheen *et al.*, 2011), 5% in Finland (Jalava *et al.*, 2012), and 40.9% in Croatia (Šeol *et al.*, 2011). Other published studies reported that the prevalence of VRE in dogs in Spain was 17% and 26% in the Netherlands (Herrero *et al.*, 2004; Van Belkum *et al.*, 1996). Previous scientific publication also confirmed that dogs with a history of antimicrobial therapy in the past year had a higher risk of being carriers of ESBL producing and plasmidic AmpC betalactamase-producing *E. coli* (Belas *et al.*, 2014).

Although we did not find ESBL or VRE in this study, great attention should be paid to these pathogens in future resistance monitoring.

We did not find an association between previous antibiotic treatment and antimicrobial resistance of enterococci. That is in line with the study done by Rantala *et al.* (2004). Another study demonstrated that the resistance to beta-lactams was more common in faecal *E. coli* strains isolated from cefovecin-treated dogs compared to untreated dogs, but the resistance of enterococci was not altered (Lawrence *et al.*, 2013). The retrospective data in our study showed that antibiotics were primarily prescribed after clinical diagnosis, but data on bacteriological investigations were missing.

The resistance of *E. coli* against tetracycline was low in our study, but we found high resistance among the enterococci isolated from dogs that were not treated with tetracycline. Several studies have shown a high tetracycline resistance of *E. coli* isolated from the intestines of healthy dogs (Leener *et al.*, 2005; Costa *et al.*, 2008; Damborg *et al.*, 2008; Türkyilmaz *et al.*, 2010), but an association between tetracycline

treatment in dogs and the development of resistance was not found (Damborg *et al.,* 2008). One possible route of distribution of resistance is food contaminated with resistant bacteria or distribution via the environment (Wu *et al.,* 2013). Possible links between tetracycline-resistant environmental bacteria and resistance of normal enteric microflora of dogs should be studied in the future.

Dog age, bodyweight and living environment was not a significant risk factor for resistance of enterococci ($p > 0.05$). Rantala *et al.* (2004) also found no significant association between dog age and the development of resistance. The potential threat posed by animals or animal food products as sources for resistant isolates cannot be ignored, but the current research has not identified the extent to which livestock and pets contribute to the spread of resistance in human microflora.

6.3. Antimicrobial resistance of mastitis pathogens (IV)

In this study, the disc diffusion method for *in vitro* antimicrobial susceptibility testing was used to determine antimicrobial resistance of clinical mastitis pathogens. This technique is the most widely used method for determination of the susceptibility of animal pathogens, especially in clinical work, when it is necessary to determine the correct treatment. The primary disadvantage of using this method when monitoring development of resistance is that outcomes are reported on a qualitative basis (sensitive, intermediate or resistant), and subtle changes in susceptibility may not be apparent. Therefore, any comparison with studies that use other methods of susceptibility testing is not acceptable (Schwarz *et al.,* 2009). Generally, in our study, the *in vitro* antimicrobial resistance of the isolates examined from samples of clinical mastitis was high. Isolates of *S. aureus* had an alarming level (more than half of the isolates resistant) of resistance to penicillin (61.4%) and ampicillin (59.5%), whereas CNS exhibited a lower degree of resistance (38.5% and 34.4%, respectively). The reported percentages for penicillin-resistant *S. aureus* in cases of clinical mastitis, detected by the disc diffusion method, were 50.4% and 35.4% in the two US studies (Erskine *et al.,* 2003; Makovec and Ruegg, 2003), 63.3% in Turkey (Güler *et al.,* 2005) and 12% in Northern Germany (Schröder *et al.,* 2005). In addition, cephalotin resistance among staphylococci was found in our study.

During the time of our study, there was little published information on methicillin-resistant staphylococci causing bovine mastitis. Nowadays there are reports confirming that methicillin-resistant staphylococci are often isolated from clinical mastitis samples and can be a threat to human health when transmitted via milk (Hata, 2016; Gopal and Divya, 2017).

In this study, both staphylococci and streptococci showed resistance to erythromycin and lincomycin, but the figures for resistance in annual reports from some other countries show a low prevalence of lincomycin and erythromycin resistance in *S. aureus* and CNS (NORM/NORMVET, 2003; SVARM, 2004; MARAN, 2008; SWEDRES/SVARM, 2017). Given that *S. aureus* and CNS were the pathogens isolated most frequently from cases of subclinical mastitis, one possible explanation for resistance to several antibiotics may be the collection and submission to the laboratory of milk samples from chronic clinical mastitis (which demonstrate poor treatment efficacy). Therefore, random sampling strategies should be used to provide a good evaluation of antimicrobial susceptibility.

The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin. Coliforms are often resistant to more than one antimicrobial (Lehtolainen *et al.,* 2002; Bengtsson *et al.,* 2009; Saini, 2012), and the number of multiresistant strains may influence the resistance figures. Coliform bacteria isolated from cases of mastitis may reflect the general situation of resistance in the herd and can be considered more as an indicator of the bacteria present than an indicator of specific pathogens from the udder (Lehtolainen *et al.,* 2002). All the bacterial species investigated in this study showed resistance to tetracycline. A possible explanation for this phenomenon could be the fact that tetracycline has been the class of antimicrobials most widely used for treatment of several infections for many years. Furthermore, tetracycline has been found in multiresistant patterns with penicillin and streptomycin (Lehtolainen *et al.,* 2002; Güler *et al.,* 2005).

7. CONCLUSIONS

- From 2006-2009, the highest resistance of *E. coli* isolates from healthy swine was detected against streptomycin, tetracycline, sulfamethoxazole and ampicillin. *E. coli* isolates from diseased swine showed the highest resistance to sulphamethoxazole, trimethoprim, tetracycline, streptomycin and ampicillin. Considerable resistance was also detected to ciprofloxacin, nalidixic acid and chloramphenicol.

- From 2010-2015, among the *E. coli* isolates from healthy swine, we found a high occurrence of resistance to streptomycin, tetracycline and sulfamethoxazole. In clinically healthy cattle, the most prevalent resistance was observed against aminoglycosides and tetracycline. *E. coli* isolates from clinical submission from swine showed the most prevalent resistance against sulfamethoxazole, tetracycline, streptomycin, trimethoprim and ampicillin. *E. coli* isolates from cattle clinical submissions were also mainly resistant to streptomycin, sulfamethoxazole, tetracycline, ampicillin and trimethoprim.

- The number of MDR *E. coli* isolates was significantly higher in clinically healthy swine compared to that in cattle.

- The antimicrobial resistance of *E. faecalis* and *E. faecium* to erythromycin and tetracycline was high in both animal species, and in swine enterococci it was also high to streptomycin and kanamycin.

- The prevalence of acquired antimicrobial resistance both in commensal bacteria of the enteric microflora of healthy animals (swine and cattle) and animal pathogens indirectly indicates the magnitude of the selective pressure from the use of antimicrobials in animal populations.

- Strains of *E. coli* and enterococci as a part of the normal enteric microflora of dogs did show different resistance to antibiotics, but the association between antimicrobial resistance and suspected risk factors was not found.

- The *in vitro* antimicrobial resistance of the isolates examined from the samples of clinical mastitis was high. Isolates of *S. aureus* had an

alarming level (more than half of the isolates resistant) of resistance to penicillin and ampicillin, whereas CNS exhibited a lower degree of resistance. The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin.

- This broad-based overview of antimicrobial resistance of these animal bacteria creates a basis for the future investigations and analyses of the resistance development in Estonia. In light of this, we strongly recommend assessment of the treatment plans in the swine industry in Estonia in order to ensure the prudent use of antimicrobials and to minimise the potential spread of resistant bacteria from swine to the environment and to humans.

- The amounts of antimicrobials used in animals in Estonia should be reduced. Appropriate guidelines for antibiotic usage were first published in 2012, while completed guidelines for antimicrobial treatment of different animal species were published and implemented in 2018. A system for effective control of the use of antibiotics in veterinary medicine in Estonia is still needed. In Estonia, a central database for the collection of reports on the use of antibiotics in animals should be created, followed by the establishment of strict regulations regarding the use of antimicrobial classes (e.g., restricted use of fluoroquinolones and cephalosporins in farm animals).

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9. SUMMARY IN ESTONIAN

Eestis aastatel 2006–2015 sigadelt, veistelt ja koertelt isoleeritud *Escherichia coli* ja *Enterococcus*'e perekonna mikroobide ning lehmadelt isoleeritud mastiidipatogeenide antibiootikumiresistentsus

Sissejuhatus

Mikroobide antibiootikumiresistentsus on olnud nii humaan- kui ka veterinaarmeditsiinis suur ja kiiresti kasvav probleem viimased paarkümmend aastat. Juba 2009. aastal avaldas Haiguste Ennetamise ja Tõrje Euroopa Keskus (European Centre for Disease Prevention and Control), et ainuüksi Euroopas sureb mikroobide resistentsuse tõttu igal aastal 25 000 inimest ning infektsioonide raviks kulub umbes 2,5 miljonit lisaravipäeva. Teadlaste hinnangul võib probleem paisuda 2050. aastaks nii suureks, et mikroobide resistentsuse tõttu ravimatuks muutunud haigustesse sureb 300 miljonit inimest ja kogu maailma mastaabis jääb tootlikkus prognoositavast 2–3,5% väiksemaks. Tõendatud on resistentsete mikroobide levik loomalt inimesele ja inimeselt loomale ning resistentsusinfot kandvate mikroobidelt pärinevate geenide levik ühtedelt mikroobidelt teistele, samuti toidu, joogivee ning loomasööda vahendusel. Kindlasti tuleb järjepidevalt jälgida nii toiduloomadelt kui ka lemmikloomadelt isoleeritud bakterite resistentsust, sest lemmikloomad puutuvad inimesega tihedalt kokku ja viibivad samas keskkonnas.

Mikroobide resistentsust antibiootikumide suhtes tuleb käsitleda nii veterinaar- kui ka inimmeditsiini siduva ühtse probleemina ning resistentsuse kujunemist saab vähendada ainult ühiste jõupingutustega. Kogu maailmas, sealhulgas Euroopa Liidus, on vastu võetud mitmeid dokumente ja juhendeid, mis käsitlevad mikroobide resistentsuse pidevat seiret ning selle tähtsust. Juulis 2017 võttis Euroopa Komisjon vastu Euroopa Liidu algatuse „Üks tervis" (One Health) tegevuskava, mis summeerib olemasolevad ning tulevikus plaanitavad tegevused resistentsuse vähendamiseks. Hetkeolukorra kindlakstegemine eri riikides võimaldab välja töötada konkreetsed meetmed, mida järgides on võimalik antibiootikumide kasutamist optimeerida.

Kirjanduse ülevaade

Bakteriaalsed nakkushaigused on levinud nii toidu- kui ka lemmikloomadel ning enamikul juhtudel vajavad ravi antibiootikumidega. Paratamatult käib antibiootikumraviga kaasas ka resistentsete mikroobide kujunemine. Erinevalt inimmeditsiinist kasutatakse loomadel sageli rühmaravi. Loomade ja loomarühmade ravimisel, eriti kui kasutatakse inimmeditsiinis kriitilise tähtsusega antibiootikume, näiteks kinoloone ning kolmanda ja neljanda põlvkonna tsefalosporiine, on oht, et loomadel kujundatakse inimesele eriti ohtlike resistentsete bakterite reservuaar.

Arvatakse, et mikroobide resistentsust kandvad geenid ja resistentsuse ülekandemehhanismid on eksisteerinud ka enne seda, kui antibiootikumid kasutusele võeti. Resistentsus on mikroobide loomulik kaitsevõime neid ohustavate ainete vastu ehk ellujäämiseks vajalik mehhanism. Antibiootikumi suhtes tundlikud bakterid hävivad, väike resistentne populatsioon jääb alles ja paljuneb. Ühe antibiootikumiga kokkupuutel võib mikroobidel resistentsus kujuneda ka teiste antibiootikumide suhtes.

Mikroobide resistentseks muutumisele võivad kaasa aidata biotsiidid ja desinfitseerimisvahendid. Kui kokkupuude antibiootikumiga väheneb või puudub, võivad mikroobid taas tundlikuks muutuda. Mõnede kirjandusallikate andmetel säilib resistentsus pikka aega ka pärast antibiootikumide kasutamise lõpetamist. Arvestades antibiootikumi-resistentsuse suurenemist kogu maailmas, on tõenäoline, et uusi mikroobivastaseid toimeaineid kasutatakse ainult inimmeditsiinis, et vältida uute toimeainete suhtes resistentsete bakterite kujunemist loomadel ning võimalikku levikut inimestele. Maailma Terviseorganisatsioon on juba viimased kümme aastat soovitanud fluorokinoloone, kolmanda ja neljanda põlvkonna tsefalosporiine ning makroliide kasutada ainult inimmeditsiinis ja vältida nende ainete manustamist loomadele.

Kuivõrd Eesti kuulub antibiootikumide kasutamise poolest pigem Põhjamaade koolkonda, on veterinaarmeditsiinis kõige sagedamini kasutatav toimeainerühm penitsilliinid. Samas kasutatakse Eestis mõnesid inimmeditsiinis tähtsaid antibiootikume veterinaarseks otstarbeks liiga palju, näiteks kolmanda ja neljanda põlvkonna tsefalosporiinide kasutamises on Eesti Euroopas esikohal, liiga palju kasutatakse ka kinoloone. Positiivse suundumusena võib siiski välja

tuua aminoglükosiidide, linkosamiidide ja makroliidide kasutamise järkjärgulist vähenemist.

Väga tähtis komponent mikroobide resistentsuse vähendamisel on bakterite resistentsuse iga-aastane seire, sealhulgas indikaatorbakterite *Escherichia (E.) coli* ja enterokokkide resistentsuse pidev uurimine. Eestis on resistentsuse jälgimine tagatud nende mikroobide osas, mille seire on nõutud Euroopa Komisjoni rakendusotsusega 2013/652/EL. Eestis puudub iga-aastane kohalik seireprogramm, järjepidevalt ei uurita haigetelt loomadelt pärinevaid baktereid ega lemmikloomade indikaatorbaktereid.

Et resistentsed mikroobid võivad loomadelt inimesele üle kanduda ka loomse toidu vahendusel, on tähtis pidevalt uurida indikaatorbakterite resistentsuse taset toiduloomadel. Üheks suuremaks ohuks peetakse enteropatogeense *E. coli* (EPEC) võimalikku levikut loomadelt inimesele, mõnede uuringute andmetel on just veised potentsiaalne reservuaar.

Nii loomadele kui ka inimestele on ohtlikud laiendatud spektriga beetalaktamaase tootvad (*extended spectrum beta-lactamases producing*, ESBL) *E. coli* tüved. Need bakterid on resistentsed penitsilliinide, esimese, teise ja kolmanda põlvkonna tsefalosporiinide ning astreonaami suhtes. Lisaks võivad ESBL-i tootvad bakterid olla resistentsed ka aminoglükosiidide, trimetoprimi, sulfoonamiidide, tetratsükliini, fluorokinoloonide ja klooramfenikooli suhtes. ESBL-i tootvate bakterite olemasolu toiduloomadel on potentsiaalne oht inimese tervisele, sest bakterid võivad inimese organismi sattuda toidu vahendusel.

Resistentsuse seire programmid keskenduvad eri riikides enamasti toiduloomade bakterite uurimisele, kuid kindlasti on tähtis ka lemmikloomade mikroobide resistentsuse uurimine, sest lemmikloomad puutuvad inimestega, eriti lastega, vahetult kokku ja mikroobide ülekandumine loomalt inimesele on väga tõenäoline.

Enterokokid on nii loomade kui ka inimeste normaalse mikrofloora osa, kuid mõned enterokokid võivad inimestel põhjustada raskeid nakkushaigusi. Väga tähtis on jälgida loomadelt isoleeritud enterokokkide kui indikaatorbakterite resistentsust ning selgitada välja vankomütsiiniresistentsete enterokokkide olemasolu ja resistentsuse trend, sest nende ülekandumine inimesele on tõenäoline.

Tähtis on ka lüpsilehmade mastiiti tekitavate mikroobide resistentsuse uurimine, kuigi nende patogeenide puhul ei ole mitme aastakümne jooksul resistentsuse drastilist suurenemist täheldatud. Erilist tähelepanu tuleb pöörata metitsilliiniresistentse *Staphylococcus (S.) aureus*'e uurimisele, sest see mikroob võib põhjustada ravile raskesti alluvaid infektsioone peale veiste ka teistel loomaliikidel ja inimesel.

Uuringu eesmärgid

Uuringu peaeesmärk: hinnata *E. coli*, *Enterococcus* spp. ja mastiidipatogeenide antibiootikumiresistentsust Eestis.

Tööülesanded:

1. Hinnata tervetelt ja haigetelt sigadelt isoleeritud *E. coli* ja enterokokkide antibiootikumiresistentsust Eestis aastatel 2006–2009 (I).

2. Hinnata tervetelt ja haigetelt sigadelt ning veistelt isoleeritud *E. coli* ja enterokokkide resistentsust aastatel 2010–2015, uurida, kas eri loomaliikidelt isoleeritud bakterite resistentsus on erinev (II).

3. Hinnata kliiniliselt tervetelt koertelt Eestis aastal 2012 isoleeritud *E. coli* ja enterokokkide antibiootikumiresistentsust ning selgitada välja selle kujunemist mõjutavad riskitegurid (III).

4. Hinnata mastiidipatogeenide jaotust ja antibiootikumiresistentsust Eestis aastatel 2007–2009 (IV).

Materjal ja metoodika

Kliiniliselt tervetelt ja haigetelt sigadelt aastatel 2006–2009 (I) ning nii tervetelt kui ka haigetelt veistelt ja sigadelt aastatel 2010–2015 (II) koguti roojaproovid riikliku salmonellaseire programmi käigus. *E. coli*, *Enterococcus (E.) faecium*'i ja *Eenterococcus (E.) faecalis*'e isoleerimiseks tervete sigade proovidest võeti uuringusse 1–3 juhuslikult valitud proovi igast farmist. *E. coli* isoleerimiseks haigete sigade ja veiste roojaproovidest võeti uuringusse kõik loomaarstide poolt uuringuperioodi jooksul veterinaar- ja toidulaboratooriumisse toodud proovid.

Uuringus III koguti roojaproovid loomakliinikusse vaktsineerimiseks või konsultatsiooniks toodud tervetelt koertelt. Koerad valiti uuringusse loomaomaniku nõusolekul, üheks valikukriteeriumiks oli antibiootikumravi puudumine viimase kolme kuu jooksul.

Mastiidipatogeenide isoleerimiseks võeti uuringusse kõik uuringuperioodi jooksul veterinaar- ja toidulaboratooriumisse toodud udaraveerandi piimaproovid.

E. coli, *E. faecalis*'e ja *E. faecium*'i isoleerimine ning tuvastamine toimus Tartu veterinaar- ja toidulaboratooriumis vastavate akrediteeritud meetodite kohaselt. Mikroobide antibiootikumitundlikkus määrati uuringutes I ja II mikrodilutsiooni meetodiga ning uuringutes III ja IV diskdifusiooni meetodiga. Uuringus I ja II hinnati *E. coli* antibiootikumitundlikkust ampitsilliini, tsefotaksiimi, nalidiksiinhappe, klooramfenikooli, floorfenikooli, tetratsükliini, tseftiofuri, gentamütsiini, kanamütsiini, streptomütsiini, tsiprofloksatsiini, trimetoprimi ja sulfametoksasooli suhtes. Uuringus II hinnati *E. coli* antibiootikumitundlikkust ka kolistiini suhtes. *E. faecalis*'e ja *E. faecium*'i antibiootikumitundlikkust hinnati ampitsilliini, erütromütsiini, virginiamütsiini, gentamütsiini, streptomütsiini, kanamütsiini, tetratsükliini, klooramfenikooli, vankomütsiini, narasiini, batsitratsiini ja linesoliidi suhtes.

Uuringutes III ja IV hinnati mikroobide antibiootikumitundlikkust diskdifusiooniga Mueller-Hintoni agaril. Uuringus III hinnati *E. coli* antibiootikumitundlikkust ampitsilliini, gentamütsiini, streptomütsiini, kanamütsiini, trimetoprimi, sulfametoksasooli, tetratsükliini, naldiksiinhappe, tsiprofloksatsiini, tsefotaksiimi ja tsefasidiini suhtes. Enterokokkide antibiootikumitundlikkust uuriti ampitsilliini, erütromütsiini, gentamütsiini, tetratsükliini, klooramfenikooli, vankomütsiini, tsiprofloksatsiini ja linesoliidi suhtes. Uuringus IV hinnati grampositiivsete bakterite antibiootikumitundlikkust penitsilliini, ampitsilliini, tsefalotiini, klindamütsiini, erütromütsiini, gentamütsiini, trimetoprim/sulfametoksasooli ja tetratsükliini suhtes. Gramnegatiivsete bakterite antibiootikumitundlikkust uuriti ampitsilliini, gentamütsiini, trimetoprim/sulfametoksasooli, tetratsükliini, enrofloksatsiini, streptomütsiini, neomütsiini ja tsefaperasooni suhtes.

Uuringus II hinnati tsefotaksiimi või tsefasidiimi suhtes resistentseid *E. coli* isolaate ESBL-i või AmpC (AmpC beetalaktamaaside) ensüümide tootmise suhtes.

Tulemused ja arutelu

Sigadelt ja veistelt isoleeritud *E. coli* antibiootikumiresistentsus

Uuringus I aastatel 2006–2009 oli tervetelt sigadelt isoleeritud *E. coli* (139 mikroobitüve) resistentsus (resistentsete isolaatide hulk protsentides) kõige kõrgem streptomütsiini (23,7%), tetratsükliini (15,8%), sulfametoksasooli (12,9%) ja ampitsilliini suhtes (12,2%). Haigetelt sigadelt isoleeritud *E. coli* (n = 94) resistentsus oli kõrgeim sulfametoksasooli (71,3%), trimetoprimi (57,4%), tetratsükliini (57,4%), streptomütsiini (51,5%) ja ampitsilliini (31,9%) suhtes. Tähelepanuväärselt kõrget resistentsust täheldati ka tsiprofloksatsiini (31,9%), nalidiksiinhappe (31,9%) ja klooramfenikooli (20,2%) suhtes. Multiresistentseid isolaate oli 60–73% kõigist isolaatidest kogu uurimisperioodi jooksul. Kõige sagedamini esines samaaegne resistentsus ampitsilliini, streptomütsiini ja trimetoprimsulfoonamiidi suhtes. Uuringu jooksul leiti üks ESBL-i tootev *E. coli* isolaat.

Uuringus II aastatel 2010–2015 oli tervetelt sigadelt isoleeritud *E. coli* resistentsus kõrgeim streptomütsiini (39,2%), tetratsükliini (32,5%) ja sulfametoksasooli (30,0%) suhtes. Kliiniliselt tervetelt veistelt isoleeritud *E. coli* oli kõige resistentsem aminoglükosiidide (7,0–8,8%) ja tetratsükliini (7,0%) suhtes. Kliiniliselt tervetelt sigadelt pärineva *E. coli* tüvede resistentsus võrreldes veiste isolaatidega oli märkimisväärselt (statistiliselt oluline erinevus) kõrgem ampitsilliini, streptomütsiini, tsiprofloksatsiini, kolistiini, sulfametoksasooli ja trimetoprimi suhtes. Tervete sigade isolaatidel leiti ka oluliselt kõrgem multiresistensuse esinemus võrreldes veiste isolaatidega.

Uuringus II haigetelt sigadelt isoleeritud *E. coli* (n = 143) resistentsus oli kõrgeim sulfametoksasooli (68,6%), tetratsükliini (60,2%), streptomütsiini (54,5%), ampitsilliini (53,9%) ja trimetoprimi (53,9%) suhtes. Veiste isolaatide resistentsus oli samuti kõrgeim streptomütsiini (63,5%), sulfametoksasooli (60,3%), tetratsükliini (58,8%), ampitsilliini (58,7%) ja trimetoprimi (55,6%) suhtes. Sigade isolaatidel oli märkimisväärselt

(statistiliselt oluline erinevus) kõrgem resistentsus nalidiksiinhappe ja oluliselt madalam resistentsus gentamütsiini suhtes.

Uuringus II testiti 16-t tsefotaksiimi ja/või tsefasidiimi suhtes resistentset *E. coli* tüve täiendavalt ESBL-i ja AmpC tootmise suhtes. ESBL-i fenotüüp leidis kinnitust ühel *E. coli* isolaadil, mis pärines kliiniliselt tervelt veiselt, ning kaheksal isolaadil, mis pärinesid haigetelt sigadelt ja veistelt. AmpC fenotüüp leidis kinnitust neljal mikroobitüvel.

Sigadelt ja veistelt isoleeritud enterokokkide antibiootikumiresistentsus

Uuringus I oli aastatel 2006–2009 tervetelt sigadelt isoleeritud enterokokkide (n = 63) resistentsus kõrgeim tetratsükliini (38,1%), erütromütsiini (38%), streptomütsiini (25,4%) ja kanamütsiini (22,2%) suhtes. Multiresistentsus esines peamiselt kanamütsiini, streptomütsiini ja tetratsükliini suhtes.

Uuringus II testiti aastatel 2010–2015 antibiootikumiresistentsuse suhtes 51 tervete veiste enterokokkide isolaati ja 60 tervete sigade enterokokkide isolaati. Mõlema loomaliigi isolaatidel oli resistentsus tekkinud peamiselt tetratsükliini (33,3% veistel, 40,4% sigadel) ja erütromütsiini (21,6% veistel, 26,7% sigadel) suhtes. Sigade isolaadid olid peale selle resistentsemad ka streptomütsiini (30,0%) ja kanamütsiini 26,7%) suhtes. Sigade isolaatide resistentsus võrreldes veiste isolaatidega oli märkimisväärselt kõrgem streptomütsiini ja kanamütsiini suhtes, samuti oli sigade isolaatide multiresistentsus palju kõrgem.

Kui võrrelda eri ajaperioodidel tehtud uuringutes (I ja II) isoleeritud tervetelt sigadelt pärinevate *E. coli* ja enterokokkide, st indikaatorbakterite resistentsust, on näha pidevat resistentsustaseme tõusu ampitsilliini, streptomütsiini, tetratsükliini ja sulfametoksasooli suhtes. See viitab pikaajalisele antibiootikumide ebaotstarbekale kasutamisele, sest antibiootikumide pideval manulusel muutuvad mikroobid resistentseks ja resistentne bakteripopulatsioon hakkab domineerima. Viimase kümne aasta jooksul on nii sigade kui ka lehmade arv Eestis vähenenud, kuid veterinaarseks otstarbeks müüdud antibiootikumikogused ei ole kahanenud. Toiduloomade tarbeks müüdav antibiootikumide üldkogus on Eestis võrreldes teiste Euroopa Liidu riikidega suhteliselt väike, siiski kasutatakse meil loomade raviks liiga palju selliseid toimeaineid,

mis peaksid Maailma Terviseorganisatsiooni seisukoha järgi jääma peamiselt inimeste ravimiseks: fluorokinoloonid, pleuromutiliinid ja tsefalosporiinid. Näiteks inimeste ravis kriitilise tähtsusega 3. ja 4. põlvkonna tsefalosporiinide loomadel kasutamise poolest on Eesti Euroopas esikohal. Olukorra parandamiseks on Eestis kõigepealt tarvis luua tsentraalne andmebaas, kus registreeritakse loomaarstide aruannete põhjal ravimite kasutamine loomadel, ning teiseks tuleks töötada välja ranged reeglid, et vähendada inimmeditsiinis kriitiliste antibiootikumide kasutamist loomadel.

Tervetelt koertelt isoleeritud *E. coli* ja enterokokkide antibiootikumiresistentsus

Uuringus III hinnati 68 *E. coli* ja 66 *Enterococcus* spp. isolaadi antibiootikumitundlikkust. Isolaadid pärinesid kliiniliselt tervete koerte roojaproovidest. *E. coli* resistentsus oli üldiselt madal, vähemalt ühe uuritud antibiootikumi suhtes olid resistentsed 10,3% tüvedest. Enterokokkide resistentsus oli märkimisväärselt kõrgem, 45,5% tüvedest olid resistentsed tetratsükliini suhtes, 21,2 % tsiprofloksatsiini suhtes ja 10,6% erütromütsiini suhtes. Uuringus ei leitud seost mikroobide resistentsuse ja eelneva antibiootikumide manustamise vahel. Samuti ei leitud seost resistentsuse ega koera vanuse, kehakaalu ja elukeskkonna iseärasuste vahel.

Mastiidipatogeenide antibiootikumiresistentsus

Uuringus IV hinnati 3058 lehmade kliinilise mastiidi piimaproovidest isoleeritud patogeeni antibiootikumiresistentsust. *S. aureus* oli peamiselt resistentne penitsilliini (61,4%) ja ampitsilliini (59,5%) suhtes. Koagulaasnegatiivsed stafülokokid (KNS) olid samuti resistentsed penitsilliini (38,5) ja ampitsilliini (34,4%) suhtes, lisaks ka erütromütsiini (14,9%) ja linkomütsiini (17,6%) suhtes. Kõik streptokokkide isolaadid olid penitsilliini, ampitsilliini ja tsefalotiini suhtes tundlikud, välja arvatud üks *Streptococcus (Str.) uberis*'e isolaat. Tetratsükliini suhtes olid resistentsed 32,2% *Str. dysgalactiae* isolaatidest ja 14,3% *Str. uberis*'e isolaatidest.

Kokkuvõte

Aastatel 2006–2009 tervetelt sigadelt isoleeritud *E. coli* oli resistentne peamiselt streptomütsiini, tetratsükliini, sulfametoksasooli ja ampitsilliini suhtes. Samal ajaperioodil haigetelt sigadelt isoleeritud *E. coli* oli resistentne peamiselt sulfametoksasooli, trimetoprimi, tetratsükliini, streptomütsiini ja ampitsilliini suhtes. Täheldati resistentsust ka tsiprofloksatsiini, nalidiksiinhappe ja klooramfenikooli suhtes.

Aastatel 2010–2015 tervetelt sigadelt isoleeritud *E. coli* oli resistentne peamiselt streptomütsiini, tetratsükliini ja sulfametoksasooli suhtes. Kliiniliselt tervetelt veistelt isoleeritud *E. coli* oli resistentne aminoglükosiidide ja tetratsükliini suhtes. Haigetelt sigadelt isoleeritud *E. coli* resistentsus oli kõrgeim sulfametoksasooli, tetratsükliini, streptomütsiini, ampitsilliini ja trimetoprimi suhtes. Haigetelt veistelt isoleeritud *E. coli* resistentsus oli kõige kõrgem streptomütsiini, sulfametoksasooli, tetratsükliini, ampitsilliini ja trimetoprimi suhtes.

Tervetelt sigadelt isoleeritud *E. coli* tüvede hulgas oli märkimisväärselt rohkem multiresistentseid isolaate võrreldes veistelt isoleeritud bakteritega.

Nii sigadelt kui ka veistelt isoleeritud *E. faecalis*'e ja *E. faecium*'i resistentsus oli kõrgeim erütromütsiini ning tetratsükliini suhtes, sigade isolaadid olid lisaks resistentsemad ka streptomütsiini ja kanamütsiini suhtes.

Mikroobide resistentsus nii tervetelt kui ka haigetelt sigadelt ja veistelt isoleeritud soolebakterite hulgas viitab kaudselt antibiootikumide kasutamise pikemaajalisele mõjule nendel loomaliikidel.

Kliiniliselt tervetelt koertelt isoleeritud soolemikroobide (*E. coli* ja enterokokid) antibiootikumiresistentsuses ei olnud erinevust, resistentsuse ja võimalike resistentsuse teket soodustavate riskitegurite vahel seost ei leitud.

Kliinilise mastiidi puhul isoleeritud patogeenide antibiootikumi-resistentsus oli kõrge. *S. aureus*'e tüvede resistentsus oli eriti kõrge penitsilliini ja ampitsilliini suhtes. KNS-i resistentsus oli veidi madalam. *E. coli* ja *Klebsiella* spp. resistentsus oli kõrge kõigi uuritud

antibiootikumide suhtes, välja arvatud tsefaperasooni ja enrofloksatsiini suhtes.

Käesolevas töös käsitletud laiapõhjalised uuringud näitavad loomadelt isoleeritud mikroobide antibiootikumiresistentsuse taset uuritud loomaliikidel ning moodustavad baasi edasiste samasuunaliste uuringute tarbeks. Uuringute põhjal saab väita, et Eestis tuleb üle vaadata ja korrigeerida eelkõige sigade antibiootikumravi tavad ja plaanid, et kindlustada antibiootikumide mõistlik kasutamine ning vähendada resistentsete mikroobide kujunemist ja levimist keskkonda ning inimestele.

Tuleb vähendada antibiootikumide kasutamist loomadel. Eesti Maaülikooli kliinilise veterinaarmeditsiini õppetooli õppejõud (sh uurimistöö autor) töötasid 2012. aastal välja esmased juhendid antibiootikumide otstarbekaks kasutamiseks loomakasvatuses, juhendeid uuendati 2018. aastal. Eestis tuleb välja töötada ja rakendada loomade antibiootikumidega ravimise nõustamis- ja kontrollisüsteem. Tuleb luua andmebaas loomadel antibiootikumide kasutamise aruannete kogumiseks ning kehtestada reeglid kriitilise tähtsusega antibiootikumide kasutamise vähendamiseks loomadel (nt fluorokinoloonide ja tsefalosporiinide kasutamine põllumajandusloomadel).

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I

ORIGINAL PUBLICATIONS

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ANTIMICROBIAL RESISTANCE OF ANIMAL PATHOGENS 2006-2009 IN ESTONIA

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Abstract
The present study describes situation of antimicrobial resistance of animal pathogens and resistance trends in Estonia in years 2006-2009. Bacterial strains isolated during period 2006-2009 were *Escherichia coli (E. coli)*, *Enterococcus faecium (E. faecium)*, *Enterococcus faecalis (E. faecalis)*, collected from healthy pigs faeces as well as from diagnostic submissions of pig samples. *Staphylococcus aureus (S. aureus)* isolates originated from cows with clinical mastitis and *Staphylococcus pseudointermedius (S. pseudointermedius)* isolates from dogs with pyoderma or otitis externa. Antimicrobial susceptibility was detected by microdilution method. Normal enteric microflora from health y pigs had resistance against streptomycin, tetracyclin, sulfametoxazol and trimethoprim. *E. faecalis* and *E. faecium* were resistant to erythromycin, tetracyclin, streptomycin and kanamycin. Multiresistance occured mainly against kanamycin, streptomycin and tetracyclin. *E. coli* strains isolated from pathological material showed high resistance to ampicillin, tetracycline, streptomycin, sulphonamides and trimethoprim. Multiresistance was detected between 60–73% during study years. In 2009, one ESBL (extended spectrum betalactamase) producing isolate was observed. *S. aureus* strains isolated from clinical mastitis samples were mainly penicillin resistant (58–86%). Meticillinresistant *S. aureus* was not found during the study. In 2009, resistance to lincomycin (30%) and fucidinic acid (22%) was detected. In *S. pseudointermedius* strains isolated from canine skin samples the prevalence of resistance to penicillin as high as 53–81% was found. Multidrug resistance was relatively stable being 38% in 2006, 29% in 2007 and 25% in 2009. In conclusion, antimicrobial resistance of animal pathogens in Estonia was high. Further improvement of prudent use of antimicrobials and infection control is needed.
Key words: antimicrobial resistance, staphylococci, enteric bacteria.

Introduction

Resistance to antimicrobial agents is an emerging problem worldwide. Antibiotic use selects for population of resistance bacteria target pathogens and normal bacterial flora, including food borne pathogens such as *Salmonella spp.*, *Campylobacter spp.* and *E. coli*. Extensive antibiotic use accelerates the development of antibiotic resistance in the population. It includes increased morbidity and mortality from treatment failures and increased health care costs as newer, more expensive ingredients are needed to treat infections. Awareness of the undesirable consequences of its widespread occurrence has led to the initiation of antimicrobial agent resistance monitoring programs in several countries. The surveillance of emerging resistance and resistance trends are identified through the national antimicrobial resistance monitoring program with the purpose of facilitating timely and appropriate public health responses (Angulo et al., 2004). Separate program for human and veterinary medicine has been developed during the last ten years in the Nordic countries (SVARM, FINRES). In addition, integrated programs have been working in Denmark and Norway (DANMAP; NORM-NORMVET) for several years.

The monitoring of antimicrobial resistance is based on three categories of bacteria: human and animal pathogens, zoonotic bacteria and indicator bacteria. Indicator bacteria are included due to their ubiquitous nature in animals, foods and humans and their ability to readily develop antimicrobial resistance in response to selective pressure in both reservoirs. Human and animal pathogens are included because these cause infections that primarily reflect resistance caused by the use of antimicrobial agents in the respective reservoirs. Zoonotic bacteria are included because they can develop resistance in the animal reservoir, which may subsequently compromise treatment effect if resulting in a human infection.

Estonia University of Life Sciences in cooperation with Estonian Veterinary and Food Laboratory started the monitoring program for antimicrobial resistance of animal pathogens in the year 2000. Indicator bacteria (*E. coli*, *Enterococcus spp.*), zoonotic bacteria (*Salmonella spp.*) and different pathogenic bacteria isolated from clinical submissions (*Staphylococcus spp.*, *E. coli*) were hence investigated annually. The objective of the present study was to give an overview of the occurrence of antimicrobial resistance in bacteria isolated from healthy and diseased animals submitted between 2006 -2009.

Materials and Methods

Sampling strategy

The faecal samples representing normal intestinal microflora (*E. coli*, *Enterococcus spp.*) of healthy pigs were collected during National Salmonellosis surveillance programme. In the laboratory, systematic random sampling (every fifth sample) was used. Also, *E. coli* isolated from diseased pigs were collected during diagnostic submissions. *S. aureus* isolates originated from cases of clinical mastitis from cattle submitted to

the laboratory during routine microbiologial diagnosis. *S. pseudointermedius* isolates from dogs pyoderma or otitis externa were also included into the monitoring programme. Systematic random sampling was used among the *S. aureus* isolates, all *S. pseudointermedius* and *E. coli* isolates were included in the analysis.

Isolation and identification of bacteria

The material for identification of *E. coli* was inoculated directly to eosin methylene blue (EMB) agar (Sigma Ltd) based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies after incubation at 37 °C overnight. Phenotypic confirmatory test for production of extended spectrum beta-lactamases (ESBLs) in *E. coli* was performed by the double disc diffusion test according to Clinical and Laboratory Standards Institute (2008) (CLSI). Genotypic screening of ESBL and AmpC positive *E. coli* was performed by using Identibact Array Tube test according to the manufacturer (www.identibact.com). A polymerase chain reaction was complementary performed for identification of plasmid-mediated AmpC and CTX-M mediated ESBL according to J. F. Perez-Perez and N. D. Hanson (2002).

For isolation of enterococci one drop of faeces suspended in 2 mL sodium chloride (0.9 g kg^{-1}) was spread on Slanetz-Bartley agar and incubated for two days at 42 °C. Up to four colonies with morphology typical of *E. faecalis* / *E. faecium* were sub-cultivated on blood agar. Colonies were identified by the following criteria: colour, motility, arginine dihydrolase testing and the ability to ferment mannitol, sorbitol, arabinose, raffinose and melibiose. All isolates of *E. faecium* and *E. faecalis* were stored (-80 °C) for the suspectibility testing.

Samples from the clinical submissions to identify staphylococci were inoculated onto ovine blood agar (Oxoid, Basingstoke, UK) and mannitol salt agar plates (Oxoid), and incubated aerobically at 37 °C for 18–24 h. Staphylococcal isolates were putatively identified by colony morphology, ability to grow on mannitol salt agar and Gram-stain characteristics. Representative isolates were then subcultured onto blood agar before being subjected to further confirmatory tests. Catalase activity was determined using 3 g kg^{-1} hydrogen peroxide (Sigma, Poole, UK). A slide test for bound coagulase was undertaken using rabbit plasma (Pro-Lab, Cheshire, UK). The absence of bacterial clumping indicated a negative result. All isolates negative for bound coagulase underwent a tube coagulase test where the formation of a clot following incubation of a bacterial suspension with an equal volume of rabbit plasma at 37 °C for 4–18 h indicated the presence of free coagulase. Coagulase-positive isolates were identified to the species level using a commercially available microbial identification

Table 1

Antimicrobial resistance of *Eschericia coli* isolated from faecal samples of healthy Estonian pigs 2006-2009

Antimicrobial agents	Breakpoint µg mL^{-1}*	2006 % (n=34)	2007 % (n=45)	2008 % (n=20)	2009 % (n=40)
Ampicillin	≥8	9	18	10	10
Ciprofloxacin	≥0.06	0	0	0	3
Nalidixic acid	≥16	3	0	0	3
Gentamycin	≥2	12	9	0	0
Ceftiofur	≥1	6	2	0	1
Streptomycin	≥16	26	31	20	15
Tetracyclin	≥8	6	27	15	13
Florfenicol	≥16	0	0	0	0
Kanamycin	≥8**	9	13	5	0
Sulfametoxazol	≥256	6	24	10	13
Trimethoprim	≥2	0	20	10	5
Chloramfenicol	≥16	3	9	0	3
Tsefotaxim	≥0.25	0	0	0	3

* Test: VetMIC™ GN-mo (version4).
Epidemiological cut-off values from EUCAST (European Committee on Antimicrobial Susceptibility Testing).
** Breakpoints from SVARM 2007 (Swedish Veterinary Antimicrobial Resistance Monitoring ISSN 1650-6332 Uppsala, http://www.sva.se).
SVARM 2007 (Swedish Veterinary Antimicrobial Resistance Monitoring ISSN 1650-6332 Uppsala, http://www.sva.se).

micro-tray kit according to the manufacturers' instructions (API ID32 Staph System; BioMerieux; Lyons, France). Oxford *S. aureus* NCTC 6571 was used as a quality control standard throughout the identification procedures. Screening for methicillin resistance in *S. aureus* from milk and S. *pseudointermedius* from dogs samples from cows was performed with microdilution according to CLSI (2008), testing oxacillin with 2 g kg^{-1} NaCl added to the broth, and oxacillin without added NaCl and cefoxitin. The *in vitro* antimicrobial susceptibility was determined by using microdilution method (VetMIC®, Sweden). Epidemiological cut-off values issued by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) were used for interpretation of results of susceptibility testing of indicator bacteria and clinical breakpoints for *S. aureus*.

For interpretation of results for susceptibility testing of indicator bacteria (E. *coli* and enterococci) epidemiological cut off values (ECOFF) issued by the EUCAST (http://www.escmid.org) were used. When no ECOFFs were issued by EUCAST, the clinical breakpoints recommended for animal pathogens by CLSI were taken into consideration. The term 'multiresistance' is used with a meaning as proposed by S. Schwarz et al. (2010). Briefly, isolates with phenotypically identified acquired resistance to three or more antimicrobial classes are deemed multiresistant.

Propotion of resistance for each measured antimicrobial agent by dividing resistant isolates with all collected isolates was calculated.

Results and Discussion

Antimicrobial resistance of indicator bacteria isolated from faecal samples of healthy pigs is presented in Tables 1 and 2.

Normal gut microflora has developed resistance against several antibiotics detected. No resistance was detected to florfenicol. The highest resistance can be detected against streptomycin, tetracyclin, sulfametoxazol and trimethoprim. In 2008, resistance to streptomycin and tetracyclin decreased, no resistance to nalidixic acid and ciprofloxacin was observed.

For both, *E. faecalis* and *E. faecium*, resistance was most frequently detected against erythromycin, tetracyclin, streptomycin and kanamycin. Resistance to vancomycin was relatively high in 2008, but no *vanE* genes were found. Multiresistance occurs mainly against kanamycin, streptomycin and tetracyclin. Trends in resistance of *E. coli* strains isolated from pathological material (faeces or organs) are presented in Table 3.

Table 2

Antimicrobial resistance of *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from faecal samples taken from healthy Estonian pigs submitted between 2006-2009

Antimicrobial agents	Breakpoint μg mL^{-1}*	2006 % (n=10)	2007 % (n=11)	2008 % (n=25)	2009 % (n=17)
Ampicillin*	≥4	0	0	0	0
Erythromycin*	≥4	40	18	44	41
Virginiamycin**	≥32	0	9	4	0
Gentamycin*	≥32	4	0	4	6
Streptomycin*	≥512	50	0	32	18
Kanamycin**	≥1024	40	18	28	6
Tetracyclin*	≥2	40	36	44	30
Chloramphenicol**	≥32	0	9	12	0
Vancomycin*	≥4	0	27	8	0
Narasin**	≥2	0	0	16	0
Bacitracin**	≥32	10	9	8	0
Linesolid*	≥4	0	0	4	0

Test: VetMIC™ E-cocci (version3).
* Epidemiological cut-off values from EUCAST (European Committee on Antimicrobial Susceptibility Testing).
** Breakpoints from SVARM 2007 (Swedish Veterinary Antimicrobial Resistance Monitoring ISSN 1650-6332 Uppsala, http://www.sva.se).

Table 3

Antimicrobial resistance of *Eschericia coli* isolated from pathological material from Estonian pigs in years 2006-2009

Antimicrobial agents	Breakpoint μg mL^{-1}*	2006 % (n=25)	2007 % (n=18)	2008 % (n=21)	2009 % (n=30)
Ampicillin	≥8	64	17	50	5
Ciprofloxacin	≥0.06	8	61	33	0
Nalidixic acid	≥16	16	22	33	50
Gentamycin	≥2	12	6	5	3
Ceftiofur	≥1	0	0	0	0
Streptomycin	≥16	56	56	62	37
Tetracycline	≥8	64	50	62	54
Florfenicol	≥16	4	0	5	0
Kanamycin	≥8	24	6	19.	7
Sulfametoxazol	≥256	72	83	62	70
Trimethoprim	≥2	72	22	57	67
Chloramfenicol	≥16	36	0	14	27
Cefotaxim	≥0.25	0	0	0	3

Test: VetMIC™ Gn-mo (version 4).
* Epidemiological cut-off values from EUCAST (European Committee on Antimicrobial Susceptibility Testing).

During the study years high resistance to ampicillin, tetracycline, streptomycin, sulphonamides and trimethoprim was observed. The resistance to gentamycin decreased till the year 2006, while resistance to ciprofloxacin increased from 8% in 2006 to 61% in 2007, also resistance to nalidixic acid increased from 16% in 2006 to 50% in 2009. Multiresistance has been detected between 60-73% during all study years. The contemporaneous resistance to ampicillin, streptomycin and trimethoprim-sulphonamides was the most common trait, occurring in 84% of the multiresistant isolates. In 2009, one ESBL producing isolate was observed.

Table 4

Resistance of *S. aureus* isolated from clinical mastitis milk samples in years 2006-2009 in Estonia

Antimicrobial agents	Breakpoint μg mL^{-1}*	2006 % (n=50)	2007 % (n=21)	2008 % (n=25)	2009 % (n=50)
Penicillin*	≥0.125	58	86	80	86
Cefalotin**	≥1	0	0	8.0	8
Oxacillin+2% NaCl	≥2	4	0	0	0
Erythromycin*	≥1	0	0	4	0
Chloramphenicol*	≥16	0	0	4	0
Clindamycin	≥0.25	0	0	0	30
Tetracyclin*	≥0.25	4	0	0	6
Fusidinic acid**	≥1	x	0	0	22
Gentamütsiin*	≥2	2	0	4	2
Kanamütsiin**	≥8	0	0	0	4
Ciprofloxacin*	≥1	0	0	0	0
Trimethoprim*	≥4	0	0	0	0

Test: VetMIC™ GP-mo (version 2).
* Epidemiological cut-off values from EUCAST (European Committee on Antimicrobial Susceptibility Testing).
** Breakpoints from SVARM 2007 (Swedish Veterinary Antimicrobial Resistance Monitoring ISSN 1650-6332 Uppsala, http://www.sva.se).

Antimicrobial resistance of *S. aureus* isolated from clinical mastitis milk samples is shown in Table 4.

The resistance levels of *S. aureus* strains were generally low, except against penicillin. Resistance to penicillin incerased over the four years from 58% to 85%. Also, 8% of cephalotin resistant isolates were detected in the years 2008-2009. Meticillinresistant *S. aureus* (MRSA) was found during monitoring program. In 2009, resistance to lincomycin (30%) and fucidinic acid (22%) was detected. Table 5 describes resistance of *S. pseudintermedius* and *S. aurues* isolated from ear and skin samples of Estonian dogs between 2006-2009.

The prevalence of resistance to penicillin due to production of betalactamases (penicillinase) in *S. pseudintermedius* is high, 52-81%. An oxacillin resistant *S. pseudointermedius* was isolated in 2007 and 2008 with presence of *meqA* gene. In 2008, resistance to ciprofloxacin was observed. Data from 2009 should be interpreted with caution due to very small amount of isolates that year. Multidrug resistance is relatively stable being 38% in 2006, 29% in 2007 and 25% in 2009. The present monitoring program describes the situation of antimicrobial resistance and trends in Estonia in the years 2006-2009.

The prevalence of acquired antimicrobial resistance in commensal bacteria of the enteric microflora of healthy animals indirectly indicates the magnitude of the selective pressure from the use of antimicrobials in animal population. The resistance level of enteric microflora in pigs is higher in Estonia in comaprison with Sweden and Norway (Bengtsson et al., 2010; Kruse and Skov, 2004), but is similar to reports from Denmark (Jensen et al., 2010) and Netherlands (Mevius et al., 2009). Both *E .coli* and enterococci showed highest resistance to tetracyclin which can be explained with wide use of doxycycline for oral treatment of pigs. Also, tylosin and sulfonamides

Table5

Resistance of *Staphylococcus spp.* isolated from dogs in case of otitis externa or from skin samples of Estonian dogs between 2006-2009

Antimicrobial agents	Breakpoint µg mL^{-1}*	2006 % (n=21)	2007 % (n=17)	2008 % (n=16)	2009 % (n=8)
Penicillin*	≥0.13 *S.intermedius* ≥ 0.13 *S.aureus*	53	71	81	63
Cefalotin	≥2 *S.intermedius* ≥1 *S.aureus*	0	0	31	0
Oxacillin+2% NaCl**	≥1 *S.intermedius* ≥2 *S.aureus*	0	18	31	0
Erythromycin*	≥1 *S.intermedius* ≥1 *S.aureus*	29	29	44	13
Chloramfenicol*	≥16 *S.intermedius* ≥16 *S.aureus*	24	12	19	13
Clindamycin**	≥4 *S.intermedius* ≥ 0.25 *S.aureus*	24	12	44	13
Tetracyclin*	≥8 *S.intermedius* ≥1 *S.aureus*	24	41	25	25
Fucidinic acid**	≥ 4 *S.intermedius* ≥ 0.5 *S.aureus*	14	12	6	13
Gentamycin*	≥4 *S.intermedius* ≥2 *S.aureus*	0	12	31	13
Kanamycin**	≥8 *S.intermedius* ≥8 *S.aureus*	0	24	44	25
Ciprofloxacin*	≥1 *S.intermedius* ≥1 *S.aureus*	0	0	38	13
Trimethoprim*	≥2 *S.intermedius* ≥2 *S.aureus*	67	76	63	38

Coagulase negative staphylococci (CNS) and *Staphylococcus aureus* (S.a.).
Test: VetMIC™ GP-mo (version 2).
* Epidemiological cut-off values from EUCAST (European Committee on Antimicrobial Susceptibility Testing)
** Breakpoints from SVARM 2007 (Swedish Veterinary Antimicrobial Resistance Monitoring ISSN 1650-6332 Uppsala, http://www.sva.se).

with trimethoprim are commonly used in Estonia where cross-resistance between macrolides and high level of sulfa/trimetoprim resistance was developed in normal microflora. As enterococci are intrinsically resistant to many antimicrobial agents, antimicrobial agents used for treatment of Enterococcus infection are limited. Although food-producing animals are not always a source of Enterococcus infection in humans, antimicrobial-resistant in animal origins may cause transmission of their resistance genes from animal to human bacteria. Therefore, prevalence of antimicrobial resistant enterococci, including vancomycin-resistant enterococci (VRE) in food-producing animals, has become a serious problem in several countries.

A higher resistance is expected in bacteria from diagnostic submissions compared to bacteria originating from healthy animals sampled on farm. For instance, *E. coli* from pathological material is more resistant than normal habitant of intestine. However, this data shows a high probability of bias towards animals with recurrent infections, previously treated with antimicrobials that could explain high levelof resistance. On the other hand, the number of isolates from animal pathogens is quite low. Veterinarians do not often send samples to the laboratory for isolation and identification of bacteria. Therefore, antibacterial treatment is initiated without bacterial diagnosis, which can lead to multidrug resistance.

In 2005, *S. pseudintermedius* in dogs, a novel staphylococcal species was described (Devriese et al., 2005). Further on T. Sasaki et al. (2007) and J. Bannoehr et al. (2009) reported that canine strains of *S. intermedius* should be classified as *S. pseudintermedius*. Therefore, it was proposed to report strains from dogs as if the strain belonging to a related species (Devriese et al., 2009). Pyoderma and ear infection are common causes for dog owners to seek veterinary consultation (Holm et al., 2002). These conditions are often treated with clindamycin or cephalosporins. Detected resistance against fluoroquinolones is probably related to frequent use of marbofloxacin and ciprofloxacin in treatment of several infections in dogs. To be able to control the resistance situation in *S. pseudintermedius*, a prudent use of antimicrobials together with an effective infection control programme is of highest priority.

The resistance of *S. aureus* strains isolated from clinical mastitis samples of cows is high against penicillins. Due to high prevalence of penicillin resistant *S. aureus*, veterinarians tend to choose lincosamides as the first choice of treatment. Statistical data from the Estonian State Agency of Medicine confirmed that all together 209880 single intramammary syringes for lactating cows and 205648 for dry cows were sold in the year 2009 for therapeutic purposes. Ampicillin and cloxacillin combinations, cephalosporins with aminoglycosides and lincomycin with neomycin were the most common choices for the treatment of mastitis in lactating cows. For example, 255 g of intramammary lincomycin (pure antimicrobial) and 44.2 g of intramammary cephalosporins per thousand dairy cows were sold for treatment of clinical mastitis in 2009. However, only 73.4 g of penicillin G was used per thousand dairy cows for intramammary treatment of clinical mastitis. The use of broad-spectrum antibiotics and antibiotic combinations may influence the resistance of mastitis pathogens.

Conclusions

Antimicrobial resistance of animal pathogens in Estonia is high. Further improvement of the implementation of prudent use of antimicrobials and infection control will be needed. In a long term perspective, the need for antimicrobials must be reduced by further improvement of animal health.

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II

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RESEARCH Open Access

Antimicrobial resistance of *Escherichia coli* and *Enterococcus* spp. isolated from Estonian cattle and swine from 2010 to 2015

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Abstract

Background: The prevalence of resistant *Escherichia coli* and *Enterococcus* spp. in food-producing animals has increased worldwide. The objective of the study was to investigate the occurrence of antimicrobial resistance of *Escherichia coli*, *Enterococcus faecium* and *Enterococcus faecalis* isolated from healthy and diseased swine and cattle in Estonia. Clinical specimen and faecal samples were collected during 2010 to 2015. The in vitro antimicrobial susceptibility was determined using the microdilution method.

Results: The most prevalent resistance of *E. coli* isolates from clinically healthy swine was observed against streptomycin (39.2%), tetracycline (32.5%) and sulfamethoxazole (30.0%), whereas in clinically healthy cattle, the resistance was the highest against aminoglycosides (7.0–8.8%) and tetracycline (7.0%). The *E. coli* isolates from clinically healthy swine showed significantly higher multidrug-resistance compared to isolates originated from clinically healthy cattle. *E. coli* isolates from diseased swine showed highest resistance to sulfamethoxazole (68.6%), tetracycline (60.2%) and streptomycin (54.6%). The proportion of resistant *E. coli* isolates from diseased cattle (clinical submissions) was highest to streptomycin (63.5%), sulfamethoxazole (60.3%) and tetracycline (58.8%). The proportion of multidrug-resistant isolates did not differ significantly between animal species. Among *E. coli* isolates, four strains representing AmpC phenotypes were found. One plasmid-encoded AmpC type β-lactamases producing *E. coli* from clinically healthy cattle was found to harbour the bla_{CMY-1} gene, and another from clinically healthy swine carried the bla_{CMY-2} gene. Among nine *E. coli* strains exhibiting an ESBL phenotype three strains was found to be the same genotype $bla_{TEM-52C}$. Enterococci from healthy swine and cattle showed high resistance to tetracycline and erythromycin. Regarding enterococci, the number of multidrug-resistant strains was significantly higher in swine isolates compared to isolates originated from cattle.

Conclusions: The antimicrobial resistance of *E. coli* isolates was high in both Estonian swine and cattle. However, swine isolates, especially *E. coli* from healthy swine, had developed a higher level of resistance. The amount of multidrug-resistant *E. coli* isolates was also significantly higher in clinically healthy swine compared to that in cattle.

Keywords: Antimicrobial resistance, Cattle, *E. coli*, Enterococci, Swine

Background

Bacterial infections are one of the most prevalent groups of diseases in production animals and are commonly treated with antimicrobial drugs. Antibacterial treatment is essential to treat diseased animals; however, one of the negative impacts is expansion of antimicrobial resistance. The antimicrobial resistance of bacterial species originating from production animals also influences human health through the transfer of resistant organisms or genes via food chain [1, 2]. The extended spectrum beta-lactamase (ESBL)-positive *Escherichia coli* isolates in food-producing animals have been frequently identified [3]. As the AmpC and ESBL producing strains are detected in cattle and swine, there is a potential risk for

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transmission of the strains to other animals and humans [1].

Intestinal commensal bacteria inhabiting both animals and humans are considered good indicators to monitor antimicrobial resistance as they are subjected to the continuous selection pressure of the antimicrobials [4]. The European Union (EU) and the European Food Safety Authority (EFSA) have provided guidelines for the harmonised monitoring and reporting of resistance of indicator *E. coli* and *Enterococcus* spp. [5]. Since 2014, the monitoring and reporting of the resistance of commensal *E. coli* is mandatory according to the EU decision (2013/652/EU).

In order to reduce antimicrobial resistance and give appropriate suggestions for the use of antibacterials, the survey of the resistance situation in certain regions is inevitable [5]. Setting out the current situation at a certain time point enables us to monitor changes and take appropriate measures to diminish the development of antimicrobial resistance. Similar data from different countries enable us to compare resistance of indicator bacteria and to consider possible transmission of resistant strains between countries.

The objective of this study was to estimate the occurrence of antimicrobial resistance of *E. coli* and *Enterococcus* spp. isolated from swine and cattle in Estonia from 2010 to 2015 and to study whether antimicrobial resistance differs between swine and cattle isolates.

Methods

Collection of study material

Faecal samples from healthy cattle and swine were collected in the course of the annual national salmonella surveillance programme carried out in Estonia in 2010–2015. According to the number of faecal samples sent to the laboratory from one herd, one to three randomly chosen samples were cultivated for the isolation of *E. coli*, *Enterococcus faecium* and *Enterococcus faecalis* as follows: one sample was selected when the total number of samples from one farm was up to 15, two samples when the sample numbers ranged between 15 and 30 samples from one farm and three samples when the number of samples from one farm varied between 31 and 50. In total, 120 *E. coli* isolates from swine and 171 *E. coli* isolates from cattle, 60 *Enterococcus* spp. isolates from cattle and 51 from swine were included in the study. The isolates originated from 38 swine (total 217 in Estonia) and 42 dairy farms (total 448 in Estonia).

Escherichia coli isolates (n = 206) from clinical material (post mortem samples, organ materials) originated from diseased cattle (n = 63) and swine (n = 143). These samples were sent to the National Veterinary and Food Laboratory (VFL; Tartu, Estonia) by veterinarians in 2010–2015 and all isolates were included in the study.

Identification of *E. coli*, *E. faecium* and *E. faecalis*

The isolation and identification of *E. coli* and enterococci were performed according to accredited methods at the VFL.

For the identification of *E. coli,* the colonies were inoculated to eosin methylene blue (EMB) agar. Based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies after incubation at 37 °C overnight, *E. coli* was confirmed by biochemical tests (IMViC—indole, methyl red, Voges–Proskauer, Simmons citrate).

For the isolation of enterococci, 1 g of faeces was incubated at 37 °C overnight in enrichment broth (6.5% NaCl Brain Heart Infusion (BHI)), and 10 µL of enrichment suspension was spread on Slanetz-Bartley agar and incubated for 48 h at 42 °C. Up to four colonies with morphology typical of *E. faecalis*/*E. faecium* were subcultivated on sheep blood agar. Colonies were identified by the following criteria: haemolysis on blood agar, aesculin hydrolysis on Edwards medium, growth in presence of tellurite and the ability to ferment mannitol, sorbitol, arabinose and raffinose All pure isolates of *E. coli*, *E. faecium* and *E. faecalis* were stored (− 80 °C) for the antimicrobial susceptibility testing.

All clinical *E. coli* isolates were serotyped using *E. coli* OK O antisera for live culture produced in rabbits and F4, F5 antisera according to the manufacturer's protocol (SSI Diagnostica A/S, Copenhagen, Denmark).

Determination of antimicrobial susceptibility

The in vitro antimicrobial susceptibility was determined using the microdilution method (VetMIC®, Sweden). The susceptibility of *E. coli* isolates was tested for ampicillin, cephotaxime, nalidixic acid, chloramphenicol, florfenicol, tetracycline, colistin, gentamycin, kanamycin, streptomycin, ciprofloxacin, trimethoprim and sulphamethoxazole. The susceptibility of *E. faecalis* and *E. faecium* was tested for ampicillin, erythromycin, virginiamycin, gentamycin, streptomycin, kanamycin, tetracycline chloramphenicol, vancomycin, narasin, bacitracin and linezolid. Ampicillin was used as a test substance, whereas ampicillin covers both antimicrobial resistance ampicillin and amoxicillin.

For the interpretation of minimum inhibitory concentration (MICs) from the susceptibility testing of *Escherichia coli, E. faecalis* and *E. faecium* cut-off values available in Swedres-Svarm 2015 report Table 7.12 [6] were used.

An *E. coli* isolate was classified as multidrug-resistant (MDR) [7] when it was resistant to three or more of the

following antimicrobials: ampicillin, tetracycline, chloramphenicol, colistin and florfenicol or to the following antimicrobial classes: trimethoprim/sulfamethoxazole, fluoroquinolones (ciprofloxacin or nalidixic acid), aminoglycosides (gentamicin, streptomycin or kanamycin), extended-spectrum cephalosporins (cephotaxime or cephtazidime). An *E. faecium* or *E. faecalis* was classified as MDR if the resistance was detected to any antibiotic in three or more of the following antimicrobials/antimicrobial classes: ampicillin, tetracycline, erytromycin, vancomycin, virginiamycin, aminoglycosides (gentamicin, streptomycin or kanamycin), narasin, bacitracin and linezolid.

For *E. coli* isolates resistant to either cefotaxime or ceftazidime, the phenotypic confirmatory test (National Veterinary Institute, Technical University of Denmark (DTU) scheme) for the production of ESBLs and AmpC was performed (CLSI M100-S21) [8]. Genotypic confirmation of ESBL and AmpC-positive *E. coli* (n = 16) was performed in the EU Reference Laboratory for antimicrobial resistance (EURL-AR) at DTU, where the presence of genes encoding bla_{TEM}, bla_{CTX} and bla_{SHV} were examined. PCR assay and sequence analysis was performed at DTU as described by Xia et al. [9].

Statistical analysis

This study material was very heterogenous, originated from clinically healthy animal and clinical submission and collected from different farms during 2010–2015. To minimize this heterogenicity, three different databases were created as follows: antimicrobial resistance of *E. coli* originated from clinically healthy animal, resistance of *E. coli* from clinical submission and resistance of *Enterococcus* spp. from healthy animal. Percentages of resistant isolates with 95% confidence intervals (95% CI) to all antimicrobial agents in both animal species (cattle and swine) were calculated. Logistic regression analysis was performed for each antimicrobial agent separately, and association between the occurrence of antibiotic resistance (0—susceptible; 1—resistant) of *E. coli* and animal species (dairy cattle *vs* swine) was studied. Due to a small number of samples, the resistance of *E. faecium* and *E. faecalis* originating from healthy animals was analysed together. Odds ratios (ORs) with 95% CIs were calculated. Similarly, the associations between multidrug-resistance (simultaneous resistance to more than three antimicrobials or antimicrobial classes) of *E. coli* from a clinically healthy animal or clinical submission and animal species were studied with logistic regression analysis. As the datasets were very unbalanced with variable number of observations from different farms and years, we fitted also logistic models considering random effects of farm and year. However, as several models corresponding to the less resistant isolates did not converge and the results of the other models were similar to the simple logistic regression analysis (including magnitude of the odds ratios and statistical significance of differences), we presented only the results of simple models. Statistical significance was assumed at ≤ 0.05. Stata 14.0 (StataCorp, Texas, USA) and SAS 9.4 (SAS Institute Inc., Cary, NC, USA) software were used for statistical analyses.

Results

Resistance profile of *E. coli* in healthy animals

Among the *E. coli* isolates from swine (n = 120), we found high occurrence of resistance to streptomycin (39.2%), tetracycline (32.5%) and sulfamethoxazole (30.0%). In clinically healthy cattle (n = 171), the most prevalent resistance was observed against aminoglycosides (7.0–8.8%) and tetracycline (7.0%) (Table 1).

The resistance of *E. coli* originated from faecal samples from clinically healthy swine compared to cattle was significantly higher to ampicillin (OR = 6.5; 95% CI 2.70–15.56; P < 0.001), streptomycin (OR = 8.5, 95% CI 4.27–17.03; P < 0.001), ciprofloxacin (OR = 10.5; 95% CI 1.27–86.76; P = 0.029), tetracycline (OR = 6.4; 95% CI 3.16–12.89; P < 0.001) colistin (OR = 5.5; 95% CI 1.7–17.3; P = 0.004), sulfamethoxazole (OR = 8.7; 95% CI 3.87–19.70, P < 0.001) and trimethoprim (OR = 8.4; 95% CI 3.33–21.04; P < 0.001).

Resistance profile of *E. coli* from diagnostic submissions

In the 143 *E. coli* isolates from swine, 136 originated from post-mortem organ material and seven isolates from animals with diarrhoea. Among the 83 *E. coli* isolates 15 different serotypes were determined. Serotyping did not show results among the rest of 60 *E. coli* isolates. The most common serotype was K88 (n = 38), followed by O138 (n = 14) and O149 (n = 12).

Out of the 63 *E. coli* isolates from dairy cattle, 18 originated from calves with signs of diarrhoea, and 45 were post-mortem samples. Among the 63 *E. coli* isolates from cattle, serotypes were confirmed in 22 isolates, where the most frequent serotype was O26.

Escherichia coli isolates from clinical submission showed the most prevalent resistance against sulfamethoxazole (68.6%), tetracycline (60.2%), streptomycin (54.6%), ampicillin (53.9%) and trimethoprim (53.9%). *E. coli* isolates from cattle clinical submissions were also mainly resistant to streptomycin (63.5%), sulfamethoxazole (60.3%), tetracycline (58.8%), ampicillin (58.7%) and trimethoprim (55.6%) (Table 1).

The resistance against gentamycin was significantly lower (OR = 0.17; 95% CI 0.06–0.47; P < 0.001) and

Table 1 Resistance of *Escherichia coli* isolates originating from faecal samples of healthy cattle and swine and clinical submissions collected from 2010 to 2015 in Estonia

Antimicrobial	Cut-off values for resistance (mg/L)*	Healthy animals				Diagnostic submissions			
		Dairy cattle (n = 171)		Swine (n = 120)		Dairy cattle (n = 63)		Swine (n = 143)	
		%	(95% CI)	%	(95% CI)	%	95% CI	%	95%
Ampicillin*H	> 8	*3.5*	*(0.8 to 6.3)*	*21.5*	*(14.3 to 29.1)*	58.7	(46.5 to 70.9)	53.9	(45.7 to 62.1)
Cephotaxime	> 0.5	1.2	(− 0.4 to 2.8)	2.5	(− 0.3 to 5.3)	7.9	(1.2 to 14.6)	4.2	(0.9 to 7.5)
Cephazidime	> 0.5	2.9	(0.4 to 5.4)	3.3	(0.1 to 6.5)	7.9	(1.2 to 14.6)	7.7	(3.3 to 12.1)
Streptomycin*H	> 16	*7.0*	*(3.2 to 10.8)*	*39.2*	*(30.5 to 40.8)*	63.5	(51.6 to 6.4)	54.6	(46.4 to 62.8)
Gentamycin*D	> 4	7.0	(3.2 to 10.8)	12.5	(6.6 to 18.4)	*20.6*	*(10.6 to 30.6)*	*5.6*	*(1.8 to 9.4)*
Kanamycin	> 16	8.8	(4.6 to 13.1)	10.0	(4.6 to 15.4)	0.0	NA	0.0	NA
Ciprofloxacin*H	> 0.06	*0.6*	*(− 0.6 to 1.8)*	*5.8*	*(1.6 to 10.0)*	38.1	(26.1 to 50.1)	32.2	(24.5 to 39.9)
Nalidixic acid*D	> 16	0.6	(− 0.6 to 1.8)	3.3	(0.1 to 6.5)	*17.5*	*(8.1 to 26.9)*	*32.2*	*(24.5 to 39.9)*
Tetracycline*H	> 8	*7.0*	*(3.2 to 10.8)*	*32.5*	*(24.1 to 40.9)*	58.5	(46.3 to 70.7)	60.2	(52.2 to 68.3)
Colistin*H	> 2	*2.4*	*(0.1 to 4.7)*	*11.6*	*(5.9 to 17.3)*	3.2	(− 1.6 to 7.6)	5.6	(1.8 to 9.4)
Chloramphenicol	> 16	2.4	(0.1 to 4.7)	5.8	(1.6 to 10.0)	9.5	(2.3 to 16.7)	18.2	(11.9 to 24.5)
Florfenicol	> 16	0.0	NA	0.8	(− 0.8 to 2.4)	0.0	NA	0.7	(− 0.7 to 2.1)
Trimethoprim*H	> 2	*3.5*	*(0.8 to 6.3)*	*22.4*	*(14.9 to 29.9)*	55.6	(43.3 to 67.9)	53.9	(45.7 to 62.1)
Sulfamethoxazole*H	> 64	*4.7*	*(1.5 to 7.9)*	*30.0*	*(21.8 to 38.2)*	60.3	(48.2 to 70.4)	68.5	(60.1 to 76.1)

* Swedres-Svarm 2015. Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. Solna/Uppsala ISSN 1650-6332, 117, Table 2.17

*H and *D Statistically significant difference ($P < 0.05$) between healthy dairy cattle and swine, and between dairy cattle's and swine's clinical submissions. Corresponding percentages are also presented in italic face

resistance against nalidixic acid significantly higher (OR = 2.24; 95% CI 1.07–4.72; P = 0.034) in swine *E. coli* isolates compare to cattle isolates.

Multidrug-resistance of *E. coli* isolates

The distribution of susceptible and MDR *E. coli* isolates from swine and cattle have shown in Table 2. The *E. coli* isolates from clinically healthy swine (n = 35; 29.2%) showed significantly higher multidrug resistance (OR = 11.2; 95% CI 4.23–29.22; P < 0.001) than the isolates from cattle (n = 6, 3.5%). The proportion of MDR isolates from clinical submission was very high both in cattle (n = 42; 66.7%) and swine (n = 93; 65.0%), without statistical differences.

Determination of ESBL- and AmpC-producing *E. coli*

All 16 *E. coli* isolates with cefotaxime and/or ceftazidime MIC above cut-off level were analysed for confirmation of ESBL and AmpC production. ESBL phenotype was confirmed in one *E. coli* isolate from clinically healthy cattle and in eight isolates from organ materials both from cattle and swine. Three *E. coli* strains out of nine exhibiting an ESBL phenotype was found to be the same genotype $bla_{\text{TEM-52C}}$. All these strains originated from swine organ material that was collected post mortem.

Table 2 Distribution of susceptible and multi-drug resistant *Escherichia coli* isolates in dairy cattle and swine

Number of antimicrobials	Clinically healthy animals		Diagnostic submissions	
	Dairy cattle (n = 171)	Swine (n = 120)	Dairy cattle (n = 63)	Swine (n = 143)
Number and proportion (%) of susceptible isolates				
Susceptible to all tested anti-microbials/antimicrobials classes*	135 (78.9)	40 (33.3)	12 (19.0)	19 (13.3)
Resistant to 1–2 antimicrobials/antimicrobial classes	29 (16.9)	46 (38.3)	9 (14.3)	31 (21.7)
Number and proportion (%) of multi-drug resistant isolates				
Resistant to 3–5 antimicrobials	6 (3.5)	33 (27.5)	40 (63.5)	85 (59.4)
Resistant to 6–8 antimicrobials	0	2 (1.7)	2 (3.2)	8 (5.6)

* Antimicrobial classes: Quinolones (ciprofloxacin and nalidix acid); Aminoglycosides (streptomycin, kanamycin, gentamycin); 3th–4th generation cephalosporines (cephotaxime + cefazidime), sulfamethoxazole + trimethoprim

In total, four strains representing AmpC phenotypes were found. One plasmid-encoded AmpC type β-lactamases producing *E. coli* from clinically healthy cattle was found to harbour the bla_{CMY-1} gene, and another from clinically healthy swine carried the bla_{CMY-2} gene.

Resistance profile of enterococci

Resistance of *E. faecalis* and *E. faecium* is presented in Table 3. Altogether 51 isolates from healthy cattle and 60 isolates from healthy swine were analysed.

Enterococci from both animal species were mainly resistant to tetracycline (33.3% in cattle, 40.4% in swine) and erythromycin (21.6% in cattle, 26.7% in swine). Enterococci from swine were also resistant to streptomycin (30.0%) and kanamycin (26.7%). Enterococci isolated from swine had a significantly higher resistance against streptomycin (OR = 4.0; 95% CI 1.46–11.14; P = 0.008) and kanamycin (OR = 8.9; 95% CI 1.91–41.66; P = 0.006) compared to isolates from cattle. The proportion of fully susceptible *Enterococcus* spp. isolates was 49% (n = 25) in cattle and 35% (n = 21) in swine. Multidrug resistance was significantly higher (OR = 4.4; 95% CI 1.17–16.78; P = 0.029) in swine isolates (n = 13) than in isolates that originated from cattle (n = 3).

Discussion

This study is the first broad-based overview of antimicrobial resistance of these animal pathogens in Estonia. Currently, there is an extensive movement of live animals and food of animal origin between countries and continents. Regarding the possible transfer of resistant microbes, overview of the situation in each region cannot be underestimated [10].

In our study, the proportion of resistant *E. coli* isolates and MDR *E. coli* isolates originating from healthy swine was higher than that of *E. coli* isolates that originated from healthy cattle. The resistance against tetracycline, ampicillin, streptomycin, sulfamethoxazole, trimethoprim, ciprofloxacin and colistin differed significantly. Monitoring programmes from Finland, the Netherlands and Denmark have also described higher resistance among the swine isolates [11–13].

Isolates originating from swine were more resistant to mainly orally administered antibiotics. For instance, doxycycline, ampicillin/amoxicillin and sulpha/trimethoprim have been used for the treatment of swine diseases in a large volume and over a long time period in Estonia [10], (unpublished data from the Estonian State Agency of Medicines). There are about 86,900 dairy cows (in total 448 farms) and 298,000 pigs (in total 217 farms) in

Table 3 Proportion of resistance of *Enterococcus faecalis* and *Enterococcus faecium* isolates originating from faecal samples of healthy cattle and swine in 2010–2015 in Estonia

Antimicrobial	Cut-off values for resistance (mg/L)*	Healthy animals			
		Dairy cattle (n = 51)		Swine (n = 60)	
		%	(95% CI)	%	(95% CI)
Ampicillin	> 4	0.0	NA	1.7	(− 1.6 to 5.0)
Erythromycin	> 4	21.6	(10.3 to 21.9)	26.7	(15.5 to 37.9)
Virginiamycin					
E. faecalis	> 32	1.9	(− 1.9 to 5.7)	5.0	(− 0.5 to 10.5)
E. faecium	> 4				
Gentamycin	> 32	1.9	(− 1.9 to 5.7)	1.7	(− 1.6 to 5.0)
Streptomycin**					
E. faecalis	> 512	*11.7*	*(2.9 to 20.5)*	*35.0*	*(22.9 to 47.1)*
E. faecium	> 128				
Kanamycin**	> 1024	*3.9*	*(− 1.4 to 9.2)*	*26.7*	*(1.5 to 37.9)*
Tetracycline	> 4	33.3	(20.4 to 46.2)	40.4	(27.6 to 52.4)
Chloramphenicol	> 32	1.9	(− 1.9 to 5.7)	6.7	(0.8 to 13.3)
Vancomycin	> 4	5.9	(− 0.63 to 9.4)	10.0	(2.4 to 17.6)
Narasin	> 2	3.9	(− 1.4 to 9.2)	3.3	(− 1.2 to 7.8)
Bacitracin	> 32	3.9	(− 1.4 to 9.2)	6.6	(0.4 to 13.3)
Linezolid	> 4	0.0	NA	1.7	(− 1.6 to 5.0)

* Swedres-Svarm 2015. Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. Solna/Uppsala ISSN 1650-6332, 117, Table 2.17

** Statistically significant difference (P < 0.05) between resistant *Enterococcus* spp. isolates from healthy dairy cattle and swine. Corresponding percentages are also presented in italics face

Estonia. In 2012–2016 the average amount of tetracycline used for the treatment of swine was about 1500 kg of pure active substance per year, for the treatment of cattle about 210 kg/year (data from the Estonian State Agency of Medicines). The same figures for ampicillin/amoxicillin were 2200/500 and for sulpha/trimethoprim 110/50, respectively. In Estonia, tetracyclines (including doxycycline), ampicillin/amoxicillin and sulpha/trimethoprim are authorised for oral treatment in swine and poultry, not in cattle (data from the Estonian State Agency of Medicines). Considering this we can say that in Estonia, there might be a link between the use of antibiotics and the level of resistance, and enteric bacteria in pigs are more often exposed to antibiotics than in cattle. There is a higher probability for commensal *E. coli* to become a reservoir of resistance when oral antibiotics are widely used in the swine farms. Several authors have confirmed that oral administration of antibiotics to pigs increases the level of antimicrobial resistance [14, 15] and there is a strong correlation between the use of antimicrobials and the extent of antimicrobial resistance in *E. coli* isolated from livestock [16, 17]. This could explain the high resistance of commensal *E. coli* strains isolated from healthy swine in our study, as oral antibiotics are not commonly used for the treatment of cattle in Estonia.

We found high resistance to ciprofloxacin and nalidixic acid in bacteria originating from diseased animals in both animal species. Enrofloxacin and other quinolones are still used quite extensively for the treatment of swine and cattle in Estonia (amounts of active ingredients 85/55 kg per year respectively (unpublished data from the Estonian State Agency of Medicines)). It is not in line with the local rules of prudent use of antimicrobials [18]. That could explain the high resistance to quinolones as there can be a link between the presence of antibiotics in the body and the number of resistant bacteria [19]. Monitoring of resistance to fluoroquinolones should be continued in future studies as well as resistance to virginiamycin and chloramphenicol—compounds which are not used in veterinary practice in Estonia and which resistance can be associated with the use of tetracyclines at low concentrations [20].

We found considerable phenotypic resistance to colistin in *E. coli* isolates from healthy swine. We did not investigate colistin genotypic resistance in this study. However, colistin resistance of swine *E. coli* should be focused in future studies as a plasmid carrying the colistin resistance gene *mcr-1* was isolated from a pig slurry sample in Estonia [21]. Some isolates of vancomycin resistant enterococci were isolated from healthy swine, the genotypic conformation and possible link with the use of antibiotics should be focused in future studies.

We did not analyse the difference in the resistance of *E. coli* isolates from healthy animals and diagnostic submissions because the origin and collection of that kind of material is different, and comparison may lead to biased conclusion, although higher number of resistant isolates among the clinical submissions were observed, which is in line with the results of other authors [12, 13, 22]. Isolates from clinical submission can be more frequently resistant than isolates from healthy animals because of the more frequent exposure to antimicrobials, and in veterinary practice we need to keep in mind that the use of antimicrobial agents may select bacteria carrying virulence genes [23].

Resistance of enterococci, as well as development of multidrug resistance was lower in cattle isolates compared to swine isolates, which is also reported in other investigations [11–13].

This was the first time in Estonia when the ESBL-producing *E. coli* harbouring the $bla_{\text{TEM-52C}}$ genotype was found in swine post-mortem tissue samples. TEM-52 and CTX-M are often the most dominant types of enzymes in swine in other countries [24–26]. Several studies [27–30] have reported that strains producing AmpC and ESBL are often resistant to multiple agents. As faecal carriage of plasmid-mediated AmpC β-lactamases was found in healthy swine and cattle, the possible development and transmission methods of antimicrobial resistance in cattle and swine must be investigated in future studies.

Conclusions

The highest percentages of drug resistance in isolates of *E. coli* were detected to streptomycin, tetracycline, sulfamethoxazole, trimethoprim, ampicillin and colistin.

The number of MDR *E. coli* isolates was significantly higher in clinically healthy swine compared to that in cattle. The antimicrobial resistance of *E. faecalis* and *E. faecium* to erythromycin and tetracycline was high in both animal species, in swine enterococci it was high also to streptomycin and kanamycin.

This broad-based overview of antimicrobial resistance of these animal bacteria creates a basis for the future investigations and analyses of the resistance development in Estonia. In light of this, we strongly recommend assessment of the treatment plans in the swine industry in Estonia in order to ensure the prudent use of antimicrobials and to minimise the potential spread of resistant bacteria from swine to the environment and to humans.

Authors' contributions

BA and PK planned the study, BA drafted the manuscript. LH was in charge of the laboratory analyses. TK and PK performed the statistical analysis. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Ethical approval was not required as all samples were collected within the framework of a national resistance monitoring program.

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III

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IN VITRO ANTIMICROBIAL RESISTANCE OF INTESTINAL *ESCHERICHIA COLI* AND ENTEROCOCCI IN CLINICALLY HEALTHY DOGS IN ESTONIA

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Abstract. The aim of this study was to estimate the antimicrobial resistance of intestinal *Escherichia coli* and enterococci and identify the risk factors that are associated with resistance of enteric microflora in clinically healthy dogs.

Fecal samples were collected from 86 clinically healthy dogs. Antibacterial susceptibility of *E. coli* and enterococci was determined using the disc diffusion assay on Mueller–Hinton agar.

E. coli was isolated in 68 of 86 (79.1 %) fecal samples, and *Enterococcus spp*. was isolated in 66 (76.7 %) cases. The resistance to at least one antimicrobial agent was found among 10.3 % (n = 7) of *E. coli* and 60.6 % (n = 40) of *Enterococcus* spp. isolates. No cefotaxime resistant *E. coli* and vancomycin resistant enterococci were found. The isolated enterococci were resistant to tetracycline (45.5 %) and ciprofloxacin (21. 2 %). Previous antibiotic treatment, dog age, bodyweight, living environment and travelling were not associated with the resistance of *E. coli* and enterococci.

This was the first study addressed to the issue of the resistance of indicator bacteria in dogs in Estonia. Although significant resistance to antibiotics was not detected and suspected risk factors did not influence the antimicrobial resistance, the potential transmission of resistant bacteria between animals and humans needs to be considered and investigated in future studies.

Keywords: antimicrobial resistance, intestinal microflora, dog

Introduction

Antimicrobial resistance is the most disturbing health problem in humans and veterinary medicine (Barton and Hart, 2001). The number of companion animals has been increasing steadily and the contact between humans and animals may promote the transmission of antimicrobial-resistant bacteria to humans (Tamang et al., 2012). Intestinal commensal bacteria, such as *Escherichia coli* (*E. coli*) and enterococci, are part of the normal enteric microflora. These bacteria act as indicators of antimicrobial selection pressure, and they may harbour a reservoir of antimicrobial resistance genes for pathogenic or zoonotic bacteria.

The resistance patterns of enteric bacteria change in response to increased antibiotic exposure (Houndt and Ochman, 2000; Maddison, et al., 2008). In dogs, after antibiotic treatment, the majority of fecal coliform rapidly developed a high level of antimicrobial resistance to enrofloxacin and amoxicillin (Boothe et al., 2011). The most active resistance among enterococci isolated from dogs and cats treated with antibiotics was observed for erythromycin and oxytetracycline, and considerable resistance was found to lincomycin, gentamycin and kanamycin (Kataoka et al., 2013). Therefore, the intestinal tract may be the basic site in organisms for the development and spread of resistant microbes (Harmoinen et al., 2004, Skurnik et al., 2006).

Antimicrobial resistance of indicator bacteria in clinically healthy animals has been studied primarily in pigs, cattle and poultry; most often during national antimicrobial monitoring programs. The antimicrobial resistance of normal microflora in healthy dogs is not often studied. The prevalence of resistant *E. coli* and enterococci is low in Finland and Canada (Rantala et al., 2004; Murphy et al., 2009), but a recent study from Portugal showed high resistance against several antibiotics (Leite-Martins et al., 2014). The colonization of healthy companion animals with different types of extended spectrum betalactamase (ESBL)-producing *Enterobacteriaceae* was found in some studies.. The frequency of isolation of ESBL ranged between 2.6 % and 3.8 % in the United Kingdom and Portugal to 12.2 % and 22 % in Tunisia and Kenya respectively (Costa et al., 2008; Wedley et al., 2011; Albrechtova et al., 2012; Sallem et al., 2013).

This study estimated the antimicrobial resistance of intestinal *E. coli* and enterococci in clinically healthy dogs in Estonia and identified the risk factors that are associated with the resistance of enteric microflora in healthy dogs.

Materials and methods

Data collection

The study was performed in clinically healthy dogs in Estonia. The dogs were selected randomly, with permission and interest of dog owners. The dogs were selected from those brought to veterinary clinics for vaccination or veterinary consultation. Only one (the oldest) dog from the same household was selected. The first inclusion criterion was that dogs were not treated with antimicrobials during the last three months. The data on the dogs' health, living environment and travelling history were collected from the dog owners. The information about the previous (last two years) antibiotic treatments was collected from the databases of veterinary clinics.

Description of the study group

A total of 86 dogs (53 females and 33 males) of 39 different breeds were included in the study. One dog was excluded due to fever (39.8ºC). Twenty-six dogs were of mixed breeds. The average age of dogs in the study group was 5.7 years (min. 3 month; max. 15 years), and the average bodyweight was 23.4 kg (min. 3.5 kg; max. 58 kg).

Clinical examination of dogs and collection of fecal samples

All dogs were examined clinically before fecal sample collection. Only clinically healthy dogs were included in the study: body temperature less <39.0ºC, heart rate <120/min, respiratory rate <30/min, and no visible enlargement of the main lymph nodes (Rijnberk et al., 2009). Five grams of fecal sample was collected immediately after defecation using a sterile spoon and collection tube. Fecal samples were placed in the refrigerator (+2-4 ºC) initially and thereafter stored at -80ºC. All collected fecal samples were sent to the Estonian Veterinary and Food Laboratory for the bacteriological analysis.

Laboratory analysis

The identification of *E. coli* and enterococci was performed according to accredited methods in the Estonian Veterinary and Food Laboratory.

The material for identification of *E. coli* was inoculated directly to eosin methylene blue (EMB) agar. The colonies of *E. coli* were identified based on the occurrence of a green-metallic sheen that appears on the surface of the bacterial colonies after incubation at 37ºC overnight.

For identification of *Enterococcus faecalis/faecium,* an enrichment broth agar (6.5% NaCl Brain Heart Infusion (BHI)) incubated at 37°C overnight was used for the stabilization of enterococci before cultivation of into Slanetz–Bartley agar.

Up to four colonies with typical morphology of *E. coli*, *E. faecalis* / *E. faecium* were sub-cultivated on blood agar. Colonies were identified according to laboratory protocols. All pure isolates of *E. coli*, *E. faecium* and *E. faecalis* were stored (-80°C) for antimicrobial susceptibility testing.

Antimicrobial susceptibility

Antibacterial susceptibility was determined using the disc diffusion assay on Mueller–Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute protocols M02-A11 and VET01-A4. Quality control strains of *E. coli* ATCC® 25922 and *Enterococcus faecalis* ATCC 29212 were included with each batch of isolates tested. Epidemiological cut-off values (ECOFF) issued by the EUCAST (http://www.escmid.org) were used to interpret the results of susceptibility testing of indicator bacteria (*E. coli* and enterococci). The clinical breakpoints recommended for animal pathogens by CLSI VET01-S2 and M100-S22 were considered in the absence of EUCAST-issued ECOFFs.

The antimicrobial susceptibility of *E. coli* was tested for ampicillin, gentamycin, streptomycin, kanamycin, trimethoprim, sulfomethoxasole, tetracycline, nalidic acid, ciprofloxacin, cefotaxime and ceftazidime. The antimicrobial susceptibility of enterococci was tested for ampicillin, erythromycin, gentamycin, tetracycline, chloramphenicol, vancomycin, ciprofloxacin and linezolid.

The criteria for the interpretation of zone diameter used in this study are described in Table 1. Isolates with phenotypically identified acquired resistance to three or more antimicrobial classes were defined as multiresistant (Schwarz et al., 2010).

Data analysis

Stata 10.0 (StataCorp, Texas, USA) software was used for statistical analyses. A logistic regression model with backward elimination procedure was used to identify associations between the resistance of enterococci and *E. coli* and different risk factors. The resistance of enterococci and *E. coli* was an outcome variable. Estimated (anticipated) risk factors were categorized before statistical analyses. The full model includes the following parameters: dog age as a 4-level category variable (less than 1 years, 1–5 years, 5–10 years, more than 10 years), dog bodyweight as a 4-level category variable (less than 10 kg, 10–25 kg, 25–40 kg, 40–60 kg), living environment as a two-level variable (living inside but going out; living only outside), visiting another country in the last year (yes, no) and visit to veterinary clinics (yes, no). The Wald test was used to evaluate the overall significance of the categorical variables with more than two levels. Odds ratios (OR) with 95 % confidence intervals (95 % CI) were calculated. Statistical significance was set at $p \leq 0.05$.

Results

Occurrence of antimicrobial resistance

E. coli was isolated in 68 of the 86 (79.1 %) fecal samples, and *Enterococcus spp*. was isolated in 66 (76.7 %) cases. Resistance to at least one antimicrobial agent was found among 10.3 % (n = 7) of *E. coli* and 60.6 % (n = 40) of *Enterococcus* spp. isolates. Two *E. coli* and two *Enterococcus spp*. isolates were multiresistant.

All *E. coli* isolates were susceptible to cefotaxime and ceftazidime. Three (4.4 %) *E. coli* isolates were resistant to ampicillin and streptomycin, and two (2.9 %) of the isolates showed resistance against tetracycline, ciprofloxacin and sulfamethoxazole. In total, 45.5 % (n =

30) of enterococci were resistant to tetracycline, 21.2 % (n = 14) to ciprofloxacin and 10.6 % (n = 7) to erythromycin. None of isolated enterococci were resistant to vancomycin.

Risk factors of antimicrobial resistance

Sixteen of the 86 dogs (18.6 %) lived only outdoors, and 70 (81.4 %) lived indoors but walked outside regularly. Out of the 86 dog owners, 28 % (n = 24) had visited other countries during the last year. The main regions visited were Scandinavia and western parts of Europe. During last three years, 76.7 % (n = 66) of dogs have visited veterinary clinics, and 66.7 % (n = 44) of these dogs were treated with antibiotics. Health records and information on antibiotic treatment were available on 36 (87.8 %) dogs. The main purpose for antimicrobial treatment was traumas and urogenital tract infections (19.4 %), followed by an equal proportion (13.9 %) of ear and skin infections and respiratory infection. The most frequently used antibiotics were amoxicillin in combination with clavulane acid (83.3 %) and cephalosporins (19.4 %). The antibiotic resistance and anticipated risk factors are presented in Table 2.

Table 1. **Zone diameter interpretive criteria**

Concentration of AB in disc (µg)	*E. coli*		*Enterococcus* spp.	
	R < mm	R ≤ mm	R < mm	R ≤ mm
	EUCAST ECOFF	CLSI	EUCAST ECOFF	CLSI
Ampicillin 2 µg	–	–	10	–
Ampicillin 10 µg	14	–	–	–
Erythromycin 15 µg	–	–	–	13
Gentamycin 10 µg	16	–	–	–
Gentamycin 30 µg	–	–	8	–
Kanamycin 30 µg	–	13	–	–
Chloramphenicol 30 µg	17	–	–	12
Linezolid10 µg	–	–	19	–
Nalidic acid 30 µg	19	–	–	–
Streptomycin 10 µg	–	11	–	–
Sulfamethoxazole 250 mg	–	12	–	–
Tetracycline 30 µg	–	11	–	14
Trimethoprim 5 µg	20	–	–	–
Cefotaxime 5 µg	23	–	–	–
Ceftazidime 10 µg	22	–	–	–
Ciprofloxacin 5 µg	25	–	–	15
Vancomycin 5 µg	–	–	12	–

Table 2. **The anticipated risk factors and antibiotic resistance of isolated enterococci** (n=66)

	Number (%) of resistant isolates	p-value	Wald-test p-value
Total number of isolated enterococci (n=66)	40 (60.6)		
Previous antibiotic treatment			
No (n=35)	22 (62.9)		
Yes (n=31)	18 (58.1)	0.93	
Visit abroad			
No (n=47)	30 (63.3)		
Yes (n=19)	10 (52.6)	0.44	
Dog weight			0.91
<10 kg (n=17)	10 (58.8)		
>10-25 kg (n=20)	13 (65)	0.94	
>25-40 kg (n=19)	10 (52.6)	0.68	
>40-60 kg (n=10)	7 (70)	0.74	
Age of dogs			0.73
< 3 year (n=16)	11 (68.8)		
>3-5 year (n=21)	14 (66.7)	0.74	
>6-10 year (n=19)	10 (52.6)	0.42	
>11-15 year (n=10)	5 (50)	0.32	
Dog living environment			
Inside, walking outside (n=51)	31(60.8)	0.73	
Only outside (n=15)	9(60)		

We did not find any significant associations between resistance of enterococci and *E. coli* and anticipated risk factors. Only seven (10.3 %) *E. coli* isolates out of 68 were resistant to antibiotics and any of estimated risk factors did not associate ($p < 0.05$) with antimicrobial resistance of *E. coli.*

Discussion

The present study investigated the antimicrobial resistance of normal enteric microflora in clinically healthy dogs. The number of microbial isolates is quite small and the results of that investigation do not represent the antimicrobial resistance situation in dog's population in Estonia, but it gives a preliminary standpoint for future discussion and investigations. The antimicrobial resistance of *E.coli* was generally low in our study, which is consistent with other studies (Costa et al., 2008), but resistance among enterococci was prevalent. *E.coli* strains developed resistance against ampicillin, streptomycin and trimethoprim, and fecal enterococci were primarily resistant to tetracycline, erythromycin and ciprofloxacin. No cefotaxime-resistant *E. coli* or vancomycin-resistant enterococci were found in this study. The prevalence of cephalosporin-resistant *E. coli* varies between countries: 12 % in Canada (Murphy et al., 2009); 6 % in the USA (Shaheen et al. 2011); 5 % in Finland (Jalava et al. 2012); and 40.9 % in Croatia (Šeol et al. 2011). Other published studies reported that the prevalence of VRE in dogs in Spain was 17 % and 26 % in the Netherlands (Herrero et al., 2004; Van Belkum et al., 1996). Despite the fact that we did not find ESBL or VRE in this study, a great attention should be paid to these pathogens in future resistance monitoring.

The disc diffusion method for *in vitro* antimicrobial susceptibility testing was used in this study. This method is widely used for determinations of susceptibility of animal pathogens, especially in clinical work to determine the correct antimicrobial treatment. The primary disadvantage of this method in monitoring resistance development is that outcomes are reported on a qualitative basis (sensitive, intermediate or resistant), and subtle changes in susceptibility may not be noticeable. Therefore, comparisons of the results of studies using different methods for susceptibility testing are not acceptable (Schwarz et al., 2010).

Due to low number of resistant *E .coli* isolates, an evaluation of estimated risk factors and resistance by logistic regression model was not possible. We did not find an association between previous antibiotic treatment and antimicrobial resistance of enterococci. Rantala et al. (2008) also did not report an association between antimicrobial treatment and the resistance of enterococci, but *E. coli* resistance against a combination of sulfonamide-trimethoprim, streptomycin and amoxicillin was higher in dogs treated with antibiotics, although these associations were not statistically significant. Another study demonstrated that the resistance to beta-lactams was more common in fecal *E. coli* strains isolated from cefovecin-treated dogs compared to untreated dogs, but the resistance of enterococci was not altered (Lawrence et al., 2013). Previous scientific publications confirmed that dogs with a history of antimicrobial therapy in the past year had a higher risk of being carriers of ESBL-producing and plasmidic AmpC betalactamase-producing *E. coli* (Belas, et al. 2014). The retrospective data in the present study showed that antibiotics were primarily prescribed after clinical diagnosis, but data on bacteriological investigations were missing. The most frequently chosen antibiotics were beta-lactams, but no conclusion can be reached because of the small sample size and large variation. Shea et al. (2012) showed that doxycycline was prescribed in 58.8 % of cases without a clinical diagnosis, antibiotics were prescribed without infection in 38.4 % of cases, and antibiotics were used after a documented bacteriological diagnosis only in 17.5 % of cases. These results confirm that antibiotic treatment should be prescribed only when bacterial infection is expected based on clinical examinations or bacterial infection is diagnosed in the laboratory.

The resistance of *E.coli* against tetracycline was low in our study, but we found high resistance among the enterococci isolated from dogs that were not treated with tetracycline. Many studies show a high tetracycline resistance of *E.coli* isolated from the intestines of healthy dogs (Costa et al., 2008; Leener et al., 2005; Türkyilmaz et al., 2010; Damborg et al., 2008; Jackson et al., 2009), but an association between tetracycline treatment in dogs and the development of resistance was not found (Damborg et al., 2008). Tetracycline resistance is encoded by the *tet*M gene, which has a wide area of distribution and occurs in both Gram positive and Gram negative bacteria (Roberts, 1996). This characteristic allows resistance to tetracycline to be transferred from one bacterial strain or animal species to another. One possible route of distribution is food contaminated with resistant bacteria or distribution via the environment (Wu et al., 2013). Possible links between tetracycline resistant environmental bacteria and resistance of normal enteric microflora of dogs should be studied in the future.

Dog age and bodyweight was not a significant risk factor for resistance of enterococci ($p > 0.05$). Rantala et al. (2004) also did not find a significant association between dog age and the development of resistance. In addition, we did not find an association between dog living environment and resistance of enteric microflora. Only a few previous studies confirmed that the resistance of normal gut microflora is higher in dogs that lived in the country compared to dogs that were kept in town (Monaghan et al., 1981). Procter (2012) showed that *E. coli* strains isolated from large half-breed dogs showed higher resistance compared to strains isolated from small purebred dogs, and the dogs' living environments may play a role here. Skurnik et al. (2006) found that 17 % of the *E. coli* isolates from wild animals living in a low human density area were resistant to at least one antibiotic versus 49 % of isolates from wild animals living in a higher human density area. The potential threat posed by animals or animal food products as sources for resistant isolates cannot be ignored, but the current research has not identified the extent that livestock and pets contribute to the spread of resistance in human microflora.

Conclusions

In this study, *E. coli* and enterococci as a part of the normal enteric microflora of dogs did show different resistance to antibiotics, but the association between antimicrobial resistance and suspected risk factors was not proven.

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Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia

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Abstract

Background: The goal of this study was to estimate the distribution of udder pathogens and their antibiotic resistance in Estonia during the years 2007-2009.

Methods: The bacteriological findings reported in this study originate from quarter milk samples collected from cows on Estonian dairy farms that had clinical or subclinical mastitis. The samples were submitted by local veterinarians to the Estonian Veterinary and Food Laboratory during 2007-2009. Milk samples were examined by conventional bacteriology. *In vitro* antimicrobial susceptibility testing was performed with the disc diffusion test. Logistic regression with a random herd effect to control for clustering was used for statistical analysis.

Results: During the study period, 3058 clinical mastitis samples from 190 farms and 5146 subclinical mastitis samples from 274 farms were investigated. Positive results were found in 57% of the samples (4680 out of 8204), and the proportion did not differ according to year ($p > 0.05$). The proportion of bacteriologically negative samples was 22.3% and that of mixed growth was 20.6%. *Streptococcus uberis* (*Str. uberis*) was the bacterium isolated most frequently (18.4%) from cases of clinical mastitis, followed by *Escherichia coli* (*E. coli*) (15.9%) and *Streptococcus agalactiae* (*Str. agalactiae*) (11.9%). The bacteria that caused subclinical mastitis were mainly *Staphylococcus aureus* (*S. aureus*) (20%) and coagulase-negative staphylococci (CNS) (15.4%). The probability of isolating *S. aureus* from milk samples was significantly higher on farms that had fewer than 30 cows, when compared with farms that had more than 100 cows ($p < 0.005$). A significantly higher risk of *Str. agalactiae* infection was found on farms with more than 600 cows ($p = 0.034$) compared with smaller farms. The proportion of *S. aureus* and CNS isolates that were resistant to penicillin was 61.4% and 38.5%, respectively. Among the *E. coli* isolates, ampicillin, streptomycin and tetracycline resistance were observed in 24.3%, 15.6% and 13.5%, respectively.

Conclusions: This study showed that the main pathogens associated with clinical mastitis were *Str. uberis* and *E. coli*. Subclinical mastitis was caused mainly by *S. aureus* and CNS. The number of *S. aureus* and *Str. agalactiae* isolates depended on herd size. Antimicrobial resistance was highly prevalent, especially penicillin resistance in *S. aureus* and CNS.

Background

Bovine mastitis is the most common disease in dairy cows worldwide, and antimicrobial therapy is the primary tool for the treatment of mastitis. The prevalence of mastitis pathogens and their antimicrobial resistance have been investigated in numerous studies around the world. The main pathogens that cause subclinical mastitis are coagulase-negative staphylococci (CNS), *Corynebacterium bovis* (*C. bovis*) and *Staphylococcus aureus* (*S. aureus*) [1-5]. Coliforms, *Streptococcus uberis* (*Str. uberis*) and *S. aureus* are the pathogens isolated most frequently from clinical mastitis samples [6-8]. *Streptococcus agalactiae* (*Str. agalactiae*) has been largely eradicated from herds in Europe [3], but in studies from the United States, 7.7% and 13.1% of samples contained *Str. agalactiae* [9,10].

Several methods, such as disc diffusion, agar dilution, broth dilution and broth microdilution are suitable for *in vitro* antimicrobial susceptibility testing. Depending on the study design and the methodology used, the antimicrobial susceptibility of udder pathogens varies greatly between studies. For example, studies from France and

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the UK have reported a high prevalence of penicillin-resistant *S. aureus* (36.2%, 56%) [11,12], whereas a low percentage of resistant isolates (4-9%) were found in the Netherlands and Norway [13,14]. The streptococci that cause mastitis are susceptible to β-lactam antibiotics; however, resistance to macrolides and lincosamides is notable [13,15]. *In vitro* resistance of *E. coli* to different antimicrobials has been reported to be low [13,14,16,17].

National studies of mastitis prevalence provide important information through the monitoring of national udder health status, and they enable national guidelines to be developed for the prudent use of antibiotics in each country [18]. During recent decades, only broad-spectrum antibiotics have been used for the treatment of clinical mastitis in Estonia. For example, in the years 2006-2009, 15 different combinations of antibiotics were available for use in 18 intramammary preparations that were authorised by the Estonian State Medical Agency [19]. Given that a large overview of udder pathogens and their antibiotic resistance has not been performed in Estonia, the goal of this study was to estimate the distribution of udder pathogens and their antibiotic resistance during the years 2007-2009 in Estonia.

Methods

Sample collection

Milk samples were submitted to the Estonian Veterinary and Food Laboratory during the period 2007-2009. Quarter milk samples were collected from cows on Estonian dairy farms by local veterinarians or farmers. Clinical mastitis was diagnosed when visible abnormalities of udder (swelling) were detected or milk from a quarter had abnormal viscosity (watery, thicker than normal), colour (yellow, blood-tinged) or consistency (flakes or clots) [20]. Normal milk appearance, together with a positive California Mastitis Test result (score greater than 1), was used to make a diagnosis of subclinical mastitis.

The samples were sent to the laboratory either for isolation of the clinical mastitis pathogen and determination of its antimicrobial susceptibility or to determine the reason for an increased somatic cell count.

Laboratory analysis

Bacterial species were identified using accredited methodology based on the National Mastitis Council [21] standards. From each sample, 0.01 ml of milk was cultured on blood-esculin agar and incubated for 48 h at 37°C. The plates were examined after 24 and 48 h of incubation. A minimum of five colonies of the same type of bacterium was recorded as bacteriologically positive, and growth of more than two types of bacterial colonies was categorised as mixed growth. No bacterial growth was recorded when fewer than five colony-forming units were detected during 48 h of incubation.

Once they had been isolated and identified, pure cultures of udder pathogens were tested for antibacterial susceptibility with the disc diffusion assay on Mueller-Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) document M31-A2 in the years 2007-2008 and M31-A3 in 2009 [22,23]. Quality control strains, *S. aureus* ATCC® 25923, *E. coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Streptococcus pneumoniae* ATCC® 49619, were included with each batch of isolates tested. The antimicrobial susceptibility of Gram-positive bacteria was tested with penicillin, ampicillin, cephalothin, clindamycin, erythromycin, gentamycin, trimethoprim/sulfa and tetracycline. The antimicrobial susceptibility of Gram-negative bacteria was tested with ampicillin, gentamycin, trimethoprim/sulfa, tetracycline, enrofloxacin, streptomycin, neomycin and cefaperazone. The list of antibiotics in susceptibility testing may vary, different veteriarians preferred different set of antibiotics in order to find accurate treatment after getting the laboratory test results.

The criteria for the interpretation of zone diameter used in this study are described in Table 1.

Data analysis

The farm, herd size and year were recorded and categorised before statistical analysis. A logistic regression model with a random herd effect for the control of clustering was used for all of the analyses in this study. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. Statistical significance was set at $p \leq 0.005$.

The influence of milk samples with mixed growth or no bacterial growth on the occurrence of clinical or subclinical mastitis was assessed. Potential interactions (no growth or mixed growth × year) were assessed in the logistic regression model. The effects of herd size and year on the pathogens that caused clinical and subclinical mastitis were analysed. These analyses were conducted using Stata 10.2 [24].

Results

Isolation of mastitis pathogens

During the study period, 3058 clinical mastitis samples from 190 farms and 5146 subclinical mastitis samples from 274 farms were investigated (Table 2).

Positive results were found in 57% of the samples (4680 out of 8204), and this proportion did not differ according to year ($p > 0.05$). The proportion of bacteriologically negative samples was 22.3% and that of mixed growth 20.6%. There was a significantly higher chance (OR = 1.15, 95% CI = 1.01, 1.33, p = 0.042) of finding bacteriologically negative samples in presence of subclinical mastitis (n = 1317, 25.6%) in comparison with

Table 1 Zone diameter intepretive criteria

Disc content in µg	*Staphylococcus* spp.			*Streptococcus* spp.			*Enterococcus* spp.			*Enterobacteriaceae* spp.		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin 10 µg	≥ 29	-	≤28	≥ 26	19-25	≤18	≥ 17	-	≤16	≥ 17	15-16	≤14
Penicillin 10 µg	≥ 29	-	≥ 29	≥24	-	-	≥15	-	≤14	-	-	-
Cephalothin 30 µg				≥		≤	-	-	-			
Cefaperazone 75 µg	-	-	-	-	-	-	-	-	-	≥21	16-20	≤15
Clindamycin 2 µg	≥ 21	15-20	≥ 14	≥19	16-18	≤15	-	-	-	-	-	-
Erythromycin 15 µg	≥ 23	14-22	≥ 14	≥21	16-20	≤15	-	-	-	-	-	-
Gentamycin 10 µg	≥ 12	13-14	≥ 15	≥12	13-14	15≤	≥10	7-9	≤6	≥ 12	13-14	≥ 15
Tetracycline 30 µg	≥ 19	15-18	≥ 14	≥23	19-22	≤18	≥19	15-18	≤14	≥ 19	15-18	≥ 14
Enrofloxacin 5 µg										≥ 20	15-19	≤14
Trimethoprim/sulfa 1,25/23,75 µg	≥ 16	11-15	≥ 10	≥16	11-15	≤10	≥16	11-15	≤10	≥ 16	11-15	≥ 10

clinical mastitis (n = 554, 16.8%). The probability of obtaining mixed growth from milk samples was also significantly higher (OR = 2.2, 95% CI = 1.9, 2.6, p < 0.001) if subclinical mastitis was found. The distribution of bacterial species isolated from samples from cows with clinical and subclinical mastitis is shown in Table 3. Among the bacteriologically positive (n = 2016) clinical mastitis samples, *Str. uberis* was the bacterium isolated most frequently (n = 371; 18.4% of the positive samples), followed by *E. coli* (n = 321; 15.9%) and *Str. agalactiae* (n = 293; 11.9%). *S. aureus* (n = 532; 20%) and CNS (n = 411; 15.4%) were the bacteria isolated most commonly from milk in cases of subclinical mastitis, followed by *Corynebacterium* spp. (n = 395; 14.8%).

The probability of isolating *S. aureus* from milk samples was significantly higher on farms that had fewer than 30 cows, when compared with farms with more than 100 cows (OR = 0.2, 95% CI = 0.11, 0.53, p < 0.005). Also, there was a significantly higher risk of diagnosing *Str. agalactiae* on farms with more than 600 cows (OR = 17.6, 95% CI = 1.2, 259.1, p = 0.034) compared with smaller farms.

Table 2 Distribution of milk samples according to herd size

	Clinical mastitis				Subclinical mastitis			
Farm size category	Farms	%	Samples	%	Farms	%	Samples	%
1 (1-30 cows)	54	28.4	98	3.2	41	15	86	1.7
2 (31-99 cows)	35	18.4	149	4.9	51	18.6	268	5.2
3 (100-299 cows)	40	21.1	378	12.4	53	19.3	541	10.5
4 (300-599 cows)	44	23.2	1472	48.1	80	29.2	2426	47.1
5 (> 600 cows)	17	8.9	961	31.4	49	17.9	1825	35.5
Total	190	100	3058	100	274	100	5146	100

Antimicrobial susceptibility testing

The percentage of *S. aureus* isolates resistant to penicillin and ampicillin was 61.4% and 59.5%, respectively. In addition, CNS showed resistance to penicillin and ampicillin (38.5% and 34.4%), but resistance to erythromycin and lincomycin was also common (14.9% and 17.6%). Six isolates (3.8%) of *S. aureus* and three isolates (3.6%) of CNS were resistant to cephalothin (Table 4).

All streptococci (Table 5) were susceptible to penicillin, ampicillin and cephalothin, except for one isolate of *Str. uberis*. Of the 90 isolates of *Str. dysgalactiae*, 19.8% were classified with intermediate susceptibility and 32.2% with resistance to tetracycline. Of a total of 151 isolates of *Str. uberis*, 7.3% with intermediate susceptibility and 14.3% with resistance to tetracycline were recorded. Among the *E. coli* isolates (Table 6), the highest percentage of isolates showing intermediate susceptibility and resistance were observed with ampicillin, neomycin, streptomycin and tetracycline. *E. coli*. was 98.4% susceptible to enrofloxacin and 100% to cefaperazone.

Discussion

The results of the present study were based on an analysis of milk samples submitted to an Estonian National Veterinary Laboratory over a three-year period. The laboratory protocols did not change during the study period. Of the samples investigated, 22.3% were bacteriologically negative. Several other studies have also demonstrated bacteriologically negative findings in 17.7-26.5% cases of clinical mastitis [12,25] and as many as 28.7-38.6% of subclinical mastitis [12,26], which is in line with our results. The possible reasons for bacteriologically negative findings in milk samples could be the presence of antibacterial substances in the milk that lead to a decrease in the viability of bacteria in the culture [27], or failures in conventional culture compared with identification of bacteria using the real-time polymerase chain reaction [28].

Table 3 Distribution of bacterial species isolated from clinical and subclinical mastitis samples in 2007-2009

	Clinical mastitis			Subclinical mastitis		
Bacteria	**2007 (n = 598)**	**2008 (n = 692)**	**2009 (n = 726)**	**2007 (n = 939)**	**2008 (n = 1063)**	**2009 (n = 661)**
S. aureus	11.7	11.7	11.7	19.2	22.8	16.6
CNS	4.8	7.1	8.5	16.1	13.6	17.4
CPS*	3.8	3.3	1.6	4.6	2.8	5.1
Str. agalactiae	9.0	11.3	14.7	13.6	9.0	10.7
Str. dysgalactiae	8.0	7.8	7.2	3.6	4.0	5.6
Str. uberis	16.1	21.8	17.1	10.2	12.3	12.9
Str. spp	3.2	3.3	1.9	1.2	2.0	2.7
Lactococcus lactis	10.9	3.9	5.7	8.9	8.2	3.9
E. coli	14.4	16.6	16.5	1.6	2.0	3.8
Klebsiella spp.	7.0	1.3	2.3	0.7	0.6	0.9
Enterococcus spp.	1.3	2.3	1.1	1.5	2.8	4.2
Corynebacterium spp.	2.2	2.6	5.0	16.5	17.3	8.5
A. pyogenes	2.2	3.8	3.6	0.1	0.6	0.6
Pseudomonas spp.	1	0.3	0.3	0	0	0.6
Proteus spp.	0.2	0	0.2	0.4	0.1	0.6
Yeast	2.3	2	1.6	1.5	1.6	5.6
Other	1.8	0.9	1	0.3	0.3	0.3
Total	100%	100%	100%	100%	100%	100%

* CPS: coagulase-positive staphylococci (other than *S. aureus*).

In the present study, *E. coli* and *Str. uberis* were the pathogens isolated most frequently from clinical mastitis, while *S. aureus*, CNS and *Corynebacterium* spp. caused mainly subclinical mastitis. The same results were shown in an Estonian study ten years ago, where *C. bovis* (47.5%), *S. aureus* (21%) and CNS (15.8%) were the pathogens isolated most commonly from cases of subclinical mastitis [29]. The isolation rate of *Str. agalactiae* was surprisingly high in our study.

We found a strong association between the isolation of *Str. agalactiae* and very large-scale farms. In total, there are 98000 dairy cows in Estonia and the mean herd size is 88 cows [30]. Rapid changes in management style (from tie-stalls to free-stalls) have occurred during the last eight years, which may explain the coexistence of environmental pathogens together with *Str. agalactiae*. Although teat disinfection and dry cow therapy is a common routine on Estonian dairy farms, proper eradication programmes for *Str. agalactiae* have not been employed. In contrast, an increased probability of finding *S. aureus* was correlated with farms with fewer than 30 cows. The average age of cows on small farms was 5.3 years, compared with 4.3 years on farms on which more than 300 cows were kept [30]. The culling policy may be different, and the owners of smaller farms may keep (possibly chronically infected) cows in the herd for a longer period of time.

Table 4 Antimicrobial susceptibility of staphylococci isolated from bovine clinical mastitis

	S. aureus				CNS			
Disc content in μg	**n**	**S* (%)**	**I * (%)**	**R* (%)**	**n**	**S* %**	**I * (%)**	**R* (%)**
Ampicillin10 μg	173	40.5	-	59.5	91	61.5	-	38.5
Penicillin10 μg	174	38.6	-	61.4	93	65.5	-	34.4
Cephalothin 30 μg	160	96.2	-	3.8	84	96.4	-	3.6
Clindamycin 2 μg	169	81.9	0	18.1	91	82.4	0	17.6
Erythromycin15 μg	83	95.2	0	4.8	47	85.1	0	14.9
Tetracycline 30 μg	147	95.9	0	4.1	86	88.4	0	11.6
Trimethoprim/sulfa 1.25/ 23.75 μg	162	96.6	0	3.4	76	97.4	0	2.6
Gentamycin 10 μg	146	93.2	0	6.8	69	98.6	0	1.4

* Propotion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

Generally in our study, the *in vitro* antimicrobial resistance of the isolates examined from samples of clinical

The disc diffusion method for *in vitro* antimicrobial susceptibility testing was used in this study. This technique is the most widely used method for determination of the susceptibility of animal pathogens, especially in clinical work when it is necessary to determine the correct treatment. The primary disadvantage of using this method when monitoring development of resistance is that outcomes are reported on a qualitative basis (sensitive, intermediate, or resistant), and subtle changes in susceptibility may not be apparent. Therefore any comparison with studies that use other methods of susceptibility testing is not acceptable [31].

Table 5 Antimicrobial susceptibility of streptococci isolated from bovine clinical mastitis

	Str. agalactiae				*Str. dysgalactiae*				*Str. uberis*			
Disc content in µg	**n**	**S* (%)**	**I* (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**
Ampicillin 10 µg	162	100	-	0	111	100	0	0	265	99.6	0	0.4
Penicillin 10 µg	168	100	-	0	111	100	0	0	267	99.6	0	0.4
Cephalothin 30 µg	143	100	-	0	101	100	0	0	254	99.6	0	0.4
Clindamycin 2 µg	161	91.9	1.9	6.2	115	92.2	0	7.8	273	92	1.4	6.6
Erythromycin 15 µg	77	96.1	2.6	1.3	60	88.3	5	6.7	134	89.6	2.2	8.2
Tetracycline 30 µg	151	78.1	7.3	14.6	90	48.9	18.9	32.2	234	79.9	3.4	19.7
Trimethoprim/sulfa 1.25/23.75 µg	140	93.6	0	6.4	103	99	0	1	223	95.9	0.9	3.2
Gentamycin 10 µg	143	63.6	11.9	24.5	88	88.6	0	11.4	210	71.9	9.5	18.6

* Propotion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

mastitis were high. Isolates of *S. aureus* had an alarming level of resistance to penicillin (61.4%) and ampicillin (59.5%), whereas CNS exhibited a lower degree of resistance to penicillin and ampicillin (38.5%; 34.4%). The reported percentages for penicillin resistant *S. aureus* in cases of clinical mastitis, detected by the disc diffusion method, are 50.4% and 35.4% in the USA [10,32], 63.3% in Turkey [33] and 12% in Northern Germany [34]. In addition, cephalothin resistance among staphylococci was found in our study. Although reports of methicillin-resistant staphylococci causing bovine mastitis are rare, those samples found in our study need further investigation in order to prove or exclude the presence of the *mecA* gene. In the present study, both staphylococci and streptococci showed resistance to erythromycin and lincomycin, but the figures for resistance in annual reports from some other countries show a low prevalence of lincomycin and erythromycin resistance in *S. aureus* and CNS [13,14,35]. Given that *S. aureus* and CNS were the pathogens isolated most frequently from cases of subclinical mastitis, one possible explanation for resistance to several antibiotics may be the collection and submission to the laboratory of milk samples from chronic clinical mastitis (which demonstrate poor treatment efficacy). Therefore, random sampling strategies should be used to provide a good evaluation of antimicrobial susceptibility.

Table 6 Antimicrobial susceptibility of *E. coli* and *Klebsiella* spp. isolated from bovine clinical mastitis

	E. coli				*Klebsiella* spp.			
Disc content in µg	**n**	**S* (%)**	**I * (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**
Ampicillin 10 µg	201	68.7	7.0	24.3	39	15.4	7.7	76.9
Cefaperazone75 µg	137	100	0	0	32	100	0	0
Tetracycline 30 µg	184	77.8	8.7	13.5	39	79.6	10.2	10.2
Trimethoprim/sulfa 1.25/ 23.75 µg	191	84.3	3.7	12.0	40	97.5	0	2.5
Gentamycin 10 µg	161	94.3	2.5	2.2	40	95.0	0	5.0
Streptomycin 300 µg	154	78.6	5.8	15.6	37	73.0	8.1	18.9
Neomycin 30 µg	155	72.9	20.6	6.5	37	83.8	13.5	2.7
Enrofloxacin 5 µg	185	98.4	0	1.6	37	100	0	0

* Proportion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin. Coliforms are often resistant to more than one antimicrobial [36,37], and the number of multi-resistant strains may influence the resistance figures. Coliform bacteria isolated from cases of mastitis may reflect the general situation of resistance in the herd and can be considered more as an indicator of the bacteria present than an indicator of specific pathogens from the udder [36]. All of the bacterial species investigated in the present study showed resistance to tetracycline. A possible explanation for this phenomenon could be that tetracycline has been the class of antimicrobial most widely used for treatment of several infections for many years. In addition, tetracycline has been found in multiresistant patterns with penicillin and streptomycin [33,37].

Statistical data from the Estonian State Medical Agency confirmed [19] that alltogether 209880 single intramammary syringes for lactating cows and 205648 for dry cow therapy were sold in the year 2009. Ampicillin and cloxacillin combinations, cephalosporins with aminoglycosides, and lincomycin with neomycin were the most common choices for the treatment of mastitis in lactating cows. For example, 255 grams of intramammary lincomycin (pure antimicrobial) and 44.2 grams of intramammary cephalosporins per thousand dairy cows were sold for the treatment of clinical mastitis in 2009 [19]. However, only 73.4 grams of penicillin G was used per thousand dairy cows for intramammary treatment of clinical mastitis. The use of broad-spectrum antibiotics and antibiotic combinations may influence the resistance of mastitis pathogens. In addition, bacteriological examination of milk samples before treatment of clinical mastitis is not a common practice in Estonia. According to

the available data in Sweden, intramammary and intramuscular penicillin G [38] are used in over 80% of cases for treatment of clinical mastitis, but the prevalence of resistance of *S. aureus* to penicillins is only 7.1% [36]. In Finland, penicillin G and some broad-spectrum β-lactam antibiotics are used in the treatment of clinical mastitis, but the prevalence of resistance in *S. aureus* is only 13% [39]. Bacteriological examination before treatment is common in both countries.

Considering these results, we can assume that the main reason for the occurrence of a high number of resistant strains in Estonian herds is the wide use of broad-spectrum antimicrobials and the long-term presence of infected cows in herds.

Conclusion

This study showed that the main pathogens that caused clinical mastitis were *Str. uberis* and *E. coli*. Subclinical mastitis was caused mainly by *S. aureus* and CNS. A relatively high number of isolates of *Str. agalactiae* were cultured from both types of case. The number of *S. aureus* and *Str. agalactiae* isolates depended on herd size. Among the bacteria investigated, the prevalence of antimicrobial resistance was extremely high, especially penicillin resistance in *S. aureus* and CNS.

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Authors' contributions

PK carried out the study, compiled the results and drafted the manuscript, BA participated in data collection and coordinated the laboratory analysis, TO participated in designing the study and statistical analysis of the data, AK performed bacteriological analysis, and KK coordinated the study. All authors were significantly involved in designing the study, interpreting data and composing the manuscript.

Competing interests

The authors declare that they have no competing interests.

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LIST OF PUBLICATIONS

1.1 Articles indexed by Web of Science

Aasmäe B, Häkkinen L, Kaart T, Kalmus P. (2019). Antimicrobial resistance of *Escherichia coli* and enterococci isolated from cattle and swine from 2010 to 2015 in Estonia. Acta Vet Scand. 61:5.

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1.2 Articles in other peer-reviewed international research journals

Aasmäe B, Volkova J, Häkkinen L, Orro T, Tenson T, Kalmus P. (2015). *In vitro* antimicrobial resistance of intestinal *Escherichia coli* and enterococci in clinically healthy dogs in Estonia. Vet Med Zoot. 72:3-8.

1.3 Articles in Estonian and other peer-reviewed research journals with a local editorial board

Aasmäe B, Kalmus P, Tiirats T. (2003). Antimicrobial resistance of pathogens causing clinical mastitis in dairy cows. Lüpsilehmade kliinilist mastiiti põhjustavate mikroobide antibiootikumiresistentsus. Agraarteadus (Journal of Agricultural Science), Akadeemilise põllumajanduse Seltsi väljaanne, XIV(3), 139-143.

3.1 Articles in proceedings published in the collections indexed by the Thomson Reuters Web of Science

Aasmäe B, Kalmus P. (2012). Antimicrobial resistance of animal pathogens 2006-2009 in Estonia. Research for Rural Development 1:181-187.

3.4 Articles/presentations published in international conference proceedings

Kalmus P, Aasmäe B, Viltrop A, Kask K. (2006). Occurrence of clinical mastitis of heifers in different housing conditions in Estonian dairy herds. In: Proceedings: XXIV World Buiatric Congress, Nice, France, October 2006, 15-19.

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