ESTONIAN UNIVERSITY OF LIFE SCIENCES
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ANTIMICROBIAL RESISTANCE OF *E. COLI* AND *ENTEROCOCCUS* SPP. ISOLATED FROM SWINE AND CATTLE IN 2016-2020 IN ESTONIA

EESTIS AASTATEL 2016-2020 SIGADELT JA VEISTELT ISOLEERITUD *E. COLI* JA *ENTEROCOCCUS* SPP. ANTIBIOOTIKUMIRESISTENTSUS

Final Thesis
Curriculum in Veterinary Medicine

Supervisor: Birgit Aasmäe

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Antimicrobial resistance is recognized as one of the major global health threats globally. It is a problem relating to "One Health" concept, therefore monitoring the resistance in bacteria from food-producing animals is necessary. The objective of this thesis was to investigate and describe the occurrence of antimicrobial resistance of *Escherichia coli, Enterococcus faecium* and *Enterococcus faecalis* isolated from healthy and diseased pigs and cattle in Estonia during 2016 to 2020. Commensal *E. coli* and *Enterococcus* spp. were collected from caecal samples of fattening pigs at slaughter and resistance was determined using microdilution method. *E. coli* from diseased animals was isolated from clinical submissions of pigs and cattle and resistance was determined using disc-diffusion assay.

Both bacteria studied were found to have developed resistance against multiple antibiotics. Commensal *E. coli* isolates had highest resistance against tetracycline (19,4%), ampicillin (17,9%), trimethoprim (16,4%) and sulfamethoxazole (14,9%). *E. coli* isolates from clinical submissions of pigs had highest resistance against amoxicillin (38,1%), tetracycline (23,8%) and trimethoprim (13,1%). *E. coli* isolates from clinical submissions of cattle had highest resistance against tetracycline (62,3%), ampicillin (49,3 %), sulfamethoxazole-trimethoprim (40,6%) and enrofloxacin (37,7%). *E. faecalis* had developed highest resistance against tetracycline (72,3%), erythromycin (46,8%) and chloramphenicol (25,5%). *E. faecium* had highest resistance against erythromycin (41,5%), quinipristin/dalfopristin (25,5%) and tetracycline (23,4%). Isolates from healthy pigs had higher susceptibility rates and lower multidrug resistance rates than isolates from diagnostic submissions. No significant changes occurred during the study years. Resistance against critically important antibiotics is generally low in commensal bacteria.

Keywords: Antimicrobial resistance, *E. coli, Enterococcus*, one health, Estonia

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Lühikokkuvõte

Mikroobide resistentsus on tänapäeval kogu maailmas üks peamisi probleeme tervishoiusüsteemis. See on probleem ka “Üks Tervis” kontseptsiooni mõistes, mistõttu on väga oluline monitoorida ka loomade mikroobide resistentsuse arengut. Selle töö eesmärk oli uurida tervetelt ja haigetelt sigadelt ja veistelt Eestis aastatel 2016 – 2020 isoleeritud Escherichia coli, Enterococcus faecium’i ja Enterococcus faecalis’e antibiootikumiresistentsust. Kommensaalsed E. coli ja Enterococcus spp. koguti tapamajades nuumsigade käärsoolest, resistentsust uuriti mikrodilutsiooni meetodiga. E. coli haigetelt loomadelt isoleeriti veterinaarleboratooriumisse toodud proovidest ning resistentsust uuriti diskdiffusiooni meetodiga.

Kommensaalsed E. coli isolaadid olid kõige resistentsemad tetratsükliini (19,4%), ampitsilliini (17,9%), trimetoprimi (16,4%) ja sulfametoksasooli (14,9%) suhtes. E. coli isolaadid haigetelt sigadelt olid kõige resistentsemad amoksitsilliini (38,1%), tetratsükliini (23,8%) ja trimetoprimi (13,1%) suhtes. E. coli isolaadid haigetelt veistelt olid resistentsed tetratsükliini (62,3%), ampitsilliini (49,3 %), sulfametoksasool-trimethoprimi (40,6%) ja enrofloksatsiini (37,7%) suhtes. E. faecalis oli kõige resistentsem tetratsükliini (72,3%), erütromütsiini (46,8%) ja klooramfenikooli (25,5%) suhtes. E. faecium oli resistentseim erütromütsiini (41,5%), quinupristiin/dalfopristiini (25,5%) ja tetratsükliini (23,4%) suhtes. Kokkuvõttes saab ütelda, et tervetelt sigadelt isoleeritud mikroobid olid antibiootikumide suhtes tundlikumad ning nende hulgas oli vähem multiresistentseid tüvesid võrreldes haigetelt sigadelt isoleeritud mikroobidega. Resistentsuses ei olnud uuringus olnud aastate jooksul erilisi muutusi. Kommensaalsete bakterite resistentsus kriitilise tähtsusega antibiootikumide suhtes oli uuringuaastete jooksul madal.

Märksõnad: Antimicrobial resistance, *E. coli, Enterococcus*, üks tervis, Eesti

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LIST OF ABBREVIATIONS

AM antimicrobial

AMR antimicrobial resistance

CIA critically important antimicrobials

CLSI clinical and laboratory standards institute

EEA European economic area

ESVAC European surveillance of veterinary antimicrobial consumption

EU European union

HGT horizontal gene transfer

ISO international standards organization

MDR multidrug resistant

MIC minimum inhibitory concentration

PCU population correction unit

VRE vancomycin-resistant *enterococci*

WHO World health organization

INTRODUCTION

Antimicrobial resistance (AMR) has been recognized as one of the major global health threats globally. In 1928 Alexander Fleming discovered the mould of *Penicillium* genus having antimicrobial properties. The substance was successfully isolated and termed penicillin, and in the 1940s mass production of penicillin started. Its widespread use for bacterial infections revolutionized medicine and resulted in increased life expectancy of the general population and reduced deaths from bacterial infections (Armstrong *et al.*, 1999). However, already in the mid-1940s penicillin-resistant *Staphylococcus aureus* strains were isolated (Kirby, 1944). The emergence of AMR was well acknowledged by Fleming himself, as he warned against the wrongful use of penicillin in his Nobel lecture in 1945. In the current day, antimicrobial resistance properties have been reported for all known antibiotics used in animal and human medicine (Boerlin & White, 2013). Although the sales of veterinary antibiotics in European countries keep declining each year (European Medicines Agency, 2021) it has been estimated that most of the antimicrobials sold globally are used in food-producing animals (Van Boeckel *et al*., 2017) mainly to prevent diseases and to promote growth. Despite their benefits, the over- and misuse of antimicrobials drives for the selection of resistant bacteria (Hao *et al*., 2014), contributing to the development of resistance, and this has been recognized in the veterinary field already. Intensifying the problem is the spread of resistant organisms and their genes via different means, and the possible spread from animals to humans and the environment. Thus, diseases that were formerly curable with antimicrobials are becoming more challenging to treat, causing a heavier burden on the economy and welfare (Cosgrove, 2006). Furthermore, it has been estimated that up to 10 million deaths a year by 2050 could occur due to AMR (O'Neill, 2014).

To understand antimicrobial resistance better and the risks involved with it, it is important to keep monitoring the occurrence of resistance. This allows for recognition of any changes regarding AMR and aids when discussing appropriate measures to take for reducing the occurrence of it, as reducing the use of antimicrobials, is fundamental to preserve the current and future benefits of antimicrobials for all.

This thesis attempts to describe the antimicrobial resistance of *E. coli* and *Enterococcus* spp. isolates from swine and cattle in Estonia. The data in this study was provided by the National Veterinary and Food Laboratory in Estonia, Tartu.

1. LITERATURE REVIEW

1.1. What is antimicrobial resistance and how it affects animal and human health?

Antimicrobials are critical tools in the battle against diseases caused by harmful pathogens, such as bacteria, viruses, fungi and parasites in both humans and animals. After the discovery and use of antimicrobials, they have rendered previously dangerous diseases easily treatable. Unfortunately, antimicrobials are becoming more and more ineffective due to the pathogens developing resistance against them, caused by many different factors, such as misuse and random chance. According to the World Health Organization (WHO), AMR is one of the top 10 urgent global health risks (WHO, 2019a). Thus, AMR could force modern medicine to take huge step-backs, as Dr Margaret Chan – the Director-General of WHO stated "a post-antibiotic era means, in effect, an end to modern medicine as we know it. Things as common as strep throat could once again kill".

AMR is a One Health issue that negatively affects animal and human health and the environment. Despite the tremendous benefits antimicrobials provide, overuse does contribute to the development of resistance, and according to Aarestrup (2005), a particularly worrying concept is the over-and misuse of AMs in production animals, and their spread being facilitated by overcrowding, poor hygiene and husbandry and movement of animals and people. Furthermore, it has been well documented that the transfer of resistant organisms and genes from animals to humans is possible by means of direct contact, the environment or by consuming contaminated foods of animal origin (Mølbak et al., 1999; Hammerum & Heuer, 2009; Larsen et al., 2010; Marshall & Levy, 2011). In addition, harmless commensal bacteria living in the human, and animal intestinal tract can act as reservoirs for the resistance genes and possibly transfer them to susceptible bacteria, making them resistant (Van Den Bogaard and Stobberingh, 2000). The interconnection between animals, humans and the environment must be recognized, in order to attempt to achieve optimal health for all, as the diseases caused by resistant organisms are a huge burden on the welfare and economy in many sectors.

Diseases caused by resistant organisms can be detrimental for humans and animals, especially if their health status has already been compromised by something. Not only is there the risk of developing a life-threatening infection because of resistant bacteria, but diseases caused by

resistant organisms are more challenging to treat, and the consequences of failed treatments and prolonged illnesses are tasking, for the welfare and the economy (Cosgrove, 2006). Infections caused by resistant organisms have generally higher mortality rates than infections caused by the susceptible ones (Cosgrove et al., 2003; Helms et al., 2002; De Kraker et al., 2011). Furthermore, deaths and illnesses lead to reduced productivity by animals (Bengtsson & Greko, 2014). According to Capita & Alonso-Calleja (2013) infections by antibiotic-resistant strains are associated among other things with reduced life quality, an increase in recurrence rates and future opportunistic infections with resistant organisms. In the year 2015, there were an estimated 33,100 human deaths due to infections with antimicrobial-resistant bacteria in the European Union (EU) and European Economic Area (EEA) alone (Cassini *et al*., 2019). Furthermore, according to the Centre for Disease Control and Prevention (CDC), more than 35,000 people die each year in the United States alone, due to antibiotic-resistant infections (CDC, 2019). And with the current trend, according to a review by O'Neill (2016) by the year 2050, the expected amount of human deaths globally due to diseases caused by resistant micro-organisms could be around 10 million each year.

It is for these reasons and the clear global health threat AMR creates, the risk factors must be well-acknowledged, and each country must take appropriate steps in monitoring and preventing AMR occurrence, to evade the catastrophic health consequences on animals and humans that these organisms entail.

1.2. What drives for antimicrobial resistance?

AMR occurs when microorganisms like bacteria undergo adaptive changes and no longer succumb to the drugs that were supposed to destroy them. For the sake of this study, it is important to understand how resistance develops, as well as the factors contributing to the increasing number of resistant micro-organisms. The emergence of AMR in organisms is a natural phenomenon, the presence of resistance genes in bacteria well before the introduction of antimicrobials into the market has been documented (D'Costa *et al*., 2011). However, we should recognize that humans have driven the selection of resistance by over- and misuse of antimicrobials. As Levy (2002) has simply stated, there are two main components to the development of drug resistance and those are the antimicrobial drug, which inhibits the susceptible organisms and promotes selection of the resistant ones, which carry the second component: the resistance gene. The resistant organisms can begin taking over by multiplying,

sharing the genetic material that allowed them to survive destruction by antimicrobials. Furthermore, what makes resistant organisms even more notorious is the ability to directly pass their resistance genes to other organisms by the means of horizontal gene transfer (HGT), thus making them resistant. The transfer is mostly mediated by bacteriophages, naked DNA, transposons, and plasmids. HGT allows for the rapid dissemination of resistant organisms (Levy & Marshall, 2004). All in all, the formation of AMR organisms is a form of natural selection and survival of the fittest, which is furthermore driven by the over-and misuse of these agents (Holmes *et al.* 2016; Hao *et al*., 2014).

Bacteria can be resistant against many antimicrobial classes and not just one, these types of bacteria are called multidrug-resistant (MDR) bacteria. When exposed to one antimicrobial, co-selection for bacteria that are resistant against numerous unrelated antimicrobials may occur. This type of selection is called co- or cross-resistance. Co-resistance means that there are many resistance genes present, against different antimicrobials, and cross-resistance means that one resistance gene or mutation has antimicrobial attributes against antimicrobials from the same class (Cantón and Ruiz-Garbajosa, 2011).

What makes the situation worse, is the fact that micro-organisms are virtually a lot more effective in gaining resistance, than humans are in developing new antimicrobials, as goes to show that there have been no novel antibiotic classes discovered since 1987 (Huttner *et al*., 2013). Despite the growing need for new antimicrobials, the time and money invested into the research, accompanied with bureaucracy is not desirable for pharmaceutical companies. Considering the risk that eventually the micro-organisms will most probably gain resistance against the new drug, the focus of pharmaceutical companies shifts to treating chronic illnesses, rather than researching new antimicrobials (Projan, 2003). Any new antimicrobials coming to the market will most likely be preserved for human use, and the ones available for animal use at the moment will remain the same, therefore making the preservation of their efficiency even more critical (Mølbak et al., 1999).

One could ask, will the resistance diminish if selective pressure is removed by simply removing antimicrobials that drive the selective pressure. Contradictory studies are showing positive and negative results to this question. For example, the agricultural ban of avoparcin in 1995 in Denmark was followed by a decrease in the occurrence of glycopeptide resistant *E. faecium* (GRE) in poultry farms from 72.7% in 1995 to 5.8% in 2000 (Aarestrup *et al*., 2001). On the contrary, three years after the avoparcin ban in Norway, the prevalence of VanA-type

vancomycin-resistant *enterococci* (VRE) was still high in poultry farms *(*Borgen *et al*., 2000). In Germany however, about two years after the discontinuation of avoparcin as a feed additive for poultry, the occurrence of VRE in poultry meat and the healthy human gut flora had decreased (Klare *et al*., 1999). In the study by Aarestrup *et al*. (2001) effect of the avoparcin ban was also studied in pigs, but no significant changes had occurred within two years after the avoparcin ban. However, in 1998 and 1999 the use of tylosin was decreased for growth promotion in Denmark, and then in the year 2000, the occurrence of GRE in pigs decreased to only 6% from being at around 20% in the previous years. It was found that the resistance first persisted because of co-selection. There are more studies showing the effect of discontinuation of certain antimicrobials for food animals has on the reduction of resistance (Bengtsson & Wierup, 2006; Boerlin *et al*., 2001).

However, there are studies showing that resistant isolates persist usually in low numbers after the removal of a certain antimicrobial. For example, a study that was done 15 years after the avoparcin ban in Denmark still showed that in 100 broiler flocks tested, VRE was present in 47 flocks. However, what creates optimism in this case, is that the study showed that the proportion of VRE from the total enterococci population in broiler faeces was low (Bortolaia *et al*., 2015). Similar results were obtained from a New Zealand study after the removal of avoparcin use in broilers (Manson *et al*., 2004). Furthermore, a study done in Norway in two poultry farms previously exposed to avoparcin showed that animal and human GRE remained in those farms, even long after the avoparcin ban (Johnsen *et al*., 2005).

Regardless of the positive results some studies exhibit, others challenge the hypothesis that once selective pressure is removed the resistant populations will then regress into a mostly susceptible state. Mechanisms behind the persistence are unclear but believed to be multifactorial (Boerlin & White, 2013). This absence of clear correlation furthermore goes to show the urgent need for surveillance and further research on the matter.

1.2.1. Antimicrobial use in food-producing animals and transmission routes of resistance

The correlation between the emergence of AMR and the use of these antimicrobials by humans and animals appears definitive. However, regardless of being debated (Phillips *et al*., 2004), the irresponsible use of antimicrobials in animals, in both veterinary medicine and agricultural settings, has been suggested to contribute to the spread of AMR bacteria from animals-to-

animals and animals-to-humans, by different means of transmission, such as food-chain, the environment and direct contact (Hamer & Gill, 2002; Marshall & Levy, 2011; Vieira *et al*., 2011; Holmes *et al*., 2016). The bacteria may be commensal in animals, but pathogenic in humans, or it can be commensal in both, but driving for resistance by means of HGT. The spread of resistance from animals to humans is likely of more concern from the point of view of the general population, however, even though there's less data on the subject, the spread of resistant organisms from humans to animals is also likely possible (Unnerstad *et al.,* 2018).

Antimicrobials are given to production animals for various reasons. Depending on the country and legislation these reasons include among other things, disease treatment and prevention, and growth promotion (Levy & Marshall, 2004). Mass medication of animals is an important factor in the emergence of resistance. As resistance among bacterial populations increases, the beneficial therapy options for animals decreases, burdening the economy and welfare of the animals and farm owners. Furthermore, as discussed earlier, the risk of dissemination of resistant organisms and their genes to humans by different transmission routes also increases.

It is possible that resistant pathogens and genes can be transmitted from animals to humans for example by means of direct contact with animals, or their excretions, but according to Van Den Bogaard and Stobberingh (2000) contaminated food products of animal origin are the most important source of human infection caused by resistant bacteria. In essence, the resistant organisms can be transferred from food-producing animals to humans via the food chain, and according to Schwartz *et al*. (2001) food-borne infections caused by resistant zoonotic bacteria *Salmonella* spp., *Campylobacter* spp. and *Enterococcus* spp. can be traced to have originated from animal sources with a high level of confidence. For instance, the transfer of resistant *Salmonella* pathogens from food of animal origin to humans has been documented (Mølbak *et al*., 1999; Lu *et al*., 2019).

It is not only pathogenic bacteria that can create problems, as the commensal bacteria such as *E. coli* and *Enterococcus* spp. can also gain resistance to antimicrobials. Consequently, like zoonotic bacteria, these resistant commensal bacteria of food-producing animals can contaminate food products like meats, which are then handled and ingested by humans, reaching their intestinal tract. A study done by Hummel *et al*. (1986), exhibits the transfer of AMR from food-producing animals to humans, by studying the spread of nourseothricin resistant *E. coli*. Nourseothricin was given to pigs for growth promotion and this class of antibiotic was not used in human medicine. Within a year of administration, nourseothricin

resistant *E. coli* were isolated from the pigs' faeces. The resistance gene was located on a transposon, and after two years, this transposon was also found in the environment of the piggery such as manure and river water, in faecal isolates of the farm employees and their family members, in the area's outpatients and in *E. coli* that caused one urinary tract infection in human. In addition, more recent studies have also suggested the spread of resistant *E. coli* isolates originating from animal food products to humans (Larsen *et al*., 2010; Kluytmans *et al*., 2012).

In modern days intensive farming techniques where the food-producing animals are usually living in a somewhat dirty environment and high population densities, the spread of bacteria among the animals occurs. There is no question whether there is the presence of antimicrobial resistant organisms in production animals as it has been widely documented (Aasmäe *et al*., 2019). Regardless of the administration route of the antimicrobial, a substantial amount ends up in the intestine of the animal, then to be excreted (Burow *et al.,* 2019). It has been estimated that a significant amount of the antibiotic administered an animal is excreted into the environment, in unmetabolized form (Kumar *et al.*, 2005). Animal manure application to soil is considered to be the main cause of the propagation of resistance genes, resistant bacteria and antibiotic residues into the environment (Checcucci *et al.,* 2020). Additionally, resistance genes can pollute the environment directly, as goes to show a study done by Wu *et al*. (2019) in which *E. coli* carrying antimicrobial genes isolated from swine faeces, could spread into the external environment and pollute nearby waters, soil and air. Thus, excretions by animals and the practice of land application of livestock manure, provides an introductory point for resistant organisms, genes and antimicrobials into the environment, despite improved waste management strategies (Checcucci *et al*., 2020). This leaking exposes organisms in the environment to antimicrobials, which allows for the selection of resistant bacteria, promoting HGT within the diverse bacterial population present in the environment (Larsson & Flach, 2021). Furthermore, according to Gullberg *et al*. (2011) the presence of antibiotics in the environment even in concentrations below the minimum inhibitory concentrations (MIC), are relevant in the selection and maintenance of resistant bacteria in the environment.

Another concerning factor in the spread of resistant bacteria from animals to humans comes from the direct contact between the farm animals and the farmworkers and animal's handlers (Aubry-Damon *et al.,* 2004; Graveland *et al.*, 2010). As most people are not in direct contact with farm animals, this form of spread does not cause a huge health threat for the general

population, but the farmworkers can play as a factor in the entry of resistance genes into the general population (Marshall & Levy, 2011).

Rather than using antimicrobials as prevention of disease in animals, more focus should be given to the good biosecurity practices. As healthy animals do not need antimicrobials, by lowering the incidence of infectious diseases with good hygiene and other biosecurity practices, the need for antimicrobials should lower.

1.3. Usage of antimicrobials in animals in Estonia

The rules and restrictions in Estonia about antimicrobial use in animals are similar to those of other European countries, especially the Nordic countries. WHO has developed a list of critically important antimicrobials (CIA) for humans. The purpose of the list is to rank antimicrobials by their significance in human and veterinary medicine respectively. It was developed due to emerging concerns the widespread use of antibiotics in food-producing animals, particularly the same classes used in human medicine, could have on public health if these resistant micro-organisms would spread to human populations. According to a 6th revision by the WHO, the highest priority CIA classes are cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides and ketolides, polymyxins and quinolones (WHO, 2019b).

According to the latest European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) report, the sales of all antibiotics to be used in food-producing animals in Europe fell by more than 43% between 2011 and 2020. Furthermore, sales of CIA's to be used in animals have reduced in each class. Between 2011 and 2020, 3rd and 4th generation cephalosporins sales have reduced by 33%, polymyxin sales have reduced by 77%, fluoroquinolones for 13% and 85% for other quinolones (European Medicines Agency, 2021). In Estonia, the findings are quite similar. The overall sales of veterinary antimicrobial agents decreased by 35.8% between 2014 and 2020. Furthermore, the percentage of sales in mg per population correction unit (mg/PCU) for solely food-producing animals decreased from 65.2 mg/PCU in 2015 (European Medicines Agency, 2016) to 49.2 mg/PCU in 2020 (European Medicines Agency, 2021). What it comes to the CIA's, the percentage of total sales in mg/PCU for food-producing animals in Estonia have fluctuated between 2015 to 2020, as Estonia is a small country, outbreaks of diseases or small changes in farms may significantly influence the sales. All the CIA's have decreased in sales from 2015 to 2020. In mg/PCU sales of 3rd and 4th generation cephalosporins

decreased from 0.6 to 0.7, fluoroquinolones from 1.8 to 1.1, macrolide sales decreased from 2.8 to 1.6 and polymyxins decreased from 1.3 to 0.3, for other quinolones, no sales have been reported since 2011 (European Medicines Agency, 2016; European Medicines Agency, 2021). According to the latest ESVAC report, 3rd- and 4th-generation cephalosporins are sold most in Estonia when compared to other European countries that were involved in the report. According to the data from the Estonian State Agency of Medicine in 2020 tetracyclines (25,1%), penicillins (24,5%) and pleuromutilins (14,8%) were sold the most in veterinary medicine. A trend in the use of monensin is seen in Estonia. The category "other antimicrobials" (Table 1.) counts for 847 720 grams of active ingredient used in Estonia in 2020, of that value, 767 070 grams come from monensin. The use of monensin in Estonia has increased each year, it is a part of the ionophore drug class. Its intended use in cattle is to be given orally before calving in a controlled-release capsule to prevent and control ketosis. The European Medicines Agency has stated that the use of ionophores does not pose a public health risk, as ionophores are not used in humans, and there's no proof of cross-resistance nor co-selection. However, the widespread use of ionophores in agriculture, especially in the United States and Canada has created concerns, and some argue that there are risks and proof of cross-resistance and co-selection, and more research on the subject is needed to ensure that it does not add to the AMR problems we are seeing (Wong, 2019).

Table 1. The use of antimicrobials (active ingredients in grams) in animals in Estonia in 2016-2020.

Antimicrobial	2016	2017	2018	2019	2020
Aminoglycosides	408 401	438 665	472 572	407 407	418 143
Amphenicols	40 750	55 490	56 341	48 957	45 265
Fluoroquinolones	148 513	150 700	143 265	131 559	129 552
Lincosamides	33 608	53 982	78 274	66 369	38 181
Macrolides	266 753	239 927	349 435	215 502	183 834
Penicillins and penicillin combinations	3 683 293	2 485 167	2 158 519	1 824 013	1 651 153
Pleuromutilins	589 362	564 736	856 382	955 560	996 679
Polymyxins	82 860	109 438	82 966	43 663	25 010
Sulphonamide and trimethoprim combinations	200 942	405 311	440 208	651 287	589 318
Tetracyclines	1 832 813	1 675 350	1 395 496	1 861 625	1 691 005
Cephalosporins 1. and 2. generation	77 977	68 986	62 196	55 464	51 104
Cephalosporins 3. and 4. generation	82 211	91 503	104 144	88 079	81 462
Other antimicrobials	434 827	591 573	655 554	819 276	847 720
TOTAL	**7 882 310**	**6 930 828**	**6 855 352**	**7 168 761**	**6 748 426**

[1]**Data from Estonian State Agency of Medicines, unpublished**

1.3.1. Monitoring of antimicrobial resistance in Europe

Most developed nations have taken steps towards restricting and reducing the use of antimicrobials, for example, the EU banned the use of antimicrobials for growth promotion in food-producing animals in 2006 and has also banned the use of human antibiotics in animal feeds. However, further education on this matter is still needed, especially in developing countries where the demands of food-producing animals continue to increase.

Monitoring the prevalence of AMR is of utmost importance, as its occurrence is not erasable, but the surveillance may allow for timely detection of resistance. Additionally, monitoring guides in the development of intervention strategies and better helps us to understand the effectiveness or the lack thereof, of these strategies. Monitoring can be done by researching the resistance in some bacteria such as *E. coli* and *Enterococci* spp., which are known as indicator bacteria. They are the natural inhabitants of the gastrointestinal tract of healthy animals and the occurrence of AMR in these bacteria reflects the selection pressure of resistance caused by the use of antimicrobials. Furthermore, indicator bacteria like *E. coli* can be reservoirs of resistance genes to other bacteria, including potentially pathogenic ones (Van Den Bogaard and Stobberingh, 2000; Blake *et al*., 2003).

According to Commission Implementing Decision (EU) 2020/1729, it is required that the EU member states monitor and report the prevalence of zoonotic bacteria *Campylobacter jejuni*, *Campylobacter coli* and *Salmonella* spp. and the prevalence of indicator bacteria *E. coli* and *E. faecalis* and *E. faecium* in certain food-producing animals and food products. Additionally, the EU has constructed an annual summary report prepared by the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) on AMR in indicator and zoonotic bacteria from animals, humans and food since 2004.

In addition to the aforementioned, the sales of veterinary antimicrobial agents for pets and food-producing animals have been monitored by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), since 2010. The number of European countries taking part in the program has increased from being 19 in 2010 to 31 in the latest report done in 2018, which includes all the EU/EEU countries and Switzerland.

From January 2022, new regulations on veterinary medicines (Regulation (EU) 2019/6) and medicated feed (Regulation (EU) 2019/4) will come into place in the EU. The main goal of

these regulations is to set measures for prudent and responsible AM use to battle against AMR. New regulations will include among other things; a ban on the preventive use of antibiotics in groups of animals, a ban on the preventive use of AMs in medicated feed, new regulations on the metaphylactic use of AMs in animals, additional restrictions on the ban of AM use for growth promotion, reservation of certain AMs to human use only and the commitment from all EU Member States to collect data on the sales and usage of AMs (More, 2020). The concept of One Health is well recognized by setting these new regulations, as animals, humans and the environment will greatly benefit from them.

1.4. Antimicrobial resistance of *E. coli* in cattle and swine

E. coli are commensal bacteria of the gastrointestinal tract of animals and humans, they are mainly harmless, but some strains are pathogenic and can cause intestinal or extraintestinal infections. *E. coli* is an important indicator organism, level of resistance is measured in *E. coli* isolates, as they reflect the selective pressure from antibiotic use. This is also important because it is believed that commensal bacteria can be a reservoir of resistant genes to pathogenic bacteria. Monitoring of prevalence of resistance in the indicator bacteria in different populations, such as people and animals is very important, as it provides us with information on the resistance levels and the transfer of resistant bacteria or genes between the populations. As stated earlier, the monitoring and reporting of resistance in indicator bacteria, such as *E. coli* is required from all EU member states, hence there's information and studies about resistance levels of *E. coli* in different populations.

A study done during a five-year period measuring the prevalence of resistance in *E. coli* isolates collected from cattle and swine populations around Estonia, offers a good review of the situation in Estonia. According to the study by Aasmäe *et al.* (2019), the AMR of the isolated *E. coli* was high in both cattle and swine but isolates from swine had significantly higher amounts of resistance, as well as multidrug resistance when compared to those collected from cattle. Reports from other European countries support the trend that *E. coli* isolated from swine has higher rates of resistance when compared to the ones collected from cattle (FINRES-Vet, 2021; SWEDRES-SVARM, 2015). The study also compared samples from clinically healthy animals and diseased ones. The diseased animals from both species had higher amounts of resistance when compared to the samples from clinically healthy animals. This can be explained by the possibly higher administration of antimicrobials to the diseased animals for treatment of

disease. The highest amounts of resistance in *E. coli* isolates from both species were against streptomycin, tetracycline, sulfamethoxazole, trimethoprim and ampicillin (Aasmäe *et al.,* 2019). Reports from other European countries show similar findings, with the highest amounts of resistance against tetracycline, sulfamethoxazole, trimethoprim, ampicillin, and sulphonamides, these are frequently used drug classes. Reports from different countries vary, in Sweden, Finland and the Netherlands almost all the indicator *E. coli* tested from cattle have remained susceptible to the antibiotics tested, and for swine, the majority of isolates tested have remained susceptible, and the resistance rates are low on an international level. In Denmark, most of the cattle isolates are also susceptible to the tested antibiotics, but less than half of the tested swine isolates were susceptible in the latest report (FINRES-Vet, 2021; FINRES-Vet, 2020; DANMAP, 2020; MARAN, 2021; SWEDRES-SVARM, 2019; SWEDRES-SVARM, 2015).

Aasmäe *et al*. (2019) suggest that there is a higher probability for commensal *E. coli* in swine to gain resistance against orally administered antimicrobials, as the isolates from swine were more resistant to antibiotics that are mostly administered orally. Other studies support this argument (Burow et al., 2014). This hypothesis however could not be confirmed by a study from Burow *et al.,* (2019), in which they indicate that the rate of resistance may rather be substance specific than depending on the route of administration. Further research on the matter is required, as a lot of antibiotics are administered orally, especially in pig production (Lekagul *et al.,* 2019).

Additionally, some *E. coli* are able to produce enzymes termed extended spectrum beta-lactamases (ESBL) and AmpC beta-lactamases, that alter the beta-lactam antibiotics, such as penicillins and cephalosporins, making them ineffective. Numerous studies have exhibited the presence of AmpC and ESBL-producing *E. coli* in animals and meat, and they are considered a health hazard, as there is the potential transmission risk from animal products to humans (Hammerum & Heuer, 2009).

1.5. Antimicrobial resistance of *Enterococcus* spp. in cattle and swine

Like *E. coli*, Enterococci are also commensal bacteria of the gastrointestinal tract of animals and humans, but some species, such as *E. faecalis* and *E. faecium* can cause infections in humans. Such infections include urinary tract infections, wound infections and even bacteraemia and endocarditis (Hammerum, 2012). Of more concern are resistant strains such

as vancomycin-resistant enterococci (VRE), gentamicin-resistant enterococci (GRE) and streptogramin-resistant *E. faecium,* as for example VRE has been studied to be selected in animals if avoparcin is present in the feed, and that it remains in the animals even after avoparcin has been removed from the use (Borgen *et al*., 2000; Bortolaia *et al*., 2015). Even after the ban of antimicrobials for growth promotion in the EU, resistant strains such as VRE are still a concern as tylosin and tetracycline, which are still widely used in animals, have been associated with high levels of VRE in production animals (Hammerum, 2012). Animals or foods of animal origin being a direct source of resistant enterococci in humans is still studied but, enterococci of animal origin can pose a risk of transferring their resistance genes, such as vanA genes, to human bacteria (Lester *et al*., 2006). Treatment of infections caused by *Enterococcus* spp. is more difficult since they are intrinsically resistant against numerous antimicrobials such as β-lactams and cephalosporins and are resistant to low levels of aminoglycosides. Furthermore, some species have intrinsically low susceptibility to streptogramins, which is of concern as quinupristin/dalfopristin are frequently used in patients with vancomycin-resistant *E. faecium* infection (Miller *et al*., 2014; Hammerum, 2012; EFSA, 2008).

In the study by Aasmäe *et al*. (2019), enterococci isolated from Estonian cattle and swine had the most resistance against tetracycline and erythromycin, and similarly to the *E. coli* isolates, pigs had more MDR *Enterococcus* strains when compared to cattle. Other studies done in Europe also exhibit *E. faecalis* and *E. faecium* to have the highest amount of resistance against tetracycline and erythromycin, additionally, gentamicin and vancomycin resistance has been reported to be low (de Jong *et al.,* 2019; DANMAP, 2015).

2. AIMS OF THE STUDY

Aim of this study was to estimate the occurrence of antimicrobial resistance of *Escherichia coli, Enterococcus faecium* and *Enterococcus faecalis* isolated from healthy and diseased cattle and swine in Estonia from 2016 to 2020.

3 MATERIALS AND METHODS

3.1. Collection of study material

3.1.1. Faecal samples and clinical submissions from swine and dairy cattle

The samples in this study were collected between 2016 and 2020 in Estonia. Samples from healthy animals were collected as a part of the annual monitoring program of zoonoses and zoonotic agents designed according to the EU Commission Implementing Decision 2013/652/EU. Samples from diseased animals were collected for laboratory diagnostics. Total amount of bacterial isolates included in this study are presented in Table 2.

For the monitoring program of commensal *E. coli*, *E. faecium* and *E. faecalis* samples were collected from healthy pigs at slaughter in 2017 and 2019. In 2017, 68 caecal samples from pigs originating from 68 different holdings were collected. From those samples, in total of 25 *E. faecalis*, 40 *E. faecium* and 67 *E. coli* isolates were collected and tested for AMR. In 2019, 74 caecal samples from pigs originating from 74 different holdings were collected. From these samples, 22 *E. faecalis*, 54 *E. faecium* and 71 *E. coli* were isolated and tested for AMR. In total, 138 *E. coli* samples from healthy swine and 141 *Enterococci* samples from healthy swine were collected in the study years.

Clinical material (organs, abortion material, discharge etc.) were collected from diseased cattle and swine by local veterinarians around Estonia. Those samples were sent to and analysed in the National Veterinary and Food Laboratory in Tartu, and selected *E. coli* isolates were included in this study. There are in total of 69 *E. coli* isolates from diseased cattle and 84 from diseased swine. Most samples originated from organs of dead animals, but some samples originated from live animals also, for example in pig's majority of the samples originated from studies of uterine/vaginal discharge and in bovine animals of different age groups the samples mostly originated from faecal samples.

All the samples were sent to and analysed in the National Veterinary and Food Laboratory in Tartu, which is accredited according to of International Organization for Standardization (ISO) 17025 quality management system.

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

Bacteria	Number of isolates during study period Years 2016-2020
E. coli from healthy swine	138
E. coli from diseased swine	84
E. coli from diseased cattle	69
Enterococcus spp. from swine	141
Enterococcus faecalis	47
Enterococcus faecium	94

3.2. Isolation and identification of bacterial species

3.2.1. Isolation and identification of *E. coli* from diseased and healthy swine and cattle

For the detection of *E. coli,* the standard operating procedures of the National Veterinary and Food Laboratory were used. For isolation of indicator *E. coli*, the test sample was suspended in phosphate buffered saline (PBS), using a cotton swab. The suspension was inoculated onto MacConkey agar (selective media) using a 10 µl inoculation loop. The Petri dishes were then incubated upside down at 37°C for 18 to 24 hours. For detection of *E. coli* from samples of diseased animals, direct inoculation onto media was done.

Preliminary evaluation was made based on the typical morphology and colour of *E. coli* colonies on MacConkey agar, which are typically red to purple colonies. Confirmation was performed with using Maldi-TOF mass spectrometry and/or different biochemical tests.

3.2.2. Isolation and identification of *E. faecium* and *E. faecalis* isolated from healthy swine

For the detection and identification of *E. faecium* and *E. faecalis* from healthy animals the standard operating procedures of the National Veterinary and Food Laboratory were used, based on the EFSA guidelines (EFSA, 2008). For the detection, the sample was incubated at an enrichment broth agar, in this study brain heart infusion (BHI) supplemented with 6.5% sodium-chloride (NaCl) was used. They were incubated at 37°C for 18-28 hours. After which, they were sub-cultured onto Slanetz-Bartley agar, incubated at 37°C for another 18-24 hours. Typical colonies are then recognized for *E. faecalis* being deep red with a golden reflection,

and pinkish to white colonies for *E. faecium*. The colonies were then sub-cultured again onto blood agar and were identified by their ability of haemolysis, biochemical tests and/or with Maldi-TOF mass spectrometry.

3.3. Determination of in vitro antimicrobial susceptibility

3.3.1. Broth microdilution method

Susceptibility of *E. coli, E. faecium* and *E. faecalis* from healthy animals was determined using the broth microdilution method according to the standards of ISO 20776. In broth microdilution method an inoculum was prepared by suspending colonies of *E. coli* or *Enterococci* spp. into a Mueller-Hinton broth with a sterile loop or a swab. The broth was then incubated at 35 ± 1°C, until the growth reached a turbidity equal to or greater than 0,5 McFarland standard, which is approximately 1.5 x 10^8 CFU/ml. The suspension was then diluted, so that the final inoculum contained approximately 5 x 10^5 CFU/ml. At this point, it is recommended to perform visible counts of the test suspension to ensure the wells contain approximately 5 x 10^5 CFU/ml. This was done by adding 10 µl of the test suspension and diluting it into 10 ml of saline or broth. 100 µl of the dilution was then spread over an agar plate and incubated overnight, if 20-80 colonies appeared on the plate, it was an acceptable test suspension.

The wells of the microdilution tray must be filled with the broth within 30 minutes of standardizing the suspension. Serial dilution was prepared of the antimicrobial of interest, so that 50 µl of antimicrobial in different concentrations was be added to the wells, and to each well another 50 µl of the bacterial suspension was inoculated. The trays were then incubated at 35 ± 1°C for 18 ± 2 hours at ambient air incubator. Results were read when there was a clear growth in the positive growth control (bacteria) and clearly no growth in the broth sterility control (antimicrobial). The amount of growth was compared to that of the positive growth control, and the MIC is the lowest concentration of antimicrobial that will inhibit visible growth of bacteria completely. For interpretation of results, MIC cut-off values according to Commission Implementing Decision 2013/652/EU were used. The values used are epidemiological cut-off (ECOFF) values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table 3).

According to Commission Implementing Decision (EU) 2020/1729, the testing of commensal bacteria must be performed using the broth micro dilution method only. The susceptibility for

E. coli was tested for ampicillin, cefotaxime, ceftazidime, meropenem, nalidixic acid, ciprofloxacin, tetracycline, colistin, gentamicin, trimethoprim, sulfamethoxazole, chloramphenicol, azithromycin and tigecycline. For *Enterococcus* spp. the susceptibility was tested for gentamicin, chloramphenicol, ampicillin, vancomycin, teicoplanin, erythromycin, quinupristin/dalfopristin, tetracycline, tigecycline, linezolid, daptomycin and ciprofloxacin.

In this study, the isolate was classified as multidrug-resistant (MDR) when it was resistant to three or more antibiotics.

Table 3. EUCAST ECOFF values for *E. coli* and *Enterococcus* spp.

	Interpretative thresholds of AMR (mg/L)	
Antimicrobial	***Escherichia coli***	***E. faecium* and *E. faecalis***
Ampicillin	> 8	> 4
Azithromycin	>16	
Cefotaxime	> 0,25	
Ceftazidime	> 0,5	
Chloramphenicol	> 16	> 32
Ciprofloxacin	> 0,064	> 4
Colistin	> 2	
Gentamicin	> 2	> 32
Meropenem	> 0,125	
Nalidixic acid	> 16	
Sulfamethoxazole	> 64	
Tetracycline	> 8	> 4
Tigecycline	> 1	> 0,25
Trimethoprim	> 2	
Daptomycin		> 4
Erythromycin		> 4
Linezolid		> 4
Quinupristin/Dalfopristin		> 1[1]/NA[2]
Teicoplanin		> 2
Vancomycin		> 4

[1]*E. faecium*, [2]*E. faecalis*

3.3.2. Disc diffusion assay

Antimicrobial susceptibility testing of clinical *E. co*li isolates from cattle and swine was determined using disc diffusion assay following the CLSI protocol document VET01 (CLSI, 2018).

In this method the surface of Mueller-Hinton agar plate was inoculated with the selected bacteria. The inoculum was prepared similarly to the inoculum in microdilution method. Firstly, a direct saline or broth suspension was prepared of isolated colonies. To standardize the inoculum, the suspension was adjusted to equal to 0,5 McFarland turbidity standard. The test plates were then inoculated with the suspension by dipping a sterile cotton swab into the suspension prepared, the swab must be rotated and pressed against the tube to remove any excess fluids, after which the surface of MHA plate was inoculated with the suspension by spreading it with the swab throughout the surface. The procedure was repeated two more times while rotating the plate to ensure even distribution of the inoculum. The lid was then left ajar for 3-5 minutes to allow any excess surface moisture to be absorbed. The paper discs containing the antimicrobials or interest were then placed on the agar. Overlapping must be avoided and maximum of 12 discs can be placed on 150mm plate and six discs on 100 mm plate. The plates were then incubated upside down at 35 ± 2 °C for 16-20 hours in ambient air incubator.

If the bacteria were susceptible for a certain antibiotic, a zone of inhibition appeared around the paper discs. When reading the results, if the inoculum concentration was correct and the plate was streaked correctly, the zones of inhibition will be uniformly circular and there will be confluent lawn of growth. If these findings are not recognized, the test must be repeated.

The diameters of the inhibition zones were measured to the nearest millimetre using a ruler or sliding calipers. To determine susceptibility or resistance of the organisms, inhibition zone diameters were evaluated using breakpoints according to CLSI. The laboratory interpreted the results as S (susceptible), I (intermediate) or R (resistant). Intermediate suggests that there is some decreased susceptibility, but the bacteria is not fully resistant, and some clinical failures can be expected at standard doses.

For clinical submissions, the antimicrobials tested vary between the animals, as the veterinarians decided which antimicrobials are to be tested. Therefore, the antimicrobial and amount of them tested for each isolate differs. Susceptibility of *E. coli* could have been tested for the following: amoxicillin, ampicillin, cefalexin, cefotaxime, ceftiofur, colistin, doxycycline, enrofloxacin, florfenicol, gentamicin, kanamycin, lincomycin, linco-spectin, marbofloxacin, nalidic acid, neomycin, oxytetracycline, tetracycline, penicillin, spectinomycin, streptomycin, sulfamethoxazole/trimethoprim, sulfisoxazole, tetracycline, trimethoprim and tylosin.

3.4. Statistical analysis

The frequency of susceptible, resistant and MDR *E. coli*, *E. faecium* and *E. faecalis* isolated from samples of diseased and healthy pigs and cows were calculated. The proportion of resistance to each antimicrobial tested was also calculated. The descriptive statistics was performed with Microsoft Excel.

3.5. Ethical consideration

The data used in the study were collected and analysed by National Veterinary and Food Laboratory in Tartu and used in an anonymous form.

4. RESULTS

4.1. Resistance profile of *E. coli* isolated from swine and cattle

4.1.1. Resistance profile of *E. coli* isolated from caecal samples of healthy swine in 2017 and 2019

In 2017 68 caecal samples from fattening pigs that originated from 68 different holdings were collected. *E. coli* was isolated from 67 of the samples and all of them were tested for AMR. Of these samples, 62,7% (n=42) isolates were fully sensitive to all tested antibiotics, 37,3% (n=25) were resistant to at least one of the tested antibiotics, and of those samples 13,4 % (n=9) were multiresistant. Most resistance was against tetracycline (19,4%), ampicillin (17,9%), trimethoprim (16,4%) and sulfamethoxazole (14,9%).

In 2019 74 caecal samples from pigs that originated from 74 holdings were taken. *E. coli* was isolated from 71 samples and all of them were tested for AMR. Of these samples 57,7% (n=41) isolates were fully resistant to all tested antibiotics, 42,2% (n=30) were resistant to at least one of the tested antibiotics, and of those 21,1% (n=15) were multiresistant. Similar to samples from 2017, most resistance was against tetracycline (32,4%), ampicillin (25,3%), trimethoprim (25,3%) and sulfamethoxazole (22,5%).

No resistance to meropenem, colistin, gentamicin, azithromycin or tigecycline was detected in either study year. In 2017 no resistance to cephalosporins was detected, and in 2019 only one *E. coli* isolate had developed resistance against cefotaxime and ceftazidime.

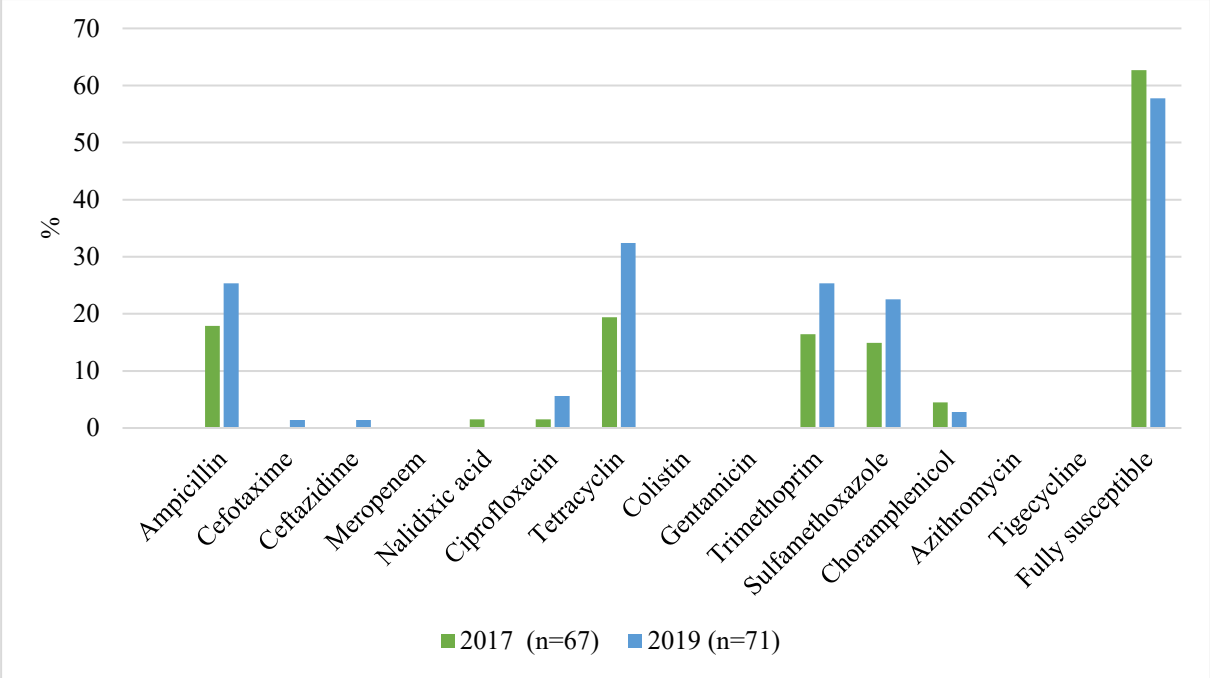

Figure 1. Proportion of resistant E. coli isolates to selected antibiotics from healthy pigs in 2017 and 2019

4.1.2. Resistance profile of *E. coli* isolated from diagnostic submissions of cattle and swine from 2016 to 2020

Between 2016 and 2020, in total 69 *E. coli* isolates were collected from diseased cows and 84 isolates from diseased pigs (Table 4). Of the 84 *E. coli* diagnostic submissions from pigs, 37 originated from post-mortem material and 43 isolates originated from studies on uterine or vaginal discharge from pigs with metritis/vaginitis. The rest of the isolates originated from abortion material (foetuses, foetal membranes), discharge from abscesses and one originated from a faecal sample. Of the *E. coli* isolates, 30,9% (n=26) were fully susceptible to all the tested antibiotics, 10,7% (n=9) were intermediate and 58,3% (n=49) of the isolates had resistance to at least one of the antibiotics tested. Of the resistant isolates 46,9% (n=23) were MDR. Most resistance was against amoxicillin (38,1%), tetracycline (23,8%), trimethoprim (13,1%) and sulfisoxazole (11,9%).

Out of the 69 *E. coli* isolates from dairy cattle, 40 faecal samples originated from cows with enteritis and 25 were post-mortem samples, the rest were from abortion material (foetuses,

foetal membranes) and uterine/vaginal discharge. Of the *E. coli* isolates, only 15,9% (n=11) were fully susceptible to all the tested antibiotics, 8,7% (n=6) isolates were intermediate and 75,4% (n=52) isolates had resistance to at least one of the antibiotics tested. Of the resistant isolates 59,6% (n=31) were MDR. Most resistance was against tetracycline (62,3%), ampicillin (49,3 %), sulfamethoxazole-trimethoprim (40,6%) and enrofloxacin (37,7%). However, what must be considered is that not all antimicrobials were tested for each isolate equally, which might cause some bias in this study. The proportion of antimicrobials tested for the isolates can be seen in Figure 2. and 3. As demonstrated, there are higher resistance rates to the antimicrobials that are tested the most. However, it must be noted that resistance towards cefotaxime and gentamicin were also tested in large numbers in cows and resistance rates for them are low. In pigs, a large proportion of the isolates were tested for marbofloxacin, colistin, enrofloxacin and ceftiofur and *E. coli* had very low resistance rates against these antimicrobials.

Table 4. Proportion (%) of resistant of *E. coli* isolates from diseased pigs and cows to selected antimicrobials

Antibiotic	Sick pigs (n=84)	%	Sick cows (n=69)	%
Ampicillin		8,3		49,3
Amoxicillin		38,1		7,2
Cefalexin		0,0		0,0
Ceftiofur		3,6		0,0
Colistin		1,2		0,0
Doxycycline		10,7		0,0
Enrofloxacin		1,2		37,7
Lincomycin		6,0		NA
Linco-spectin		1,2		0,0
Marbofloxacin		1,2		1,4
Neomycin		0,0		0,0
Oxytetracycline		1,2		0,0
Penicillin		7,1		1,4
Spectinomycin		1,2		4,3
Trim-Sulfa		10,7		40,6
Sulfisoxazole		11,9		NA
Tetracycline		23,8		62,3
Trimethoprim		13,1		NA
Tylosin		8,3		NA
Cefotaxime		0,0		11,6
Gentamicin		0,0		15,9
Kanamycin		NA		2,9
Nalidixic acid		NA		1,4
Streptomycin		NA		1,4

[1]Not assessed (NA)

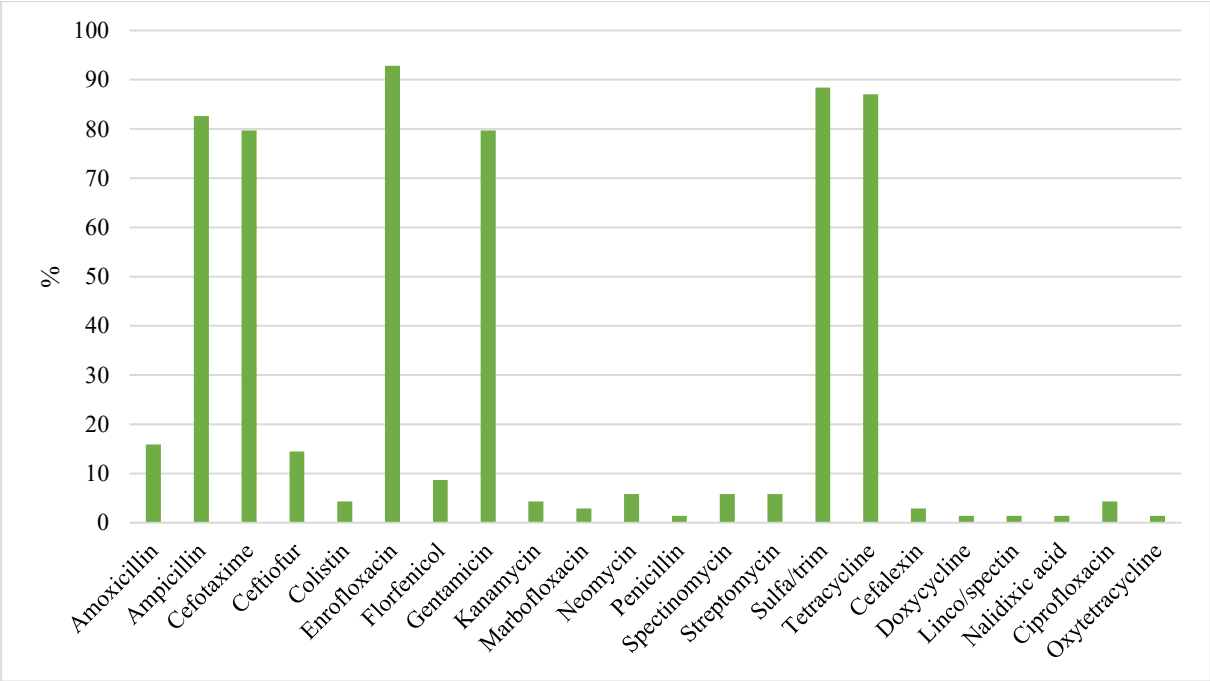

Figure 2. The proportion (%) of antibiotics tested against *E. coli* isolates from diseased cows (n=69)

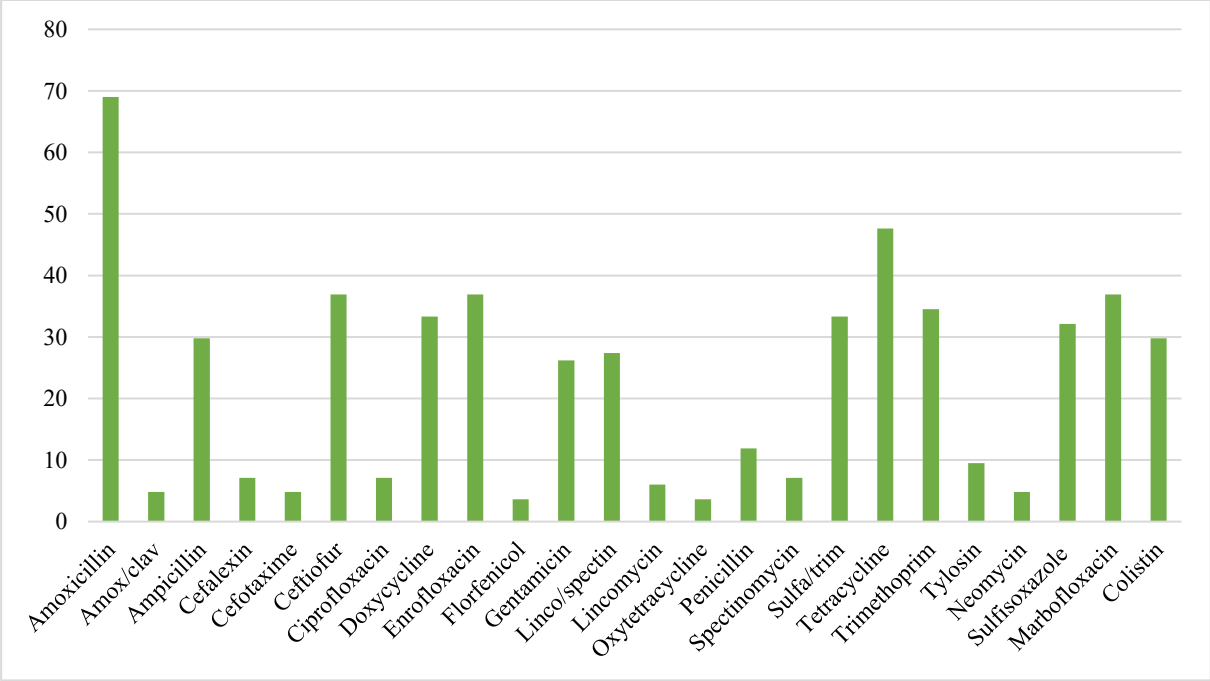

Figure 3. The proportion (%) of antibiotics tested against *E. coli* isolates from diseased pigs (n=84)

4.1.3. Multidrug resistance profiles of *E. coli* isolates

To be classified as a multidrug resistant (MDR) isolate, *E. coli* had to be resistant to three or more antibiotics. The proportion of susceptible, moderately susceptible, resistant and multiresistant *E. coli* isolates is shown in Table 5. Diagnostic submissions from cows have higher proportion of MDR *E. coli* when compared to isolates from pigs (pigs; 27,4%, cows;

44,9%). Isolates from heathy pigs have significantly lower proportion of MDR (17,3%) when compared to isolates collected from diseased pigs.

Table 5. Distribution of susceptible, resistant, and multi-drug resistant *E. coli* isolates from cows and pigs

	Clinically healthy animals	Diagnostic submission	
	Pigs (n=138)	Pigs (n=84)	Cows (n=69)
Susceptible to all tested antimicrobials	83 (60,1%)	26 (31%)	11 (15,9%)
Moderately susceptible isolates	NA	9 (10,7%)	6 (8,7%)
Resistant to 1-2 antimicrobials	31 (22,5%)	26 (30,9%)	21 (30,4%)
Resistant to 3-5 antimicrobials	21 (15,2%)	22 (26,2%)	26 (37,7%)
Resistant to 6-8 antimicrobials	3 (2,1%)	1 (1,2%)	5 (7,2%)

4.2. Resistance profile of *E. faecalis* and *E. faecium* isolated from caecal samples of healthy swine in 2017 and 2019

In 2017 and 2019 a total of 47 *E. faecalis* and 94 *E. faecium* were isolated from fattening pigs at slaughter, and all of them were tested for AMR.

In *E. faecalis* isolates (n=47), total of 27,6% (n=13) were fully sensitive to all tested antibiotics. In total 72,3% (n=34) isolates were resistant to at least one of the tested antibiotics, and of them 29,8% (n=14) were multiresistant. Resistance was mostly against tetracycline (72,3%), erythromycin (46,8%) and chloramphenicol (25,5%). Proportion of resistance rates between study years can be seen in Figure 2.

In *E. faecium* isolates (n=94), a total of 31,9% (n=30) isolates were fully susceptible to all the tested antibiotics and 68,1% (n=64) isolates were resistant to at least one of the tested antibiotics, and of them 8,5% (n=8) were multiresistant. Most resistance was against erythromycin (41,5%), quinupristin/dalfopristin (25,5%) and tetracycline (23,4%). Proportion of resistance rates between study years can be seen in Figure 3.

Neither *E. faecium* nor *E. faecalis* had resistance against vancomycin, teicoplanin, tigecycline or linezolid. Low resistance rates were for ampicillin, gentamicin and daptomycin.

E. faecalis is intrinsically resistant to streptogramins (quinupristin/dalfopristin), but *E. faecium* is usually susceptible at low concentrations. Therefore, the resistance rates of *E. faecalis* was not determined in this study.

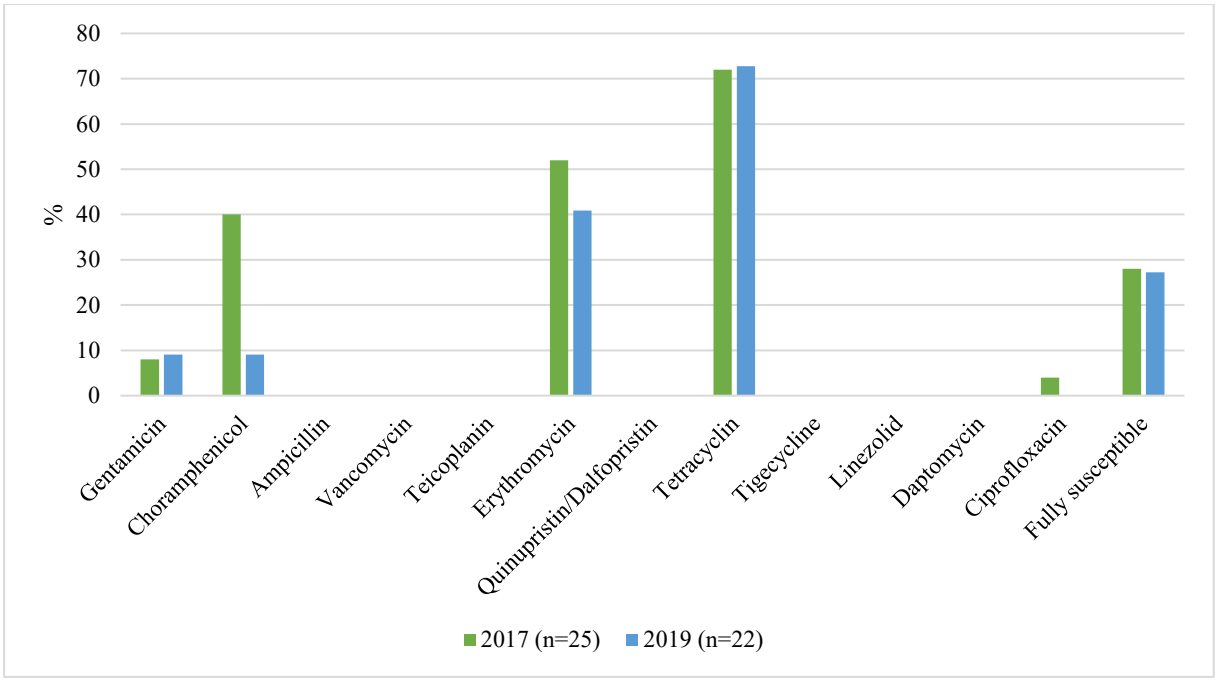

Figure 4. Resistance profile of E. faecalis from healthy pigs in 2017 and 2019

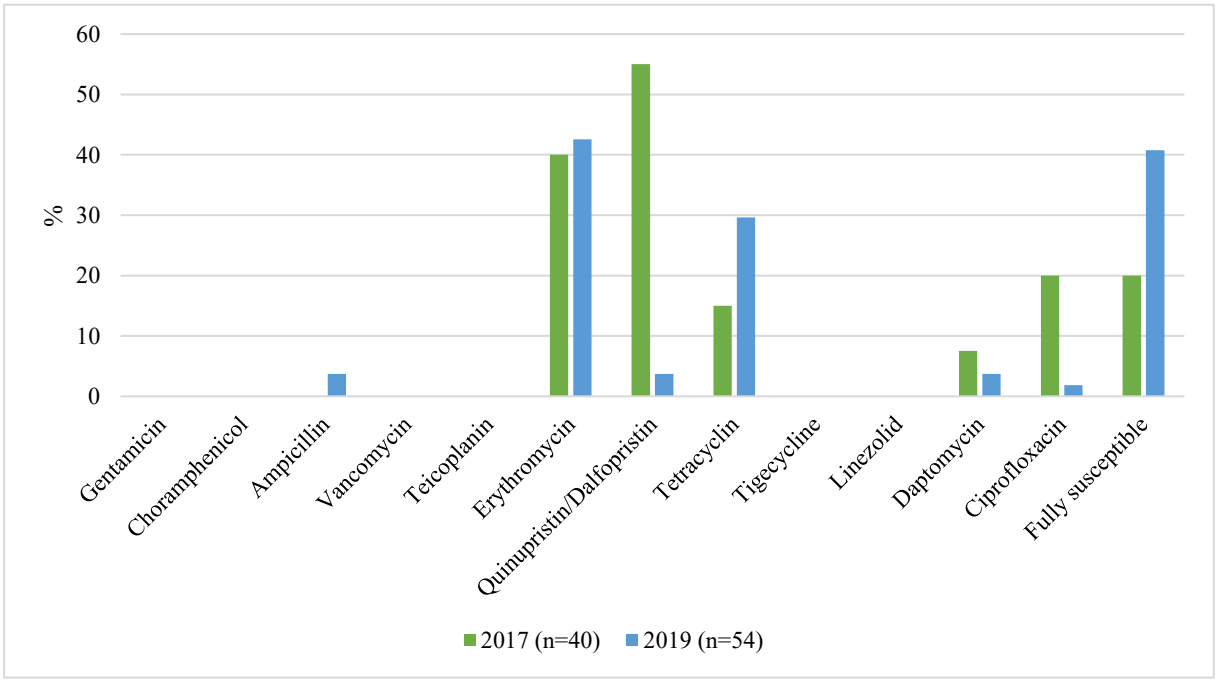

Figure 5. Resistance profile of *E. faecium* from healthy pigs in 2017 and 2019

5. DISCUSSION

The study shows that commensal *E. coli, E. faecalis* and *E. faecium* isolated from healthy fattening pigs have developed resistance against several antibiotics. Commensal *E. coli* had most resistance against tetracycline, ampicillin, and trimethoprim/sulfamethoxazole. For *E. faecalis* and *E. faecium,* resistance was most frequently detected against tetracycline and erythromycin. The highest frequencies of resistance in *E. coli* and *E. faecium* from pigs and cows were against tetracycline, this correlates with previous studies suggesting the extensive use of tetracyclines has resulted in high prevalence of tetracycline resistance (Burow *et al*., 2014).

Any significant changes in resistance profile of *E. co*li did not occur between the study years, but resistance rates to antibiotics had increased slightly as well as the proportion of MDR isolates. When the resistance rates in commensal *E. coli* are compared to other EU countries, we can observe fairly uniform resistance patterns against tetracycline, ampicillin, trimethoprim, sulfamethoxazole, and sulphonamides. Common traits in resistance suggests that due to long-term usage of antimicrobials, the resistant populations will remain while susceptible ones will be eliminated (Levy & Marshall, 2004). Respectively, similar trends are recognized in *E. coli* having low to no resistance at all towards cefotaxime, ceftazidime, colistin, gentamicin, meropenem, azithromycin and tigecycline (FINRES-Vet 2019; SVARM 2019; DANMAP 2019; Aasmäe *et al.,* 2019).

More than half (2019 57,7%; 2017 62,7%) of the *E. coli* isolates were fully susceptible to all the antibiotics tested, the results are better when compared to the European median from pigs, which was 38,8% in 2019 (EFSA, 2021). However, the levels in this study are a bit poorer when compared to the Nordic countries that have the top susceptibility rates in Europe, in 2019 in Sweden, Finland and Norway being 71%, 77,6% and 91,2% respectively (SVARM 2019; FINRES-Vet 2019; NORM-Vet 2019). Nordic countries have the lowest levels of antimicrobials sold in the EU in mg/PCU, which possibly contributes to the highest susceptibility rates. In the EU, the median MDR levels for *E. coli* from pigs was 34,2% in 2019, but the results ranged from 2,8-85,3% (EFSA, 2021). The results in this study are better with MDR from indicator *E. coli* being 13,4% in 2017 and 21,1% in 2019.

Some authors have stated that the oral administration of antibiotics to pigs, directly increases the risk of AMR of *E. coli* in pigs (Burow *et al.*, 2014). Although the use of antibiotics in the

sampled animals was not investigated in this study, according to Estonian State Agency of Medicines tetracyclines and penicillins are majorly sold antimicrobial classes for veterinary use in Estonia in 2015-2020. In this study, there is a high proportion of resistance towards tetracycline in both bacterial species studied, and *E. coli* having high resistance rates towards ampicillin/amoxicillin, which supports the theory. Respectively, the bacteria studied have the least to no resistance at all to the groups sold the least, such as amphenicols, lincosamides, polymyxins and cephalosporins (Estonian State Agency of Medicines, unpublished).

For *E. faecalis* there is a slight decreasing trend in the resistance and MDR rates from 2017 to 2019. Although the use of chloramphenicol has been banned in food-producing animals in the EU since 1994, chloramphenicol resistant isolates were frequently isolated from *E. faecalis* in 2017, but in 2019 the resistance was low. When comparing results of *E. faecium* during the study years, we can see a positive trend in the full susceptibility rates. *E. faecium* isolated in 2017 showed high resistance against dalfopristin/quinupristin (55%), whereas in 2019 the amount was significantly less (3,7%). Quinupristin/dalfopristin is frequently used for infections caused by VRE in critically ill patients, thus maintaining susceptibility of *E. faecium* towards quinupristin/dalfopristin is of importance. Resistance against quinupristin/dalfopristin of *E. faecalis* was not determined due to having intrinsically low susceptibility rates to streptogramins. As *Enterococcus* spp. are intrinsically resistant to numerous antimicrobials such as β-lactams and cephalosporins and are resistant to low levels of aminoglycosides they present a considerable therapeutic challenge (Miller *et al*., 2014), which is why further monitoring is required. Resistance against ciprofloxacin was low, which is of importance due fluoroquinolones being CIAs. Additionally, vancomycin resistant isolates were not detected in this study, which is of importance because vancomycin can be used as a last resort antibiotic in infections caused by enterococci in humans. Full susceptibility rates of *E. faecium* and *E. faecalis* to all tested antibiotics is quite similar to other studies. For instance, a study performed in Estonia in 2010-2015 found that 35% *of Enterococcus* spp. isolated from healthy pigs were fully susceptible. Most resistance was against tetracycline, erythromycin, streptomycin, and kanamycin (Aasmäe *et al*., 2019).

The *E. coli* isolates originating from diseased swine and cattle both show resistance to multiple antibiotics. With diagnostic submissions, there are no specific rules on which antimicrobials must be tested for resistance in this study and the decision was made based solely on the individual cases, thus the isolates were tested with different antimicrobials, and comparing the

two species and results between study years is not desirable. When compared to other studies it is common to find higher resistance against antibiotics that can be used to treat *E. coli* infections in pigs. A study in Finland of 50 *E. coli* isolates from pigs with enteritis found highest resistance rates towards tetracycline, streptomycin, sulfamethoxazole, trimethoprim, ampicillin and nalidixic acid (FINRES-Vet, 2020). Previous study in Estonia shows *E. coli* isolated from diseased pigs having most prevalent resistance against sulfamethoxazole, tetracycline, streptomycin, ampicillin, and trimethoprim. As for cattle, most resistance was against streptomycin, sulfamethoxazole, tetracycline, ampicillin, and trimethoprim (Aasmäe *et al*., 2019). The findings correlate to the results found in this study. Additionally, there was high resistance against nalidixic acid and ciprofloxacin from diseased animals (Aasmäe *et al*., 2019). In this study, resistance for nalidixic acid and ciprofloxacin was hardly studied from either species. But high resistance to enrofloxacin was found in isolates from cows. As fluoroquinolones are widely used in human medicine and are the highest priority CIA, it's a bit worrying. However, the number of isolates is quite low from both animal species, thus the results are not representative of the whole *E. coli* population in Estonia.

Comparing the levels of full susceptibility from healthy and diseased submissions, some bias might occur due to different collection and analysis methods and differing number of isolates. However, the amount of fully susceptible *E. coli* isolates is higher from healthy animals when compared to diseased animals. We can assume, that the diseased animals have been treated priorly with antibiotics, and this could be the reason for higher resistance rates due to selection pressure by usage of antimicrobials. Other studies have reported similar findings (FINRES-Vet 2020; SVARM 2019). Samples from diseased animals should be analysed for bacterial infection and undergo susceptibility testing, prior to initiating treatment with antibiotics.

Cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides and ketolides, polymyxins and quinolones are categorized as the highest priority CIAs by WHO. Therefore, monitoring resistance against them is of particular interest due to risk of spread to humans. In this study, cefotaxime and ceftazidime resistance was tested in all *E. coli* isolates from healthy pigs, and cefotaxime was tested from some *E. coli* isolates from diseased cattle. In indicator *E. coli* only one isolate had developed resistance against cefotaxime and ceftazidime. The EU median is similarly very low (EFSA, 2021). In *E. coli* isolates from diseased cattle, eight isolates had developed resistance against cefotaxime during 2016-2020. Resistance against colistin and azithromycin was not detected from commensal *E. coli* in this study, which reflects

to the European median levels that are generally prescribed as “low” and “very low”. Only one isolate from diseased pig was resistant to colistin. Resistance against ciprofloxacin in commensal *E. coli* and *Enterococci* spp. was found to be low in this. Generally, resistance levels to ciprofloxacin are a lot higher when compared to the other CIAs, the median level in Europe in indicator *E. coli* from pigs was 11,2%. Resistance against vancomycin was not found.

Of importance is the absent resistance rates of commensal *E. coli* to colistin in this study. Colistin is considered as one of the last resort antimicrobials used to treat infections caused by MDR gram-negative bacteria in humans. The colistin resistant mcr-1 gene was discovered in plasmids of *E. coli* in 2015. Liu *et al*. (2016) have hypothesized that there is a clear correlation in usage of colistin in food-producing animals to resistance to colistin found in *E. coli* isolated from human patients. The plasmid carrying mcr-1 gene has also been isolated in Estonia from three *E. coli* isolates from a single pig slurry sample (Brauer et al., 2016).

Monitoring of antimicrobial resistance in bacteria from animals should be carried on annually, and there should be reports made of the findings that are collected into one database. Additionally, more regulations on the use of antimicrobials, especially should be put in place.

CONCLUSIONS

E. coli, E faecium and *E. faecalis* isolates from pigs and cows have developed resistance to numerous antimicrobials. *E. coli* isolates from healthy pigs had developed highest resistance to against tetracycline, ampicillin, trimethoprim, and sulfamethoxazole. *E. col*i isolates from clinical submissions of pigs had highest resistance against amoxicillin, tetracycline, and trimethoprim. *E. coli* isolates from clinical submissions of cattle had highest resistance against tetracycline, ampicillin, sulfamethoxazole-trimethoprim and enrofloxacin. *E. coli* isolates from healthy pigs have higher susceptibility rates than isolates from diseased animals. Proportion of MDR *E. coli* isolates was higher in diseased cows than in diseased pigs. *E. faecalis* had developed highest resistance against tetracycline, erythromycin, and chloramphenicol. *E. faecium* had highest resistance against tetracycline, erythromycin and quinipristin/dalfopristin. Resistance against CIAs was found to be generally low in commensal bacteria.

Prevalence of resistance in commensal and pathogenic bacteria with common traits in resistance in previous years and other countries suggests the use of antimicrobials drives for selection pressure and that resistant bacteria are maintained while the susceptible one’s regress. Due to

risks that resistant bacteria create for humans and animals alike, further constant monitoring is required. For future investigations, monitoring the total antimicrobial use in food-producing animals would be warranted, to make connections between usage and occurrence of resistance and for easier implementation of prudent use guidelines.

The inappropriate and excessive use of antimicrobials in humans and animals has come with a cost. The impact that AMR has on the health and economy is significant, and it is a phenomenon affecting animals, humans, and the environment equally. Thus, the problem of resistance is complex and cannot be addressed by one country or continent only and requires a multisectoral response. Globally, the prudent use of antimicrobials to date has not been met. Stopping the occurrence of AMR is likely not feasible, but it is possible to try to cease the rate at which it's occurring. Antimicrobials should only be used when justified with the most suitable antimicrobial with correct dose and course of treatment, thus the overall use of AMs in animals should be reduced and monitored more strictly in Estonia, which could aid in slowing down the rate at which AMR occurs. Additionally, what should be created in the near future, is a central database to which all reports of AMR from animals are collected into. The matter of lacking it has been discussed in an earlier study from Estonia as well.

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