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MAILIS LAHT

Using the One Health approach for mapping the spread of antibiotic resistant bacteria in Estonia

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LIST OF ORIGINAL PUBLICATIONS

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Publication III
Telling K, **Laht M**, Brauer A, Remm M, Kisand V, Maimets M, Tenson T, Lutsar I. (2018) Multidrug resistant Pseudomonas aeruginosa in Estonian hospitals. BMC Infectious Diseases, 18 (1), ARTN 513.
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Publication IV
Telling K, Brauer A, **Laht M**, Kalmus P, Toompere K, Kisand V, Maimets M, Remm M, Tenson T, Lutsar I. (2020) Characteristics of Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae and Contact to Animals in Estonia. Microorganisms, 8 (8), ARTN 1130.
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Publication V
Laht M*, Karkman A*, Voolaid V, Ritz C, Tenson T, Virta M, Kisand V. (2014) Abundances of Tetracycline, Sulphonamide and Beta-Lactam Antibiotic Resistance Genes in Conventional Wastewater Treatment Plants (WWTPs) with Different Waste Load. PLoS ONE, 9 (8), e103705.
http://doi.org/10.1371/journal.pone.0103705. * – Shared first authors.

Publication VI
Laht M, Telling K, Kalmus P, Corander J, Lutsar I, Tenson T, Kisand V. (manuscript in revision) Pseudomonas aeruginosa distribution among humans, animals and the environment in the same local geographical region

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The author's contribution to each article is as follows:

Publication I
I performed laboratory work (isolation of bacteria, DNA extractions and quantification, 16S rRNA gene PCR, preparation of whole genome sequencing) and bioinformatic analysis (MLST) and wrote the manuscript.

Publication II
I participated in experiments (sampling and communication with WWTPs in Estonia, analysis of cefotaxime-resistant E. coli), analysis of results, and reviewing the manuscript.

Publication III
I participated in experiments (DNA extractions, 16S rRNA gene PCR, preparing the bacterial isolates collection and plating strains for phenotypic susceptibility testing), bioinformatic analysis (MLST) and wrote the manuscript.

Publication IV
I participated in experiments (DNA extractions, 16S rRNA gene PCR), bioinformatic analysis (MLST) and wrote the manuscript.

Publication V I
participated in the study design and experimental work (sampling, DNA extractions, qPCR). Participated in data analysis and co-wrote the manuscript.

Publication VI
I performed laboratory work (isolation of bacteria, DNA extractions and quantification, 16S rRNA gene PCR, preparation of whole genome sequencing) and bioinformatic analysis (MLST) and wrote the manuscript.

ABBREVIATIONS

AMC	antimicrobial consumption
DNA	deoxyribonucleic acid
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
MLST	multi locus sequence typing
16S rRNA	16S ribosomal ribonucleic acid
WHO	World Health Organization
AMR	antimicrobial resistance
ARB	antimicrobial resistant bacteria
API	active pharmaceutical ingredient
AST	antimicrobial susceptibility testing
EU	European Union
ESBL	Extended Spectrum β-Lactamases
ESAC-Net	European Surveillance of Antimicrobial Consumption Network
EARS-Net	European Antimicrobial Resistance Surveillance Network
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EFSA	European Food Safety Authority
EMA	European Medicines Agency's
EFSA	European Food Safety Authority
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MIC	minimum inhibition concentration
EQS	environmental quality standard
VMP	veterinary medicinal products
PCU	population correction unit
CIA	critically important antimicrobials
ICS	inter-sectoral steering committee
PNEC	predicted no effect concentration
AWaRe	Access, Watch and Reserve classification of antibiotics
EEA	European Economic Area
ECDC	European Centre for Disease Prevention and Control
MRSA	Methicillin resistant Staphylococcus aureus
MIC	minimum inhibitory concentration
WWTP	waste water treatment plant
MDR	multi drug resistance
WGS	whole genome sequencing
ST	sequence type
ARG	antimicrobial resistant gene

1. INTRODUCTION

Bacterial infections have threatened humanity throughout history. Despite the efforts of mankind, infectious diseases are still widespread and, combined with antimicrobial resistance (AMR), are a rising public health concern. We are losing the possibility of using antibiotics for our benefit and protection against infectious diseases. One of the driving forces for raising AMR is our enthusiasm to over-exploit the beneficial effects of antibiotics in multiple fields: human healthcare, veterinary medicine, and food production. This widespread use of antibiotics has led to a global problem. The WHO has stated that AMR is one of the biggest threats to global health, food security, and development today; it can affect anyone of any age in any country; it occurs naturally, but misuse of antibiotics in humans and animals is accelerating the process; a growing number of infections are becoming harder to treat as the antibiotics used to treat them become less effective and leads to longer hospitalisation, higher medical costs and increased mortality (World Health Organization, 2023a). Still, we lack multidisciplinary actions in pathogenesis and prevention pathways that are more integrated and connect epidemiology to ecology, environmental reservoirs and pathways. We underestimate the risk of spreading antibiotic resistance through the reuse of nutrients (manure, sludge) and water (wastewater, stormwater, untreated natural water). We know that many settings can harbour antimicrobial-resistant bacteria (ARB), including fresh water, soil, food products and feed. Critical pathways for the spreading of ARB are organic waste and wastewater. Some nations have taken actions to prevent and slow the increase of ARB in different areas, but a broader approach is needed to avoid worst-case scenarios and prevent new mistakes. Sustainable use of resources raises new challenges related to the spread of antibiotic resistance.

Collaboration between different parties and coordinated data collection and analysis, or creating a "One Health" surveillance system, is to understand the extent of the problem with antibiotic use and antimicrobial resistance relationships. One Health approach connects people and animal health concerns with the environment and searches for solutions for ARM-related health concerns as a complex epidemiological issue. "A European One Health Action Plan against Antimicrobial Resistance" was developed in 2017 (European Commission, 2017). The strategy was updated in 2023 by the EU Council's Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach (2023/C 220/01)(European Commission, 2023). The spread of AMR among pathogens is a good example of a One Health matter in which human health is connected to that of animals and the environment. Only a multidisciplinary effort can provide an adequate response. There is a significant lack of knowledge about the release and spread of resistant organisms in the environment and the threats and risks this poses to human and animal health. The transfer of resistance between different bacterial species, including pathogens, is frequently documented, as well as the transfer of pathogens between species and environ-

ments. Undoubtedly, ecological systems affected by human activities contain residues of antimicrobial agents and resistant bacteria. Less knowledge exists about how resistance spreads through the environment and affects organisms, ecosystems and humans. All further activities related to the sustainable reuse of resources should be based on knowledge of the spread of antibiotic resistance. The reuse of materials that could be part of antibiotic resistance spreading pathways should be assessed with suitable antimicrobial resistance risk assessment methods. We must develop new technologies to enable efficient and rapid degradation of antimicrobials in wastewater treatment plants, organic waste streams and the environment. The advantages of reuse are saving water resources and sustainable circulation of nutrients. Both activities reduce the usage of clean water supply and reduce the need for new fertilisers. In addition to the water resources, it is also necessary to protect the fertility and safety of arable land so that it will be possible to produce food in the future.

Resistant bacteria and infectious diseases do not recognise borders between countries or species. Microorganisms are the carriers in the distribution cycle of AMR, capable of changing knowledge and inhabiting several environments and hosts. We have to fill in the gaps in the knowledge about the spreading pathways of AMR microorganisms between human activity and the natural environment. Infectious disease management, biosecurity measures and their implementation are critical to reducing the need for antibiotic treatment by reducing the occurrence of infectious diseases. Effective adherence to infectious disease control systems is essential to curbing the spread of resistant microbes in hospital environments and veterinary clinics.

The Estonian authorities have started to use the One Health approach to tackling AMR issues. The necessary national One Health AMR action plan will be finalised by 2024 (Sotsiaalministeerium, 2024). Detailed plans for the prevention and spread of AMR are missing in human health and the environment. In the veterinary field, the AMR action plan has been in place since 2019 and renewed for a period of 2021–2026 (Regionaal- ja Põllumajandusministeerium, 2023).

We support implementing the One Health approach in Estonia with our work by filling in the knowledge gaps in different spreading routes of antibiotic resistance. That knowledge will be used to develop a monitoring program for AMR. To achieve such an ambitious goal, we established a multidisciplinary work group that includes genetics researchers, medical doctors, environmental specialists, and veterinarians to study the spread of antibiotic resistance in our area. We collected bacterial isolates from hospitals and volunteers, veterinary clinics and farms, and soil, water and wastewater for potential relationship detection. We studied the possible spread of antimicrobial resistance in Estonia over 14 years (2007–2021) in several environmental matrices related to human activities.

This dissertation focuses on mapping the distribution of antibiotic-resistant bacteria and medically relevant pathogens in human-related areas to support the One Health development in Estonia and fill in the knowledge gaps for AMR spreading routes in Estonia.

2. REVIEW OF LITERATURE

2.1 Antimicrobial resistance as an epidemiological issue

AMR is a complex epidemiological issue as resistant microorganisms exist in humans, animals, food, and the environment. The main cause of AMR is the use of antimicrobials. Antimicrobial resistance (AMR) remains a major public health concern in the WHO European Region, with estimates showing that each year, more than 670 000 infections are due to bacteria resistant to antibiotics. Approximately 35 000 people die as a direct consequence of AMR in the European Union/European Economic Area (EU/EEA) alone (European Commission, 2024). A need for comprehensive, collaborative and coordinated data collection and analysis from multiple domains was recognised. In July 2022, the European Commission and the Member States declared AMR one of the top three priority health threats. They adopted the EU Council Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach (2023/C 220/01) (European Commission, 2023).

2.1.1 One Health as a necessary bridge between activities

Risk factors for the spread of antibiotic-resistant bacteria exist in three major areas: humans, animals (food production, agriculture) and the environment. In all those areas, the benefits of antibiotics are needed, but misuse has led to problems. Therefore, all activities are directly or indirectly related to each other and need a unified approach to solve the problems. One Health integrates human and animal health and environmental determinants at the local, national, and global levels and was systematically introduced in 2017 (Xie et al., 2017). This concept has been increasingly used to understand the complex interactions between the different dimensions related to health. This understanding requires multi-, inter-, and transdisciplinary perspectives. Pungartnik et al. noted in their review about the uses of One Health that the main areas where One Health has been studied and is critical to use are zoonosis, emerging infectious diseases, antimicrobial resistance and food security/safety (Pungartnik et al., 2023). While health, food, water, energy and environment are all broader topics with sector-specific con-cerns, collaboration across sectors and disciplines is needed to address health challenges such as the emergence of infectious diseases, antimicrobial resistance, and food safety and promote the health and integrity of our ecosystems (WHO, 2024b). The concept of One Health is gen-eral, and at the international policy levels, it covers all necessary aspects. However, due to its complexity, it is practical to focus on specific problems and involve more detailed approaches depending on the questions raised.

One of the topics where the detailed One Health concept has been globally used is the prevention of the spread of antibiotic resistance. WHO has established a framework for international cooperation and coordinated actions that ensures

that sectors collaborate in the design of pro-grammes, policies, legislation and research to improve human health outcomes (World Health Organization, 2024c). The spread of ARB is caused by the widespread use of antibiotics in human medicine, animal husbandry and food production, in veterinary medicine of companion animals and distributed by reuse of resources like water (biological wastewater treatment) and nutrients (manure, sludge).

European Commission has been working towards AMR prevention. The European Council made an important update on June 13th 2023, where adopted the "Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach" (European Commission, 2023) that states the major actions needed for AMR prevention:

1) National Action Plans against AMR:
   - Have it in place by 14. June 2024, and regularly update and implement National Action Plans against AMR ('National Action Plans'), based on the One Health approach and taking into account the objectives of the World Health Organization Global Action Plan and the 2016 Declaration of the United Nations high-level meeting of the General Assembly on AMR.

2) Surveillance and monitoring of AMR and antimicrobial consumption (AMC):
   - Close existing surveillance and monitoring gaps and ensure completeness of data, including real-time data and timely access to data where appropriate by 2030, on both AMR and AMC at all levels (e.g. community, hospitals and long-term care facilities) to support the prudent use of antimicrobials in human health;
   - Continue to assess, based on opinions of the European Food Safety Authority (EFSA) animal diseases caused by bacteria resistant to antimicrobials to ascertain if it is needed to list any of those diseases in Regulation (EU) 2016/429 to categorise them for any regulatory surveillance, control or other management measures.
   - There is no coordinated system for AMR's environmental monitoring. However, the importance of the environmental dimension (including aquatic) should be monitored in line with the One Health approach in groundwaters and surface waters, including coastal waters, wastewater, and agricultural soils, which is essential to understand further the role played by the presence in the environment of antimicrobial residues in the emergence and spread of AMR, the levels of environmental contamination and the risks posed to human health (COM (2022) 540 final (European Commission, 2022). Although the environmental aspect has not been as focused as AMR on human or animal health, growing evidence shows that the natural environment may be a major reservoir and driver of AMR.

3) Infection prevention and control:
   - Ensure that infection prevention and control measures in human health are put in place and continuously monitored to limit the spread of antimicrobial-resistant pathogens. Develop, in coordination with ECDC, EU infection prevention and control guidelines in human health, taking into account a cost-effective approach, notably for hospitals and long-term care facilities.
   - Take measures to improve the health and welfare of food-producing animals to decrease the occurrence and spread of infectious diseases in farming and reduce the need for antimicrobial use.
   - Use good, evidence-based manure management practices and good sewage sludge management practices addressing their application in agriculture to reduce environmental exposure to substances with antimicrobial properties and to AMR determinants.

4) Antimicrobial stewardship and prudent use of antimicrobials:
   - Ensure that measures are put in place in human health to support the prudent use of antimicrobial agents in healthcare settings, including primary healthcare settings, long-term care facilities, and community care.
   - Have programs for collecting and safely disposing of unused, expired and leftover antimicrobials from the community, hospitals and long-term care facilities, farms, veterinary medicine providers, veterinary premises and manufacturing facilities of antimicrobials.

5) Recommended targets for antimicrobial consumption and antimicrobial resistance.

6) Awareness, education and training.

7) Research and development and incentives for innovation and access to antimicrobials and other AMR medical countermeasures.

8) Global cooperation.

2.2 Responsible use of antibiotics

Antibiotics are used for treatment purposes in human medicine and veterinary medicine. Unfortunately, not only treatment of diseases has been the field of use for many antimicrobial substances globally (growth regulators). Overusing antimicrobials in the fields other than treatment of infected animals (prevention activities like the whole herd treatment) has led to the loss of treatment possibilities (WHO, 2022b). The consumption of antimicrobials must be wise, or we will lose the possibility of benefiting from them.

2.2.1 Antibiotics for human treatment

One of the critical health targets of the 2030 Sustainable Development Goals is access to safe, effective and affordable medicines for all. In the case of antibiotics and other antimicrobials, using them carefully is vital to maintaining their effectiveness. To promote the responsible use of antibiotics and slow the spread of antibiotic resistance, the World Health Organization (WHO) developed the Access, Watch, and Reserve (AWaRe) classification system of antibiotics in 2017, and it is updated every two years (WHO, 2023b). The WHO AWaRe framework categorises antibiotics according to their spectrum of activity and potential to develop resistance (WHO, 2022):

- The **Access group** contains antibiotics used in the first- and second-line treatment of infections.
- The **Watch group** contains broad-spectrum antibiotics with a higher potential of developing resistance.
- The **Reserve group** contains last-resort antibiotics used for multidrug-resistant infections.

2.2.2 Antimicrobials for veterinary use

Similar targets as for human antimicrobials use have been set for veterinary medicine. Guidelines for the prudent use of antimicrobials in veterinary medicine were implemented in the EU in 2015 (2015/C 299/04). These principles on the prudent use of antimicrobials have been applied as a routine on farms and in veterinary practices. The new EU Regulations on veterinary medicinal products (2019/6) and medicated feed (2019/4) on the use of antibiotics in farms are now in place, and all the previously only voluntary measures are mandatory to implement (Article 107 in regulation 2019/6).

- All forms of routine antibiotic use, particularly the use of antibiotics to compensate for inadequate husbandry and poor hygiene, promote growth, or increase yield, are now forbidden.
- Prophylaxis is only allowed in exceptional cases for individual animals. Metaphylaxis should be avoided.
- The prescription of antimicrobials should be issued only after a clinical examination or any other proper assessment of the health status of the animal or group of animals by a veterinarian (2019/6).

EU established general regulations. Additional country-specific regulations were needed. For Estonia, the adjusted requirements for prescribing, issuing and using medicine in the provision of veterinary services and the form of the veterinary prescription have been regulated in national law since 2022 (Maaeluministeerium, 2022).

The Antimicrobial Advice Ad Hoc Expert Group (AMEG) was set up to provide guidance on the impact on public health and animal health of the use of antibiotics in animals and on the measures to manage the possible risk to humans (EMA, 2024). The division is based on the principle that antibiotics are crucial in human treatment and should be avoided for animal treatment to prevent the development of resistance. EMA has made categorisation of antibiotics for use in animals for prudent and responsible use:

- Category A – **Avoid** – antibiotics in this category are not authorised as veterinary medicines in the EU; they should not be used in food-producing animals; they may be given to companion animals under exceptional circumstances.
- Category B – **Restrict** – antibiotics in this category are critically important in human medicine, and use in animals should be restricted to mitigate the risk to public health; they should be considered only when there are no antibiotics in Categories C or D that could be clinically effective; use should be based on antimicrobial susceptibility testing, wherever possible.
- Category C – **Caution** – for antibiotics in this category, there are alternatives in human medicine for some veterinary indications; there are no alternatives belonging to Category D; should be considered only when there are no antibiotics in Category D that could be clinically effective.
- Category D – **Prudence** – should be used as first-line treatment whenever possible; as always, it should be used prudently, only when medically needed (EMA, 2020).

In the "CVMP strategy on antimicrobials 2021–2025", the CVMP's mission is stated – ensure the availability of effective antimicrobial medicines for the treatment of infectious diseases of animals while, at the same time, minimising the risks to animals, humans and the environment arising from their use (EMA, 2021). In 2022, a new EU regulation (2022/1255/EU) was introduced (entered into force in January 2023) designating antimicrobials or groups of antimicrobials reserved for the treatment of certain infections in humans and shall not be used in veterinary medicinal products or medicated feed.

2.3 Clinically relevant bacterial species and antibiotics they are most commonly resistant to

The data collection and surveillance programs for the most common bacterial species that cause infections are developed on the European and World levels (ECDC and WHO, 2022). European Antimicrobial Resistance Surveillance Network (EARS-Net) is Europe's largest publicly funded antimicrobial resistance (AMR) surveillance system. The European Centre for Disease Prevention and Control (ECDC) has been carrying out AMR monitoring for humans within EARS-Net since 2001(ECDC, 2017) and annual epidemiological reports about

antimicrobial resistance in the EU/EEA are made (latest for 2022 (European Centre for Disease Prevention and Control, 2023b)). The AMR surveillance for humans focuses on invasive isolates of eight key bacterial species (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter species, Streptococcus pneumoniae, Staphylococcus aureus, Enterococcus faecalis* and *Enterococcus faecium*). Other notifiable diseases caused by microorganisms with AMR, such as *Campylobacter spp*., *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, and *Salmonella spp*., are also monitored by ECDC but are not included in EARS-Net(ECDC, 2017; ECDC, 2023). WHO prioritises fluoroquinolone-resistant *Salmonella spp.*, *Campylobacter spp.* and *Neisseria gonorrhoeae* (WHO, 2017). EARS-Net includes only data from invasive (blood and cerebrospinal fluid) isolates. Starting with the data collected for 2019, EARS-Net has only accepted data generated using EUCAST clinical breakpoints.

European Food Safety Authority (EFSA) is responsible for monitoring AMR in zoonotic and commensal organisms focused on the animal populations to which the consumer is most likely to be exposed through food (EFSA, 2024). The European Centre for Disease Prevention and Control (ECDC) monitors human infections (based on routine clinical antimicrobial susceptibility data). For EU reporting, susceptibility (CS) is defined as susceptibility to each of the nine antimicrobial classes tested in the harmonised panel described by the ECDC methods (ECDC, 2017). On the EU level, we have now developed a system in which both authorities are reporting data in one joint report, and it is easier to make decisions based on data (EFSA and ECDC, 2024). WHO has prioritised this (WHO, 2017).

Our studies focus on bacterial species with clinical relevance and possible connection routes to the other One Health domains. We focused on *Escherichia coli*, *Enterococcus spp*., *Pseudomonas aeruginosa* and *Staphylococcu aureus.*

2.3.1 *Escherichia coli* and *Klebsiella pneumonia* strains that produce extended-spectrum-lactamases (ESBL)

E. coli and *K. pneumoniae* are commonly found in the gut of animals and humans and may act as a reservoir of resistance genes, which may be transferred to other bacteria.

E. coli is the most common cause of community-acquired bloodstream infections and urinary tract infections (ECDC and WHO, 2022). WHO prioritises third-generation cephalosporin-resistant *Enterobacteriaceae* (WHO, 2017). Beta-lactamases are enzymes that open the beta-lactam ring, inactivating the antibiotic. Extended-spectrum-lactamases (ESBLs) are enzymes that hydrolyse most penicillins and cephalosporins, including oxyimino-β-lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but neither cephamycins nor carbapenems. This distribution results from the clonal expansion of producer organisms, the horizontal transfer of ESBL genes on plasmids and, less commonly, their emergence de novo. The most clinically important groups of ESBLs are CTX-M enzymes, emerging since the early 2000s, followed by SHV- and TEM-derived ESBLs (EUCAST, 2017).

Diseases caused by *K. pneumonia* are liver abscesses, bacteremias, pneumonia, and urinary tract infections. Historically, immunocompromised patients have been the main target of serious infections induced by *K. pneumoniae*. However, with the recent appearance and dissemination of hypervirulent strains, healthy individuals have also become susceptible to infection (Abbas et al., 2024). *K. pneumoniae* is easily transmitted between patients, leading to nosocomial outbreaks. Third-generation cephalosporin resistance in *K. pneumonia* has become widespread in the WHO European Region (ECDC and WHO, 2022).

E. coli are monitored as indicator bacteria for antimicrobial resistance in healthy food-producing animals and food-specific monitoring of extended-spectrum beta-lactamases (ESBL)-, AmpC beta-lactamases (AmpC) (EFSA, 2024). Nationally recommended targets for the incidence of third-generation cephalosporin-resistant *E. coli* bloodstream infections (number per 100 000 people) have been set by EU recommendations, and a 0–12% reduction should be met by countries by 2030. Estonia has a target of a 10% reduction compared to the 2019 starting point (2023/C 220/01).

2.3.2 Carbapenemase producing *Pseudomonas aeruginosa*; *Klebsiella pneumoniae* and *Escherichia coli*

WHO has a priority list for Carbapenem-resistant bacterial species: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae* (WHO, 2017). Many carbapenemases are encoded on plasmids, facilitating the spread of resistance genes among organisms of the same species or even different bacterial species.

P. aeruginosa is a common cause of infection (including hospital-acquired pneumonia, bloodstream and urinary tract infections) in hospitalised patients, especially those with compromised immune defences. *P. aeruginosa* is intrinsically resistant to many antimicrobial agents and is challenging to control in healthcare settings (ECDC and WHO, 2022). National recommended targets on the incidence of carbapenem-resistant

K. pneumoniae bloodstream infections (number per 100 000 population) from 2019, and the target reduction per cent by 2030 is from 0–5%. Estonia had no *K. pneumoniae* bloodstream infections incidence in 2019, and the reduction target is zero per cent (2023/C 220/01). Hopefully, we can keep the situation as it is.

Specific monitoring of carbapenemase-producing *E. coli* has been mandatory since 2021 (EFSA and ECDC, 2024). Based on the data reported, resistant *E.coli* isolates have not been found in Estonia (EPI-Net 2020).

2.3.3 Methicillin resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *S. aureus* is a major cause of morbidity and mortality worldwide. The mortality of MRSA bloodstream infections is double that of similar infections caused by methicillin-susceptible strains due to delayed adequate treatment and inferior alternative treatment regimens. MRSA infections are endemic in hospitals and communities in most parts of the world (EUCAST, 2017).

WHO has studied the global prevalence of *S. aureus* methicillin resistance situation worldwide. Based on the data in 2011–2014, among the collected isolates in the USA,>50%; in Africa, 12–80%; in the Western Pacific Region, 4–84%; and 17% in Europe were MRSA (WHO, 2017). Newer data from Europe (EU/EAA 2020) shows that the situation has not improved much, with the range of MRSA infections in different countries between 1.4–49.1% (mean value 16.7%) (ECDC and WHO, 2022).

Methicillin, a β-lactam antibiotic, acts by inhibiting penicillin-binding proteins (PBPs) involved in synthesising peptidoglycan, an essential mesh-like polymer that surrounds the cell. *S. aureus* can become resistant to methicillin and other β-lactam antibiotics through the expression of a foreign PBP, PBP2a, that is resistant to the action of methicillin but can perform the functions of the host PBPs. The MecA gene is in a genome (Stapleton and Taylor, 2002). *S. aureus* mainly causes skin, soft tissue, bone, and bloodstream infections. It is the most common cause of postoperative wound infections (ECDC and WHO, 2022).

The EU has set the target for member states for the reduction of incidence of MRSA bloodstream infections between 3–18% depending on the level of incidence per 100 000 population in 2019. Estonia has a 3% target (2023/C 220/01). EFSA coordinates voluntary monitoring of MRSA in food production in the EU (EFSA, 2024). MRSA is defined based on AST (antimicrobial susceptibility testing) results for cefoxitin or, if unavailable, oxacillin. AST results reported for cloxacillin, dicloxacillin, flucloxacillin or meticillin are accepted as a marker for oxacillin resistance if oxacillin is not reported (EARS-Net, 2022; ECDC, 2023b).

2.3.4 Vancomycin resistant *Enterococcus faecium* and *Enterococcus faecalis*

Enterococcus spp. (*E. faecium, E. faecalis*) are monitored as indicator bacteria for antimicrobial resistance in healthy food-producing animals. They are commonly present in the gut of animals and humans. They may act as a reservoir of resistance genes, which may transfer to other bacteria (EFSA) and ECDC, 2024).

E. faecium belongs to the normal bacterial microbiota of the human gastrointestinal tract. It is usually not pathogenic but can, under certain circumstances, cause severe diseases such as bloodstream infections, endocarditis and peritonitis (ECDC and WHO, 2022). *Enterococcus* strains resistant to vancomycin (VRE) have vancomycin MIC >4 mg/L isolates harbouring VanB (ECDC, 2023b). Vancomycin resistance has a higher prevalence in the USA (80–85% isolates vancomycin resistance) compared to Europe (8%) (WHO, 2017). The latest data about the EU/EEA (excluding the United Kingdom) were concerning as the increasing trend appeared in the population-weighted mean percentage of vancomycin-resistant isolates of *E. faecium* (from 11.6% in 2016 to 16.8% in 2020) (ECDC and WHO, 2022).

WHO is prioritising vancomycin-resistant *Staphylococcus aureus* (WHO, 2017).

2.4 Anthropogenic factors influencing levels of antimicrobial resistance

Three important factors behind the increased resistance are:

1) increase in the use of antibiotics
2) more frequent exposures to pathogenic bacteria due to increased population
3) resistance mechanisms (the ability of bacteria to adapt)

The increase in resistance is related to anthropogenic activities, and risks must be mitigated through conscious action. Below, we list the most relevant transaction points we tackled in this work.

Healthcare-acquired infection and spreading in the hospitals (hospital hygiene). Hospitals are the most critical environment where resistance and multi-resistance can develop. In a closely related space, all the presumptions are met: infectious bacterial strains, weak host (patient), high levels of antimicrobials, and genetic material with already developed resistant properties. Unfortunately, hospital outbreaks occur everywhere, and around 70% of cases of infections with antibiotic-resistant bacteria are healthcare-associated (European Commission. Directorate General for Health and Food Safety. et al., 2023). It is necessary to learn from them so that the spread of infection within the hospital does not repeat itself for the same reasons.

Globalisation and fast travelling. Already developed resistant strains can be spread all over the globe fast; in 24 hours, a person carrying the strain can be everywhere. In combination with gene transfer (antimicrobial properties in mobile elements), spreading resistance can be fast and cause serious treatment failures in the future. In the 2000-nds, the isolates with high resistance were identified, and for example, the NDM-1 enzyme was characterised from an infection caused by a carbapenem-resistant Klebsiella pneumoniae strain (Yong et al., 2009). The spread was fast as the first isolates with the NDM-1 enzyme were detected in the UK in 2008, and it became the predominant carbapenemase-producing *Enterobacteriaceae* in 2009 (Kumarasamy et al., 2010).

Insufficient wastewater treatment and surface water pollution (drinking water). Environment-related activities like recycling biologically active materials (livestock production waste, sewage sludge, slurry) and using biological treatment technologies in wastewater treatment plants create possible risk factors for spreading antibiotic resistance. Wastewater treatment can not remove all the substances, and wastewater sanitation is not the standard procedure in many countries. There is a risk of spreading the resistance when the drinking water sources are closely connected to the waste water outlets (same water body). The use of manure and slurry as nutrients in crop production is generating new contact points and the possible spread of AMR for larger areas by polluting the surface and groundwater.

Agricultural activities and food production

Industrial livestock production uses high levels of antibiotics in animal husbandry and creates several risk points for secondary contamination. The global food and feed market is a possible transfer route of resistance between countries and continents. For example, in the case of the mrc-1 gene, the extensive use of colistin in livestock drove the gene's spread among pigs. We also found the gene among our isolates in the pig farm (Brauer et al., 2016). After the ban, the decrease was noticed (Shen et al., 2020). The highest risk of transmission of AMR is direct transmission from animal to animal. In food production, antibiotics are used for the animals in three ways depending on the need:

1) Treating infections (antimicrobial therapy) – treating animals with antimicrobials diagnosed with clinical evidence of infectious disease.
2) Controlling the spread of infections (metaphylaxis) – the administration of a medicinal product to a group of animals after a diagnosis of clinical disease in part of the group has been established to treat the clinically sick animals and control the spread of the disease to animals in close contact and at risk and which may already be subclinically infected (i.e. not yet showing clinical signs).
3) Preventing infections (prophylaxis) – administering a medicinal product to an animal or group of animals before clinical signs of a disease to prevent the occurrence of disease or infection (EFSA, 2024).

Care must be taken where antibiotics are used, and additional measures must be taken to continue activities but resistance does not spread.

2.4.1 Human aspect of AMR in Estonia compared to the EU – consumption and resistance rates

Human antibiotic consumption in Estonia

Estonian competent authority (The Republic of Estonia Agency of Medicines) regularly reports antibiotic consumption data to the European Network (ESAC-Net). The Estonian consumption data are based on total wholesaler data aggregated at the country level, with separation of antibiotics bought by community and hospital pharmacies. According to a 2022 report, the use of antibiotics in Estonia is well under control (ECDC, 2023a).

All antibiotics are prescription drugs in Estonia. Antibiotics are used more in hospitals and less for home treatment. Estonia has a lower community level and average hospital consumption than other EU/EEA countries. WHO set a global target that 60% of antibiotics prescribed at the country level must be from the Access antibiotics (according to WHO AWaRe classification) by 2023(WHO, 2022)). Estonia has met this target as 64% of the AB used were from the Access group in 2021 (WHO 2021 data (WHO, no date a)) (Figure 1). In the EU, with new recommendations from 2023, a target for EU members has been that at least 65% of AB used should be from the AWaRe access group by 2030 (2023/EC 220/01).

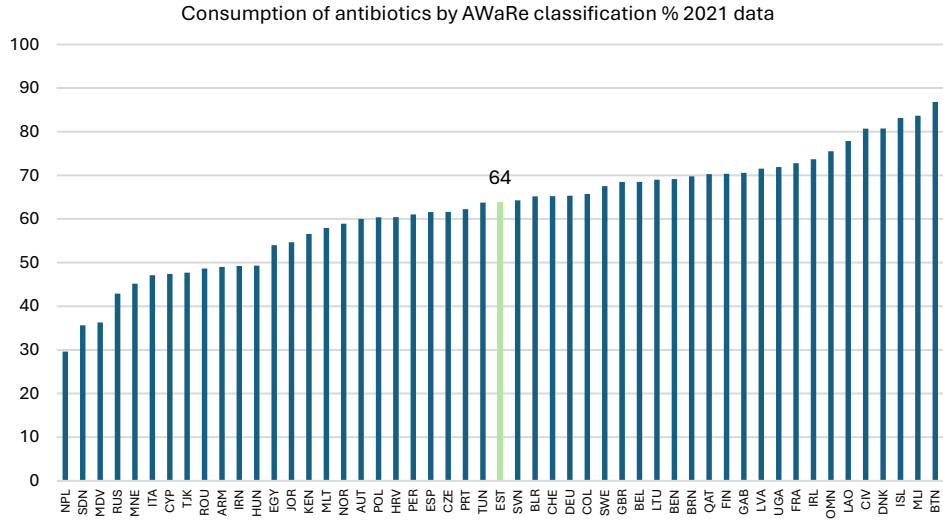


Figure 1. Access group antibiotic consumption by countries % from total consumption in 2021. Consumption of antibiotics by AWaRe classification Global AMC data. The global GLASS-AMC report summarizes the annual antibiotic consumption data reported by Countries, territories, and areas (CTAs) to GLASS by the end of 2022 for the selected year (Data from Global AMC data Web Page).

Antibiotic resistance among human pathogens in Estonia

EARS-Net has done antimicrobial resistance (AMR) surveillance in Europe. From Estonia, three institutions report the data to the EARS-Net: Estonian Health Board, East-Tallinn Central Hospital, and Tartu University Hospital (ERAS-Net, 2022). Estimated national population coverage (mean population coverage (%)) of laboratories reporting *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Enterococcus faecium* to EARS-Net for Estonia is 100%. Figure 2 shows the highest percentage of isolates with resistance phenotype of invasive isolates tested in species-antibiotic combination in Estonia according to the EARS-Net 2022 data (ERAS-Net, 2022). All the resistance rates in Estonia are lower than the EU average.

Estonia has an Infectious Disease Information System (NAKIS) where drug-resistant infectious agents from humans are recorded based on the phenotypic resistance of isolates. For example, in 2020, the number of drug-resistant infectious agents in humans was 1443 notifications (the majority of them: *E. coli* ESBL+ 932; *K pneumonia* ESBL+ 286 and *S. aureus* MRSA +174) (Tenson et al., 2024).

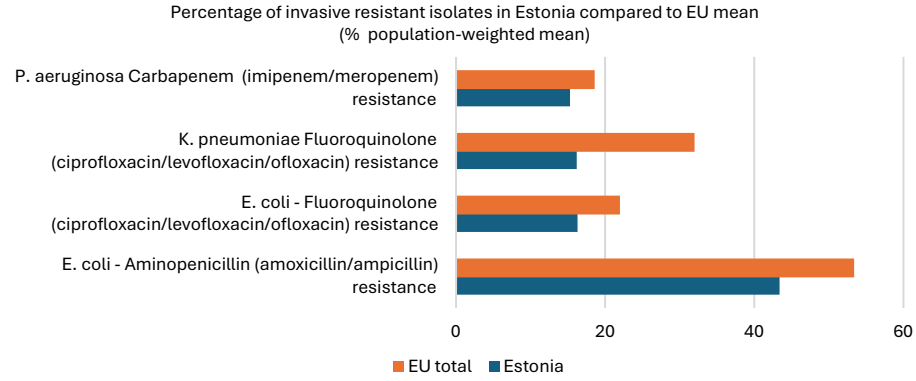


Figure 2. The percentage of invasive resistant isolates in Estonia compared to EU mean (% population-weighted mean) (ERAS-Net, 2022).

Multidrug resistance among human isolates

Multidrug resistance is commonly defined as an isolate resistant to at least three antimicrobials. Figure 3 shows the comparison of MDR for invasive human isolates in Estonia and EU/EAA in 2022 (ERAS-Net, 2022).

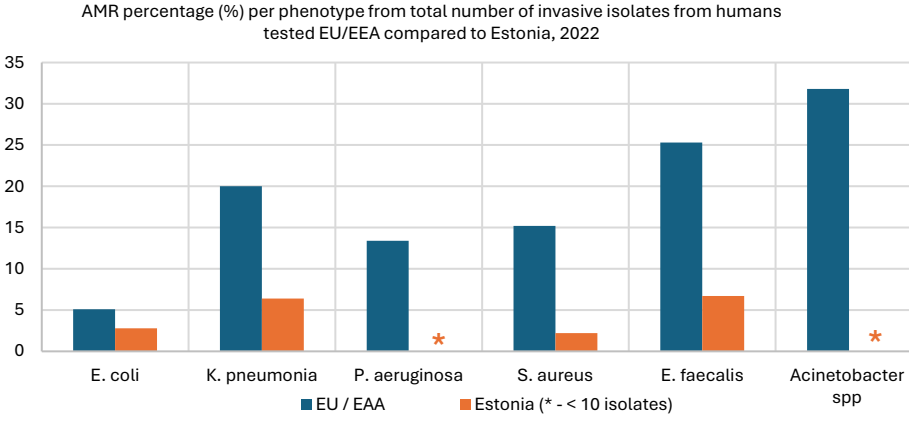
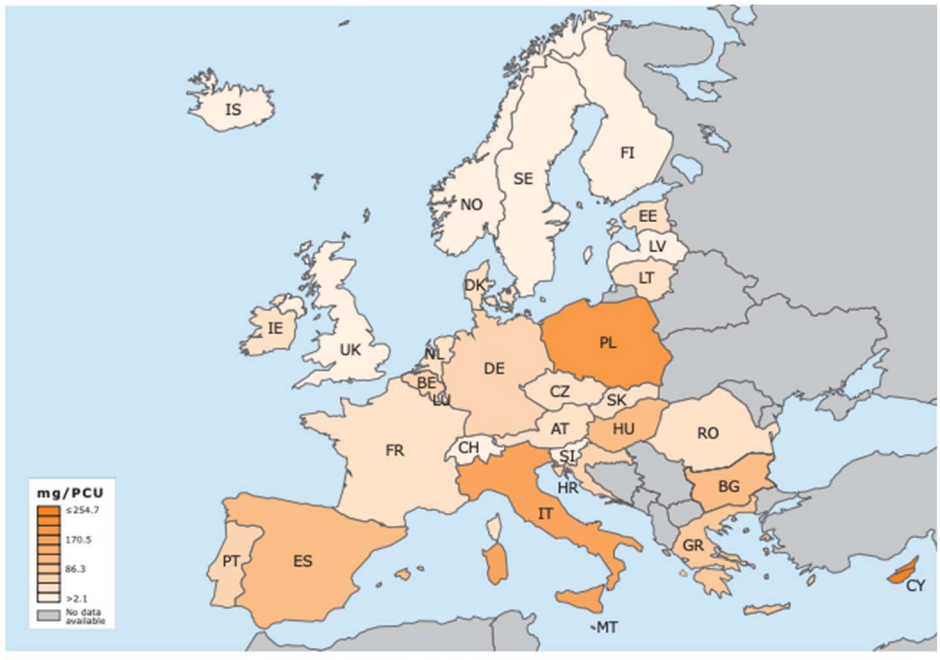


Figure 3. AMR percentage (%) per phenotype from total number of invasive isolates from humans tested EU/EEA compared to Estonia, 2022 (ERAS-Net, 2022). According to the bacterial species, multi-resistance has been identified for the following antibiotics: *E. coli* – combined resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides; *K. pneumonia* – combined resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides; *P. aeruginosa* – combined resistance to ≥3 antimicrobial groups (among piperacillin-tazobactam, ceftazidime, carbapenems, fluoroquinolones and aminoglycosides; *S. aureus* – cefoxitin or, if unavailable, oxacillin; *E. faecalis* – gentamicin resistance; *Acinetobacter spp.* – combined resistance to carbapenems, fluoroquinolones and aminoglycosides; *E. faecium* – vancomycin resistance.

2.4.2 Veterinary aspect of AMR in Estonia compared to the EU – consumption and resistance rates

Veterinary antimicrobial consumption in Estonia

The level of total sales of antimicrobials in the veterinary sector (in the food-producing animal population (including all horses and excluding companion animals)) in Estonia is moderate based on sales of veterinary antimicrobial agents in 31 European countries in 2022 (Figure 4) (EMA, 2022). Sales of antibiotic veterinary medicinal products (VMP) for use in food-producing animals represented 98.4% of total sales. They ranged from 2.1 mg/PCU to 254 mg/PCU in the 31 participating countries (Estonia 45.8 mg/PCU) in 2022.



[1] ESVAC-participating countries' codes according to ISO 3166 — Codes for the representation of names of countries and their subdivisions.

Figure 4. Spatial distribution of overall sales, in mg/PCU, of antibiotic VMPs for food-producing animals in 31 European countries in 2022 (European Medicines Agency, 2022). (PCU – The population correction unit, referred to as PCU, has been established as a denominator for the sales data and serves to normalise the total quantities of active substances sold in each country by the animal population that could be potentially treated with these in each country. The PCU only includes food-producing animals, including horses and farmed fish, as population data of companion animals such as dogs and cats are not available for all participating countries. 1 PCU = 1 kg of animal biomass.)

The use of antibiotics in the treatment of farm animals is prevalent in Estonia, as the proportion of bacterial infections among all diseases is high. It has been found that the length of antibiotic treatment, the choice of active substances used, and the indication for treatment varied among farms in Estonia. The most common active substances used for animals are beta-lactams and tetracycline (Tenson et al., 2022).

Category B antimicrobials are critically important in human medicine (EMA, 2020). Third- and fourth-generation cephalosporins and fluoroquinolones belong to the category B antimicrobials and have been extensively used in Estonia for food-producing animals. For the third and fourth-generation cephalosporins, Estonia has been the top user of the EU for the years 2016–2020 (Kalmus, Aasmäe and Sammul, 2022).

The regulation for using these antimicrobials for food-producing animals only when susceptibility tests have proved impossible to use first-line treatment medicines (category D) has been in place for years in national law. For fluoroquinolones, the trend has been reversed. In 2020, the amount used in Estonia was equal to EU median sale numbers (1.1 mg/PCU) and lower in 2022 (EU mean 1.9 mg/PCU Estonia 0.79 mg/PCU) (EMA, 2022). The changes have been noticed for less usage of enrofloxacin (oral form) and increased use of marbofloxacin (injectable form) (Kalmus, Aasmäe and Sammul, 2022). For the third and fourth-generation cephalosporins, also a significant decreasing trend has been achieved (from 0.91 mg/PCU in 2018 to 0.46 mg/PCU in 2022), but still above the EU mean of 0.1 mg/PCU in 2022(EMA, 2022). The survey was conducted in 2019 to determine the reasons for Estonia's higher-than-EU average use of third and fourth-generation cephalosporins. The survey aimed to determine how much category B antibiotics are used and which diseases are treated with them in cattle and pigs in Estonia. 38.7% of Estonian dairy cows were studied (50 different farms). A total of 24 active pharmaceutical ingredients (API) were used to treat cows. In the previous 12 months, at least one animal from all study herds was treated with 3.– 4. generation cephalosporins in 47 farms (92.1%) and quinolones in 40 farms (78.4%). The reason was not clear why veterinarians preferred this category B API-s in a situation where alternatives for the treatment of bovine bacterial infections (mastitis, inflammation of the uterus and inflammation of the hoof) are available. For the pig farms, data collection was problematic due to the incomplete registration of antibiotic usage (Kalmus, Aasmäe and Sammul, 2022).

Antibiotic resistance among veterinary pathogens in Estonia

AMR monitoring for animals has been carried out in Estonia according to the European program on monitoring and reporting antimicrobial resistance in zoonotic and commensal bacteria (The rules were revised (2020/1729/EL) and applied since 2021). Data is collected about *Salmonella spp*. (including strains producing the following enzymes: Extended Spectrum β-Lactamases (ESBL), AmpC β-Lactamases (AmpC), Carbapenemases (CP)); *Campylobacter coli* (*C. coli*); *Campylobacter jejuni* (*C. jejuni*); commensal *Escherichia coli* (*E. coli*)

(including strains producing ESBL, AmpC, CP); commensal *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium (E. faecium*). The following pathogens mainly cause animal diseases in Estonia: *E.coli*, *Staphylococcus spp*., *Brachyspira hyodysenteriae* and *P. aeuruginosa* (EFSA and ECDC, 2024).

The problematic trend in the use of 3.- and 4.-generation cephalosporins in veterinary treatment has led gram-negative environmental bacteria to develop resistance to extended-spectrum beta-lactams. The possibility of isolating the ESBL *E. coli* in bovine farms using higher amounts of cephalosporins per year is 2.5 times higher (Tenson et al., 2022). The prevalence of presumptive ESBL-/AmpC-producing *E. coli* in broiler meat has been reported to have statistically significant decreasing trends in Estonia. However, a significant increase from 2015 to 2021 of presumptive ESBL-/AmpC-producing *E. coli* has been reported in pig meat and cattle meat in Estonia (EFSA and ECDC, 2024). There is no database for information about the AMR veterinary pathogens in Estonia.

Harmonised antimicrobial resistance monitoring

Food is one possible resistance gene transfer route from animals to humans. Efforts to prevent the resistance transfer will only bear fruit if international cooperation exists. For example, studies have shown that *E. coli* is more frequently found in imported meat than locally produced meat in Estonia (Kalmus, Aasmäe and Sammul, 2022). Some β-Lactamases were only detected in imported meat products (blaCARB-2, blaCTX-M27, blaCTX-M32), of which blaCTX-M27 was found from 11.4% human isolates (Tenson et al., 2022). Intersectoral data sharing on the spread of resistance is important. For that purpose, a harmonised joint report of the food and human antimicrobial-resistant isolates 2021–2022 AMR monitoring in *Salmonella spp.*, *Campylobacter jejuni* and *Campylobacter coli* from humans and food-producing animals (broilers, laying hens and fattening turkeys, fattening pigs and cattle under one year of age) and relevant meat was prepared. For animals and meat thereof, AMR data on indicator commensal *E. coli*, presumptive extended-spectrum beta-lactamases (ESBL)-/AmpC beta-lactamases (AmpC)–/carbapenemase (CP)producing *E. coli*, and the occurrence of methicillin-resistant *Staphylococcus aureus* (MRSA) are also analysed. Relevant findings for Estonia from that report were: Tetracycline resistance in human *C. coli* is high in the EU (average 71%) but even extremely high in Estonia (94%). A significantly increasing trend of resistance to tetracycline in *C. coli* from fattening pigs for the period of 2014–2022 was observed in Estonia. Estonia has been reporting the highest % of *C. coli* ciprofloxacin isolates in the EU for 2021 and 2022. On the other hand, no erythromycin-resistant *C. jejuni* was isolated in Estonia (EFSA and ECDC, 2024).

Companion animals

Human health risks associated with using antibiotics for companion animals are often underestimated. In Europe, approximately 68 million households own cats,

and 78 million households own dogs (Statista, 2022). Approximately 58 000 dogs and 24 000 cats are registered in Estonia (https://lemmikloomaregister.ee/) 37% of households in Estonia have cats, and 27% have dogs (If Kindlustus, 2021). The amount of antimicrobial active substances used in companion animals was 2022 70.8t in 31 European countries (Estonia 0.19t) (EMA, 2022). In Europe, carbapenem-resistant Enterobacterales are sporadically but regularly reported in companion animals despite the rarity of use of carbapenems. The type of enzyme causing the resistance in pets often mirrors the human epidemiology in the same country (EFSA and ECDC, 2024).

2.4.3 Environmental aspects of AMR in Estonia – AB resistance rates and AB residues in the environment and the role of the environment in the spread of antibiotic resistance

Water environment

Contamination of **drinking water** with antibiotic-resistant bacteria and getting an infection from the water is relatively small in Estonia because the majority of the population is connected to the public water supply (83% in 2022), and most of the drinking water is produced from groundwater (58% in 2021) (Keskkonnaagentuur, 2022).

Rivers, lakes and Baltic Sea water

The spreading of potentially disease-causing bacteria (indicators of microbial pollution) in the environment (surface water, groundwater, soil) is not monitored regularly. There are no current plans to monitor the presence of resistant microorganisms in the environment in the near future. Still, the new direction in European legislation is towards monitoring microorganisms, genes or genetic material reflecting the presence of microorganisms resistant to antimicrobial agents, particularly microorganisms pathogenic to humans or livestock in the water environment. The directive 2000/60/EC annexe VIII update states the need to monitor hygiene parameters. Antimicrobial resistance genes are included in the indicative list of the main pollutants (European Commission, 2022).

Concentrations of API residues in the water environment have been monitored for some years. Only nine antimicrobial substances have been regularly analysed in Estonian environmental samples. Three of them (azithromycin, erythromycin, clarithromycin) have been monitored in Estonian environmental monitoring programs and related development projects since 2015. They are now determined annually in national surface water and groundwater monitoring. Amoxicillin, ciprofloxacin, tetracycline, ofloxacin, clindamycin and sulfamethoxazole have been added later to the monitoring programs, and only project-based data has been collected so far. Figure 5 presents summarised results of antimicrobial residues in Estonian surface water samples (inland and marine).

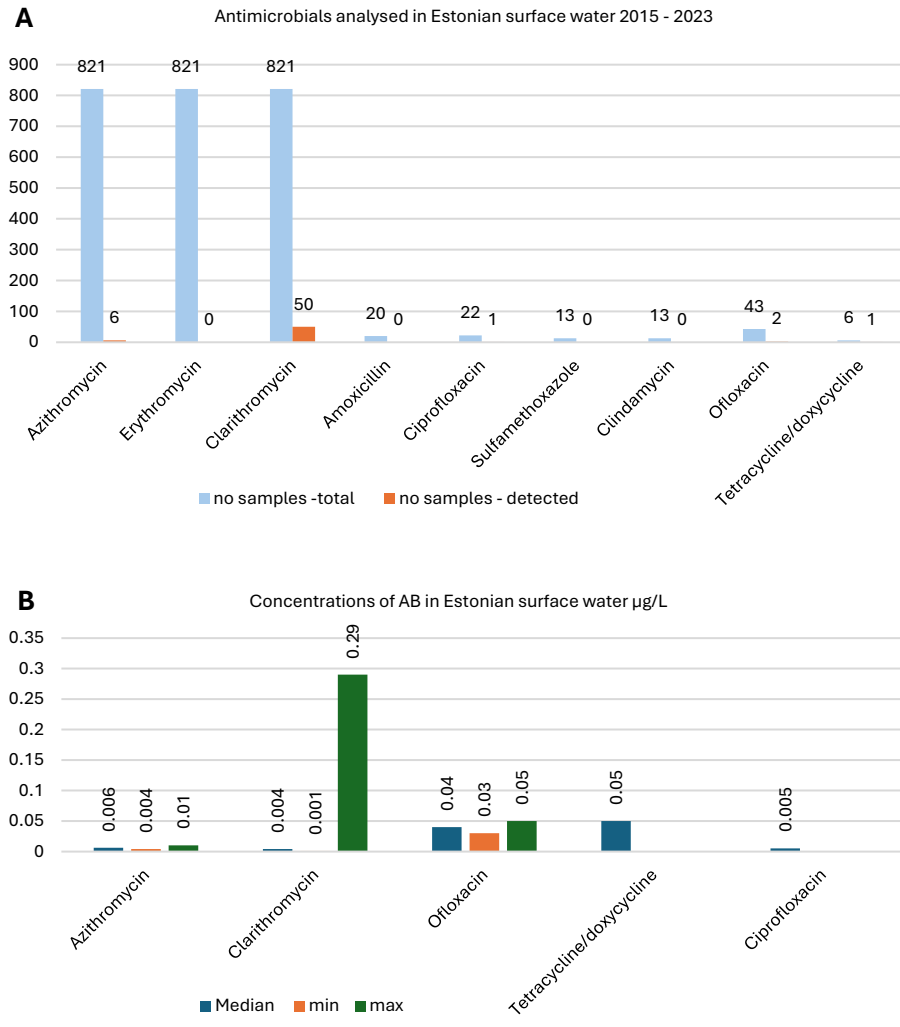


Figure 5. Antimicrobial residues in Estonian surface water samples. Data from Estonian Environmental monitoring Database (Keskkonnaagentuur (2023a), Keskkonnaseire infosüsteem). Panel A shows the number of total samples analysed (2015–2023) and the number of samples where antibiotics were detected. Panel B shows detected concentrations.

Although the overall levels of ABs are low in the Estonian water environment, the detected AB concentrations in the environment in some areas still show risks at the local level. Predicted no-effect concentrations (PNEC) values assess potential environmental risk. API concentrations above PNEC have an ecotoxicological effect on the ecosystem, and the microbial community is more likely to develop and keep antimicrobial properties and resistance genes.

When comparing the results for three antibiotics (azithromycin, erythromycin, clarithromycin) from surface water with the new proposed environmental quality

standards (EQS), the most problematic is azithromycin, where all the results above the detection limit exceed the proposed limit value of 0.0019 µg/l. Erythromycin and clarithromycin levels are lower than the proposed EQS (0.13 and 0.5 µg/l, respectively) (European Commission, 2022). For clarithromycin, the median detected concentration (0.004 µg/l) in water exceeds the PNEC value. Ofloxacin exceeded the PNEC both times it was detected (LOQ equals the PNEC 0.02µg/l). Used PNEC values are from the work of Kalinowski and Kondzielski, 2020.

The sediment risk assessment in the Baltic Sea region revealed that the levels of ciprofloxacin, clarithromycin and the sum concentration of doxycycline and tetracycline may pose environmental risks in many of the Baltic Sea estuaries (Ek Henning et al., 2020). In Estonian river sediments, nine antibiotics were detected (trimethoprim, clarithromycin, fluconazole, lincomycin, ofloxacin, azithromycin, tetracycline, norfloxacin, ciprofloxacin) (Ek Henning et al, 2020; Keskkonnaagentuur, 2023a). When compared to the PNEC value (Lowest PNEC values for the sediment from NORMAN Ecotoxicology Database – Lowest PNECs (05.2024) were used (NORMAN Association, 2024)) in Estonia, the most problematic AB in sediment are ciprofloxacin, norfloxacin and tetracycline maximum concentration exceed the PNEC value 22.8, 6.4 and 1.3 times, respectively.

Bathing water

The possible risk of getting the infection through bathing water is low in Estonia because the water temperatures in the region are low (up to 20 °C in summer), and there is no beach weather most of the year. All these climate conditions do not favour the spread of pathogens living in warm-blooded organisms. *E. coli* and *Enterococcus spp*. are monitored in bathing water as quality criteria (2006/7/EC) (European Parliament, 2014). In 2022, 64.6% of Estonian bathing waters were of excellent quality (EU total 85.7%). Resistance is not monitored among bathing water isolates (EEA, 2023).

Wastewater effluence and sewage sludge

Wastewater effluents inherently pose a higher risk of spreading resistance. However, in Estonia, only a few wastewater recycling activities are carried out, and the spread of resistance via wastewater is low. Wastewater effluents are discharged to rivers, lakes and coastal seas. There is currently no requirement in Estonia to disinfect wastewater or to remove antibiotic residues from the wastewater. Therefore, microbiological quality indicators such as monitoring the total number of bacteria or the number of *E. coli* and the presence of antibiotic residues are not monitored in the wastewater effluent samples. The main reason is the separation of effluent systems from water intakes in Estonia. Some information about the microbiological indicators has been collected in studies that map the situation of drinking water sources in Estonia. The Spread of AMR as an additional risk factor may be the reason for considering adding disinfection stages to

wastewater treatment. The tertiary treatment obligation will be in place for WWTP plants of over 100 000 pe (European Parliament, 2022).

The possible risks from pharmaceuticals for the Baltic Sea were mapped in 2017 (The United Nations Educational, Scientific and Cultural Organization and HELCOM, 2017). Detection of removal rates for antimicrobials from the wastewater is not always possible due to the raw wastewater's matrix effect. From the water phase, approximately 50% of antimicrobials can be removed (Ek Henning et al., 2020). Antimicrobials are one group of pharmaceuticals that stay in the solid phase (sludge) and can enter the environment (Figure 6) (UNESCO and HELCOM, 2017).

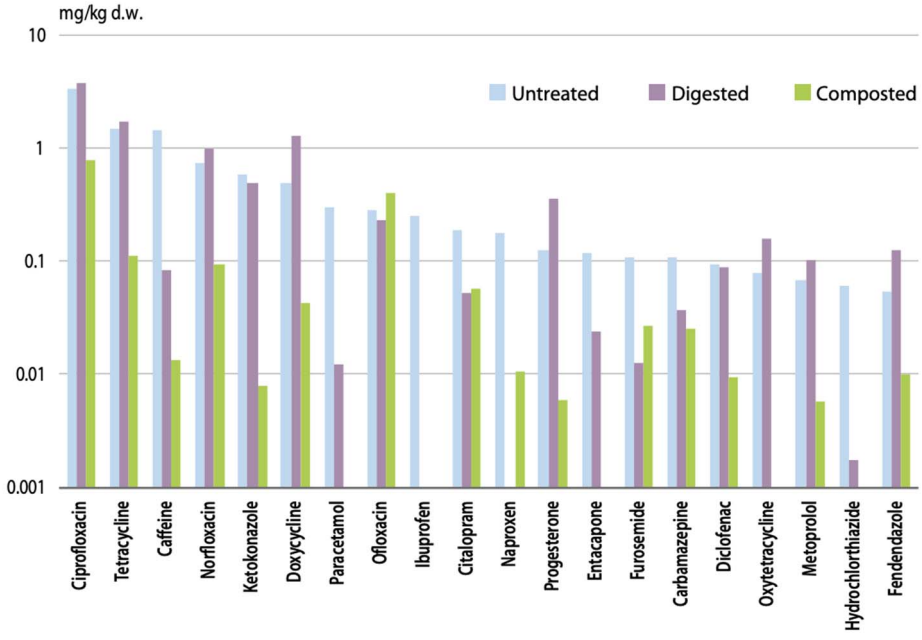


Figure 6. Average concentrations of pharmaceuticals in untreated, digested and composted sludge in the Baltic Sea region for the period 2003–2014. Denmark, Estonia, Finland, Germany, Poland, Russia and Sweden provided data. (UNESCO and HELCOM, 2017).

Figure 6 compares detected antibiotics in untreated, digested and composted sewage sludge in the Baltic Sea region. Figures 7 and 8 show the overall situation for the antimicrobials in the Baltic Sea region wastewater-related matrixes. Concentrations in effluent in Estonia compared to other Baltic Sea region countries were higher in Estonia for clarithromycin (2.2 µg/l), tetracycline+doxycycline (SUM) (0.55 µg/l), erythromycin (5 µg/l). Norfloxacin was detected in all Baltic Sea region countries (average 13 µg/l; highest in Estonia 19 µg/l) (Ek Henning et al., 2020). Figure 9 summarises the results of the WWTP-s for the Baltic Sea region. Antimicrobials pose risks in water and solid phase (Baltic Marine Environment and Protection Commission (HELCOM), 2022).

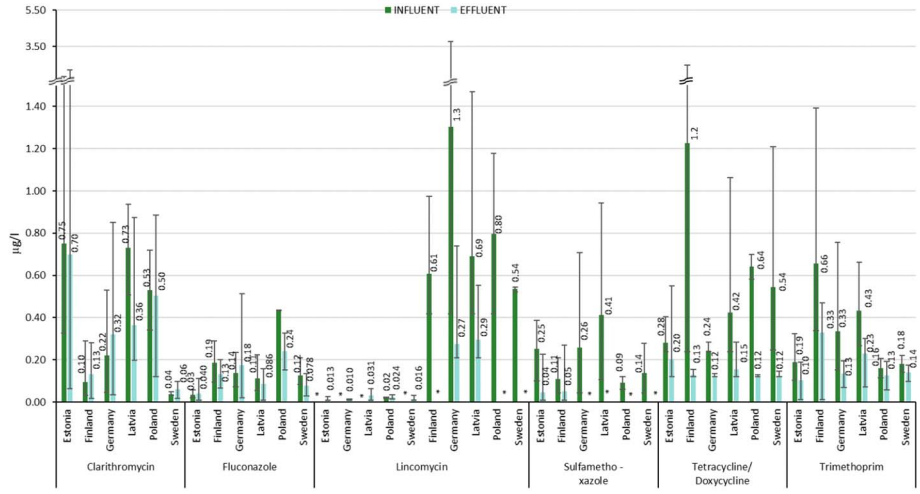


Figure 7. Average concentrations of antibiotics in WWTP influent and effluent. Data from Interreg CWPharma project 2017–2018 (Ek Henning et al., 2020).

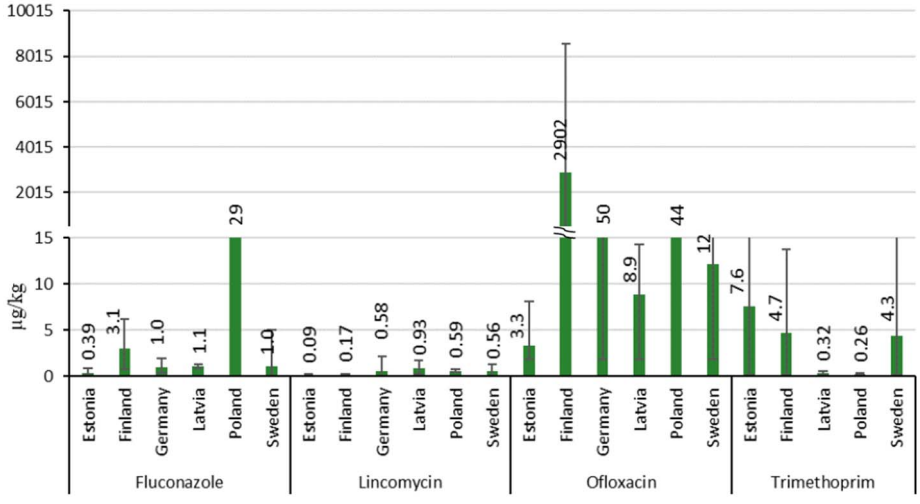


Figure 8. Average concentration of antibiotics in sludge in Baltic Sea region 2017–2018. Only 4 AB were measured from the sludge and all were detected (Ek Henning et al., 2020).

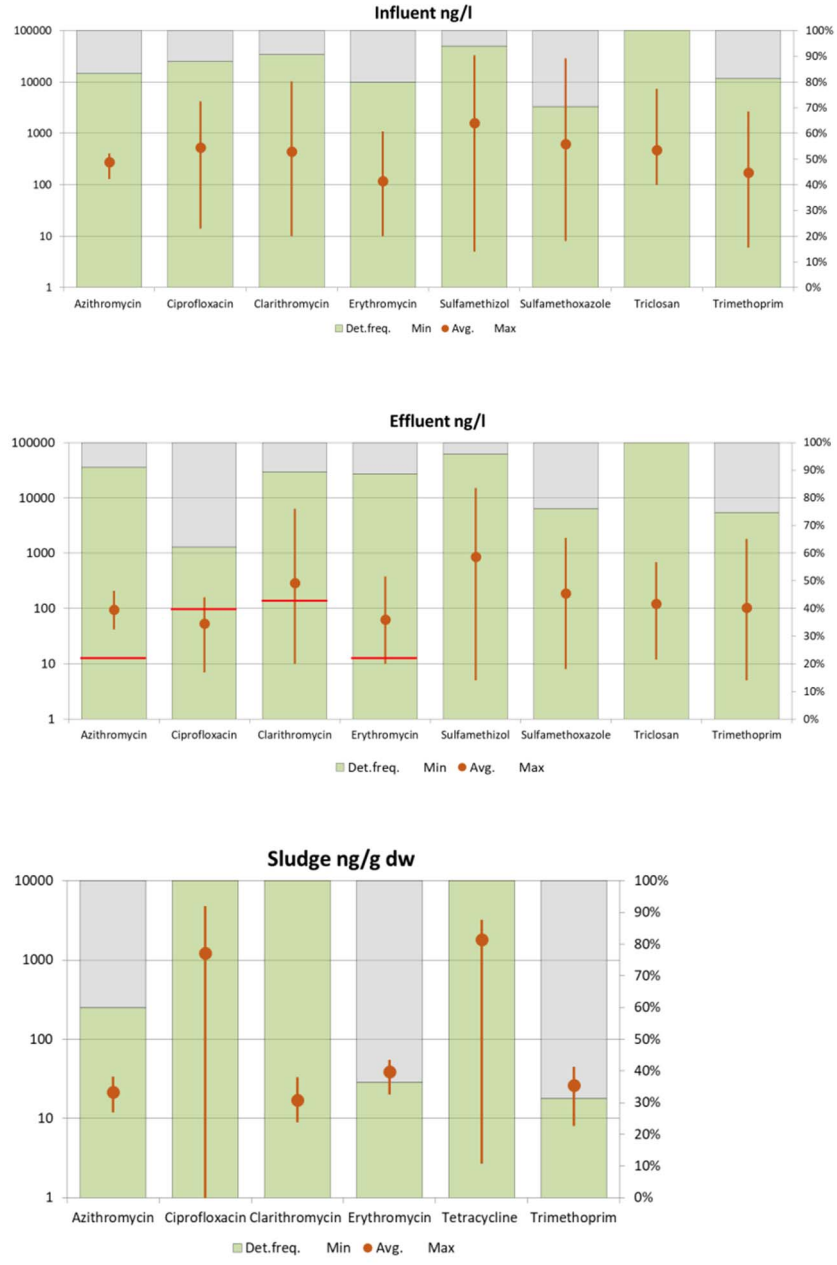


Figure 9. Concentrations of antimicrobial and antiparasitic APIs in WWTP influent, effluent and sludge samples. Only concentrations above the limit of quantification (LOQ) are reflected. Red lines indicate AA-EQSs for inland surface waters (2013/39/EU). Data on pharmaceuticals in wastewater treatment plants in the Baltic Sea region was compiled through HELCOM data calls (2015 and 2018) and processed within the CWPharma project. The final dataset contained over 10 000 measurements from Denmark, Estonia, Finland, Germany, Latvia, Poland and Sweden, covering over 100 WWTPs (HELCOM, 2022).

Terrestrial environment

Soil is an important link in AMR development. Biological balance and soil biodiversity should be maintained. Anthropogenic activities like fertilising the fields with manure or using wastewater and sewage sludge on land can be risk factors. Manure is commonly used to fertilise fields in Estonia. Approximately 3 million tons of manure are produced in Estonia annually (Dataset KK94. Statistikaamet, 2021), and all will be used for fertilisation in fields. The composted sewage sludge is applied to the fields and used for recultivation and landscaping. The amount of sewage sludge has been stable over the years approximately 30 000t dry weight/year), but the uses have changed from landfilling to soil improvement (uses in agriculture and recultivation). In 2022, the majority (71%) was used in agricultural applications (Figure 10).

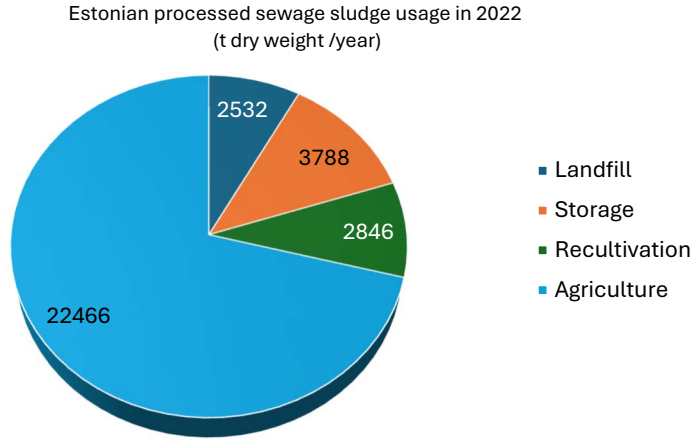


Figure 10. Processed sewage sludge usage in Estonia 2022 (Keskkonnaagentuur, 2023b).

In Estonian national soil monitoring, API-s are not monitored (Keskkonnaagentuur 2023a; METK 2023). Possible risks and concentrations close to the source have been studied in soil fertilised with sewage sludge or manure and sewage sludge (Ek Henning et al., 2020; Tenson et al., 2024). The concentrations of fluoroquinolones and tetracyclines in liquid manure are comparable to those in uncomposted sewage sludges. In addition, enrofloxacin (10mg/kg dw), oxytetracycline (124 mg/kg dw), and doxycycline (480 mg/kg dw) were detected in manure. In sewage sludge, ciprofloxacin (74 mg/kg dw) and ofloxacin (23 mg/kg dw) residues were detected in 2020 (Tenson et al., 2024). Trimethoprim (0.15 mg/kg dw) and norfloxacin (15 mg/kg dw) were detected in soils where manure was applied (dairy cattle manure was applied on the field five weeks before sampling; actively used antibiotics in the farm where tetracycline, sulfamethoxazole and lincomycin hydrochloride September 2018). Trimethoprim concentrations have been detected in sewage sludge in Estonia up to 41 µg/kg dw, which was higher than in other Baltic Sea countries. For other detected antibiotics in the sewage sludge, fluconazole

(0.39 mg/kg dw), lincomycin (0.09 mg/kg dw) and ofloxacin (3.3 mg/kg dw), the concentrations were lower in Estonia than the other Baltic Sea countries. The detection patterns vary by country and align with differences in usage patterns (Ek Henning et al., 2020). Guidelines for detecting risk and residues in manure have been in place since (Committee for Medicinal Products for Veterinary Use (CVMP), 2016) A more comprehensive need for soil protection on the European level has now been addressed. The importance of soil as a vital, limited, non-renewable and irreplaceable resource has been noticed, and a new EU directive will be enforced on Soil Monitoring and Resilience (Soil Monitoring Law) (European Parliament, 2023). The directive will change the principles of how to use and protect soil health in the future. By "soil health", the soil's physical, chemical and biological condition determining its capacity to function as a vital living system and provide ecosystem services has been defined. As a measurable descriptor, the loss of soil biodiversity has been set. There is a need on the member state level to establish criteria that can support the goals for better soil quality, and preventing antimicrobial resistance from spreading is one of them (European Parliament, 2023).

Estimation of AMR distribution and concentration in the environment using consumption and general environmental data

As environmental monitoring is resource-demanding, other combined estimations about the environmental concentration of antimicrobials can be used. The possible risk for antimicrobial resistance can be evaluated based on environmental concentration prediction. Figure 10 shows an example from CWPharma project data. This figure illustrates how complex the antimicrobial agent distribution system is, but this model did not include potential emissions from landfills, veterinary medication, and sewage sludge. For the Baltic Sea catchment area, the comprehensive analysis combining the data about human and veterinary consumption, emissions from wastewater treatment plants, manufacturing facilities, hospitals, fish and livestock farms, and environmental levels of antibiotics were determined and used in a model to predict the loads for two antibiotics (clarithromycin CLM and ofloxacin OFL) to the Baltic Sea were calculated (Äystö et al., 2020). The overall sales of clarithromycin in the Baltic Sea drainage area were estimated to be 14 tonnes, resulting in a total load of 3.1 tonnes being emitted into the aquatic environment annually. The estimated load from Estonia to the Baltic Sea was 87 kg/annually. Regionally, the highest per capita emissions were estimated to originate from coastal areas in Estonia (68 mg/annually) (Äystö et al., 2020).

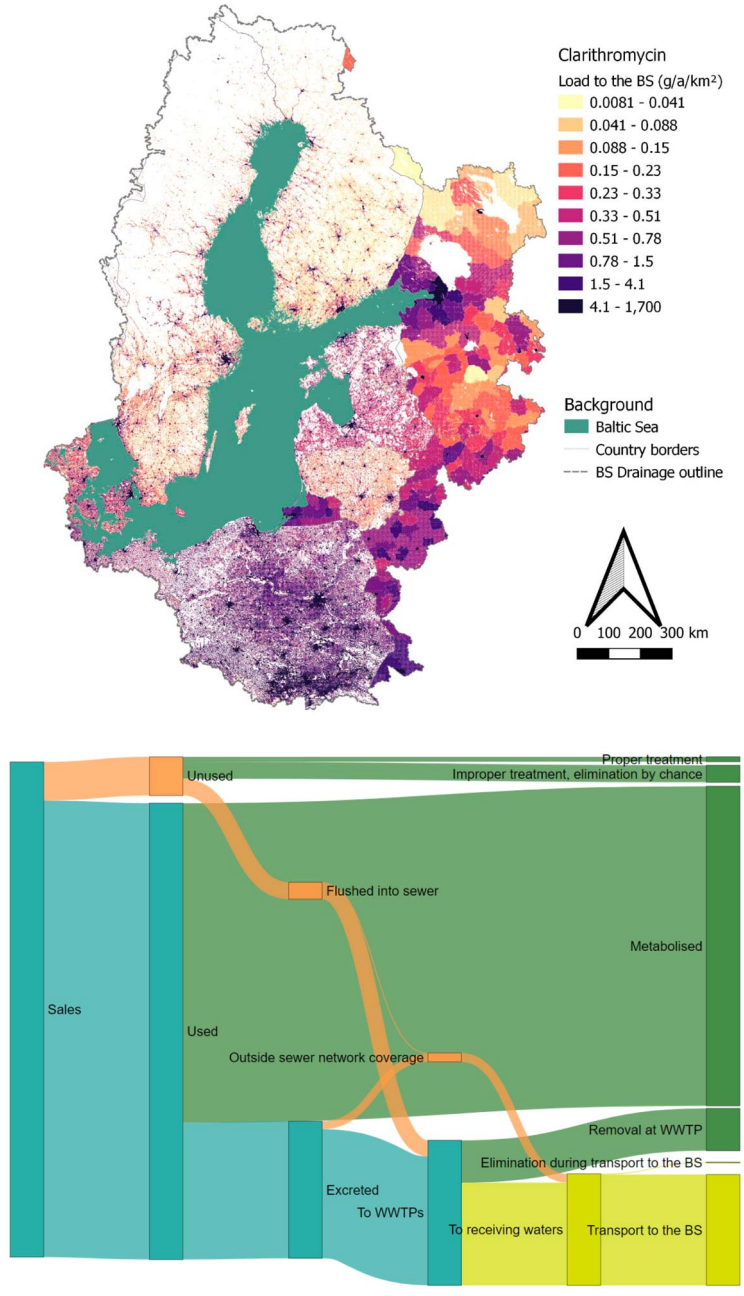


Figure 11. Clarithromycin estimated loads and model flow of clarithromycin entering the Baltic Sea. Based on 2015–2017 average sales data. On the map (Baltic Sea catchment area) – clarithromycin load (g/annualy/km^2) reaching the Baltic Sea, originating from individual grid cells. On the flow diagram – clarithromycin flows for Baltic Sea area are calculated. Green boxes present end-points/processes, where the load is destroyed before reaching the environment. Orange boxes present suboptimal management practices, while yellow boxes present emissions into the water environment (Äystö et al., 2020).

2.4.4 Methods to study the distribution of antibiotic resistance to understand the action mechanisms

The action mechanisms of resistance spread must be understood on multiple levels (bacterial population, isolate, antimicrobial resistance gene) to prevent the spread of antibiotic resistance. The availability and analysis of good quality research data is essential to create new measures to combat AMR and to improve existing measures. Antibiotic resistance spreading can be assessed using several methods. There are still no internationally agreed cross-sectorial methods for monitoring resistant microorganisms. Resistant pathogenic bacteria can be analysed using traditional culture-based methods or molecular methods. All the tested methods have advantages and disadvantages, and choices must be made based on the most critical parts of the work (time, mechanism info, gene info, resistance info, etc.). From a scientific point of view, DNA-based methods are preferred, but when it comes to the global practical needs in everyday risk assessment, much cheaper and simpler solutions are needed. Combined methods and data collection on a strain basis are also needed as phenotypic and genotypic resistance do not always coincide (not all genetic possibilities are used; some are dormant). The choice of the analytical method should depend on the purpose of the study.

Phenotypic methods for detection of resistance

Phenotypic resistance is a type of resistance determined by antimicrobial susceptibility testing methods such as disk diffusion, broth microdilution, and agar dilution. It is considered the reference standard for the detection of antibiotic resistance. Susceptibility/resistance is determined based on the ability of defined concentrations of antibiotics to inhibit growth. It can usually not determine the cause of the resistance (e.g. beta-lactamases versus efflux pumps), information that may be relevant for choosing the correct antibiotic for treatment. European Committee on Antimicrobial Susceptibility Testing (EUCAST) uses the concept of the epidemiological cut-off value (ECOFF) to describe the minimum inhibition concentration (MIC) above which bacterial isolates have phenotypically detectable acquired resistance mechanisms. Susceptibility is divided into three categories by – EUCAST:

- S (susceptible, standard dosing regimen),
- I (susceptible, increased exposure),
- R (resistant) (European Committee on Antimicrobial Susceptibility Testing, 2017).

In routine susceptibility tests, phenotypic detection methods are applied. The main categories of methods are the combination disk test method, colourimetric assays based on hydrolysis of antibiotics (biochemical tests), detection of AB hydrolysis with MALDI-TOF (mass spectrometry device) or immunochromatographic lateral flow assays.

Methods for detection of genotypic resistance

Genotypic resistance is detected using the data of known resistance genes. There are gaps in interpretation of the information as the action mechanisms and functions are not known for all resistance genes. Different options are available for the genotypic confirmation of the presence of AMR genes from PCR, directed sequencing, whole-genome sequencing, in silico mapping of resistance genes to different microarrays available (EUCAST).

PCR based gene detection methods are common in everyday diagnostics (meca, van, etc.). PCR and gene based methods are useful and cost-effective when the mechanisms are already confirmed and can be used as diagnostic tools. For example EUCAST has the recommendations for some resistance confirmations using marker genes like ampc, meca, vana, vanb etc. (EUCAST, 2017). However, screening hundreds of genes on hundreds to thousands of isolates may be resource-demanding, and the possibility of missing important genetic patterns is high. Choosing genes to be analysed in advance sets limitations, and only known genes will be targeted. No information about the carrier bacteria and gene transfer among species is obtained when the individual resistance genes are detected with PCR-based methods.

Gene chips (microarrays) have been used as alternative methods of detecting the expression of thousands of genes simultaneously. Nevertheless, if the individual resistance genes are detected with PCR-based methods, no information about the carrier bacteria and gene transfer among species is obtained. Novel technologies in sequencing have appeared, and whole genome sequencing (WGS) brings together the advantages of gene-based detailed analysis and connection to species. The limitation of WGS is the need for pure culture, but it allows the analysis of the whole pool of genes in a microbial isolate. It is possible to see the differences in the genome and it is possible to see what are the genetic differences in different populations using WGS.

3. AIMS OF THE STUDY

Risk factors for the spread of antibiotic-resistant bacteria exist in all three major areas: humans, animals (food production, agriculture) and the environment. The benefits of antibiotics are needed. Unfortunately, misuse of antibiotics has led to problems in infection control and treatment of inflammation for humans and animals. Ensuring the availability of safe drinking water and food has become a problem. All activities involved in the spread of antibiotic resistance are directly or indirectly related and need a unified approach to find a solution to limit the spread.

In our work, we combined different disciplines related to the study of antibiotic resistance and covered them with unified data. We compiled a collection of AMR pathogenic bacterial isolates originating from human (infected and carriers), animal (farm animals, pets (infected and not)) and environmental (wastewater, river, manure, soil and forest birds) samples. For humans, we analysed the AMR of strains collected in hospitals for diagnostic purposes and looked for carriers among healthy populations. In the veterinary field, we collected surveillance samples with increased frequency from farm animals and combined the diagnostic samples from pets with possible carrier samples. We helped to fill a gap in mapping the distribution of resistant bacteria among environmental strains, as these samples are not collected routinely. We analysed all the collected isolates for AMR using the same phenotypic and genetic methods. This dissertation aims to characterise antimicrobial resistance among pathogenic bacteria in Estonia and assess potential links between anthropogenic sources and resistant genes found in bacterial populations from the surrounding environment. Throughout the work, the aim has been to combine the knowledge of different fields and, by using them in combination, reach a more collaborative and comprehensive one-health approach for antimicrobial resistance in Estonia.

The specific goals of this research were:

- to investigate the occurrence and transfer of antibiotic resistance in a specific geographic area (Estonia) (**I, II, III, IV, V, VI**)
- to determine the occurrence of multidrug resistance in bacteria isolated from different hosts and sources: humans – infected and healthy (**III, VI**); animals – pets (dogs, cats), farm animals (including poultry, swine and cattle) (**I, IV, VI**); the environment – influenced by anthropogenic activities and without direct impact (wild-living birds) (**I, II, V, VI**)
- to create a collection of environmental isolates and fill the gap of information on antimicrobial-resistant bacteria behaviour in the environment and related anthropogenic activities (**I, III, IV, VI**)
- apply genetic methods for supporting a better understanding of resistance and find interrelationships among bacterial isolates routinely collected in hospitals, during veterinary controls in farms and pet clinics (**I, III, IV, VI**)
- to evaluate the antimicrobial testing methodologies that could be suitable for the screening and monitoring needs of determination and risk assessment of antimicrobial resistance in Estonia (**I, II, III, IV, V, VI**)

4. MATERIALS and METHODS

In this dissertation, I summarised the work on the field of AMR bacterial spread using different research approaches from One Health point of view. All the technical details about the methodologies are presented in original publications or reports. Studie was part of the research project "Transfer routes of antibiotic resistance" in Estonia (ABRESIST) (University of Tartu, 2015).

4.1 Study areas and institutions involved in the studies

Institutions involved in the studies covered different sectors to encourage collaboration to get the One Health principle to work in practice. The University of Tartu and the Estonian University of Life Sciences were leading the comprehensive studies of AMR. Estonian hospitals that routinely detect resistance among human patients and are responsible for AMR reporting were also involved in the studies. The National Centre for Laboratory Research and Risk Assessment (LABRIS) was involved in veterinary surveillance for AMR and their monitoring sites (programs). Estonian Environmental Research Centre (responsible for environmental monitoring in the water environment and related activities (state control for the WWTPs)) and the University of Tartu were doing the environmental sampling and analysis. Wastewater treatment plants from Estonia and other countries were associated as partners and involved in the studies (qPCR, plate count). Most Estonian WWTP-s were studied over the years using different methods (qPCR, plate count, isolation of ARB). We participated in two international collaboration studies for WWTP analysis: one with neighbouring country Finland and a global study (57 WWTPs from 22 countries: 47 WWTPs were located in Europe, one was located in Africa, six were in Asia, one in Australia, and two WWTPs were in North America) (Marano et al., 2020). Areas of study in Estonia for AMR bacterial collection were selected based on anthropogenic impacts: wastewater treatment plants, agricultural land fertilised with manure, and water environment connected to agricultural land or WWTP-s. Figure 12 shows the study areas.

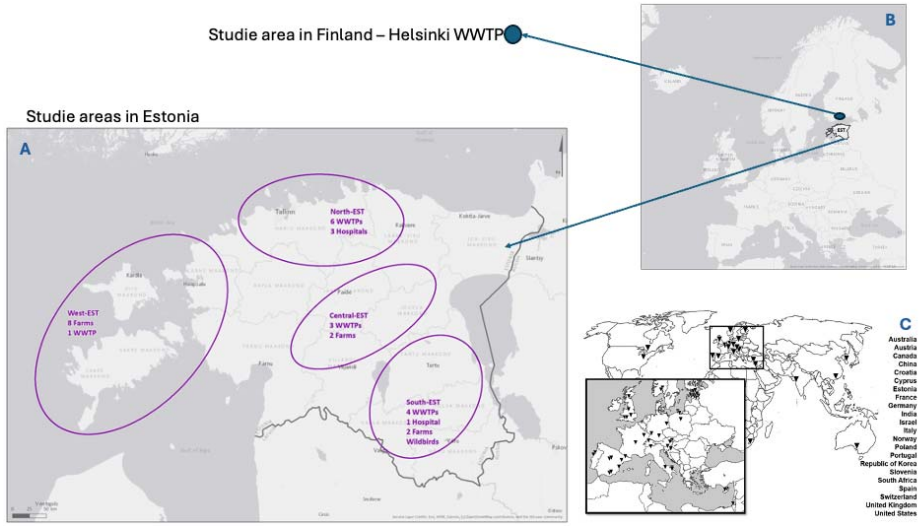


Figure 12. Study areas in our work. A – areas in Estonia (publications I, III, IV, VI). B – WWTPs in Estonia and Finland (publication V) C Study areas in a global study (publication II).

4.2 The collection of antimicrobial-resistant bacterial isolates

Our primary focus was to compile an isolate collection that covers all three One Health domains: humans, animals and the environment and where it would be possible to analyse main AMR distribution patterns. We collected bacterial strains during diagnostics and indicator organisms under surveillance programs, and we widened the range of isolates by controls from environments that were not influenced by AMR risk factors and hosts (healthy volunteers) that were not directly influenced by AMR risk factors. We collected isolates from water and soil as these matrixes are not monitored regularly, and isolates and data from other sources are unavailable. We collected the first set of isolates during the survey of the spread of antibiotic resistance in Estonia from 2011 to 2014 in frames of the ABRESIST project (University of Tartu, 2015). Over the years, the collection of isolates was supplemented with isolates from different studies. Now, the antibiotic-resistant bacterial collection (biobank) at the University of Tartu includes almost 2000 isolates from different sources and species) 400 *Esherichia. coli*, 230 *Pseudomonas aeruginosa*, 60 *Enterococcus faecalis* and 30 *Enterococcus faecium* strains, 132 *Klebsiella pneumoniae*, 136 *Staphylococcus aureus*, 11 *Staphylococcus epidermidis*, 7 other *Staphylococcus spp*., 51 *Acinetobacter baumannii*. Bacterial isolates are stored in the –80 °C freezer. The genetic information collected is added to WGS NCBI Genbank (The National Institutes of Health (NIH)).

4.2.1 Isolates from humans

Samples from humans were collected from two primary sources in 2012–2013. Firstly, isolates from the clinical samples of the patients from 4 hospitals in Estonia. Diagnoses for humans were colonisation, cholangitis, pneumonia, bloodstream infection, urinary tract infection, soft tissue infection, otitis externa, tracheobronchitis, bronchitis, and peritonitis.

The second source of strains isolated from humans was the volunteers for potential colonisation with ARB. We collected faecal samples from healthy volunteers, including three groups of people with contact with animals: veterinarians (n = 29), pig farmers (n = 29) (from farms with more than 1000 animals), and dog owners (n = 80). In addition, the general community group (n = 69) (patients admitted for elective orthopaedic surgery or pediatric patients older than six months, who had had no antimicrobial treatment nor hospitalisation in the previous three months and who had been hospitalised for <48 h) was studied for potential ARB carriage.

4.2.2 Samples from the domestic animals

Infected animals and healthy animals were included in the study from two animal groups: food-producing animals (swine, cattle, broiler) and companion animals (dogs, cats).

Samples from food-producing animals were collected from healthy animals faecal samples (during regular veterinary visits) and diseased animals from clinical material (post-mortem samples, organ materials). The isolates of healthy animals originated from 38 swine and 42 dairy farms in Estonia and were collected during the annual national surveillance program in Estonia in 2010–2015 (Aasmäe et al., 2019). The faecal samples from healthy poultry were collected post-mortem in slaughterhouses during the national Salmonella surveillance program (random 20 samples) (Põllumajandus- ja toiduamet, 2023). A total number of samples from diseased cattle (n = 63) and swine (n = 143) were analysed (Aasmäe et al., 2019). All farm animal samples were sent to the National Veterinary and Food Laboratory for isolation and identification of different bacterial species and resistance detection. For our AMR study, *Enterococcus spp*., *E. coli* and *Staphylococcus aureus* (MRSA) isolates with phenotypic resistance were added for further genetic analysis.

One study group was composed of companion animals, healthy dogs, and diseased animals (dogs, cats, etc.). Clinically healthy dogs (in total 86 dogs) were selected randomly, with permission and interest of dog owners, during veterinary visits in clinics for vaccination or veterinary consultation. The first inclusion criterion was that dogs were not treated with antimicrobials during the last three months before the sampling (Aasmäe et al., 2015). Isolates collected during diagnostics of infected animals were collected for the study. Isolates collected during diagnostics of infected animals were collected for the study. A total of 81 isolates from infected pets were added to the study.

4.2.3 Samples from the environment

We collected the environmental samples (n = 66) during 16 sampling campaigns from water, soil, manure and slurry. Sampling covered various seasons.

We collected agriculture-related environmental habitat samples at three farms: from slurry and manure, from the soil at the fields fertilised with manure, and surface water from streams and rivers connected to the fields.

We collected city-related water samples from a city of 100 000 inhabitants: from wastewater treatment plant effluent, from a stream receiving the WWTP effluent, from a river inside the city and from an artificial outdoor bathing lake connected to the river.

We included the samples from wild-living raptors in the environmental samples. Raptor samples were collected from the nestlings using cloacal swabbing during a national wildlife monitoring program (Kotkaklubi, 2024). We selected three raptor species foraging in an agricultural landscape for our study: the goshawk (*Accipiter gentilis*) – feeds mainly on birds in the study area, lesser spotted eagle (*Clanga pomarina*) – a generalist hunting primarily small mammals, and common buzzard (*Buteo buteo*) – a generalist with a broad spectrum of diet.

4.3 Methods used for identification of resistance patterns and identification of connections between hosts and environments

There is no universal method that would fit all the purposes and answer the questions that need to be answered in detecting antibiotic resistance spread. Antibiotic resistance can be defined in many ways. Depending on the need (is it enough to know the resistance to certain antibiotics or is the resistance mechanism important, or multi-resistant data) and limitations (time, specific equipment, specialists, standardised methods (internationally agreed for the surveillance monitoring, for example) combination of methods used can vary. Different fields and their everyday practices can be combined in one health approach, and better results can be obtained from the synergies. Over time, we have had different goals in our studies and different analytical abilities to get the needed answers. In the following subsections, I will describe all the main methods used in our studies to map the resistance spread in Estonia.

Three questions about the methods have been discussed:

- main principle of the method main uses;
- advantages, and disadvantages for describing the spread of resistance;
- arguments for the choice of use in our work (questions to be answered with the information gathered with the described method).

4.3.1 Phenotypic methods to detect the antibiotic resistance

Selective culture media plate count with added antibiotic

The most classical method is cultivating bacteria from the samples on a solid (agar) culture medium. Selective media will be used to detect specific bacteria and antibiotics. Known bacteria and predicted antibiotic resistance can be tested. The most common routine tests used in diagnostics are ESBL *Enterobacteriaceae*, VRE *Enterococcus*, etc. Phenotypic AMR detection is possible with that method and has been used for diagnostics for a long time. Standardised methods are available in many fields (human, veterinary diagnostics, etc.). There is no possibility of finding relationships between different strains. Only a few antibiotics and concentrations can be used at the same time. The method is time-consuming as it involves the need for bacterial growth, and depending on the species, it usually varies between 18–72 hours.

In our study, we used the plate count method mostly in combination with other methods, such as screening and purifying the isolates. Collected samples were plated on selective media for isolation of ESBL-producing organisms (Brilliance TM ESBL Agar, Oxoid, Basingstoke, UK – cefotaxime); VRE (Oxoid Brilliance VRE Agar – vancomycin) or MRSA (Oxoid Brilliance MRSA Agar – methicillin). *Pseudomonas aeruginosa* from environmental samples was selected using CN selective agar plates (Scharlau CN Selective Agar Base for Pseudomonas supplemented with Nalidixic Acid 0.015 g/L, cetrimide 0.2 g/L and 10 ml/L glycerol). During diagnostics, the Estonian Veterinary and Food Laboratory uses blood agar and McConkey agar to grow the possible disease-causing bacterium. *Pseudomonas spp*. was identified using a combination of colony morphology, Gram staining, biochemical tests and *Pseudomonas* selective media. *P. aeruginosa* was identified using the API NE (bioMérieux) identification system and MALDI-TOF (Bruker Daltonics In addition, we took part in a global survey of cefotaxime-resistant coliforms in urban wastewater treatment plants. The study targeted cefotaxime-resistant (CTX-R) coliforms as indicators and used agar plates for the quantification of CTX-R coliforms on mFC Agar plates (supplemented with cefotaxime sodium salt (Sigma®) at a final concentration of 4 μg/mL). Cultivation was done using filtration through 0.45mm pore-size filters, followed by incubation of the filters on the selective media. We collected some isolates from selective media without extra antibiotics to look at connections and the spread of resistance even in groups where strains with phenotypic resistance were not obtained (for example, raptors for some bacterial species). That gave a more comprehensive overview of resistance distribution and genetic relationships between host groups.

Susceptibility to antibiotics – detection methods of minimum inhibitory concentration

Disk diffusion and broth dilution methods have been used to detect minimum inhibitory concentration (MIC). The principle is to find out the concentration of AB that inhibits the growth (different concentrations are added to the disk). MIC

tests are a standard procedure in the EU for MIC testing in food-producing animals. Disk diffusion is used in medicine. Our study used the hospital standard procedure E-test (disk diffusion) for MIC detection (bioMérieux).

4.3.2 Detection methods of genotypic resistance

Genotypic resistance detection methods have several steps, starting with DNA extraction and amplifying the DNA (PCR, WGS, etc.) The applied conditions (primers, temperatures, detection technologies, etc.) can affect the final result.

DNA extraction is the first step for most of the gene detection methodologies. The choice of DNA extraction protocol is a crucial step influencing the DNA yield from the extraction. DNA can be extracted from a sample or a single isolate. Today, many DNA extraction methods are available as ready-to-use kits. For many of them, inhibitor removal steps have also been added. Depending on the matrix, the further steps of DNA amplification can be inactivated with residues from the purification chemicals or matrix.

For most of our studies, we used the method of DNA extraction from a single bacterial colony grown on blood-agar (human isolates) or LB agar for other isolates using a GuSCN-silica protocol (Boom et al., 1990) modified with bead beating (publication V).

For resistance gene detection in WWTPs, we extracted DNA from total filtered material using part of the samples and MoBio PowerWater DNA isolation kit (MoBio Laboratories, Inc., CA, USA) (publication V).

Quantitative PCR (q-PCR) approach for resistant gene detection

Quantitative polymerase chain reaction (qPCR) adds two elements to the standard PCR process ((DNA amplification): fluorescent dye and fluorometer for fluorescence detection in real-time as the thermal cycler runs, giving readings throughout the amplification process of the PCR. Combined with appropriate standard curves and reference values, this real-time information about reaction rates and times translates into information about relative and absolute amounts of DNA present.

We used traditional PCR to screen potential genes for further studies with the qPCR method as the number of ARGs is around 100 000, and testing all these genes quantitatively would be prohibitively difficult. We tested 12 bla, 6 tet, 3 sul, 3 qnr (fluoroquinolones) and 1 vancomycin-resistant gene using traditional PCR. After that, we studied seven resistance genes sul1, sul2, tetm, tetc, blashv-34, blactx-m-32, and blaoxa-58 with q-PCR for antibiotic-resistant gene detection and quantification of ARG copy number in influent and effluent of the three city WWTPs (Helsinki, Finland; Tallinn and Tartu Estonia). We collected samples over one year from December 2010 to December 2011 at five different time points, each representing a different season (four seasons; winter was sampled twice). We performed qPCR using a Dynamo Flash SYBR Green qPCR kit (Thermo Scientific, Lithuania) and a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) (publication V).

Using 16S rRNA gene for phylogenetic marker and normalisation tool

The 16S ribosomal RNA gene codes (16S rRNA) for the RNA component of the 30S subunit of the bacterial ribosome and is widely present in all bacterial species (depending on the bacterial species, one to multiple copies of the 16S rRNA gene are present). 16S rRNA gene sequencing (~1500 bp) is one of the most common methods targeting housekeeping genes to study bacterial phylogeny and genus/species classification. Detection of relative abundances of ARGs in samples using 16sRNA gene to evaluate the abundance of the total bacterial community, we quantified 16S rRNA gene in the samples using qPCR. To avoid inconsistencies among qPCR assays, including sub-optimal efficiency in some cases, we used 16S rRNA gene-normalised values. This data analysis quantifies the relative changes in ARG abundances, whether more or fewer ARGs appear per microbial genome.

Whole genome sequencing (WGS)

Following the manufacturer's protocols, one nanogram of sample DNA was processed for the sequencing libraries using the Illumina Nextera XT sample preparation kit. Libraries were validated by qPCR with Kapa Library Quantification Kit to optimise cluster generation. ssDNA Nextera XT libraries were pooled and sequenced on one high-output lane of HiSeq2500, with paired-end, 150 bp reads. Demultiplexing was conducted using CASAVA 1.8.2. (Illumina, San Diego, United States), allowing one mismatch in the index reads (publication I, III, IV).

After that, all Illumina reads were assembled de novo with the SPAdes genome assembler (ver 3.5.0) using MismatchCorrector (Bankevich et al., 2012).

4.3.3 Identification of genetic links between isolates using bioinformatics analysis

Genotypic information interpretation involves large-scale data analysis. The developments in computer technologies have made it possible to develop different bioinformatics tools to help us better understand and interpret biological data.

Multilocus sequence typing (MLST)

Since the MLST protocols are widely used to find relationships between strains in medicine, we used in silico MLST to make a global comparison. Currently, 139 organisms are listed in pubMLST where the MLST protocols are used (pubMLST https://pubmlst.org/organisms).

MLST only uses five to seven (depending on the bacteria) housekeeping genes. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles. For each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST) (https://pubmlst.org/multilocus-sequence-typing).

MLST is a valuable tool for adding information about the connection between isolates and the phenotypic data collected for diagnostics. Depending on the organism, it can be a robust and simple genetic method. MLST data can be collected from PCR (housekeeping genes) or available whole genome sequences. MLST ST does not give information about resistance.

In traditional MLST analysis, the first step is to detect the genes involved in a sequence typing protocol (usually 5–7 genes) using PCR, followed by sequencing of the genes and data analysis to detect the sequence type. After the WGS became more common in bacterial analysis, the MLST protocols were also used from the whole genome data. Using WGS data eliminates several time-consuming technical steps but needs additional bioinformatic tools.

Our work used traditional PCR-based MLST analysis for the bacterial species *E. coli* and *P. aeruginosa*. MLST analysis of *P. aeruginosa* was made according to the schemes provided on the PubMLST website (http://pubmlst.org/paeruginosa/) (Curran et al., 2004). In brief, profiling is based on genes: acsA, aroE, guaA, mutL, nuoD, ppsA, and trpE. PCR conditions and primers were used precisely as in the original protocol. For *E. coli* MLST we used seven housekeeping genes: adk, fumC, gyrB, icd, mdh, purA, recA (so-called Achtman 7 Genes MLST or Warwick University (WU) scheme) (Wirth et al., 2006), available at https://enterobase.warwick.ac.uk/species/ecoli/download_data).

Before the next analytical steps, we used an enzymatic PCR clean-up method to remove the remaining primers and dNTP left from a PCR reaction. We used an ExoI/FastAP combined reaction mix where FastAP (Thermo Scientific) thermosensitive Alkaline Phosphatase catalyses the release of 5'- and 3'-phosphate groups from DNA, RNA, and nucleotides, and this enzyme also removes phosphate groups from proteins and exonuclease I (Exo-I – Thermo Scientific) degrades single-stranded DNA in a 3'→5' direction. Exo-I releases deoxyribonucleoside 5'-monophosphates in a stepwise manner and leaves 5'-terminal dinucleotides intact. It does not cleave DNA strands with terminal 3'-OH groups blocked by phosphoryl or acetyl groups. FastAP is an alkaline phosphatase which dephosphorylates all types of DNA ends (blunt, 5'- and 3'-overhangs) in 10 minutes at 37 °C. The enzyme is inactivated in 5 minutes at 75 °C.

Sequencing was performed on Applied Biosystems 3730xl DNA Analyze). Sequence data were analysed with Geneious Pro 5.5.8 c (Biomatters). Sequence type (ST) was identified with PubMLST tools available on the PubMLST web. Only 20 isolates had (decent-quality) sequencing results for all 7 genes. However, no ST-s was possible to ascertain with all 7 genes. The most problematic gene was the nuoD. Results obtained for the genes: acsA 92%; aroE 42%; guaA 75%; mutL 77%; nuoD 23%; ppsA 83%; trpE 90%. The possible need for modification of primers and PCR conditions was too time-consuming. Other researchers have done that by designing new primers, changing annealing temperatures and amplification conditions (van Mansfeld et al., 2009; Nemec et al., 2010; Maatallah et al., 2011; Eusebio et al., 2013), but these modifications are not implemented in pubMLST scheme and may remain unnoticed. All those studies were conducted independently, and no information on the PubMLST website was given to change

the original protocols. Our study did not investigate the issue and changes to the ST identification from WGS data. From WGS results, we identified all 7 loci gene fragments (48 isolates were tested using both methods). Based on WGS results, we found 30 different *P. aeruginosa* ST-s. We also performed traditional PCR-based MLST for *E. coli* isolates with the protocol from the PubMLST, and no problems were detected there.

Sequence type identification

In our study for *in silico* identification of sequence types, a BLAST-based tool from https://cge.cbs.dtu.dk/services/MLST/ was run to annotate the MLST fragments within the WGS data (Larsen et al., 2012). Sequence type (ST) identification was done with the MLST software (Seemann T, https://github.com/tseemann/mlst) using MLST schemes from the PubMLST website (https://pubmlst.org/). ST identification software MLST which scans the contig files against traditional PubMLST typing schemes based on the sequence of seven house-keeping genes (gdh, gyd, pstS, gki, aroE, xpt, and yqiL) for E. faecalis and (atpA, ddl, gdh, purK, gyd, pstS, and adk) for *E. faecium* (Jolley, Bray and Maiden, 2018) (publication I). Same in silico procedure was used for *P. aeuruginosas* even housekeeping genes (acsA, aroE, guaA, mutL, nuoD, ppsA, trpE) and *E. coli* (adk, fumC, gyrB, icd, mdh, purA, recA) (publication III, IV). We used the data obtained to describe the connections between isolates in our collections and literature, and database data was used to find global matches.

Testing for Resistance Genes from genome data

Antibiotic resistance genes from *P. aeruginousa* were identified using a homology search against the collection of antibiotic resistance protein sequences from The Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.mcmaster.ca/,version 1.1.0) and beta-lactamases from http://www.lahey.org/studies/. GyrA, parC, oprD, rmt and arm sequences were retrieved from the NCBI protein database. Identity and coverage thresholds were set to 90%.

The presence of acquired and intrinsic resistance genes was determined using ResFinder 3.2 software and the ResFinder database as of October 1, 2019 (Zankari et al., 2012). The search was conducted against all AMR classes in the database, with the ResFinder's minimum coverage cutoff raised from a default of 0.6 to 0.8 and the minimum identity per cent cutoff raised from a default of 0.9 to 0.95. As the *Enterococcus* resistance to last-resort antibiotic linezolid often results from point mutations in polyclonal chromosomal 23S rRNA gene, we used the LRE-Finder software tool (Hasman et al., 2019), which is dedicated to detect these mutations and other linezolid resistance-associated genes (optrA, cfr, cfr(B), and poxtA) on the sequencing raw reads of our isolates.

The plasmid or chromosomal origin of the detected virulence and antimicrobial resistance factors was determined by combining the results of the PlasmidFinder software tool with default parameters (Carattoli et al., 2014) and

BLASTn homology search of the corresponding contigs against the ad hoc compiled database of plasmid sequences derived from NCBI RefSeq database as described in Roosaare et al. (Roosaare et al., 2018). The BLAST search was conducted using an identity threshold of 70% and a plasmid coverage threshold per hit of 10%. The PlasmidFinder results were ignored if the hit for plasmid replicon was found in the complete chromosomal sequence. The genomic regions containing multiple resistance genes were studied in more detail, and the organisation of the genes in these regions was reconstructed by the prediction of gene and corresponding protein sequences in these regions using Prodigal software (Hyatt et al., 2010). The predicted genes were annotated by comparing the corresponding protein sequences to available annotated sequences in public databases using BLASTp (Altschul et al., 1997).

Beta-lactamases were detected using a homology search against the ResFinder database. Contigs containing ESBL-genes were further analysed using BLAST (Altschul et al., 1997) against the NCBI nt/nr databases to specify possible relation to known plasmid sequences and to check for the presence of mobile elements near the ESBL-genes (publication III). Beta-lactamase genes chromosomal or plasmid origin was determined using a BLASTn search of corresponding contigs against the NCBI nt/nr database. Top matches were also examined manually to determine whether they were plasmid or chromosomal sequences. We could not decide whether the beta-lactamase gene was located in the plasmid or chromosome for very short contigs and/or contigs with low coverage matches or matches against both chromosomal and plasmid sequences. Contigs matching only against chromosomal genome sequences were not considered to be plasmid-related, whereas contigs matching with high coverage (>95%) and identity (>98%) to only plasmid sequences were considered as possibly originating from the plasmids.

Phylogenetic analysis is important for gathering information on biological diversity and genetic classifications and learning developmental events that occur during evolution. Phylogenetic trees were conducted to examine the relatedness of our isolates. For phylogenetic analysis, recombination-free alignments were created by masking all significant recombinant segments as missing data in the input alignment. These alignments were used to reconstruct a maximum likelihood phylogenetic tree with RaxML using the GTR-GAMMA model (Stamatakis, 2014). As core genome alignment and MLST analysis resulted in similar clustering, the data are presented according to STs of MLST.

5. RESULTS AND DISCUSSION

The spread of resistance is important in human-impacted areas in Estonia, and regardless of the method we used, all showed the need for further action. AMR is present in all one health domains, and genetically closely related isolates are shared in between the domains.

5.1 Distribution of genetically closely related antibiotic-resistant bacteria

The species-based distribution of isolates based on host and source was studied using sequencing information. Depending on the bacterial species, the distribution of AMR isolates between One Health domains was different. Genetic variability and possible connections were detected using MLST analysis. In our datasets, core genome alignment and MLST analysis resulted in similar clustering. Results in the discussion are presented according to STs of MLST.

5.1.1 *E. coli* strains origin and genetic variability (IV)

The collection of *E. coli* strains was specifically targeted at extended-spectrum-lactamase (ESBL) producers, and the first isolation was made on ESBL plates. In total, 347 *E. coli* strains were collected, and 278 of them were studied with WGS. ESBL-resistant *E. coli* strains originated as follows: 94 isolates were from patients getting antimicrobial treatment, and 23 isolates were collected from healthy volunteers. From the people with contact with animals, colonisation with ESBL-resistant *E. coli* was detected 10 times: 2 veterinarians, 7 pig farmers, 1 dog owner. From the general community, 4 people were carrying the ESBL-resistant *E. coli*. Isolates from animals: fattening pigs (*Sus scrofa* = 23), broilers (*Gallus gallus* = 25), bovine (*Bos taurus* = 6), cats (*Felis catus* = 4), dogs (*Canis familiaris* = 15).

E. coli ESBL strains from the environment: from wastewater (n = 27), manure (n =11), soil (n = 4), surface water (n = 17), (13 agricultural land-related ditches and 4 from the river). Fifteen isolates (no phenotypic resistance) from wild birds (*Aquila pomarina* = 5; *Accipiter gentilis* = 3; *Columba livia* = 5).

MLST ST131 was prevalent in our *E. coli* dataset. Frothy six *E. coli* isolates were belonging to the ST131. Most of these were from hospital patients, with two isolates from the WWTP effluent downstream of the hospital, 1 isolate from a pig farm worker, and 1 isolate from the general community. The isolates sharing the ST131 displayed high variability in resistance patterns. One ST131 isolate was resistant to 5 antibiotic groups (pneumonia patient) and one to 6 (long term hospitalised patient).

Five previously not detected ST-s were found. The strains from not-hospitalised human hosts were distributed into 11 STs, and only two STs, ST10 and ST131, were identified more than once (both, with two strains from two individuals each) (publication IV). ST533 was obtained only from broilers (16 isolates). ST10 had a much more variable host and origin pattern. In total, 15 ST10 isolates were analysed, and they originated from all three One Health domains: 5 from the environment(soil, manure, wastewater), 7 from humans (hospital patients, veterinarians, pig farm workers) and 3 from the animals (healthy broiler, healthy bovine, infected swine). Five more sequence types (in addition to ST10) were shared among all the One Health domains: ST108, ST38, ST58, ST88, and ST117. ST108 was found in isolates unrelated to infections: wild birds, healthy humans and surface water from agricultural areas. ST88 was shared among human, manure and pig isolates. ST177 was shared among human, pig, cat and found also from the wastewater.

5.1.2 *Enterococcus spp.* strains origin and genetic variability (I)

In total, 61 *E. faecalis* isolates were recovered from the collected samples and involved in our study. These included eight isolates from environmental sources (river = 3, manure = 5), six isolates from farm animals (*Bos taurus* = 4, *Sus scrofa* = 2), isolates from poultry (*Gallus gallus* = 17) and nine isolates from human samples (clinical = 6, healthy = 4). Our study tested 21 isolates from wild raptors (*Buteo buteo* = 5, *Accipiter gentilis* = 7, *Clanga pomarina* = 9). No phenotypic antibiotic resistance was the screening criteria (isolates were obtained from the regular *Enterococcus* plates, not the VRE plates.). WGS results did also not detect any resistance genes (publication I).

In total, 30 sequence types (STs) were detected, of which a single isolate represented 18. The most abundant sequence type among *E. faecalis* was ST49, with eight isolates from poultry (*Gallus gallus*), followed by ST936 (six isolates *Accipiter gentilis*), ST287 (*Buteo buteo* five isolates), and ST4 (four isolates *Clanga pomarina*). For *E. faecalis*, ST287 was found in *Accipiter gentilis* and *Clanga pomarina*, ST4 was found in *Gallus gallus*, *Clanga pomarina*, and *Bueto buteo*, ST49 was found in *Gallus gallus* and *Homo sapiens*, and ST16 was found in *Homo sapiens* and *Bos taurus* and also in manure.

One (ST32) was detected from an environmental sample (manure) and *Sus scrofa*. Nine novel *E. faecalis* sequence types were detected (ST933–ST941 and ST943) and submitted to the PubMLST database.

For *E. faecium*, 34 isolates were recovered from the collected samples. These included four isolates from environmental sources (river = 3, manure = 1), three isolates from farm animals (*Bos taurus* = 1, *Sus scrofa* = 2), 23 isolates from *Gallus gallus*, one isolate from free-living *Columba livia*, and three isolates from human samples (clinical = 2, healthy = 1).

E. faecium isolates were resolved into 24 STs, of which a single isolate represented 19. The most abundant ST was ST258, with six isolates from poultry (*Gallus gallus*). The only human-colonising *E. faecium* isolated from healthy

volunteers was ST822, and two isolates from hospitalised patients were ST117. Six novel *E. faecium* STs were discovered and submitted to the PubMLST database (ST1634–ST1639). *E. faecium* isolates in our dataset were found within the same host species or environmental origin (publication I).

5.1.3 *Pseudomonas aeruginosa* strains origin and genetic variability (VI)

In total, 238 *P. aeruginousa* isolates were collected. 147 *P. aeruginousa* strains were collected from humans majority were hospital isolates. Five isolates originated from volunteers (dog owners = 4; general community = 1). 63 strains were from various animals: bovine (*Bos taurus* =18), cat (*Felis catus* = 2), dog (*Canis familiaris* = 41), elephant (*Loxodonta africana* = 1) and Chinese water dragon (*Physignathus cocincinus* = 1), 26 from environment: manure = 7, waste water = 10, soil = 3, surface water = 5, potato (*Solanum tuberosum* = 1).

Two STs (313, 395) were obtained from all the One Health domains. ST 313 was shared among three isolates: an infected dog diagnosed with dermatitis, an infected human diagnosed with bronchitis, and an isolated from the wastewater effluent. The antibiotic resistance pattern was different for the isolates: human isolate was MDR (resistance to 4 out of 6 antibiotic groups tested meropenem MIC 16mg/L; piperacillin/tazobactam MIC 48 mg/L; ciprofloxacin MIC 32 mg/L; imipenem MIC 32 mg/L; susceptible to colistin, aminoglycosides). Wastewater isolate was resistant only to meropenem (MIC >32 mg/L). Dog isolate has no phenotypic resistance data. Isolates were from different years and locations. Isolates grouped into ST 395 were collected from three places: a potato studied by the Estonian Crop Research Centre (sensitive to all tested antibiotics) from a human patient with a soft tissue wound infection hospitalised in Tallinn (isolate resistant to imipenem MIC 32 mg/L), and from two dogs, one from Tartu diagnosed with dermatitis (resistance pattern not tested) and another dog from Pärnu with a wound infection (isolate resistant to imipenem MIC 16 mg/L). The human and dog wound infection strains have a similar resistance pattern, resistant to imipenem (MIC 16 and 32 mg/L) and meropenem (MIC 3 and 6 mg/L).

104 different MLST sequence types/genotypes were found, among which 29 were identified as novel sequence types and submitted to the PubMLST database (ST2449 – 2477). The most abundant ST was ST108, shared by 33 hosts (32 humans and one dog). The phenotypic resistance among the ST108 isolates varied from susceptible to all to MDR (four isolates resistant to four groups and one to five groups). The most resistant isolate was susceptible to colistin, but we also had one isolate from the same ST108 group resistant to colistin as all the others were susceptible. There is a high possibility that this hospital clone can harvest genetic information and become resistant to all six groups of antibiotics. Five susceptible isolates from the most abundant hospital clone, ST108, were all isolated at the beginning of treatment when the patients were first admitted to the hospital. Dog isolate (otitis externa) was found to be resistant only to an

imipenem (MIC >32 mg/L). Twenty-one human patients shared a second hospital clone (ST260). This ST was even more resistant than the ST108. No isolates susceptible to all antibiotics were found. In total, 14 MDR isolates were found in that ST: four isolates were resistant to 5, and five isolates were resistant to 4 ABs. No colistin resistance was detected.

The most abundant number of shared STs was observed between humans and dogs (n = 9.7% of all STs). However, none of the human-dog connections were from our sampling of dog–owner pairs. Several isolates were collected from populations inhabiting the same locality, such as the most significant hospital outbreak sequence type in Tartu, ST108, originating from 26 hospitalised human patients. The same ST was found in a dog from the same area with highly similar genotypes (number of SNPs from 8 to 18). ST 274 was carried by two dogs, and three hospitalised humans (variation of pairwise SNP number from 39 to 96) from the same city. Other possibly shared sequence types, such as ST245, were carried by three humans and two dog hosts but originated from various localities. The same was true in the case of ST319 isolates, which two human hosts and one dog carried, while ST251 and ST270 were isolated from one human and one dog host. Different animal species shared novel ST 2450 among three isolates, one cat and two bovine. All the isolates were from infected animals but from different years and locations.

5.1.4 *Staphylococcus spp*. strains origin and genetic variability

In total, 156 *Staphylococcus spp*. isolates were collected. We have 107 hospital isolates collected from infected humans and 6 environmental isolates from the manure. In total, 43 MRSA isolates were collected from animals (*Canis familiaris* = 19, *Sus scrofa* = 24). Most isolates were obtained from sow nose secretion samples (24 isolates showed phenotypical resistance on MRSA plates). Twenty-one of them were confirmed by meca PCR as MRSA. All 21 isolates were from the same farm, representing spread in one location. Isolates from dogs were collected as follows: infected tissue = 15 phenotypical MRSA isolates and from healthy dogs = 4. PCR confirmation detected 6 MRSA isolates from infected dogs and 2 from healthy dogs. 86 *S. aureus* isolates were further analysed with WGS. Bioinformatic analyses will be performed in the future.

5.2 The spread of globally relevant clones, our dominant clones compared to the global data and their antibiotic resistance

Previous research has found several genetically similar antibiotic-resistant bacteria spread globally. Below, I will highlight the possible spread of such global strains in Estonia using the example of *E. coli* and *P. aeruginosa*.

The population of *P. aeruginosa* was confirmed to be heterogeneous in Estonia. *P. aeruginosa* high-risk clones are disseminated worldwide and are common causative agents of hospital-acquired infections (Kocsis, Gulyás and Szabó, 2021). Globally, the most frequently spreading *P. aeruginosa* sequence type (ST235) was isolated in Estonia only once from a human patient who was hospitalised in Germany before treatment in an Estonian hospital (MRD isolate). Seven isolates belonged to the ST233, also known as high-risk clone. Four of them were MDR isolates. Other previously described globally distributed relevant infection outbreaks caused by genotypes ST111 (Nemec et al., 2010; Cabot et al., 2012; Liakopoulos et al., 2013; Haenni et al., 2015), ST175 (Cholley et al., 2011; García-Castillo et al., 2011; Ji et al., 2013), ST277, ST357, ST654, ST773 (Kocsis, Gulyás and Szabó, 2021) were not observed among our isolates.

On the other hand, the most abundant STs in this study, ST108 and ST260 (hospital outbreak (publication III)), were previously reported only in a few clinical studies and have never been reported as dominant outbreak sequence types (van Mansfeld et al., 2009; Nemec et al., 2010; Cholley et al., 2011; Gomila et al., 2013). In addition, ST108 was observed outside a hospital, being shared between humans and dogs, thus inferring possible transmission. This ST has been reported from animal infection previously (isolated from dogs, horses and cattle) in France (Haenni et al., 2015). A third abundant hospital clone, ST 233 (with eight isolates), has been reported previously as an outbreak clone in Japan and South Africa (Tsutsui et al., 2011; Mudau et al., 2013). The same ST was found in three different hospitals in our study. The fifth most abundant ST in our data set, ST274, seems to be a worldwide spread genotype (van Mansfeld et al., 2009; Nemec et al., 2010; Cholley et al., 2011; García-Castillo et al., 2011; Ji et al., 2013). ST274 has been found within animals (Kidd et al., 2012) and healthy humans (Estepa et al., 2014) before and was carried by seven hosts in our dataset – two dogs and five humans (from the hospitalised group).

Our MDR isolate, resistant to all antibiotics tested (including colistin), belonged to ST362, which is not a commonly known high-risk clone (Vanegas et al., 2014; Yamaguchi et al., 2014).

In healthcare institutions, highly pathogenic multi-resistant *E. coli* ST131 carrying blaCTX-M-15 has been spreading globally (Pitout and Finn, 2020). In our study, 16% of isolates were from ST131. ST131 MDR isolates were twice isolated from the general community (publication IV). ST10 is globally widespread (Google Scholar search more than 6000 articles about *E. coli* ST10 (May 2024), in MLST database (EnteroBase https://enterobase.warwick.ac.uk/species/ecoli/ 1.05.2024) 18253 registered isolates with ST10) and studied sequence type. Among our isolates, 15 ST10 isolates were present from all One Health domains from very different origins and hosts: 5 environmental (soil, manure, wastewater), 7 isolates from humans (MDR isolate from the hospitalised patient, three veterinarians, a farm worker from pig farm), three from animals (bovine, swain, broiler). Such wide variation in isolates' origin suggests excellent transmission mechanisms. ST38 has been considered highly virulent and widespread (Fonseca et al., 2022). ST38 was found in our dataset from humans (4 patients), broilers and wastewater.

5.3 Phenotypic and genotypic resistance patterns among the isolates

Depending on the purpose, the resistance patterns were detected using phenotypic (susceptibility testing MIC values) or genotypic resistance from the WGS or PCR results.

MDR *E. coli* and *P. aeruginosa* strains sharing the ST isolated from hospitals were found in sewage and downstream surface water bodies.

Phenotypically multi-drug resistant (MDR) isolates were found in all sampling sources, and 33% of all tested *P. aeruginosa* isolates and 18% of *E. coli* isolates were MDR. On the other hand, 30% of *P. aeruginosa* isolates and 44% of *E. coli* isolates were susceptible to all tested antibiotics. MDR isolates were observed in all one health domains (humans, animals, environment) (Figure 13). We observed a high resistance rate among clinical isolates from humans (50% of the isolates were MDR (64 out of 128)) because antibiotic resistance was the selection criteria for those isolates (publication III). One *P. aeruginosa* isolate (ST362), collected from a patient with a urinary tract infection, was resistant to all antimicrobial agents we tested. In the case of *P. aeruginosa*, no genotype was more multi-drug resistant than the others. Genetic analyses revealed that local hospital outbreak clones can evolve from several strains with substantial genetic variability. Selection driven by antibiotic pressure in the surrounding environment is a plausible reason for the repeated resistance gaining in the *P. aeruginosa* population. For *E. coli*, eight different antibiotics were tested, and two isolates were resistant to six of them, and they originated from human (wound infection) and broiler meat.

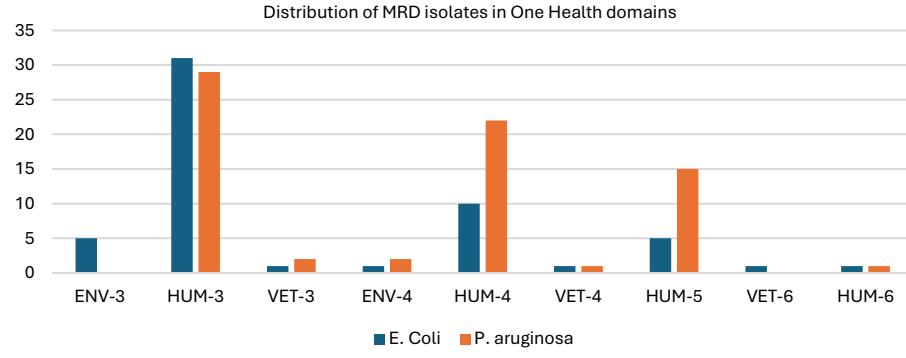


Figure 13. Distribution of multi-drug resistant *P. aeruginosa* and *E. coli* isolates among One Health domains: (HUM – human; VET – animal; ENV – environmental). The number in a legend represents the number of antibiotic group isolates are resistant to.

For *Enterococcus*, only vancomycin resistance was phenotypically tested. Two highly resistant (vancomycin MICs of 256 and 32 mg/L) human isolates from ST774 of the *E. faecalis* were found. They carried vanb gene clusters in the genomes. None of the *E. faecium* isolates were vancomycin-resistant according to EUCAST 2018 breakpoints (MIC ≤ 4, sensitive, MIC >4, resistant) (publication I).

Wildlife currently has a low prevalence of resistance genes in *Enterococcus* isolates (publication I). Six *E.coli* isolates obtained from wild birds were susceptible (phenotypical resistance) to all tested antibiotics. *P. aeruginosa* isolates were not obtained from the wild birds.

Carriage of antibiotic-resistant bacteria for non-infected human hosts is generally low. For humans, 37 *E. coli* isolates were tested, and three of them were MDR (resistant to 3 groups). MDR isolates were from veterinarians (n = 2) and healthy volunteers from the general community (n = 1). 51% of the *E. coli* isolates from healthy humans were susceptible to all antibiotics. In the population of humans in contact with animals, ESBL-*Enterobacteriaceae* carriage is low. However, pig farmers may pose a threat to transferring resistant microorganisms to other vulnerable groups (publication IV).

5.4 Antibiotic resistance in Estonian wastewaters (II, V)

Wastewater is a collection of different anthropogenic sources, and resistance carriers arrive there from various sources. Wastewater treatment plants are an important link in the spread of resistance, where, depending on the circumstances, the resistance can increase and decrease. There has been a question about whether and which processes take place in WWTPs regarding AMR and the possibilities of using it to our advantage. Wastewater is like an information point. Today, it is used to monitor many diseases and habits (legal and illegal drug use) that spread in the population. For example, worldwide surveillance of infectious disease outbreaks as an early warning system wastewater monitoring is used for Polio (World Health Organization, 2024a), SARS-CoV-2 (World Health Organization, 2020) including Estonia (Kisand et al., 2023; Terviseamet, 2024). Estonia is currently monitoring for residues of narcotic and psychotropic substances in wastewater (Tervise Arengu Instituut, 2020; Justiitsministeerium, 2024). We have used WWTPs in our studies to map the spread of antibiotic resistance and compared it with other countries (publication II, V).

5.4.1 Screening of antibiotic resistant genes in waste water (V)

In order to obtain a first overview of the situation of the spread of resistance, we used wastewater samples PCR and qPCR methods for screening of resistance genes in our study (2009–2011). The advantage of the method is that it provides extensive information about the presence of selected genes and does not have to be used to isolate bacterial strains (whole DNA present in the sample will be tested). PCR screening methods and gene-based approaches will not provide

important information about the sources of resistance and possible spreading patterns. Another disadvantage is that the genes to be studied have to be selected in advance, significantly narrowing the amount of information available. We conducted a two-stage preliminary study to identify resistance genes in sewage treatment plants in Estonia and Finland. Traditional PCR was used in the first step, and qPCR was used in the second. Based on previous information and literature, the screenings of 25 resistant genes were selected. The most important criteria for selection were clinically relevant genes (risk to human health) previously detected in WWTPs, genes found in various mobile elements, demonstrating their potential for transfer between bacteria and high-consumption antibiotics – sulphonamides, tetracyclines, and beta-lactams. Using traditional PCR we tested from wastewater 6 tetracycline-related resistance genes (tetm, tetc, teta, tetg, tete, tetw), 3 sulphonamide-related resistance genes (sul1, sul2, sul3), 12 beta-lactam antibiotic related resistance genes (blashv-34, blactx-m-32, blaoxa-58, blaoxa-40, blaoxa-m-48, blaoxa-50, blaimp2, blaimp9, blactx-m-27, blactx-m-4, ampc, meca), 3 fluoroquinolone related resistance genes(qnr, qnr-b4, qnr-b1) and 1 vancomycin-resistant gene (vana). The seven resistance genes (sul1, sul2, tetm, tetc blashv-34, blactx-m-32, and blaoxa-58) with the highest frequency of detection in the screening were selected for quantification with qPCR in inflow and outflow water of the WWTPs. All seven tested genes were present in influent and effluent. Our qPCR results showed that there is neither considerable enrichment nor purification of antibiotic resistance genes in studied conventional WWTPs. Screening for the gene indicated that we have a potential transmission problem, and further research is needed. It is important to fill the gaps in information about which species carry the resistance genes found and whether there is a connection with the spreading pathogens in the population.

5.4.2 Abundance of AMR in waste water compared to the global scale (II)

We participated in a global study to determine the relative abundance of cefotaxime-resistant (CTX-R) *coliforms* in wastewater influent and effluent. In this study, a more traditional antibiotic-supplemented plate count method was used. Only phenotypic resistance was detected. We participated in a study explaining the resistance level in sewage treatment plants using a globally comparable method and seeing Estonia's position in the general picture. While most of the effluent samples contained low levels of CTX–R *coliforms*, it is troubling that 37% (89 out of 243 individual sampling points) exceeded the WHO cut-off formulated for faecal *coliforms* in water reuse (<10 E.coli cfu/ml). In 2020, new EU regulation 2020/741/EU on minimum requirements for water reuse was adopted, and limit values for *E. coli* are from <10 cfu/100ml up to <10 000 cfu/100ml) to be used for four reuse classes suitable for different applications. On the global scale (WWTPs from 22 countries across Europe, Asia, Africa, Australia, and North America), the results varied due to the differences in technologies and population density. Estonia has average results compared to the other countries

with similar technologies without disinfection. One conclusion from the global study was that a subset of the isolates obtained should be subject to screening for cefotaxime-resistance genes (i.e. those encoding CTX-M enzymes) using PCR and/or whole genome sequencing and comparative analyses of ARGs and MGEs to detect possible geographic patterns. Estonia did not collect isolates from that study (publication II). However, this is a good suggestion for future global study planning, as at least some isolates should be collected to get more information from the studies.

None of the Estonian treatment plants has disinfection steps in the treatment process for wastewater effluences. Direct water reuse is not an issue in Estonia; our study showed that the number of *E. coli* indicators in a recipient water body directly influenced by WWTP was low <5 cfu/ml and no CTX-R *coliforms* were detected during the samplings.

5.4.3 Removal of AMR genes and indicator microorganisms from waste water (II, V)

The average removal rate of faecal *coliforms* in WWTPs was 97.9% and 99.9% CTX-R *E. coli*. Figure 14 shows the results of *E. coli* and cefotaxime-resistant *E. coli* in Estonian treatment plans during three sampling rounds in the winter of 2016–2017. The removal rates were lower in smaller plants with simpler technologies and higher in bigger plants with tertiary treatment technologies. Low-capacity treatment facilities are more vulnerable to changes in inflowing wastewater composition and flow rates. Previous studies have shown that wastewater treatment systems with greater complexity (TEC >5) were more successful in removing hazardous substances. A comprehensive study of the most used wastewater technologies in Estonia and the performance of the WWTPs for hazardous substances removal showed that the removal efficiency of organic hazardous substances had a significant (p-value <0.05) linear correlation with removal efficiencies of chemical oxygen demand (COD) and total suspended solids (TSS) (Kõrgmaa et al., 2020). As most bacteria are attached to the solids in the wastewater, better removal of suspended solids will also affect removing bacteria from the wastewater effluences.

The highest CTX-R numbers were in smaller treatment plants, where local healthcare facilities have a bigger proportion of the wastewater loads. CTX-R *E. coli* was present in all the effluent samples in Estonia. Similarly, all seven tested ARGs were present in all samples (publication V). We can conclude from our studies that AMR is common in our effluences, and for sustainable water use (protection of drinking water sources; reuse of wastewater and recreation opportunities in natural water bodies (bathing, fishing, etc.), we need an additional sanitation step for WWTP-s before discharges the effluent water to the environment. The situation has stayed similar as there have not been many changes in WWTP technologies in recent years. A new EU regulation (Urban Wastewater Directive) will be in place in 2024 to remove micropollutants from wastewater.

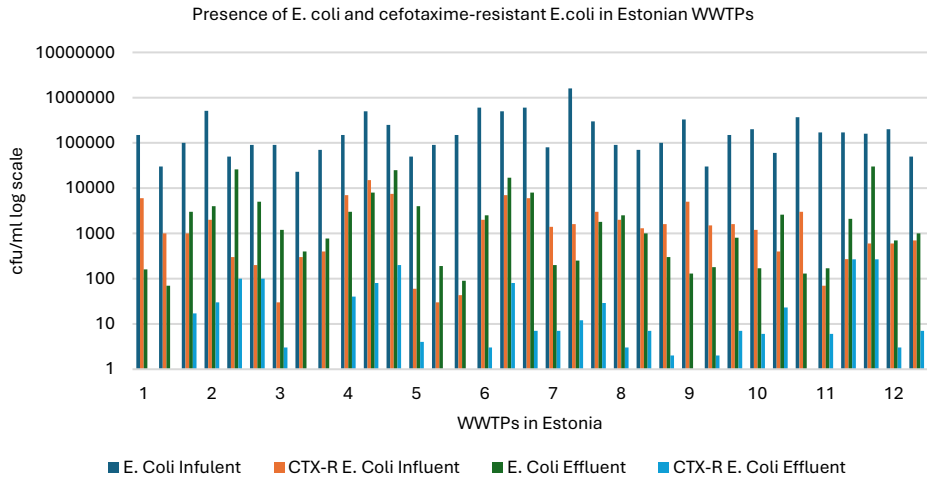


Figure 14. *E. coli* and CTX-R *E. coli* in Estonian WWTPs 2016–2017. Estonian data collected for the global *E.coli* study (publication II).

Using the qPCR method, we evaluate the abundance of the total bacterial community in the WWTP by counting the 16S rRNA gene in the samples. The raw gene copy numbers were initially used to estimate the general changes in bacterial levels during wastewater purification. The copy number of the 16S rRNA gene was several orders of magnitude lower in the effluent (EF) than in the inflow (IF). The raw gene copy numbers of ARGs/ml decreased during processing in the WWTP water phase. The levels of ARGs detected in the effluent were lower than influent in all three plants. Differences obtained mainly were based on the treatment capacity of the plants. The raw gene copy numbers of ARGs/ml decreased during processing in the WWTP water phase. The levels of ARGs detected in the EF were lower than IF in all three plants (Figure 15) (publication V).

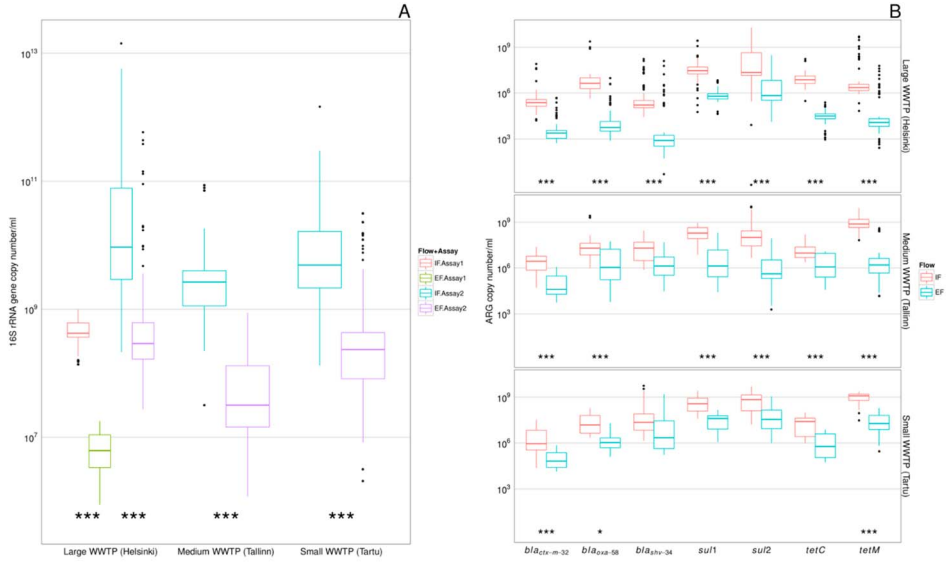


Figure 15. The abundance of the total bacterial community in the WWTP by quantifying 16S rRNA gene and seven ARG-s. Estonia, Finland samples from 2010–2011 (EF – effluent water; IF – influent water) (publication V).

CONCLUSIONS

Our research significantly contributes to the One Health approach for combating antibiotic resistance by fostering an interdisciplinary network encompassing human medicine, veterinary science, genetics, and environmental science. We established a comprehensive baseline for Estonia regarding the spread of antibiotic resistance, including areas not typically monitored. While progress has been made, especially in implementing the One Health framework, there is still a need for more data and measures, particularly in the environmental sector.

The key findings of performed studies are the following:

- We established a robust interdisciplinary network and created a comprehensive collection of isolates and genetic data, facilitating further research and monitoring efforts.

Transfer between One Health domains:

- *Escherichia coli*: Six shared sequence types (STs) (10, 38, 58, 88, 108, 117) were found across one health domains (human, animal, and environment). These isolates originated from diverse sources, but no clear transmission patterns were detected (publication IV).
- *Pseudomonas aeruginosa*: Two shared STs (313, 395) were detected, with human-dog-environment connection/transmission (publication VI).
- *Enterococcus faecalis*: ST16 was shared across humans and cattle and manure, indicating potential transmission (publication I).
- *Enterococcus faecium*: No cross-species transmission was observed (publication I).
- Multi-resistance was widespread at the gene level and confirmed phenotypically. (publication I, III) Notably, one P. aeruginosa isolate (ST362) from a human patient was resistant to all six tested antibiotic groups (publication III).
- Generally, there are low carriage rates of antibiotic-resistant bacteria among non-infected hosts, with low prevalence in healthy humans and almost non-existent in wildlife, indicating opportunities for preventative measures (publications I; IV).
- Estonian wastewater treatment plants (WWTPs) showed average antibiotic resistance levels compared to global data. Our study results suggest incorporating additional sanitation measures into wastewater treatment processes to meet higher biohazard prevention standards and ensure the sustainable use of water and soil (publications II, V).

Further studies should focus on collecting isolates from various sources for comprehensive PCR and whole genome sequencing screening. In addition, it is recommended that more robust policies and monitoring frameworks be developed and implemented to address gaps, particularly in the environmental sector. In conclusion, our study underscores the importance of a unified One Health approach in addressing antibiotic resistance. By bridging human, animal, and environmental health, we can develop more effective strategies to prevent and control the spread of antibiotic-resistant bacteria, ultimately protecting public health and the environment.

SUMMARY IN ESTONIAN

„Üks Tervis“ lähenemine antibiootikumidele resistentsete bakterite leviku kaardistamisel Eestis

Antibiootikumiresistentsuse teema on mitmekülgne ning vajab erinevaid teadmisi, lähenemisi ja koostööd, et ka tulevikus oleks võimalik nakkushaiguseid ravida. Me ei taha pöörduda tagasi aega, kus bakterinakkused olid sageli surmavate tagajärgedega. Antibiootikumide (AB) suhtes resistentseid baktereid elab nii inimestel, loomadel kui keskkonnas. Resistentsed bakterid võivad levida loomadelt inimestele ja vastupidi: otsekontaktide, toidu või ümbritseva keskkonna kaudu. „Üks Tervis“ on lähenemine, mis arvestab tervikpilti ja teadvustab, et antibiootikumide kasutamine ükskõik millises valdkonnas mõjutab antibiootikumiresistentsuse levikut kõikjal. Varasemad üksikute valdkondade sisesed probleemide lahendused ei ole jätkusuutlikud. Uus globaalne terviklähenemine põhineb koostööl ja ühise jagatud keskkonnaga arvestamisel resistentsuse leviku pidurdamisel. Keskkond olulise vahelülina on ühelt poolt koht kuhu koguneb inimtegevuse tulemusel oluline hulk antibiootikumide jääke, aga sinna satuvad ka juba multiresistentsuse saavutanud bakterid. „Üks Tervis“ lähenemine eeldab seotud tegevusi antibiootikumiresistentsuse (AMR) vähendamiseks kõikides sektorites sama aegselt: meditsiinis, veterinaarmeditsiinis, toidutootmises ja keskkonnas. Rahvusvaheliselt on sõlmitud mitmeid „Üks Tervis“ põhimõtteid järgivaid kokkuleppeid, et jõuda antibiootikumide resistentsuse vähendamiseni (WHO; Euroopa Liidu 2017.aastal koostatud tegevusplaan jne.). Antibiootikumiresistentsuse leviku piiramiseks on vajalik järgida antibiootikumide vastustundlikku kasutust. Mida rohkem me kasutame antibiootikume, seda suurem on surve resistentsuse tekkeks. Antibiootikume tuleb tarbida alati diagnoosi põhiselt (teada on haigust tekitav bakter), parajal hulgal st. õiges ravidoosis ja ettenähtud aja jooksul ning seda tuleb jälgida nii inimeste kui loomade ravis. Sugugi mitte kõik antibiootikumid ei mõju kõigile bakteritele ühtemoodi, sest nende geneetilised kaitsemehhanismid on erinevad. Ala- ja üleannustamine annab bakteritele võimaluse resistentsuse väljakujundamiseks, kas otseselt ravitava organismi juures või hilisemalt AB jääkide mõjul keskkonnas. Bakterinakkuste raviks piisavate valikute tagamiseks nii inimestel kui loomadel on kehtestatud ülemaailmsed reeglid, milliseid antibiootikume kasutatakse inimeste ja milliseid loomade ravis. Loomakasvatuses ei ole antibiootikumid olnud kasutuses ainult ravieesmärgil, vaid ka nn kasvuedendajatena ja ka mitte diagnoosi põhist ravi kasutati enne piiranguid laialdaselt (nn ennetavad ja kogu karja ravimeetodid). Nende tegevuste tulemusel suurenes oluliselt kasutatud antibiootikumide kogus ja antibiootikumide jääke jõudis keskkonda rohkem. Loomakasvatuse otsene seos keskkonnaga on tekkivate väljaheidete kasutamine väetisena ja kui lisaks toitainetele on sõnnikus ka AB jäägid olemegi tekitanud olukorra, kus keskkonnas loomulikult elavad bakterid peavad hakkama otsima ellujäämiseks võimalusi ja arenevad välja resistentsusmehhanismid. Ka inimeste tarbitud antibiootikumidest jõuab enamus jääke kesk-

konda väljaheidete kaudu. Reoveepuhastite arenguga suudame küll puhastada olulise osa tekkivatest saasteainetest, aga bioloogilist ohutust tänased tehnoloogiad veel ei taga. Lisaks on bakterid nutikad ja suudavad omavahel infot vahetada (sh resistentsusgeene) ja selleks on reoveepuhasti bioloogilised protsessid ideaalsed kohad.

Alustasime Eesti olukorra kaardistamisega juba 2012. aastal kui moodustati ühine töörühm meditsiini, veterinaaria, keskkonna ja geneetika spetsialistidest antibiootikumide resistentsuse uurimiseks. Meditsiinis ja veterinaarias kogutakse haigust tekitavate bakterite tüvesid ja neid puudutavat fenotüübilist resistentsuse infot juba pikaajaliselt. Uute geneetiliste uurimismeetodite kasutuselevõtuga avardusid võimalused tüvede päritolu ja nakkuste leviku teadasaamiseks ja resistentsuse täpsemaks uurimiseks. Keskkonna seos levikus on tõendatud, aga kohapõhiseid andmeid regulaarselt ei kogutud ja see lünk vajas täitmist võimalike resistentsuse ülekandumiste kaardistamisel. Kõigi kolme üks tervis valdkonna osas (inimene, loomad ja keskkond) võimalike resistentsuse levikute ja seotud ohukohtade leidmiseks kogusime kõigist antibiootikumidele resistentseid baktereid, mida uurisime erinevate meetoditega sh kasutasime täisgenoomide sekveneerimist. Uurimiseks valisime meditsiiniliselt ja veterinaarselt olulised haigustekitajad, millel on juba teada antibiootikumiresistentsused ja mis seetõttu alluvad halvasti ravile. Olulisemad antibiootikumide bakterite paarid resistentsuse levikus olid: ESBL (laiendatud toimespektriga beetalaktamaas) *E.coli*; vankomütsiini resistentne *Enterococcus* (VRE); metitsillini resistentne *Staphylococcus* (MRSA); karbapeneemi resistentne *Pseudomonas aeruginosa*. Oluliseks üldise leviku ja kokkupuute kohtade kaardistamise osaks oli tervete inimeste ja loomade kaasamine uuringusse, et näha võimalikku varjatud edasikannet. Keskkonnas leiduvaid tüvesid kogusime loomakasvatuse ja reoveepuhastuse mõjudega piirkondadest nii veest kui pinnasest. Oma töödes olen keskendunud ühelt poolt olukorra kaardistamisele, et mõista kas ja kus on Eestis resistentsuse leviku probleemsed kohad. Teisalt otsinud lahendusi, kuidas ja milliste meetoditega oleks võimalik antibiootikumiresistentsuse levikut keskkonnas uurida, et olukorrast oleks piisavalt infot mille alusel planeerida edasisi tegevusi. Keskkond on „Üks Tervis" valdkondadest seni olnud kõige vähem vaatluse all ja regulaarselt ei koguta andmeid tänini. Uued rahvusvahelised nõuded resistentsuse jälgimiseks on küll kehtestamisel, aga nende rakendamiseks on vajalik eelneva info ja olemasoleva olukorra teadmine ja sobivate metoodikate juurutamine keskkonnaproovide jaoks.

Meie uuringud aitavad oluliselt kaasa antibiootikumiresistentsuse leviku tõkestamisele „Üks Tervis" lähenemisviisil, edendades interdistsiplinaarset võrgustikku, mis hõlmab meditsiini, veterinaariateadust, geneetikat ja keskkonnateadust. Töö annab lähtekoha antibiootikumiresistentsuse leviku kohta, sealhulgas valdkondades, mida tavaliselt ei jälgita. „Üks Tervis" lähenemise rakendamisel on tehtud edusamme, kuid endiselt on vaja rohkem andmeid ja tegevusi, mis resistentsuse levikut aitaksid piirata, seda eriti keskkonna valkdonnas.

Doktoritöö peamised tulemused ja järeldused:

- Loodud on geneetilise info andmebaas ja bakteritüvede kogu antibiootikumidele resistentsetest bakteritest. Edasistes uuringutes on võrdlusena võimalik kasutada nii bakterite tüvesid kui ka nende kohta kogutud geneetilist infot, mis on edastatud rahvusvahelistesse andmebaasidesse.
    - Leidsime geneetiliselt sarnaseid antibiootikumiresistentseid baktereid erinevatelt peremeestelt ja keskkondadest, mis viitab tõenäoline ülekandele. Geneetiliselt sarnaste tüvede levik erinevate peremeeste ja keskkonda vahel ei ole Eestis laialt levinud.
    - Multiresistentsus geneetilisel tasemel on laialt levinud ja enamasti avaldub see ka fenotüüpilise resistentsusena.
    - Üldiselt on antibiootikumiresistentsete bakterite levik tervete peremeesorganismide seas madal sh metsloomadel peaaegu olematu. Mis näitab ennetusmeetmete võimalusi.
    - Antibiootikumidele resistentsete bakterite hulk Eesti reoveepuhastites võrreldes globaalsel skaalal kasutusel olevat indikaatorit – CTX-R kolilaadsed heitvees – on keskmine, aga ületab WHO soovitust. Bioloogiliselt ohutu heitvee keskkonda juhtimiseks on vajalikud uued tehnoloogilised lahendused.

„Üks Tervis“ põhimõtte rakendamisega on Eestis alustatud ning valdkondadevaheline koostöö on oluliselt paranenud, moodustatud on riiklik AMR juhtrühm, mis koostab rahvusvahelist õigusraami arvestades riikliku AMR tegevuskava (2023/C 220/01), millest juhindumine võiks lähiajal antibiootikumide resistentsuse leviku vähendamisele oluliselt kaasa aidata.

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