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Wastewater Surveillance: An Early Warning Tool for Multidrug-Resistant Bacterial Pathogens

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DOCTORAL DISSERTATION
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Abstract

Antimicrobial resistance (AMR) is one of the global public health threats that is associated with significant mortality and has a substantial social and economic burden. Significant differences exist globally in burden, impact, diagnostic approach, and interventions to control AMR, with the highest burden being recorded in resource-limited settings. Several key drivers are associated with the emergence and spread of AMR bacteria; the extensive use of antimicrobials is a key factor. Resistance can be intrinsic or acquired; thus, AMR bacteria transcend human, animal, wildlife, and their shared ecosystem. This interconnectedness of AMR makes it a One Health issue.

AMR surveillance mainly relies globally on passive reporting of phenotypic laboratory results for specific pathogens isolated from humans, animals, and food products, with limited environmental data. This passive surveillance approach is typically targeted towards priority multidrug-resistant (MDR) pathogens. Wastewater-based surveillance (WBS) for AMR and priority pathogens is a public health tool that could enable a shift from reactive disease surveillance to proactive population health assessment. WBS provides a cost-effective and sensitive early-warning tool that is independent of the AMR surveillance system. It offers a community view for AMR into the hidden reservoir of resistance, guiding stewardship and containment efforts. WBS is poised to become a cornerstone of 21st-century public health infrastructure as technology and methodologies continue to evolve. Finland has a relatively low burden of multidrug-resistant bacterial pathogens. Despite this, routine surveillance is crucial for tracking their spread, assessing public health risks, and informing effective control measures. Routine human surveillance and WBS will help to identify emerging threats like increased incidence in certain hospital districts, potential for outbreaks in vulnerable populations such as the elderly in long-term care facilities, or their introduction into Finland from high-burden countries.

This thesis is based on three studies that focused on WBS for three pathogens identified in the 2024 bacterial priority pathogen list of the World Health Organization: methicillin-resistant *Staphylococcus aureus* (MRSA), Extended Spectrum Beta Lactamase (ESBL)-producing *Escherichia coli*, and vancomycin-resistant *Enterococcus faecium*. The thesis specifically aimed to study the genomic epidemiology of these pathogens and evaluate the utility of WBS as an early warning tool to identify these clinically relevant AMR pathogens.

MRSA's isolation rate in the Finnish population has been increasing over recent years, with more MRSA cases being detected in blood samples. An increase in Panton-Valentine Leukocidin (PVL)-positive and CC398 (livestock-associated MRSA) strains is also being seen in humans. Hence, the study evaluated WBS as an early warning tool by selectively

culturing MRSA and detected a prevalence of 27.5% (n=22/80) with seasonal and temporal variations across the ten wastewater treatment plants (WWTPs). WBS revealed that most of the isolates belonged to the community-associated ST8-*t*008 (USA300 clone) and hospital-associated ST6-*t*304 spa types. Genomic epidemiology of wastewater isolates revealed significant similarity with circulating human strains in Finland. A low number of livestock-associated CC398 complex and PVL-positive isolates were also detected in wastewater. Wastewater (WW) isolates also clustered with other human mecA-ST6/t304 isolates, and wastewater (WW) PVL+ ST8/*t*008 clustered with PVL+ human isolates.

The genomic epidemiology of AmpC/ESBL-producing *E. coli* revealed its high abundance (97.5%, n=75/77) in municipal wastewater in Finland. The in-silico analysis revealed clonally diverse isolates comprising 30 sequence types (STs), including the globally distributed pandemic ST131 clone. Several clinically relevant resistance genes, including the *bla*$_{\text{CTX-M}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{OXA-1}}$, *bla*$_{\text{KPC-2}}$, *bla*$_{\text{NDM-1}}$, and *mcr-1.1*. Core genome MLST (cgMLST) revealed the circulation of distinct clonal lineages of AmpC/ESBL-producing *E. coli* across Finland and detected closely related clinical and wastewater ST131 isolates, underscoring the utility of WBS for monitoring the hypervirulent ST131 strain.

Vancomycin-resistant *E. faecium* - a hospital-associated pathogen - is another priority AMR pathogen. The culture-based approach enabled the isolation of the pathogen from 17 samples (22%), with no significant difference in the isolation rate across the ten WWTPs. All wastewater isolates were genetically very close to those detected in national human surveillance, particularly within the outbreak-associated CC17 lineage, mostly differing by fewer than ten allelic differences. WBS identified circulating clusters of the pathogen across Finland.

The three studies show that isolates obtained from WBS closely align with human clinical and screening isolates. Put together, these studies support the utility of WBS as a sensitive tool for population-level surveillance and a complementary source of data for public health decision-making. This study also contributes to the One Health approach, needed to fully understand the transmission dynamics of pathogenic bacteria and effectively manage the AMR challenge. The future of WBS lies within a One Health approach that intentionally designs interconnected wastewater surveillance networks and unified diagnostic assays. This will enable us to have an unparalleled, real-time understanding of health threats that emerge at these interfaces, ultimately leading to a more proactive, predictive, and resilient global health system.

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Helsinki, February 2026

Ahmad I. Al-Mustapha

“When a man says I cannot, he has made a suggestion to himself. He has weakened his power of accomplishing that which otherwise would have been accomplished.”
[Muhammad Ali]

List of abbreviations

AMR	Antimicrobial resistance
ARG	Antibiotic resistance genes
AST	Antimicrobial susceptibility testing
BPW	Buffered peptone water
CDC	Centers for Disease Control and Prevention
cgMLST	Core genome multi-locus sequence typing
CGE	Center for genomic epidemiology
CLSI	Clinical and Laboratory Standards Institute
CPE	Carbapenemase producing Enterobacterales
ECDC	European Centre for Disease Prevention and Control
ECOFF	Epidemiological cut-off values
ESBL	Extended-spectrum beta-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
GAP	Global Action Plan
GLASS	Global antimicrobial resistance and use surveillance system
KPC	*K. pneumoniae* carbapenemases
LMICs	Low- and middle-income countries
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time of flight mass spectrometry
MGE	Mobile genetic elements
MIC	Minimum Inhibitory Concentration
MLST	Multi-locus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
NGS	Next-generation sequencing
PBPs	Penicillin-binding proteins
PCR	Polymerase chain reaction
ST	Sequence type
THL	Finnish Institute for Health and Welfare
VFDB	Virulence factor database
VREfm	Vancomycin-resistant *Enterococcus faecium*
WBE/WBS	Wastewater-based epidemiology/surveillance
WGS	Whole-genome sequencing
WHO	World Health Organization
WOAH	World Organization for Animal Health
WW	Wastewater

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List of original publications

This thesis is based on the following publications:

I **Al-Mustapha, A. I.,** Tiwari, A., Johansson, V., Heljanko, V., Lehto, K.M., Lipponen, A., ... & WastPan Study Group. (2024). Characterization of methicillin-resistant *S. aureus* in municipal wastewater in Finland. One Health, 19, 100881. https://doi.org/10.1016/j.onehlt.2024.100881.

II **Al-Mustapha, A. I.,** Tiwari, A., Laukkanen-Ninios, R., Lehto, K. M., Oikarinen, S., Lipponen, A., ... & Heikinheimo, A. (2025). Wastewater based genomic surveillance key to population level monitoring of AmpC/ESBL-producing *Escherichia coli*. Scientific Reports, 15(1), 7400. https://doi.org/10.1038/s41598-025-91516-9

III **Al-Mustapha, A. I.,** Lindholm, L., Laukkanen-Ninios, R., Johansson, V., Tiwari, A., Heljanko, V., ... & WastPan Study Group. (2026). Comparative core-genome MLST of vancomycin-resistant *Enterococcus faecium* supports the utility of wastewater-based surveillance: a pilot study. BMC Microbiology. https://doi.org/10.1186/s12866-025-04610-3.

1 Introduction

The effectiveness of antimicrobials is in jeopardy because microorganisms have become resistant to them. The concept termed antimicrobial resistance (AMR) is associated with increased infectious disease morbidity, mortality, and a substantial economic burden; AMR also threatens the attainment of the United Nations' Sustainable Development Goals (SDGs) (WHO, 2023). Resistance to crucial antimicrobials such as HIV medications, anti-tuberculosis medications, anti-helminthics, and antibiotics could threaten decades of gains in control of these infectious diseases.

Antibiotic resistance is a subset of AMR that focuses on resistance among bacterial pathogens. Of these pathogens, more attention has been focused on World Health Organisation (WHO) and European Union (EU) priority pathogens, amongst which are methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococcus faecium*, and third-generation cephalosporin-resistant *E. coli* (WHO, 2024). These pathogens cause a variety of infections, including urinary and respiratory tract infections, and are ranked in the top three bacterial species causing the largest global burden of disease (ECDC, 2020; 2023). Resistance among these priority pathogens could be intrinsic or due to acquired resistance determinants. This has been associated with several factors: illicit antibiotic usage, selective pressure, poor diagnostics, and poor infection prevention and control measures, especially in resource-limited settings.

Understanding AMR dynamics, trends, burden, and evaluation of interventions requires active surveillance and reliable data. Such data (clinical and surveillance) provide information on emerging resistance mechanisms and public health threats (WHO, 2015). The One Health approach is invaluable for monitoring AMR in the epidemiological triad: humans, animals, and their shared environment. The approach could be crucial for appraising microbial source attribution, risk assessment, transmission dynamics, and the interconnectedness of human, animal, and environmental health (Mettenleiter et al., 2023). Most AMR surveillance systems are currently fragmented based on sector: human, food-producing animals, and, less commonly, the environment. Wide variability also exists in AMR surveillance systems for targeted pathogens, mode of operation, ideal sample types, ideal identification methods, and data interpretation and compatibility.

Wastewater (and environmental)-based epidemiology of AMR pathogens offers a promising approach to obtaining community-level data on disease burden. Wastewater-based surveillance (WBS) in the genomic era offers a more integrated, non-invasive, relatively cheaper, and optimisable approach than the traditional patient-based AMR surveillance systems. WBS has the potential to produce complementary, comparable, and multi-target data on AMR pathogens and their association with other health and sociodemographic markers (Tiwari et al., 2022). This thesis characterised three priority pathogens in wastewater and evaluated their utility as an AMR preparedness or early warning tool. These offer an integrated, reliable, reproducible, and globally acceptable surveillance system enabling the detection of a pathogen's introduction into an area, correlation with clinical cases, and population-level data. Wastewater has a significant potential to assess and survey AMR at the population level, simultaneously avoiding ethical issues and the practical and logistical barriers associated with individual sampling (Hendriksen et al., 2019; Larsson et al., 2023; Pruden et al., 2021). Wastewater surveillance could also hold potential in the AMR surveillance of food-producing animals.

2 Review of the literature

2.1 Burden of Antimicrobial Resistance

Antimicrobial resistance (AMR) is a phenomenon used to describe the ability of microbes to counteract, endure, and proliferate when exposed to an antimicrobial agent to which they were previously sensitive. It is an important threat to the control of diseases (infectious and communicable) and is needed for medical interventions. AMR (or antibiotic resistance) is resistance among bacteria. Other forms include resistance to anthelmintics (anthelmintic resistance), viruses (antiviral resistance), and fungi (antifungal resistance) (WHO, 2023). AMR is a threat to the global control of infectious diseases such as tuberculosis, HIV/AIDS, and several bloodstream infections. AMR is also a significant threat to animal husbandry, because several infectious diseases also threaten animal production and husbandry. More global research has been done in understanding AMR burden, spatial and temporal spread, and mechanisms of resistance among bacterial pathogens. Generally, resistance in bacteria is usually acquired, or it could be innate in nature (Reygaert, 2018).

There has been an increase in the global emergence of multidrug-resistant (MDR) pathogens over the past decades in humans and animals. This increased burden resulted in prioritising AMR as one of the top ten global public health threats facing humanity (WHO, 2023). Regarding global burden, it is very difficult to have a reliable evaluation of the global burden due to poor quality (insufficient) data in several parts of the world, poor diagnostic facilities in resource-limited settings, and a lack of standardisation of AMR identification techniques, amongst others. Despite these challenges, several studies have estimated the global AMR burden (WHO, 2025). Murray et al. (2022) estimated that there were around 4.95 (95% UI: 3·62–6·57) million deaths associated with AMR. This burden included 1·27 million (95% UI: 0·91–1·71) deaths attributable to bacterial AMR in 2019. Another study by Naghavi et al. (2024) estimated a similar global burden and reported an estimate of 4·71 million (95% UI: 4·23–5·19) deaths associated with bacterial AMR, including 1·14 million (95% UI:1·00–1·28) deaths attributable to bacterial AMR in 2021. The study, like others before it, reported that an estimated 1·91 million (95% UI: 1·56–2·26) deaths attributable to AMR and 8·22 million (95% UI: 6·85–9·65) deaths associated with AMR could occur globally in 2050. This estimated burden by 2050

was similar to that reported over a decade ago in the O'Neill report (O'Neill 2016). Global realities in health inequalities have shown an increased burden (impact) in certain regions of the world. The "super-regions" with the highest all-age AMR mortality rate in 2050 are forecasted to be South Asia, Latin America, the Caribbean (Naghavi et al., 2024); and low- and middle-income countries (LMICs), particularly countries in western Sub-Saharan Africa, have the highest AMR burden (Murray et al., 2022; Al-Mustapha et al., 2025).

Another key AMR impact, aside from the health impact (death and collapse of medical interventions), is the significant economic toll, especially at the microeconomic (hospitals, farms, care homes, etc.), macro level (societies, national economies), and the global level. Again, the true economic impact is difficult to estimate due to a lack of sufficient data, especially in resource-limited settings. Despite this, estimates reported that AMR could cause a global loss of USD 300 billion to USD 1 trillion with a resultant decrease in global GDP by 1% and 5-7% for developing countries by 2050 (Pulingam et al., 2021; Pantea et al., 2023). This includes US$98.6 billion in direct inpatient costs associated with AMR infections. The WHO made an earlier case for investment in AMR by estimating AMR's economic burden and reported a 1.1–3.8% reduction in global gross domestic product by 2050. The reduction means a shortfall of 1–3.4 trillion United States dollars annually by 2030 (World Bank, 2017). These and other economic estimates have utilised data on the hospital costs (direct, indirect and total hospital charge, including the cost of antibiotics, the costs of stay, and the cost of all other medical services provided) and offer forecasts based on factors such as an anticipated drop in national global domestic product, trade (mostly exports), labour productivity, and labour supply. AMR was estimated to cost between US$34.3-98.6 billion between 2030 and 2050 in inpatient costs. Additionally, the world's gross domestic product (GDP) could be boosted by US$386.2 (2030) to US$989.7 (2050) when five AMR interventions are instituted. These intervention scenarios are better treatment of bacterial infections, innovation and rollout of effective new gram-negative antibiotics, better treatment and innovation, combined interventions, and accelerated rise in resistance (McDonnell et al., 2024). AMR's economic impact also challenges/disrupts the attainment of seven of the 17 United Nations' Sustainable Development Goals. The seven SDGs threatened by AMR are: SDG 1 (No Poverty), SDG 2 (Zero Hunger), SDG 3 (Good Health and Well-being), SDG6 (Clean Water and Sanitation), SDG 8 (Decent Work and Economic Growth), SDG 12 (Responsible Consumption and Production), SDG 13 (Climate Action), and SDG 15 (Life on Land) (Aslam et al., 2024).

Several factors have generally been reported to be associated with the emergence and spread of MDR bacterial pathogens. These include misuse of antimicrobials (underdosage, overuse, extra-label use) in humans, animals, and agriculture, which increases the selective pressure on microbes. This misuse could be failure to adhere

to prescription drugs (a significant cause of MDR tuberculosis strains), the over-the- counter and unprescribed use of antimicrobials, including last resort ("reserved") antibiotics, especially polymyxins (such as colistin), carbapenems (such as meropenem), and newer generation cephalosporins (such as cefepime) (Al-Mustapha et al., 2025; Klein et al., 2021). Misuse of antimicrobials can also be used as meta-phylaxis or prophylaxis in animal husbandry, where drug compounds are used as growth promoters to increase the feed conversion rate, reduce the morbidity, and mortality of endemic diseases.

Other factors that promote AMR spread include increasing global travel, inadequate infection prevention and control, poor sanitation and hygiene, environmental contamination, and other mechanisms such as horizontal gene transfer (Klein et al., 2021). Other factors, such as a lack of awareness, could also result in the misuse of antimicrobials and promote the emergence and spread of resistant microbes and resistant determinants (Hendriksen et al., 2019; Holmes et al., 2016). AMR depicts a typical cross-sector challenge that requires a multidisciplinary (transdisciplinary) approach to tackle all sectors because of the threat it poses to the three key components of the epidemiological triad: human, animals, and ecosystems (Winkler et al., 2025). Hence, the One Health approach has been described as an all-inclusive approach that involves several sectors and a multitude of stakeholders to achieve optimised health for all.

2.2 Mechanisms and spread of antimicrobial resistance

Two main mechanisms of antibiotic resistance have been reported in bacteria. The first is the intrinsic resistance that is conferred when an organism is innately resistant to antibiotics (associated with microbes' intrinsic characteristics that arise from inherited structural or functional features). For example, Gram-negative bacteria are inherently resistant to vancomycin and other glycopeptide antibiotics because of the impermeability of the Gram-negative outer membrane to these large antibiotic molecules (Reygaert, 2018). Several mechanisms of intrinsic resistance are further depicted below: reduced permeability, drug efflux, alteration of the drug target site, target alterations, and modifications (Cox & Wright, 2013; Reygaert, 2018).

2.2.1 Intrinsic mechanisms of antibiotic resistance

1. **Reduced permeability:** Gram-negative bacteria's outer membrane is a complex and sophisticated organelle that has evolved to operate as a barrier and offer protection while enabling nutrient intake. Many antimicrobials must penetrate the bacterial cell membrane to reach their

target and start working. Their double-membrane structure renders their cellular envelope relatively impermeable, so Gram-negative bacteria pose a significant challenge to the development of new drug molecules that can bypass the cell envelope and offer intrinsic resistance to many antibiotics that are effective against Gram-positive pathogens (Darby et al., 2023). AMR can also arise as a result of changes to the envelope structure, such as loss of porin or modifications to the phospholipid and fatty acid composition of the cytoplasmic membrane, which might impact a drug's capacity to enter the cell. Gram-positive bacteria are inherently more susceptible to many antibiotics due to the absence of their outer membrane; however, it has been demonstrated that modifications to the cytoplasmic membrane's composition, which impact fluidity, play a significant role in lowering antibiotic permeability (Mishra et al., 2012). Studies have shown that reductions in porin expression significantly contribute to resistance to newer (reserve) antibiotics such as carbapenems and cephalosporins, whose resistance is usually mediated by enzymatic degradation (Blair et al., 2015; Wozniak et al., 2010).

2. **Active transport of antibiotics:** Despite the reduced permeability to reduce the influx of antibiotic molecules into the bacterial cell, bacteria can actively export them in a process known as efflux, achieved using efflux pumps. Antibiotics are among the many harmful substances that efflux pumps, which are transmembrane proteins, can move across bacterial membranes in an energy-dependent fashion. All bacteria have many efflux pumps, but they play a crucial role as AMR mediators in Gram-negative bacteria. They complement the impermeable double barrier in a way that makes these viruses naturally resistant to a wide range of medications. Efflux functions as a vital "platform" mechanism that permits the majority of other resistance mechanisms to have an effect, while the effects of various efflux systems on medications vary, with some providing high levels of resistance and others modest levels (Darby et al., 2023; Nazarov, P., 2022). Efflux transporters are generally categorised into six families. The resistance–nodulation–division (RND) family exhibits the highest clinically significant levels of resistance among Gram-negative bacteria. A wide variety of physically and chemically different antibiotics can be exported by RND pumps, and their overexpression leads to multidrug resistance (MDR) in clinical isolates (Ebbensgaard et al. 2020). The Gram-negative bacteria's inherent resistance to many medications used to treat Gram-positive bacterial illnesses is mostly caused by bacterial efflux pumps. Additionally, efflux pumps that are overexpressed might confer significant levels of

resistance to antibiotics that were once clinically beneficial (Blair et al., 2015).

3. **Target alteration, modification, and protection:** Another key bacterial resistance mechanism is the ability of these microbes to alter or modify their genome or the receptor profiles to protect themselves. This is because most antibiotics have high specificity for important bacterial cellular targets (which they use to selectively kill the bacteria). Antibiotics from a variety of classes have a significant affinity for their primary target, which frequently inhibits an essential cellular function and stops its development (bacteriostatic) or causes its death (bacteriocidal). Antibiotic binding may become inefficient and lead to antibiotic resistance if extra chemical moieties are added to the primary target to change its structure or shield it. Microbes could add moieties to the drug target as part of protecting themselves, eventually preventing antibiotic access and thus protecting the target, ultimately resulting in resistance (Bhujbalrao and Anand, 2019). This is a well-known mechanism of resistance to macrolides. High-level resistance to aminoglycoside drugs is also being increasingly attributed to methylation of the 16S rRNA (Doi et al., 2016). It has also been established that target alteration leads to resistance to polymyxins, including colistin. Colistin is a last-resort antibiotic that has been reserved as one of the few effective drugs left for Gram-negative infections that exhibit resistance to traditional first-line therapy. Colistin's mechanism of action is complex, although its ability to target lipopolysaccharide (LPS), which causes membrane damage and cell death, is essential to its effectiveness. Bacteria decorate the LPS with moieties that alter the charge of the overall molecule and inhibit interaction between the drug and its target to counter this (Sabnis et al., 2021).

4. **Inactivation and modification of the drug:** Aside from reducing cell permeability, increasing efflux of antibiotics, and target alteration and modification, microbes could also inactivate or modify antimicrobial drug molecules. This is a widespread resistance mechanism in many pathogenic bacteria (Darcy et al., 2022). Drug inactivation or modification is mostly achieved using enzymes; hence, no fundamental bacterial cell components are altered, which can be advantageous because fitness costs are less likely to be involved than with other mechanisms like drug target mutation or alteration. Antibiotic modification can be generally divided into two mechanisms: modification by the transfer of a chemical group or inactivation of the antibiotic by degradation. Typical examples are the hydrolytic effect of β-lactamases

on β-lactam antibiotics and the binding of tetracycline hydroxylases to inactivate tetracyclines. β-lactamases are enzymes that give resistance to β-lactam medications by breaking down the amide link of the β-lactam ring (Darby et al., 2023). The list of characterised β-lactamases continues to increase, with around 7,000 distinct β-lactamases being currently curated (Naas et al., 2017). In addition to the β-lactamase production, the emergence of resistance to carbapenems via the production of an inactivating enzyme - carbapenemases – is a concern to public health and clinicians. Carbapenem resistance can be mediated by carbapenemases or by the production of an extended-spectrum β-lactamase in combination with porin loss (Darcy et al., 2022). Additionally, the continuous emergence of novel variants such as the NDM-19 (which can hydrolyse β-lactams even in low-zinc conditions) and KPC-55 (particularly effective at catalysing aztreonam and meropenem) poses public health threats (Yoon et al., 2020). Microbes also become resistant by modifying antibiotic compounds through the transfer of a chemical group, which renders the drugs ineffective. This resistant mechanism has been documented for several classes of antibiotics, including aminoglycosides, macrolides, rifamycins, streptogramins, lincosamides, and phenicols (Bordeleau et al., 2021).

5. **Target bypass:** The "target bypass" is another resistance mechanism that confers resistance by creating a different channel that avoids antibiotics by rendering the original target unnecessary. This can happen through the acquisition of a different gene that the initial antibiotic is unable to effectively suppress, but that can give the cell the necessary characteristics (Blair et al., 2015; Darby et al., 2023). The most well-known instance of a target bypass is probably the emergence of methicillin-resistant *S. aureus* (MRSA). The bacterial cell wall is disrupted when β-lactam antibiotics, like methicillin, attach to penicillin-binding proteins (PBPs) and block the transpeptidase domain. *S. aureus*, although it has a decreased affinity for β-lactam antibiotics, can acquire an exogenous PBP (PBP2a) that is identical to the original target. Methicillin resistance is conferred by the mecA gene, which encodes this protein. It is found in the staphylococcal cassette chromosome mec (SCCmec), a mobile genetic element (Munita & Arias, 2016). This resistance mechanism is also involved in the resistance to vancomycin as well as trimethoprim & sulfamethoxazole, a combination antibiotic routinely used to treat urinary tract infections and a prophylaxis for pneumonia in immunocompromised patients (Grammatikos et al., 2020).

2.2.2 Extrinsic (Acquired) mechanisms of antibiotic resistance

Contrary to intrinsic resistance, which is a bacteria-induced resistance mechanism, resistance to antimicrobials could be a result of acquired (extrinsic) determinants that are carried on mobile resistance genes. The presence of an intrinsic resistance mechanism does not preclude the acquisition of any mobile elements that also harbour antibiotic resistance genes (ARGs). The capture, accumulation, and dissemination of resistance genes are due largely to the actions of mobile genetic elements (MGE), a term used to refer to elements that promote intracellular DNA mobility (e.g., from the chromosome to a plasmid or between plasmids) as well as to those that enable intercellular DNA mobility (Partridge et al., 2018). The MGEs that mediate the acquisition of external ARGs are usually plasmids, Insertion sequences (hereafter called IS elements), Transposons (Tn), integrons (especially class 1), and gene cassettes.

The IS elements and Tn are distinct DNA segments that can practically relocate themselves (along with related resistance genes) to new positions within the same or other DNA molecules or between a donor and a recipient bacterial cell. Other components, such as integrons (In), employ site-specific recombination to transfer resistance genes between designated sites. These MGE types are frequently found in numerous copies at various locations throughout a genome; thus, they can also promote homologous recombination, which is the exchange of sequences between segments that are identical or related.

1. **Resistance plasmids:** Plasmids, which range in size from less than a kilobase (kbases) to many megabases (Mbases), are crucial carriers of other MGE and acquired antimicrobial resistance genes linked to these elements in both Gram-positive and Gram-negative taxa (Shintani et al., 2015; Partridge et al., 2018). Plasmids can also spread horizontally through conjugation or mobilisation functions (Holmes et al., 2016). The genes that encode these tasks work together to produce a "backbone", a core of plasmid housekeeping operations to which "accessory" niche-adaptive activities can be added that may help the host cell (and thus the plasmid itself) in a particular environment. In addition to vertical transmission by cell division, plasmid replication is also generally aided by horizontal transmission to neighbouring bacterial cells. Conjugative plasmids have genetically intricate systems for horizontal plasmid transfer that greatly expand the size of their conserved backbone. Among members of the Enterobacterales, the known resistance plasmids include large (up to at least 200 kb), usually conjugative, and small, often mobilisable, plasmids. Three approaches are used for plasmid typing, of which the first two are laborious: The PCR-based Replicon typing (PBRT), MOB typing, and tools that characterise plasmids based on

sequence alignment, e.g., PlasmidFinder Tool (Caratolli et al., 2005; Alvarado et al., 2012; Partridge et al., 2018). Clinically important plasmid replicons are generally those incompatibility groups belonging to the following groups: *Inc*C, F, H1, H2, M, N, Q, X, and Y (Fernandez-Lopez et al., 2016). According to Gilmour et al. (2004), the HI plasmids are larger than the majority of the other conjugative plasmids covered here, encode serologically related pili that resemble the F pilus, and may also encode colicin, phage, and/or heavy metal resistance.

2. **IS Elements and Composite Transposons:** Aside from plasmids, IS elements are another very diverse group amongst the mobile genetic elements that transmit ARGs extrinsically. Usually carrying one or two transposase (tnp) genes, IS elements are classified as tiny mobile elements. They can be categorised in part by Tnp active site motifs, which are identified by important amino acids that assemble in the active site. These include DDE (Asp, Asp, and Glu), DEDD and HUH (two His residues separated by a large hydrophobic amino acid) (Hickman & Dyda, 2015). They can also be classified based on whether transposition is a replicative or a conservative mechanism, in which the IS element is simply removed from the donor and inserted into the recipient. Replicative transposition can occur by a copy-and-paste mechanism or a copy-out-paste-in mechanism (Chandler et al., 2015). The web-based, open-access ISFinder tool (https://www-is.biotoul.fr/) provides a comprehensive database of IS elements and includes BLAST search tools. According to Kamruzzaman et al. (2015), insertion upstream of an intrinsic chromosomal gene can also affect antibiotic resistance, and many IS elements have a potent promoter that stimulates expression of the captured gene. As an alternative, an IS might just offer a −35 region, which can form a hybrid promoter by joining with a nearby −10-like sequence. The most common types of IS elements involved in the transfer of ARGs can be obtained from Partridge et al. (2018).

3. **Unit Transposons:** Several types of transposons are associated with ARGs transmission. The Tn3 family transposons, whose members are identified by ~38-bp terminal IR, are frequently linked to ARGs. Tn3 family transposons also contain a tnpR resolver gene along with a resolution (res) site comprising two or three subsites and, in certain instances, passenger genes. TnpA catalyses the creation of a cointegrate structure, which comprises directly repeated copies of the transposon that separate the original donor and recipient molecules, allowing transposition to occur by a replicative mechanism (Harmer and Hall, 2016). Members of a different transposon superfamily, known as Tn7-

like transposons, are also linked to the transfer of antibiotic resistance determinants. Examples of these include Tn552 in Staphylococcus and Tn7 and Tn402-like elements in Gram-negative bacteria. Members of this group, which have various transposition processes, share certain characteristics, such as having numerous genes producing products (including a transposase regulator) involved in transposition instead of the single lengthy tnpA gene found in the Tn3 family. Members of this group may also target a specific site or sites, in contrast to transposons of the Tn3 family. The list of fully characterised Tn3 family and Tn7-like transposons, which have been reported to be possible elements for antibiotic resistance in the two prioritised gram-positive bacteria, Staphylococci and Enterococci, can be obtained from Partridge et al. (2018).

4. **Gene cassettes and Integrons:** Gene cassettes and integrons are also mobile genetic elements that could transmit ARGs between bacterial populations. Gene cassettes are tiny, mobile elements (0.5–1 kb) that contain one gene (often two), usually without a promoter or an *att*C recombination site. Gene cassettes, which are non-replicative and typically found inserted into an integron, can exist in a free circular form. They are distinguished by an *int*I gene, an *att*I recombination site, and a promoter (Pc). Based on the sequence of *int*I, several classes of integron have been identified (named *Int*I1, *Int*I2, *Int*I3, etc., with cognate *att*I1, *att*I2, and *att*I3 sites). Members of the Class 1 Integron are the most prevalent and are initially described in clinical isolates that are resistant to antibiotics. The Tn402 transposon seems to have resulted from the capture of the *int*I1/*att*I1/Pc combination, found on the chromosomes of betaproteobacteria in association with a *qac*E cassette, by a Tn5053 family transposon (Gillings et al., 2008). The term “class 1 In/Tn” has been suggested to encompass structures with *int*I1/*att*I1/Pc and either a full or truncated tni region. More recently, most whole genome sequence-based studies utilise the web-based, open access INTEGRALL tool (http://integrall.bio.ua.pt/) to identify the gene cassettes in bacteria (Moura et al., 2009).

2.3 One Health Hotspots of Antimicrobial Resistance

The usage of antimicrobials for therapeutic and non-therapeutic purposes increases the chances of the emergence of resistance. This is attributed to the chances of a few bacteria surviving the selective pressure posed by these compounds. These bacteria that survive eventually multiply and form a community of resistant bacteria. This could occur at any point where antibiotics are used, hence giving rise to hotspots

where MDR bacteria could emerge. Understanding the hotspots of AMR emergence could help public health decision makers to design health advisories that could help limit the spread of these pathogens. Amongst the epidemiological triad, the hotspots of AMR bacteria (genes) emergence are human (hospitals, community, pharmaceutical companies); animal (farms, livestock markets, food production facilities, slaughterhouses, companion animals, wildlife), and the environment (soils fertilised with manure or sludge, aquatic environments, rivers, lakes, oceans) (Table 1).

Empirical evidence exists that the hospital environment and healthcare workers (HCW) are key players in the large-scale dissemination of healthcare-associated infections (HAI)-related MDR bacteria (Otter et al., 2013; Facciolà et al., 2019; Shams et al., 2019; Rozman et al., 2020; Sereia et al., 2021). For example, a number of microbiome studies have been carried out utilising high-throughput sequencing (HTS) techniques in healthcare facilities (Laço et al., 2017; Christoff et al., 2020). According to Christoff et al. (2020), a number of collection locations exhibit diverse levels of hospital-associated resistant bacteria in hospital equipment, including NICU computers, infusion bombs, ICU phones, door handles, and access buttons. Around 50% of patient samples and 70% or more environmental samples in the intensive care unit contained *S. epidermidis, P. aeruginosa*, *K. pneumoniae*, *E. coli*, *E. faecium*, *E. faecalis*, and *S. aureus* were also found in the study (they were found in high proportions in both environmental and patient samples). Heljanko et al. (2023) also detected Carbapenemase-producing *Citrobacter freundii* in sink traps and toilets in the selected hospital in Helsinki, Finland, but not in the other sampling sites. Another study also indicated that the hospital plumbing system and the hospital environment are a source of outbreaks of carbapenemase-producing Enterobacterales in Heidelberg, Germany (Nurjadi et al., 2021). Further research should focus on microbial abundance and diversity among key healthcare setting actors (especially patients, healthcare workers, and the hospital environment) and their individual roles in the spread of resistant bacteria and resistance determinants. Hayword et al. (2025) reported that drinking water plumbing systems outside of the hospital environment are hot spots for antimicrobial-resistant pathogens: They detected MDR *P. aeruginosa*, *A. baumannii*, and *S. aureus* in household plumbing systems in Australia.

Table 1. Hotspots for the emergence and dispersal of AMR bacteria and genes.

Hotspot	Description	Key Drivers	Examples	References
Hospital wastewater and urban sewage	High antibiotic use leads to the selection and release of resistant bacteria and genes into the environment.	Human antibiotic use, inadequate wastewater treatment	*K. pneumoniae*, ESBL-*E. coli* in treated hospital effluent	*Lan et al., (2025); Mehanni et al., 2023*
Livestock farms and aquaculture	Widespread use of antibiotics for growth promotion and disease prevention in animals selects for AMR.	Veterinary antibiotic overuse, manure spreading	Tetracycline, colistin, and fluoroquinolone resistance in *Salmonella*, *E. coli*	Panicker et al. (2025); He et al. (2020)
Slaughterhouses and meat-processing plants	Interface between animal and human bacteria; possible transmission of AMR through meat.	Poor hygiene, contaminated equipment	MRSA, ESBL-*E. coli* in meat products	Ahmed et al. (2025)
Live animal markets and wet markets	High-risk mixing of animal species and humans facilitates HGT and zoonotic transmission.	Crowding, poor biosecurity, mixed species	Zoonotic diseases, *Campylobacter* and *Salmonella,* with AMR profiles	Hasan et al. (2025)
Wastewater from pharmaceutical industries	Discharge of antibiotic residues into water selects for multidrug-resistant bacteria in aquatic systems.	Improper waste management	Antibiotics in river water above therapeutic levels	Yan et al. (2025)
Companion animal clinics and pet waste	Antibiotic use in pets and close contact with humans can spread AMR.	Overprescribing antibiotics for pets	*E. coli* and *Staphylococcus* in pets	Guardabassi et al. (2004).
Soils fertilised with manure or sludge.	Environmental reservoir for resistant bacteria and resistance genes.	Land application of animal waste or sewage sludge	ARGs (e.g., tet, sul, *bla*) in agricultural soils	Lima et al., 2020.
Aquatic environments (rivers, lakes, oceans)	Sink for antibiotics, resistant bacteria, and resistance genes from multiple sources.	Runoff, effluent, aquaculture	Detection of NDM-1 in urban rivers and coastal waters	Tiwari et al., (2022).
Wildlife–livestock–human interfaces	Wildlife can carry resistant bacteria acquired from environmental	Habitat fragmentation, interaction with livestock	AMR in birds, rodents, and wild ungulates	Mitchell J. (2023).

	exposure or scavenging.

Moving to livestock, the second member of the epidemiological triad, studies have revealed several hotspots for the emergence of AMR/MDR pathogens in livestock. This is also of public health significance, especially because 73% of all antimicrobials produced are used in animals meant for food (Van Boeckel et al., 2019). A study by Zhao et al. (2024) reported several countries where their food animals were hotspots for the emergence of resistance. The study defined resistance hotspots as regions with resistance prevalence higher than the 95% percentile of all pixels on the map and recommended that the identified hotspot regions should immediately focus on reducing antimicrobial use and strengthening biosecurity in farms. According to recent studies by Xin et al. (2022) and Gao et al. (2022), livestock farms were also a major source of airborne AMR.

The AMR genes were consistently enriched in the air environments of animal farms, particularly in swine and cow farms. The potential for airborne ARGs to spread to their potential pathogenic host was reinforced. Some AMR genes have a significant load that can be breathed, similar to the load found in drinking water. Additionally, a study reported that the continuous evolution of novel livestock-associated *S. aureus* (mecC-MRSA ST130) in animal farms has been shown to facilitate its host jump (Aires-de-Sousa, M. 2017). In summary, animals and their production systems serve as significant hotspots (reservoirs) for the emergence and spread of resistance determinants within animal populations, with spill-over effects on human and environmental health. This is typified by the emergence of mobile colistin resistance in pigs (colistin was only chromosomally transmitted vertically prior to 2016), with subsequent spread to humans and the environment (Liu et al., 2012; Liu et al., 2016).

Aside from livestock, AMR can be transmitted amongst several points during the journey from farm to fork, with the entire food chain possibly serving as an interconnected reservoir. Resistant bacteria inevitably emerge in animal intestines and spread through faeces to soil, water, and crops when antibiotics are administered to livestock for growth promotion or disease prevention (Galarce et al., 2023). During slaughter and processing, these resistant pathogens contaminate meat surfaces, processing equipment, and workers' hands (Almansur et al., 2023; Galarce et al., 2023). Studies have documented that antibiotic-resistant bacteria can be easily transferred to humans through meat, fruit, or vegetable consumption (Kaur et al., 2024; Mediouni et al., 2025).

Food processing facilities, with their moist environments and biofilm formation on surfaces, provide ideal conditions for AMR bacteria to persist (Zeng et al., 2021). Biofilms form collections of sticky substances that glue bacteria to surfaces in food processing facilities, protecting them from chemical cleaning and allowing bacteria to persist. The possibility of cross-contamination is dependent on sanitary

standards and can occur at multiple points: during transport, in retail settings, and through improper handling in home kitchens. Consuming undercooked contaminated food or touching contaminated surfaces transfers resistant bacteria to humans, where they can colonise the gut or cause infections that fail to respond to standard antibiotics (Chavan & Vashishth, 2025). This demonstrates the fact that livestock, food processing chains, and the entire agricultural ecosystem could be sources of AMR bacteria, creating a persistent public health challenge.

The third member of the epidemiological triad, the environment, can also be a hotspot for the emergence and dispersal of resistance determinants. Firstly, anthropogenic activities, including animal husbandry, all affect environmental health. Secondly, naturally occurring in the environment, antibiotic-producing bacteria/fungi can be found in plants, soil, aquatic plant debris, and animals. The ideal ecological and selective conditions for the emergence of new resistant strains are created by the extensive mixing of these environmental bacteria with exogenous bacteria from anthropogenic sources like waste processing and farm drainage; as a result, soil, water, and other nutrient-enriched habitats can serve as hotspots for horizontal gene transfer (Wellington et al., 2013).

Various ways exist that humans can come into contact with antibiotics, their residues, resistance genes, or resistant bacteria: (1) contaminated sludge, manure, and slurry-exposed crops; (2) livestock that have accumulated veterinary medications and resistant plants through the food chain; (3) fish exposed to pharmaceuticals released to surface waters, either purposefully (aquaculture treatments) or accidentally; (4) extracted groundwater and surface water that contain pharmaceutical residues and are subsequently used as drinking water; and (5) coastal waters used for recreation or shellfish production. Studies by D'Costa et al. (2006), Allen et al. (2010), Lau et al. (2017), and Sun et al. (2024) reported that soil itself harbours a diverse range of microbes and reported novel antibiotic resistance determinants from agricultural soil exposed to antibiotics widely used in human medicine and animal farming. The majority of bacteria pass through the human gut innocuously after consumption; however, plenty of chances exist for horizontal gene transfer, which enables ARGs to join the gut microbiota.

Antimicrobial resistance is an ideal template for studying the interconnectedness between the three wheels of the epidemiological triad and how interdependent our health is. The One Health approach acknowledges the interdependence of ecosystems, animals, and human health. These three domains come together to form hotspots of antimicrobial resistance (AMR) that allow resistance to evolve, be amplified, and spread (Mettenleiter et al., 2023).

2.4 Antimicrobial Resistance Surveillance: Approaches

Robust, timely, representative, complete, and standardised (comparable) data are needed to control AMR. These data can be obtained from surveillance systems (active or passive). AMR surveillance data are needed at the global, regional (continent), national, and local levels to provide information that could help monitor AMR trends, understand the spatial spread and burden across geographical landscapes, identify risk factors, identify hotspots, evaluate success and effectiveness of established control strategies, measure economic impact, develop policy, support national action plans, and evaluate the One Health threats (WHO, 2015). Surveillance data are also crucial to establishing the bacterial priority and essential medicine lists, amongst other global guidelines.

Various approaches to AMR surveillance exist. The approach to be adopted depends on numerous factors, among them available funding, the targeted pathogen(s), purpose, scope, data sources, and the sector involved (human, animal, environmental). Most AMR surveillance systems (global and regional) are passive in nature, because it is cheaper and relatively easier to set up using existing clinical/surveillance AMR protocols that exist in-country (Table 2). Global consortia such as the AMR Action Fund and Fleming Fund also upgrade in-country laboratory networks and make use of passive surveillance in LMICs. On a global level, as the One Health concept is being institutionalised, WHO GLASS is encouraging its 120 participating countries to switch from one approach to a tiered approach, which encompasses a passive + active + integrated surveillance approach. This will give more detailed information on vital parameters such as source attribution of bacterial pathogens, epidemiological linkages, and other variables of interest. National AMR surveillance systems are evolving from lab-based, passive surveillance to active and One Health systems in certain parts of the world.

Table 2. Approaches to AMR surveillance in human, animal, and environmental sectors.

Surveillance Type	Focus	Example (s)	Strength (s)	Limitations
Passive	Routine data	GLASS, EARS-Net	Cost-effective, easy to integrate into existing systems.	Often incomplete, biased toward hospital-based or severe cases.
Active	Targeted collection	ACORN	Provides more accurate, complete, and targeted surveillance	Resource intensive
Sentinel	Selected sites, especially in resource-limited settings	ReLAVRA+, RISLNET	Tracks AMR trends at low cost, mostly in resource-limited settings	-
Population-based	Whole population trends, incidence	CDC's ABCs program	Provides accurate epidemiological indicators.	Expensive and complex to operate.
Integrated (One Health)	Cross-sectoral across human, animal, food, and ecosystems	WHO Tricycle, NARMS	Tracks transmission across sectors and assesses resistance drivers.	-
Laboratory-based	Priority pathogen identification and AST results from labs	GLASS, EARS-Net, national reference labs	Provides pathogen-specific resistance profiles.	Often lacks clinical context or patient-level data.
Clinical	Clinical outcomes + prescribing practices + microbiological screening	ACORN, CANWARD	Correlates resistance with treatment failure or complications.	-
Molecular/Genomic	Resistance genes, mobile elements, mechanisms of AMR spread	GenomeTrackr, GLASS WGS pilot sites	Detects emerging resistance mechanisms and tracks transmission pathways.	Requires technical capacity, relatively more expensive

AST – Antimicrobial susceptibility testing; GLASS – Global Antimicrobial Resistance and Use Surveillance System; EARS-Net – European Antimicrobial Resistance Network; ACORN - A Clinically-Oriented Antimicrobial Resistance Surveillance Network; Red Latinoamericana de Vigilancia de la Resistencia a los Antimicrobianos; RISLNET - Regional Integrated Surveillance and Laboratory Network; CDC- US Centers for Disease Control and Prevention; ABC – Active Bacterial Core Surveillance; WHO – World Health Organization, NARMS – National Antimicrobial Resistance Surveillance System; Canadian Ward Surveillance System; WGS – Whole Genome Sequencing. Some of the parameters from this table were extrapolated from Cheng et al. (2024).

Those approaches to AMR surveillance can utilise one of two main methodologies:

- Culture-dependent
- Culture-independent approach

The culture-dependent approach depends exclusively on the use of selective culturing techniques to isolate the target microbes (or group of microbes), followed by phenotypic and genotypic approaches to characterise the isolates to evaluate the susceptibility to antibiotics and the molecular features conferring phenotypic resistance. According to Bliem et al. (2018), the sensitivity and specificity of culture-based techniques are comparable to those of PCR-based techniques. The culture-based approach has certain drawbacks, although it is also appealing for a variety of reasons. The approach is generally cheaper to implement, requires less technical expertise, is generally standardised, identifies viable pathogens, and is used by most AMR surveillance programs in the world. It is easiest to set up in resource-limited settings. These factors make the culture-based approach more widely available (Pruden et al., 2021). Conversely, the approach is comparatively time-consuming, usually targeted to priority pathogens, and is restricted to culturable organisms only (Aarestrup & Woolhouse, 2020). This makes it poor for studying microbiomes and identifying unculturable bacteria. The culture-dependent approach is usually not tentative in nature and requires further confirmation, because several genera of bacteria could have a similar colonial morphology. This further confirmation could be by biochemical confirmation or the use of other protocols, such as the MALDI-ToF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) mass spectrometry.

This approach allows for specificity of treatment, is vital to patient empirical treatment, and can produce comparable data that is the basis for passive surveillance databases such as the AMR ATLAS database (https://atlas-surveillance.com/login). This approach also allows the utilisation of several downstream analytical tools, generally referred to as single-cell omics. For instance, following the isolation of a bacterial colony, other molecular methods can then be incorporated. This enables an in-depth genotypic profiling of the isolate, allowing for genomic epidemiological studies, source tracking, and transmission dynamics. Such genotyping could also enable the linkage of the bacterial clones with the resistance genes (Pruden et al., 2021).

However, while the initial step in the culture-dependent approach is easy and cheap, the other downstream analysis is not. For instance, the detection and description of all relevant clones is extremely laborious and requires plenty of resources and laboratory capacity. This complexity is further made difficult during a multi-country outbreak of an infection. A typical example is the 2022 monophasic *Salmonella Typhimurium* ST34 outbreak across 15 European countries caused by Kinder chocolate (EFSA, 2023a). Furthermore, culture-based methods require, at least for now, benchmarking against clinical surveillance data to ensure that the

detected organisms and their resistance features reflect those present in the studied population (Larsson et al., 2023). This PhD utilised the culture-dependent approach to understanding the genomic epidemiology of three priority AMR pathogens in Finland (see section 2.5 below).

The culture-independent approach identifies pathogens without the time-consuming process of bacterial culture and identification. Such methods usually involve one of these methods:

- Gene-based methods: This method directly amplifies the conserved gene(s) in clinical, environmental, food, feed, or other sample types. It utilises basic and universal techniques such as PCR (quantitative or real-time) for targeted or 16S rRNA amplification. This method has the added advantage of allowing the quantification of each targeted gene. As expected, the gene-based approach can be done for each targeted gene (simplex) or several genes together (multiplex). According to Larsson et al. (2023), they are also thought to be useful for evaluating the effectiveness of wastewater treatment. PCR-based techniques are limited to describing the resistance genes or gene variations in the predetermined target panel (Chau et al., 2022), although they can characterise a wide variety of AMR genes in the wastewater (Pärnänen et al., 2019). The main advantages are the tests' speedy nature, high specificity, and wide utilisation over a wide range of resistance genes and profiles. The main disadvantages of the gene-based approach are the possibility of false negatives due to PCR inhibition, especially in samples with low bacterial (pathogen) load or in complex microbial communities, such as wastewater samples and environmental samples, among others. It is also impossible to know the specific pathogen harbouring a particular resistance profile (Hendriksen et al., 2019; Larsson et al., 2023; Pärnänen et al., 2019). For instance, the same macrolide resistance determinant, msr(C) or multidrug efflux pumps, can be found in all members of the Enterobacterales.

- The metagenomic method is another culture-independent approach that can be used for AMR surveillance. It involves direct sequencing of samples (irrespective of source) to detect the diversity and abundance of resistance genes (resistome), viruses (virome), virulence-associated genes (virulome), metabolites (metabolomics), lipid profiles (lipidomics), and mobile genetic elements (mobilome), especially plasmids (plasmidome). The main advantages are the diversity and depth of data (information) that can be obtained from metagenomic surveillance for AMR. Most metagenomic sequencing is based on newer sequencing techniques and utilises their platforms. Hence, the most common techniques are the shotgun metagenomics (Illumina sequencing platform), long-read metagenomics (Oxford Nanopore sequencing, PacBio, etc.), or slightly modified to achieve

certain improvements in output (interpretation), such as pyrosequencing, proximity ligation, etc. The main advantages of the metagenomic approach are its ability to provide data across the entire bacterial (viral) taxa, including the unculturable organisms, which are missed with the culture-dependent approach.

This makes it more useful for research (exploratory) purposes at the moment and can be used for personalised medicine (for instance, understanding bacterial diversity and abundance prior to faecal microbiota transplantation in the treatment of Clostridium difficile). AMR surveillance using the metagenomic approach also might not require clinical surveillance data. The main disadvantages of this approach, however, include the high cost of metagenomic sequencing, the need for technical expertise to analyse data, the need for specialised equipment (such as supercomputers) for large-scale analysis, and the non-specificity for clinically relevant resistance determinants, pathogens, or mobile elements.

It is widely used in wastewater surveillance as a tool to evaluate the population carriage of pathogens, diversity of resistance genes, and other variables of interest. More recently, there has been a significant improvement in the methodological approaches to metagenomic sequencing analysis, with several dozen tools being used at several analytical stages, such as taxonomic profiling and annotations (Gardner et al., 2019; Han et al., 2025; Poussin et al., 2022). More recently, some new metagenome-based approaches, such as culture-enriched metagenomics, could also amplify the utility of metagenomics by sequencing all colonies obtained during bacterial culture. This enables an in-depth understanding of clinically relevant microbes and narrows the taxonomic scope (Heljanko, V. 2024). Other approaches, such as the HI-C (proximity) ligation, deep- and long-read sequencing, and single-cell metagenomics, also aim to tackle the challenges in current methodologies (Arikawa et al., 2021; Jain et al., 2018).

The AMR surveillance approaches could influence some of the surveillance methodologies that would be used. The surveillance approaches and methodologies could also be influenced by the surveillance's aim. The two methodologies can generally be used for AMR surveillance across the three wheels of the epidemiological triad (human, animal, and environment). Most global AMR surveillance datasets are currently based on the culture-dependent isolation and susceptibility testing of bacterial isolates.

2.4.1 Surveillance of antimicrobial resistance in humans

Prior to the 2015 declaration of antimicrobial resistance (AMR) as a public health crisis and further deliberations at the World Health Assembly, there were

very few coordinated AMR surveillance projects across the globe. Thereafter, the AMR global action plan highlighted the need for surveillance in evaluating its spatial and temporal spread, differential burden, and the need for interventions. The phenotypical antimicrobial susceptibility testing (AST) of invasive clinical isolates and non-invasive surveillance isolates is currently the primary basis for AMR surveillance in humans (WHO, 2015). Despite the establishment of national, regional, and international platforms for AMR surveillance in humans, a significant disparity in AMR surveillance remains among nations (Larsson et al., 2023). An illustration: Whereas surveillance systems in the US and Europe are well advanced, many nations worldwide do not routinely gather data on antimicrobial resistance. Due to differences in the quantity and quality of data, as well as the variety of drug-bug combinations that are included in the surveillance, it is frequently thought to be difficult to integrate and harmonise AMR surveillance (Aarestrup & Woolhouse, 2020; Larsson et al., 2023).

The Global Antimicrobial Resistance and Use Surveillance System (GLASS) was established on a global scale by the WHO in 2015. GLASS seeks to coordinate worldwide human AMR surveillance in addition to promoting and supporting national surveillance systems in nations with inadequate AMR surveillance. The phenotypical AST data of isolates derived from various collection types, including blood, urine, faeces, and urethral and cervical swabs, provide the basis of GLASS. *Acinetobacter spp., E. coli*, *K. pneumoniae*, Neisseria gonorrhoeae, *Salmonella spp., Shigella spp., S. aureus*, and *Streptococcus pneumoniae* are the eight bacterial pathogens that are the specific focus of GLASS (WHO, 2015).

GLASS is based on passive surveillance data from their contact at national and sub-national levels among the 120 participating countries (https://www.who.int/glass). GLASS is laying the groundwork for integrated data gathering, although the paucity of resources still makes it difficult to conduct globally coordinated and integrated AMR surveillance (Larsson et al., 2023). Aside from GLASS, several other global-scale AMR surveillance activities have been conducted as part of antimicrobial regulatory frameworks or as part of concerted efforts to better understand AMR's burden. The AMR Vivli dashboard (https://amr.vivli.org/resources/research-programs/) currently has 12 dashboards containing around two million anonymised AMR surveillance data from multinational projects that focus on several aspects of AMR, including antifungal resistance surveillance. Several regional and continent-wide surveillance initiatives (programs) monitor AMR at the regional level (Table 3). These include:

1. Africa: Africa CDC AMR Surveillance Network (AMRSNET), which launched the Regional Integrated Surveillance and Laboratory Network (RISLNET) initiative (http://africacdc.org/rislnet/). AMRSNET, in collaboration with the WHO AFRO regional office, focuses on strengthening lab capacity,

standardising AMR data collection, linking with GLASS, and human resource training.

2. Asia-Pacific: This region's ACORN (A Clinically-Oriented Antimicrobial Resistance Surveillance Network) project evaluates AMR by conducting sentinel site-based hospital surveillance with clinical and microbiological data in LMICs such as Cambodia, Laos, Indonesia, and Nepal (https://acornamr.net/#/).

3. Europe: The European Antimicrobial Resistance Surveillance Network (EARS-Net) is ECDC's continent-wide AMR surveillance system (https://www.ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/ears-net). EARS-Net is the most advanced regional AMR surveillance program: It comprises 30+ countries and evaluates antibiotic susceptibility data from invasive isolates (blood and cerebrospinal fluid) across the continent. EARS-net covers several priority bacterial pathogens such as *E. coli*, *K. pneumoniae*, *P. aeruginosa, Acinetobacter spp*., *S. pneumoniae*, *S. aureus*, *E. faecalis*, and *E. faecium* (ECDC, 2023).

4. North America: The US National Antimicrobial Resistance Monitoring System (NARMS) is a collaborative and integrated surveillance project that utilises the One Health concept in evaluating the AMR burden across the epidemiological triad. It is a collaborative effort of the US CDC, FDA, and USDA. Like EARS-Net, it focuses on priority enteric pathogens such as *Salmonella spp., Campylobacter spp., Shigella spp.* and *E. coli*. (https://www.cdc.gov/narms). North of the US, the Canadian Ward Surveillance System (CANWARD), an ongoing, national, Public Health Agency of Canada (PHAC)-partnered surveillance study, assesses antimicrobial resistance among bacterial pathogens in Canadian hospitals. It's a collaborative effort with the Canadian Antimicrobial Resistance Alliance (CARA). The project focuses on hospital-based AMR surveillance for respiratory, bloodstream, and wound pathogens (http://www.can-r.com).

5. Latin America and the Caribbean: The ReLAVRA+ (Red Latinoamericana de Vigilancia de la Resistencia a los Antimicrobianos) is an AMR surveillance system coordinated by the Pan American Health Organization (PAHO). It focuses on AMR surveillance across 20+ countries and focuses on select bacterial pathogens and harmonisation of data with GLASS (https://www.paho.org/en/topics/antimicrobial-resistance/latin-american-and-caribbean-network-antimicrobial-resistance).

6. Australia: The Australian Commission on Safety and Quality in Health Care Antimicrobial Use and Resistance in Australia (AURA) program manages the Australian Commission on Safety and Quality in Health Care. The program's scope is a comprehensive national surveillance system in Australia that focuses on AMR in human pathogens and on antimicrobial prescribing patterns in hospitals and the community (Link: https://www.safetyandquality.gov.au/aura).

Table 3. Antimicrobial resistance surveillance programs in humans.

Program	Region	Focus Areas	Scope
GLASS	Global	Human AMR, lab data, consumption	>120 countries
ATLAS	Global	Human AMR	>80 countries
EARS-Net	Europe	Invasive infections, hospital data	30+ EU/EEA countries
AMRSNET	Africa	Network labs, NAPs, One Health	Continental African
ReLAVRA+	Latin America	Human AMR, lab capacity building	20+ countries
AURA	Oceania	AMR trends, prescribing, stewardship	National (Australia)
ACORN	SE Asia	Clinical-lab integration, hospital-based	LMICs in Asia

GLASS – Global Antimicrobial Use and Resistance Surveillance (http://who.int/initiatives/glass); ATLAS – Antimicrobial testing, leadership, and Surveillance (https://atlas-surveillance.com); EARS-Net – European Antimicrobial Resistance Surveillance Network (https://www.ecdc.europa.eu/en/about-us/networks/disease-networks-and-laboratory-networks/ears-net-data); AMRSNET – Africa CDC AMR Surveillance Network (https://africacdc.org/download/africa-cdc-framework-for-antimicrobial-resistance/); ReLAVRA+ - Red Latinoamericana de Vigilancia de la Resistencia a los Antimicrobianos (https://www.paho.org/en/documents/relavra-leading-fight-against-antimicrobial-resistance-latin-america-and-caribbean); AURA - Antimicrobial Use and Resistance in Australia (https://www.safetyandquality.gov.au/our-work/antimicrobial-resistance/antimicrobial-use-and-resistance-australia-aura); ACORN - A Clinically-Oriented Antimicrobial Resistance Surveillance Network (https://acornamr.net/#/).

Several countries have a comprehensive antimicrobial use and resistance surveillance program at the country level. Some EU countries are already implementing whole-genome-based genomic profiling in addition to phenotypic assays. The Finnish Institute for Health and Welfare (THL) in Finland is responsible for maintaining FINRES, the country's human AMR surveillance system. The Finnish Study Group for Antimicrobial Susceptibility Testing gathers and reports phenotypical AST data, which THL uses to create the annual FINRES reports. Isolates of several bacterial species, mostly from infections but sometimes

noninvasive isolates, comprise the national surveillance data. The National Infectious Disease Registry also has information on several priority pathogens, such as MRSA, VRE, third-generation cephalosporin-non-susceptible *E. coli* and *K. pneumoniae*, and carbapenem-non-susceptible Enterobacterales isolated from all specimen types, in addition to FINRES (THL, 2024).

2.4.2Surveillance of antimicrobial resistance in food-producing animals and food products

Most AMR surveillance programs in animal health are not as developed (funded) as their human health counterpart. At the moment, the FAO-ATLASS (Assessment Tool for Laboratories and AMR Surveillance Systems) is a tool designed to assess and define targets to improve national AMR surveillance systems in the food and agriculture sectors. The tool is country-administered, and it is composed of two modules: the surveillance module and the laboratory module. The tool's structure is based on five main areas of an AMR surveillance system: governance, data production network (laboratories), data collection and analysis, communication, and sustainability. The tool, which is currently being deployed and used in 40+ countries, focuses on surveillance in animals, food, and the environment and assessment of lab capacity for animal sector surveillance (https://www.fao.org/antimicrobial-resistance/resources/tools/fao-atlass/en/).
Another FAO tool, InFARM (International FAO Antimicrobial Resistance Monitoring), has also been deployed to improve global, regional, and local collection, collation, and analysis of AMR and AMU data (https://www.fao.org/antimicrobial-resistance/resources/infarm-system/en/).
The tool is also crucial for properly disseminating information at the national and international levels. The InFARM tool also integrates the data with the World Organization for Animal Health's Animal Antimicrobial Use Global Database – ANIMUSE, currently available in over 160 countries) and GLASS-AMR in animals, which is under development by the WHO, which hopes to improve the integrated surveillance for AMR pathogens on a global scale (https://amu.woah.org/amu-system-portal/amu-data), maintained by the World Organization for Animal Health (WOAH).

Several regional/continental surveillance activities are performed in animals meant for human consumption, food products, animal feed, ingredients, and the environment. The EFSA–ECDC–EMA Integrated AMR Surveillance, which produces the EARS-Vet (European Antimicrobial Resistance Surveillance network in Veterinary Medicine), is a successful program in animals in Europe. The EARS-Vet, like its human counterpart, EARS-Net, focuses on priority pathogens such as multidrug-resistant *E. coli*, *Salmonella spp., and Campylobacter* spp., from

animals and food (Table 4). The Africa CDC AMR Surveillance Framework on the African continent also supports surveillance activities in animals, food products, and the environment, utilising the One Health principle. The Fleming Fund Regional Grants have also been crucial to AMR surveillance in LMICs because they have supported regional collaborations in AMR capacity building and AMR surveillance infrastructure in 19+ African countries (https://www.flemingfund.org/grants-funding/). Several Fleming Fund Country Grants are also being implemented in LMICs and aim to generate evidence-based surveillance data on priority pathogens, especially zoonotic pathogens like *Salmonella spp., Campylobacter spp.,* cephalosporin-resistant *E. coli*, as well as indicator organisms like non-resistant *E. coli* and *Enterococcus spp*. Depending on the country's implementation mechanism, some countries also monitor non-zoonotic animal pathogens like *Pasteurella spp*. and *Mycoplasma spp*. and monitor antibiotics of interest such as tetracyclines, beta-lactams, fluoroquinolones, colistin, and macrolides.

Table 4. Antimicrobial resistance surveillance programs in animals and food products.

Region	Key Programs	Scope
Global	FAO-ATLASS, FAO-InFARM; WOAH-ANIMUSE,	One Health, AMU/AMR data
Europe	EARS-Vet	Food chain surveillance
Asia	Thailand iAMRSS, China MARA reports	National One Health systems
Africa	Africa CDC, Fleming Fund, OHCEA	Capacity-building, integrated AMR
Latin America	ReLAVRA+ (PAHO)	Integrated AMR in animals/humans
USA	NARMS	AMR in humans, animals, and retail meat
Canada	CIPARS	AMR and AMU across sectors
Japan	JVARM	Animal AMR monitoring

FAO-ATLASS – FAO Assessment Tool for Laboratories and AMR Surveillance Systems (https://www.fao.org/antimicrobial-resistance/resources/tools/fao-atlass/en/); WOAH-ANIMUSE – WOAH Animal Antimicrobial Use (https://amu.woah.org/amu-system-portal/home); FAO-InFARM – International FAO Antimicrobial Resistance Monitoring (https://www.fao.org/antimicrobial-resistance/resources/infarm-system/en/); EARS-Vet – European Antimicrobial Resistance Surveillance Network in Veterinary Medicine (https://eu-jamrai.eu/surveillance/ears-vet/); NARMS- The National Antimicrobial Resistance Monitoring System (https://www.fda.gov/animal-veterinary/antimicrobial-resistance/national-antimicrobial-resistance-monitoring-system); CIPARS – Canadian Integrated Program for Antimicrobial

Resistance Surveillance (CIPARS) (https://www.canada.ca/en/public-health/services/surveillance/canadian-integrated-program-antimicrobial-resistance-surveillance-cipars.html); JVARM - Japan Veterinary Antimicrobial Resistance Monitoring System (https://www.maff.go.jp/nval/english/AMR/Monitoring/index.html).

AMR surveillance in food-producing animals and foods in the European Union is integrated and has a strong legislative foundation. The first EU legislation regarding AMR surveillance in multiple bacterial pathogens in food-producing animals and retail meat was laid in 2003 (Directive 2003/99/EC) and harmonised in 2013 (Commission Implementing Decision 2013/652/EU). This legislation included the AMR surveillance in *Salmonella* spp., *Campylobacter jejuni, Campylobacter coli,* indicator commensal *E. coli,* and ESBL-, AmpC, and/or Carbapenemase-producing *E. coli* and *Salmonella* spp. AMR surveillance of food-producing animals and foods is currently based on Commission Implementing Decision (EU) 2020/1729, which includes the same target organisms as the previous legislation but has some changes, for example, in the sampling and antimicrobials used in AST (EU Commission, 2020).

The surveillance is based mainly on the phenotypical AST of the isolates, and samples are mostly collected from healthy individuals in the abattoirs. The current surveillance includes broilers, laying hens, fattening turkeys, bovines under one year of age, fattening pigs, and pork, beef, broiler, and turkey meat. The results of the surveillance are reported in the annual European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals, and Food (EFSA/ECDC, 2024).

The AMR surveillance of animals started in the 1980s in Finland by studying the resistance in *Salmonella* spp. and expanded in the 1990s to the surveillance of *Campylobacter* spp. and indicator bacteria. Systematic AMR surveillance of animals and food has occurred since 2002 and has included AMR surveillance in zoonotic and indicator bacteria in animals, animal pathogenic bacteria, and *Salmonella* from foodstuffs. The surveillance is executed by the Finnish Food Authority and is called FINRES-Vet (Finnish Food Authority, 2021). The FINRES-Vet surveillance program was integrated with EU legislation in 2014 by including the targets defined by EU legislation (Commission Implementing Decision (EU) 2020/1729). Furthermore, the FINRES-Vet surveillance includes occasional MRSA surveillance in fattening pigs and pork, as well as in companion animals. The results are compiled in the annual FINRES-Vet report (Finnish Food Authority et al., 2022).

Country-level AMR surveillance in food-producing animals varies greatly on a global scale. For example, in Japan (Japan Veterinary Antimicrobial Resistance Monitoring), the United States (National Antimicrobial Resistance Monitoring System), the United Kingdom (**UK-VARSS** - Veterinary Antibiotic Resistance and Sales Surveillance), Canada (Canadian Integrated Program for AMR Surveillance), and most countries in the European Union. AMR surveillance in food-producing

animals and foods is systematic, well-developed, and has been established for years (EFSA, 2023b). Some countries are also progressing to whole-genome sequencing-based genomic surveillance.

Much work is needed across the three wheels of the epidemiological triad to attain standardised, comparable, robust data on key AMR pathogens. Despite this, significant resources have been dedicated to human surveillance of antimicrobial production, importation, consumption, usage, and the emergence and spread of AMR pathogens. Inadequate surveillance data can result from a lack of resources, poor documentation, insufficient regulations, or poor implementation of surveillance measures. AMR surveillance is well coordinated and produces reliable data in Europe, but some deficiencies still exist, especially in the coordination and data sharing among the different One Health sectors (WHO, 2021).

2.4.3 Surveillance of antimicrobial resistance in the environment

Amongst the three wheels of the epidemiological triad, the environmental AMR surveillance remains comparatively underdeveloped despite mounting evidence that aquatic environments, soil, and wastewater serve as significant reservoirs and transmission pathways for ARGs and resistant bacteria. The environment is a critical component in the development, transmission, and dissemination of resistant pathogens and plays a multifaceted role in AMR ecology. Waste streams and wastewater from human activities act as potential reservoirs and vehicles of transmission, creating AMR environmental hotspots (Punch et al., 2025). WWTPs have been identified as critical points in the wastewater-environment continuum, where the circulation of AMR bacteria between human populations and the environment occurs (Pandey et al., 2023).

The United Nations Environmental Programmme (UNEP), as part of the quadripartite joint secretariat, has been involved with coordinating multisectoral approaches to AMR management and response (https://www.unep.org/topics/pollution-and-health/antimicrobial-resistance-amr/management-and-response-amr). The UNEP launched the Wastewater Surveillance for Africa Initiative in 2024 to strengthen systems for wastewater and environmental surveillance across the African region (https://www.unep.org/technical-highlight/african-countries-protect-environmental-and-public-health-through-improved). This initiative aims to help reduce wastewater and nutrient pollution, reduce risks for ecosystems and human health, and improve socio-economic and environmental conditions through expanded wastewater surveillance programs. The JPI-AMR (now OH-AMR in the EU) environmental surveillance network in the EU brought together 23 partners from 15 countries to identify robust, measurable surveillance indicators and methodologies for environmental AMR. This initiative aims to provide a global, multisectoral framework that can deliver comprehensive data for use by public health entities, environmental protection agencies, and the scientific community. The network produced a comprehensive technical report outlining surveillance

objectives, target selection, and methodological approaches specifically adapted for complex environmental microbial communities in soil and aquatic ecosystems (JPI-AMR, 2022).

Several countries have initiated environmental AMR monitoring programs at the country level, though with varying degrees of coordination and public data accessibility. The United States has established a strong collaborative interagency working model that monitors surface water at a national scale through the Surface Water Ambient Monitoring (SWAM) program (Ligouri et al., 2022). Water represents the most frequently sampled environmental medium in surveillance studies, including wastewater, surface water, catch basins, and recreational waters, while research also encompasses aerosol studies, soil sampling, and wildlife as sentinel species. Other sample types include wildlife, migratory birds, soil samples, air samples, etc.

Several factors make environmental surveillance more challenging than its counterparts - humans or livestock. A major challenge for implementing routine environmental AMR monitoring is the decision of which resistance genes to select as targets in surveillance assays, necessitating comprehensive background data on abundances and prevalence of ARGs in both pristine and human-impacted environments (Knight et al., 2025). No clear consensus exists regarding which indicators to measure for the environmental sector, highlighting the urgent need to identify robust, measurable surveillance indicators that allow the generation of comparable data in a coordinated manner. Another major challenge is the slow rate of its integration with AMR National Action Plans (NAPs) across the globe. The integration of environmental surveillance into comprehensive One Health approaches remains essential but underachieved globally. Hence, significant investment in methodological standardisation, data platform development, funding mechanisms, and international coordination is required to establish routine, globally comparable environmental AMR surveillance datasets that can inform evidence-based interventions and policy development (Pandey et al., 2023).

2.5 Wastewater surveillance of pathogens and antimicrobial resistance

2.5.1 Concept of wastewater surveillance for population-level health monitoring

The concept and utility of wastewater (sewage) surveillance lie in its ability to screen certain pathogens, including antimicrobial-resistant bacteria, from sewage samples. Wastewater-based surveillance (WBS) has been used for decades to monitor the circulation of poliovirus in the population to supplement clinical poliovirus surveillance (Hovi et al., 2012). WBS could be a crucial tool for detecting the introduction of a novel pathogen into a naïve population, monitoring population-level carriage of a pathogen, helping determine pathogen hotspots,

monitoring disease trends, and evaluating intervention activities. For instance, in the global fight against polioviruses, wastewater and environmental surveillance (WES) activities are a crucial basis for the administration of the oral polio vaccine (OPV) to communities where the polioviruses were detected. Quantification of biomarkers such as viruses or ARGs allows the establishment of a baseline of these parameters for certain diseases, and a sudden unexplained increase in such baseline levels would indicate the beginning of an epidemic, suggesting the utility of WBS as an epidemic/pandemic preparedness tool.

WBS is conducted for antimicrobial resistance (AMR) mainly for two reasons: The first is the population-level carriage of AMR pathogens circulating in the community by screening untreated sewage; the second is the evaluation and surveillance of the environmental burden and efficiency of wastewater treatment by studying the AMR pathogens or AMR genes present in the treated wastewater or sludge (Clarke et al., 2024; Larsson et al., 2023; Pärnänen et al., 2019). Wastewater surveillance has also been used for other purposes, including surveillance of health markers, such as obesity, and surveillance of illegal substance use (Lin et al., 2024; Yi et al., 2023). Nevertheless, the number of research initiatives and the interest in wastewater surveillance exploded during the COVID-19 pandemic when the circulating virus variants and ongoing and upcoming epidemic spikes were monitored with wastewater surveillance (Figure 1).

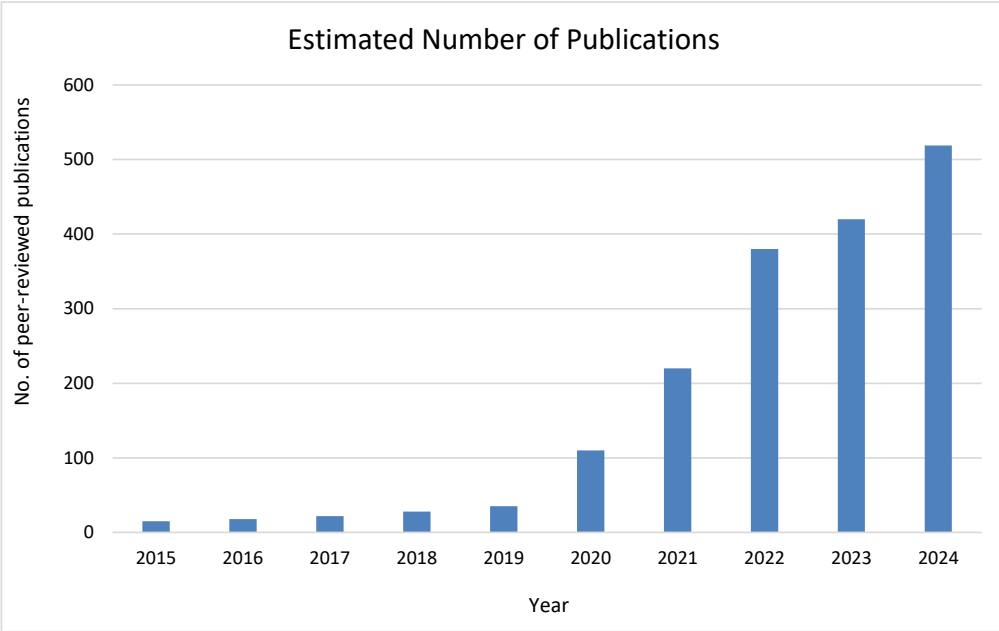


Figure 1. Wastewater-themed research publications, 2015-2024. Data were obtained from four databases: Google Scholar, Web of Science, Scopus, and PubMed. The

following search terms were used: Wastewater based surveillance pathogens, WWS, WBS, WES, WBE, WBS wastewater epidemiology, Pathogen detection wastewater, Wastewater monitoring infectious disease, Sewage surveillance, WBS viral pathogens, WBS bacterial pathogens, WBS emerging pathogens, WBS antimicrobial resistance, WBS SARS-CoV-2, WBS influenza, WBS polio, WBS pathogen detection methods, Wastewater surveillance PCR pathogens, Wastewater surveillance public health, Wastewater surveillance case studies amongst others.

Most of the peer-reviewed academic publications in high-impact journals on wastewater-based epidemiology (WBE) post 2020 were associated with the screening of SARS-CoV-2 and its variants. Researchers realised soon after that other pathogens of public health interest could also be screened using WBS. Figure 2 shows that AMR was the second most-studied pathogen during WBS post COVID-19. Now, other priority pathogens such as community vaccine-derived polioviruses (CVDPV2), Influenza, RSV, Noroviruses, amongst others. The 2022 Mpox pandemic also showed the utility of WBS for evaluating population-level carriage of the Mpox DNA virus.

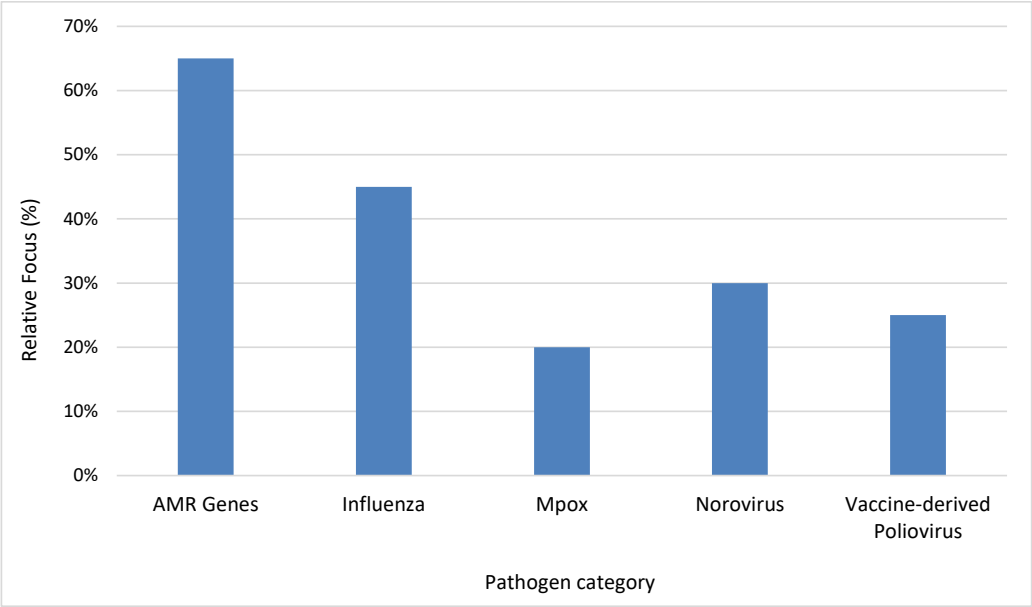


Figure 2. Proportion of peer-reviewed publications on non-COVID pathogens screened via wastewater surveillance.

This PhD thesis is based on the premise that we could determine the population-level carriage of three important bacterial pathogens: methicillin-resistant *S. aureus* (MRSA), ESBL-producing *Escherichia coli* (ESBL), and Vancomycin-resistant *Enterococcus faecium* (VREfm). WBS also has the advantage

that it encompasses the burden of these pathogens in the community because AMR pathogens may be carried asymptomatically, not all symptomatic patients receive healthcare, not all cases are correctly diagnosed, and reporting of the diagnosed cases can vary (Chau et al., 2022). WBS, while it provides some complementary data that could be useful for public health decision makers, cannot and is not designed to replace clinical surveillance.

Furthermore, wastewater surveillance can not only uncover important AMR drivers and hotspots, but it can also detect new emergent dangers, such as outbreaks (Larsson et al., 2023; Pruden et al., 2021). Several research workers reported that the AMR profiles found in wastewater are similar to those seen in clinics, and a strong association exists between estimates of AMR prevalence in humans and wastewater AMR (Chau et al., 2022; Pärnänen et al., 2019). Additionally, research indicates a correlation exists between resistance rates in clinical *E. coli* isolates and related wastewater isolates, and wastewater surveillance can effectively track the diffusion of Carbapenemase-producing Enterobacterales (CPE) in the population (Blaak et al., 2021; Hutinel et al., 2019). However, there are still issues with wastewater surveillance. The wastewater's bacterial composition is quite complex, and AMR happens in a variety of organisms, many of which cannot be linked to humans.

WBS could have some limitations despite its utility as a preparedness tool. The environment, animals, and the sewage network itself, in addition to the human population, can all produce bacteria that end up in wastewater. Furthermore, although wastewater surveillance shows promise in reflecting AMR in human populations, the association may be stronger for certain organisms and AMR mechanisms than others. Additionally, the survival and persistence of clinically relevant AMR bacteria in sewage systems are poorly understood (Chau et al., 2022). Therefore, additional research is required to assess wastewater surveillance's performance capacity against various AMR pathogens (Hendriksen et al., 2019; Tiwari et al., 2022).

WBS data from environmental, animal, and human AMR monitoring should also be integrated in the future (Pruden et al., 2021). Wastewater surveillance is now mostly unregulated, so a variety of data types from different research viewpoints are generated. According to Tiwari et al. (2022), the data are likewise inconsistent and dispersed. The European Union's Urban Wastewater Treatment Directive (Council Directive 91/271/EEC) has mandated member states to incorporate wastewater surveillance of AMR at WWTPs that serve more than 100,000 people. EU member states are currently free to design monitoring procedures, and monitoring can be conducted on either treated or untreated wastewater. This will yield data from the standpoint of environmental transmission risk, wastewater treatment efficiency, or population monitoring. The Centers for Disease Control and Prevention (CDC) in the United States is also expanding their ability to investigate AMR in untreated wastewater (CDC, 2021; Larsson et al., 2023).

At the time of writing this thesis, there are none or only very few global or regional platforms/databases dedicated solely to wastewater AMR monitoring. Below are large-scale research projects that monitored AMR/pathogens using wastewater.

1. The Global Wastewater Initiative (GWWI) is coordinated by the UN Environmental Program (UNEP), a platform designed for knowledge and capacity building on wastewater monitoring, including pathogens and AMR,

under a One Health framework. GWWI, while broader than just AMR, is a key global driver for standardising wastewater-based surveillance (WBS). It works to connect countries, share protocols, and advocate for the use of WBS for public health, explicitly including AMR. It is a central hub for policy and guidance rather than a raw data repository. UNEP publishes reports, guidance documents, and case studies through the initiative. It facilitates the network for data sharing rather than hosting a database itself.

2. The Global Wastewater Consortium (GLOWACON) was launched by the European Commission (in collaboration with global partners) to help improve global wastewater and environmental surveillance for public health. This partnership, consisting of both public and private partners, has the potential to increase pandemic preparedness by greatly increasing the surveillance of new health threats around the world. For example, GLOWACON seeks to establish early warning systems for cross-border health concerns by integrating data from community-based surveillance with wastewater surveillance at key sites like transportation hubs, such as airports and aeroplanes. Additionally, GLOWACON will be a tool for locating and matching financing opportunities. The European Commission also established the EU wastewater observatory for public health.
3. The COMBAT-AMR Project is a multinational public health project designed to combat AMR through wastewater surveillance in the Philippines and Southeast Asia. This is another prime example of a regional operational platform aside from the previously-mentioned EU projects. It involves routine sampling of wastewater from healthcare facilities and communities across the Philippines and other Southeast Asian countries. The goal is to generate actionable data on AMR hotspots and trends to guide interventions. The project generates context-specific data that it shares with public health authorities. It may not be fully public, but it represents a dedicated operational model.
4. National Wastewater Surveillance Dashboards & Visualization Tools: Several public health-focused platforms present wastewater-based surveillance data and present it in an accessible, visual format for public health use. These include:
    a. COVIDPoops19 Dashboard, an open-access dashboard by researchers at the University of California, summarised the SARS-CoV-2 wastewater monitoring efforts across the globe. The dashboard demonstrated a model that could be used as an analogue for AMR-based WBS activities. The dashboard doesn't host raw data but aggregates metadata on thousands of sampling sites globally (location, frequency, and responsible agency). It demonstrates the feasibility of tracking and visualising global WBS efforts. The infrastructure and partnerships built for COVID-19 wastewater surveillance are now being adapted for AMR and other pathogens.
    b. The Swiss WBE Platform was developed by the Swiss Federal Institute of Aquatic Science and Technology (Eawag) and the Federal Office of Public Health (FOPH). It is a national platform for Wastewater-Based Epidemiology (WBE). Like most other national surveillance activities, it gained prominence during COVID-19; it is explicitly designed as a multi-pathogen platform

and includes AMR as a core component. The platform showed time-series graphs, showing trends of specific antimicrobial resistance genes (ARGs) over time at different WWTPs across Switzerland. The platform also incorporates geographical maps, allowing for comparison of ARG levels between different regions. Pathogen Tracking can correlate ARG data with pathogen data (e.g., *K. pneumoniae*) from the same samples. The Swiss WBE is one of the most advanced operational, national-level public health platforms that integrates AMR data alongside other pathogens. It serves as a gold standard model for other countries.

c. The Wastewater-based surveillance as pandemic preparedness tool (WastPan) project in Finland also developed a WastPan Portal where open-access data on priority pathogens in Finnish wastewater are deposited. The dashboard will focus on data obtained from > 9000 results obtained from screening 32 microbes. This thesis was conducted as a part of the WastPan project, and the findings were incorporated into the development of the WastPan portal.
d. Other platforms are in development, such as the JPIAMR AMR insights platform. This initiative of the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) was active between 2011 and was officially concluded on May 31, 2025. The project was succeeded by the European Partnership on One Health Antimicrobial Resistance (EUP OHAMR) in June 2025. It aims to be a global, integrated knowledge platform for AMR data, encompassing data from human, animal, and environmental (including wastewater) sectors into a unified One Health dashboard. The dashboard aims to provide visualisation for the integrated One Health Dashboard, which allows for side-by-side comparisons of clinical AMR prevalence, animal husbandry data, and wastewater ARG abundance across the same regions. The dashboard also aims to allow overlaying of global maps, such as enabling visualisation of layers for wastewater surveillance sites, clinical reporting rates, and agricultural antibiotic use within the same geographical region. Another platform in development is the Antibiotic Wastewater ARG Environmental Dashboard (AWARE dashboard). The unique profile of this dashboard is its proposed inclusion of ARG removal efficiency calculators.

2.5.2Wastewater-Based Surveillance for *Escherichia coli*

Escherichia coli is a common commensal bacterium in the gastrointestinal tract of humans and warm-blooded animals and is a predominant facultative anaerobe in the microbiota of the human colon. The pathogen is the most studied bacterium in most clinical and non-clinical studies. A decade ago, the WHO selected the extended-spectrum Beta Lactamase producing *E. coli* as the model organism for the One Health Integrated AMR surveillance in collaboration with the WAOH and the FAO (Appling et al., 2023; WHO, 2021b). There has been a significant increase since then in ESBL-*E. coli* surveillance as a screening tool for faecal contamination

in municipal water systems, to monitor the progress in AMR intervention activities, and to evaluate spatial-temporal trends in the AMR burden globally, regionally, and locally. The prevalence of ESBL–producing *E. coli* has been increasing in Finland (Ilmavirta et al., 2023).

WBS for ESBL-*E. coli* and other bacterial pathogens were generally considered important because of the reasons itemised in Table 5 below. There has also been a significant increase in wastewater monitoring of ESBL-*E. coli* across the globe (Table 6). These studies have identified ESBL-producing *E. coli* in wastewater samples using different methodologies (culture, qPCR, and metagenomics), reported several STs, including the hypervirulent ST131, evaluated the diversity of resistance genes, and, in a few studies, the impact of disinfection on the prevalence of ARGs and resistant bacteria in effluents.

Table 5. Utility and Features of Wastewater-Based Surveillance for Antimicrobial Resistance Surveillance.

Aspect	Key Findings	References
1. Overall Concordance with Clinical Data	High concordance between wastewater and human AMR prevalence estimates. Wastewater resistance rates were lower than clinical rates but followed similar geographic trends.	Chau et al., 2022; Hutinel et al., 2019; Huijbers et al., 2020
2. Optimal Surveillance Methods	Composite sampling of influent, longitudinal sampling >12 months, and time-/location-matched sampling of wastewater and human compartments improve agreement.	Chau et al., 2022; Oliveira et al., 2023.
3. Prevalence of bacterial pathogens	High concentrations in WWTP influent; reduced but detectable in effluent.	Davidova-Gerzova et al., 2023
4. Phenotypic Resistance Patterns	Resistance to aminopenicillins, fluoroquinolones, aminoglycosides, cephalosporins, sulfonamides, and tetracyclines. MDR phenotypes are common.	Hutinel et al., 2019; Oliveira et al., 2023; and Abdelgalel et al., 2025.
5. Determine Sequence Types (STs) and Clones	ST131 (pandemic clone), ST2797, ST648, ST10, ST38, ST405. ST2797 was found to be abundant in wastewater with MDR patterns.	Martak et al., 2020; Paulshus et al., 2023.
6. Evaluate the Impact of WWTP Treatment	Treatments reduce but do not eliminate ARB/ARGs. Effluent remains a source of resistance. Disinfection methods (chlorination, UV, PAA/UV) vary in efficacy.	Oliveira et al., 2023; Abdelgalel et al., 2025,
7. Determine Phylogroups and Pathogenicity	Phylogroups A and B1 (commensal), B2 and D (pathogenic) are prevalent. Human-associated strains are dominant in wastewater.	Martak et al., 2023; Abdelgalel et al., 2025
8. Evaluate Regional and Temporal Variations	Significant differences in AMR gene diversity between cities/countries. Resistance rates in municipal sewage are more stable than in hospital sewage.	Huijbers et al., 2020
9. Make Methodological Improvements	Use of WGS for comprehensive AMR profiling. Culture-based methods combined with genotypic detection. Standardized sampling and reporting are needed.	Chau et al., 2022

Table 6. Peer-reviewed studies on sewage (Wastewater)-Based Surveillance for *Escherichia coli*.

Country/Region	Matrix	Method(s)	Prevalence metric	Most common isolate features (phylogroup/ST)	Dominant AMR mechanism(s)	Reference
Brazil	WWTP (various tech)	Culture	NR	NR	NR	Machado, et al., 2023
Canada	WWTP	Culture + MLST	ST131 (3762 samples)	ST131 clade A common	NR	Finn, et al., 2020
China	WWTP	Culture + WGS	NR	Multiple STs	CTX-M variants	Jiang, et al., 2019
Czech Republic	WWTP influent/effluent	Culture + MLST	ESBL-*E. coli* in 34/45 samples	B2-O25b-ST131	CTX-M-15	Dolejska, et al., 2011
Czech Republic	Hospital & community wastewater	Culture + WGS	High ESBL proportion	NR	CTX-M-15 dominant	Davidova-Gerzova, et al., 2023
Finland	Hospital & municipal wastewater	Culture + WGS	NR (detection study)	Clinically relevant STs in CP-*E. coli*	Carbapenemases (KPC/NDM/OXA-48-like)	Heljanko et al., 2024
France	Wastewater network (11 sites)	Culture + MLST	ESBL-Ec quantified network-wide	Multiple, incl. clinical-like clones	CTX-M (esp. CTX-M-15)	Bréchet et al., 2014
France	Spring impacted by WWTP	Culture	ESBL-Ec concentrations tracked	NR	NR	Henriot, et al., 2023
France	WWTP outflows to wetlands	Culture + phylogeny	Human-associated phylogroups B2/D are prevalent	Phylogroup B2/D common	NR	Martak et al., 2020
Global (12 WWTPs)	Influent & effluent	Metagenomics	NR (behaviour classes for pathogens)	NR	ARG profiles including beta-lactamases	Garner, et al., 2024

Country/Region	Matrix	Method(s)	Prevalence metric	Most common isolate features (phylogroup/ST)	Dominant AMR mechanism(s)	Reference
Global (reviewed sites)	Tricycle protocol implementations include wastewater	Mixed	Varies (protocol-based)	NR	ESBL-*E. coli* focus (CTX-M)	Milenkov, et al., 2024
India	Hospital wastewater vs local sewer	Culture + qPCR	Up to 9-log higher CRE & bla_{NDM-1}	NR	NDM-1 predominant	Lamba et al., 2017
India	Hospital wastewater	Metagenomics	NR	NR	High ARG loads incl. ESBLs	Talat et al., 2023
Indonesia	Wastewater (Tricycle pilot)	Culture	ESBL-*E. coli* detected in wastewater	NR	CTX-M predominant	Puspandari, et al., 2021
Iran	Municipal WWTP effluent	Culture + PCR	NR (AMR burden monitored)	NR	Class 1/2 integrons; ESBL genes	Shamsizadeh, et al., 2024
Iran	Hospital & municipal wastewater	Culture + PCR	CRE 33% in hospital wastewater	NR	NDM/OXA-48-, like in the *E. coli* subset	Khavandi et al., 2024
Ireland	Hospital effluent & municipal wastewater	Culture	CPE detected in hospital effluent	NR	KPC/NDM/OXA detected	Cahill et al., 2019
Ireland	Hospital wastewater system	Metagenomics	NR	NR	ARG burden incl. beta-lactamases	Kelly et al., 2023
Japan	Hospital wastewater & WWTP	Culture + WGS	NR (presence confirmed)	Clinically important lineages.	CTX-M variants	Gomi, et al., 2017
Japan	Hospital effluent	Culture + PCR	NR	NR	CRE and ESBL presence	Azuma, et al., 2022
Japan	Wastewater over time	Culture + PCR	Temporal ESBL-*E. coli*	NR	ESBL; carbapenemases	Tanabe, et al., 2023
Morocco	Influent & effluent	Culture + PCR	ESBL-*E. coli* detected pre-/post-treatment	NR	CTX-M variants	El Garraoui et al., 2025

Country/Region	Matrix	Method(s)	Prevalence metric	Most common isolate features (phylogroup/ST)	Dominant AMR mechanism(s)	Reference
Nepal	Communal & hospital wastewater	Culture	ESBL-*E. coli* in wastewater (baseline)	NR	ESBL (CTX-M)	Acharya, et al., 2024
Norway	Pump station (sewage)	Culture + WGS	Repeated ESBL-*E. coli* isolation	ST648, ST131 recurrent	CTX-M-15 predominates	Paulshus, et al., 2019
Norway	Population sewage	Culture + beta-lactamase typing	NR	NR	Diverse β-lactamases in *E. coli*	Grevskott, et al., 2021
South Africa	WWTP & receiving river	Culture + QMRA	*E. coli* levels; risk quantified	NR	NR	Mbanga et al., 2020
Spain	WWTP influent/effluent	Culture and quantification using MPN	*E. coli* measured; reductions quantified	NR	NR	Cuevas-Ferrando, et al., 2022
Sweden	Hospital sewage	Culture + WGS	NR (method evaluation)	NR	Rare carbapenemases detectable	Flach, et al., 2021
Switzerland	WWTP influent/effluent; surface water	Culture + WGS	ESBL-*E. coli* detected; ST131 frequency variable	ST131 prominent sub-lineages	CTX-M (esp. CTX-M-15/27)	Biggel, et al., 2023
Uganda	Sewage + faecal sludge	Culture + WGS	NR (focus on genomics)	Diverse sequence types	Multiple ESBLs	Gomi, et al., 2024
United Kingdom	Municipal sewage	Culture + WGS	NR	Multiple STs incl. clinical lineages	Various ESBLs	Raven, et al., 2019
USA	Municipal wastewater	Culture	Presumptive ESBL among coliforms 12–19%	NR	NR	Appling, et al., 2023
USA	WWTP influent and effluent	qPCR vs culture	*E. coli* reductions quantified	NR	NR	Lavender, et al., 2009

Country/Region	Matrix	Method(s)	Prevalence metric	Most common isolate features (phylogroup/ST)	Dominant AMR mechanism(s)	Reference
Nepal	WWTP	qPCR	STEC genes stx1/stx2 prevalence tracked	NR	NR	Sthapit, et al., 2022

NR – Not reported. MPN- Most probable number. Although generative AI was used in this table, all the content (and references) were verified.

2.5.3 Wastewater-Based Surveillance for Methicillin-resistant *S. aureus*

Staphylococcus aureus (*S. aureus*) is also a commensal member and normal flora of the respiratory tract, as well as other organs such as the skin. Unlike *E. coli*, it is a Gram-positive coccus, and *S. aureus* is the most common cause of skin and soft tissue infections (SSTIs), which can range from minor conditions like impetigo and folliculitis to severe invasive diseases such as cellulitis and abscesses. The bacterium uses various virulence factors to cause infection, leading to an immune response that includes neutrophil recruitment and abscess formation to contain the pathogen.
Methicillin-resistant *S. aureus* (MRSA) strains confer resistance against beta-lactam antibiotics. MRSA has caused over 100,000 deaths as of 2019 and is amongst the priority AMR pathogens tagged by WHO as ESKAPE. It is the second highest-ranked Gram-positive bacterium on the WHO priority pathogen list (WHO, 2024). There are three epidemiological classes of *S. aureus*: Hospital-associated (HA-MRSA), Community-associated (CA-MRSA), and Livestock-associated (LA-MRSA) strains (Junnila et al., 2020). Most MRSA strains are zoonotic, although some strains of LA-MRSA are more specific to their animal hosts. Only a few studies have generally detected MRSA in wastewater due to its lower prevalence in wastewater. MRSA generally poses a significant public health challenge due to its increasing incidence and the rise of antibiotic resistance. Table 7 shows some studies that have evaluated MRSA's prevalence from wastewater sources. These studies have identified MRSA in municipal wastewater in several countries and reported mostly *mec*A genes in cultured MRSA isolates from influents and effluents. A study used a four-step quantitative microbial risk assessment to determine the risk of infection and disease burden from MRSA in river water and to evaluate the required water treatment for achieving health benchmarks (Azuma et al., 2022).

Table 7. Peer-reviewed studies on sewage (wastewater)-Based Surveillance for Methicillin-Resistant *S. aureus*.

Country	Matrix	Period	Methods	MR Mechanism	Other findings	References
Sweden	Influent & effluent	2007–2009	Culture + PCR	mecA	Seasonal peaks observed	Börjesson, 2009
USA	Influent & treated sewage	2014–2016	Culture + PCR	mecA	Early U.S. MRSA sewage report	Boopathy, 2017
South Africa	Influent & effluent	2021–2022	Culture + PCR + AST	mecA	MDR strains persisted	Oladipo, 2023
Japan	WWTP & river	2019–2020	Culture + qPCR + QMRA	mecA	First QMRA for MRSA in water	Azuma, 2022
Portugal	Hospital WW	2019–2020	Culture + WGS	mecA, SCC mec IV	Pandemic EMRSA-15 detected	Silva, 2022
Spain	Influent, sludge, effluent	2021–2023	Culture + qPCR	mecA	ARB surveillance, including MRSA	Pino-Hurtado, 2024

2.5.4 Wastewater-Based Surveillance for Vancomycin-Resistant *Enterococcus faecium*

Vancomycin-resistant *Enterococcus faecium* (VREfm) is a major global nosocomial pathogen; *Enterococcus faecium* predominates in healthcare settings, and *Enterococcus faecalis* is more prevalent in the community and animals. Prevalence varies significantly by region and context. VRE infection rates in hospitals range widely (1–55%), with higher colonisation rates in high-risk groups like livestock workers (11%) compared to the general public (2%) (Caddey et al., 2025; Eichel et al., 2023). Risk factors include prolonged antibiotic use (especially vancomycin), immunosuppression, invasive medical devices, and hospitalisation (O'Driscoll & Crank, 2015). The *vanA* genotype is most common globally (80–90% of cases), though regional shifts occur, such as the emergence of *vanB*-ST117 in Germany and *vanA*-ST1421 in Australia (Weber et al., 2020; Wagner et al., 2023).

The prevalence of VREfm in Europe increased from 8.1% (95% CI: 6.7–9.7) in 2012 to 19.0% (95% CI: 16.8–21.5) in 2018 (Ayobami et al., 2020). The prevalence of VRE in Finland is low. The incidence ranged from 0.4 to 5 cases per 100,000 inhabitants per year between 2016 and 2023 (Lindholm et al., 2022). The majority (97%) of isolations were *Enterococcus faecium*, and both *vanA* and *vanB* gene-positive isolates have been detected. Seven VREfm outbreak clusters have been identified by whole-genome sequencing since 2016 (THL, 2022; THL, 2023; THL, 2024). VREfm exhibits remarkable genomic plasticity, acquiring resistance through mobile genetic elements like plasmids and transposons (e.g., Tn1546 for *vanA*). Key resistance mechanisms include Glycopeptide resistance, which involves

the substitution of peptidoglycan precursors (e.g., d-Ala-d-Lac for *vanA*), reducing vancomycin affinity, and multidrug resistance, which is conferred by high-level resistance to β-lactams, aminoglycosides, and other antibiotics.
Predominant sequence types (STs) include ST17 (historically dominant), ST117, ST1421, and ST796, with regional dynamics influenced by plasmid types and antibiotic pressure (Iqbal et al., 2024). VRE infections - particularly bloodstream infections - are associated with high mortality (risk ratio 1.46 for *vanA E. faecium* vs. susceptible strains) (Eichel et al., 2023), prolonged hospitalisation, and increased healthcare costs. Treatment options for VREfm are limited to agents like linezolid, daptomycin, and tigecycline. VRE represents another critical One Health challenge. Along with MRSA, both pathogens account for over 100,000 deaths annually (Murray et al., 2022). Both pathogens similarly have unique links between livestock, food chains, and human infections, underscoring the need for integrated surveillance and antibiotic stewardship programs**.** Table 8 shows some of the very few studies that have characterised VREfm from wastewater. Most WBS of VREfm revealed the higher prevalence of *van*A genes, and only a few studies identified the *van*B genes in cultured isolates from wastewater. Most studies identified *E. faecium*, while a few reported both *E. faecium* and *E. faecalis,* as we also found in municipal wastewater in Finland.

Table 8. Peer-reviewed studies on Sewage (Wastewater)-Based Surveillance for Vancomycin-Resistant *Enterococcus faecium (*VREfm*)*.

Country	Matrix	No. of samples	Method(s)	Prevalence metric (as reported)	Most common isolate features (species/ST)	Dominant resistance mechanism(s)	References
Brazil	Influent	2018–2020	Culture + WGS	VREfm isolates recovered and genomically characterised	*E. faecium* (*vanA*)	*vanA* was predominant in the study set	de Farias et al., 2022
Czech Republic	Influent/effluent surface water	2019	Culture + chemical analysis	VRE detected in 11% of wastewater samples	*E. faecalis* and *E. faecium* reported	*vanA*/*vanB* screening;	Hricová et al., 2021
Germany	Influent (24h composites)	Sep 2022–Aug 2023	Culture + AST	High enterococci count; VRE present	*E. faecium* is dominant among VRE	*vanA*/van genes detected	Geissler et al., 2024
Italy	Influent & effluent	2022	Culture + WGS	VREfm isolated frequently; clonal lineage reported	*E. faecium*, clonal lineage (recently identified)	*vanA* dominant	Valenza et al., 2024
Iran	Hospital effluent	2020–2021	Culture + AST + PCR	High prevalence of drug-resistant *E. faecalis*/*E. faecium* in HWW	*E. faecalis* is common in HWW isolates	*vanA* was detected in the subset	Jannati et al., 2023
South Africa	Influent & effluent & river	2019–2020	Culture + PCR	VRE detected: 202 VRE isolates (202 total VRE)	*E. faecium*, *E. faecalis*; STs not always reported	*vanA* prevalent	Adegoke et al., 2022
Spain	Hospital effluent & WWTP influent	2010–2012	Culture + molecular typing	Hospital effluent supplies VRE to WWTPs; VRE detected in influent	*E. faecium* & *E. faecalis* reported	*vanA* reported	Varela et al., 2013

UK	Influent	2014-2015	Culture +WGS	Both *vanA* and *vanB* were detected.	*E. faecium* (*vanA*)	*vanA*	Gouliouris et al., 2019
USA	Surface water & sediment	2015	Culture + PCR	*vanA E. faecium* culturable up to days after spill	*E. faecium* (*vanA*)	*vanA*	Young et al., 2016
USA	Influent (longitudinal)	2023–2024	qPCR + culture + WGS	Longitudinal VRE signals tracked; both *vanA* and *vanB* detected	*E. faecium* dominant	*vanA* & *vanB* detected	Au et al., 2025

AST – Antibiotic sensitivity testing; PCR _Polymerase Chain reaction; qPCR -quantitative polymerase chain reaction; WGS- Whole genome sequencing; WWTP – Wastewater treatment plant; MALDI-ToF – Matrix Associated Laser desorption Ionization – Time of Flight. HWW- Healthcare wastewater.

3 Aims of the study

This research work aimed to study the genomic epidemiology of antimicrobial-resistant pathogens in municipal wastewater systems in Finland. I also evaluated the phylogenetic relatedness of wastewater surveillance isolates to human isolates from the Finnish Institute for Health and Welfare (THL) and other databases to evaluate the utility of Wastewater-based surveillance (WBS) to detect clinically relevant strains of these pathogens.

The specific aims of individual studies were to:

1. Study the genomic epidemiology of Methicillin-resistant *Staphylococcus aureus* (MRSA) in wastewater (I)
2. Determine the prevalence as well as spatial and temporal spread of AmpC/ESBL-producing *Escherichia coli* (II).
3. *Conduct a comparative core-genome MLST of wastewater and clinical* vancomycin-resistant *Enterococcus faecium* (III).
4. Determine if clinically relevant clones of MRSA, AmpC/ESBL-producing *E. coli, and* Vancomycin-resistant *E. faecium,* known to be circulating in the Finnish population, can be detected through wastewater surveillance (I, II, III).
5. Evaluate the utility of WBS as an early warning tool to identify these clinically relevant antimicrobial resistance (AMR) pathogens.

4 Materials and methods

4.1 Sample collection

The wastewater samples used in these studies were collected from ten wastewater treatment plants as part of the WASTPAN project in Finland (https://www.thl.fi/episeuranta/jatevesi/wastpan/en/) (Figure 3). The samples were collected at eight intervals between 2021 and 2022. Samples were specifically collected in February, April, May, July, August, October, November, and January of 2022. Each 1 L sample is a 24-hour composite sampling of flowing wastewater (influent) through each of the wastewater treatment plants (WWTPs) (THL, 2022). These samples were immediately chilled with dry ice and then transported to the Department of Food Hygiene and Environmental Health at the University of Helsinki.

Figure 3. Illustration of wastewater treatment plants included in the studies (n=10).

4.2 Isolation of targeted AMR pathogens

4.2.1 Primary enrichment (I, III)

A primary enrichment protocol was used for the *S. aureus* and *Enterococcus faecium*. Wastewater (WW) samples were pre-enriched for study I (MRSA) by inoculating 1 mL into 9 mL of Mueller-Hinton Broth (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 6.5% Sodium chloride (NaCl). This was compared with non-enriched direct plating of WW samples.
The samples were pre-enriched for study III by inoculating 1 mL into 9 mL of Mueller-Hinton Broth (Oxoid, Basingstoke, Hampshire, United Kingdom) supplemented with 6.5mg of vancomycin. All enrichments were incubated at 37°C for 18–24 hours and were then used for selective isolation of the targeted pathogens.

4.2.2 Selective isolation

We used CHROMagar (CHROMAgar, Paris, France). It is selective and differential because it combines antibiotics (methicillin or vancomycin) and chromogens into the agar to inhibit susceptible organisms and distinguish species, respectively. The differentiation of different bacterial species on these agar plates is also easier due to different colours. This is crucial in complex samples such as WW. The selective isolation of the pathogens from pre-enriched and non-enriched WW samples was performed using the following selective media:

- CHROMAgar MRSA – Study I
- CHROMAgar ESBL – Study II
- CHROMAgar VRE – Study III

A loopful (10 microliters) of the suspension was spread, after the just-described pre-enrichment, on respective chromogenic agar plates and incubated for 24 h at 37°C. However, a volume of 333 µl of freshly thawed and undiluted WW samples was used for the selective isolation of AmpC/ESBL *E. coli* using the CHROMagar ESBL (for Study II). The plates were also incubated at 37°C for 18-24 hours.
The preliminary species identification on CHROMagar is based on colonial morphology and colour, which is usually a result of the metabolic properties of the species. So:

- CHROMAgar MRSA – Mauve to purplish pink, medium to large, round, smooth colonies
- CHROMAgar ESBL – pink/red colonies, medium-sized, smooth edges
- CHROMAgar VRE - Colonies are typically 1–2 mm in diameter, purple to mauve (*Enterococcus faecium*) or blue-green (*Enterococcus faecalis*).

Typical colonies for the targeted bacteria on the plates were subsequently selected and sub-cultured on the respective selective agar media, incubated at 37°C for 18-24 hours, and stored for phenotypic antibiotic susceptibility testing and genomic extraction (Figure 4). The differences in the VREfm isolation rate between the ten WWTPs were tested using logistic regression with Bonferroni correction and adjusted for population size using SPSS *v*.29®.

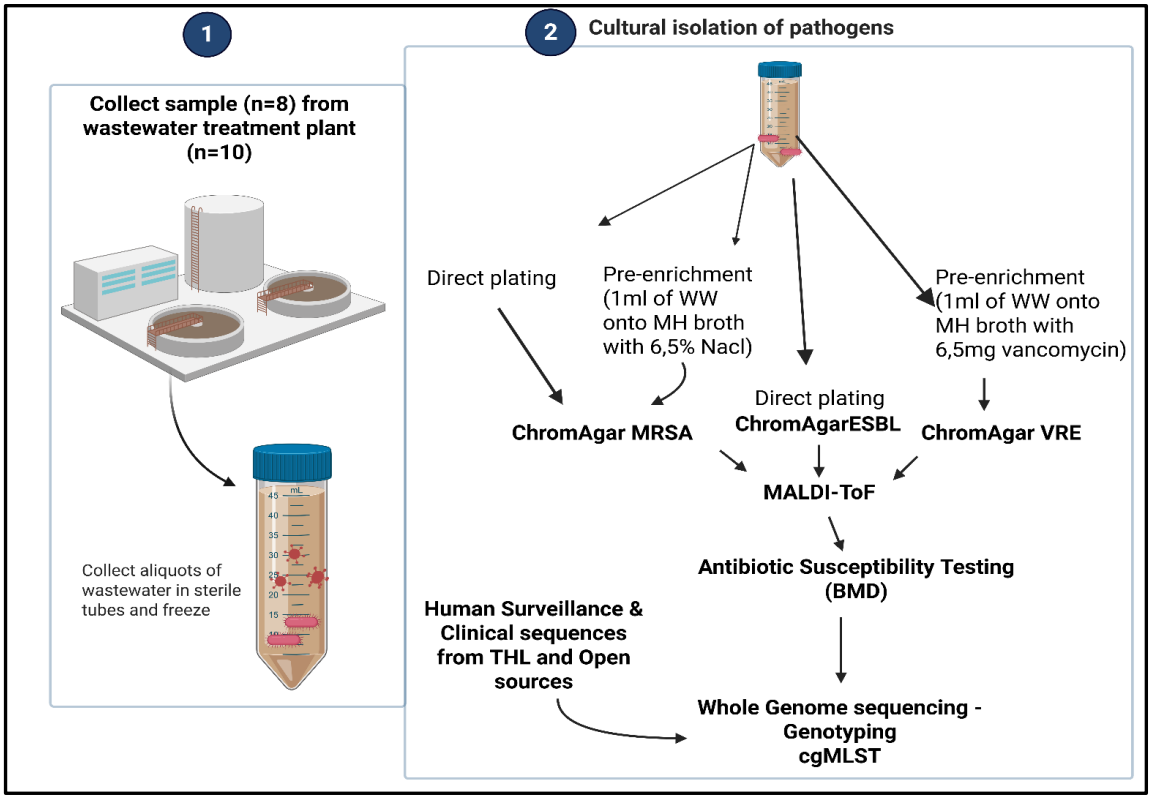


Figure 4. Illustration of workflow describing the enrichment, selective culturing, species determination, and antimicrobial susceptibility testing utilised in studies I–III. MH - Mueller Hinton Broth. MALDI-TOF MS - Matrix-assisted laser desorption ionisation-time of flight mass spectrometry. The figure was created in BioRender.com.

4.2.3 Species determination (I–III)

Purified isolates were sub-cultured on sheep blood agar plates (Columbia Blood Agar Base, Oxoid Ltd., Basingstoke, United Kingdom) and incubated at 37°C for 18 to 24 hours. Bacterial species were determined with a matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) -based Bruker Microflex LT/SH (Bruker Daltonics GmbH & Co. KG, Bremen, Germany) instrument. A best match score value of > 2.30 on MALDI-ToF was considered a high confidence threshold for reliable species-level identification for all isolates. Only isolates that were identified as MRSA, *E. coli*, and *Enterococcus faecium* were stored at -70°C for downstream analysis.

4.3 Antimicrobial susceptibility testing

Two assays were used to test the Phenotypic resistance profiles of all isolates:

- Broth microdilution assay – Study I, III
- Disk diffusion assay – Study II

Study I utilised the EUSTAPF Sensititre plates (Thermo Scientific, Vantaa, Finland) to test MRSA isolates against different dilutions of ten classes of antibiotics. The EUSTAPF Sensititre plates contained the following antibiotics: Cefoxitin, Ceftarolin, Clindamycin, Daptomycin, Erythromycin, Fusidic acid, Gentamicin, Levofloxacin, Linezolid, Moxifloxacin, Mupirocin, Norfloxacin, Rifampin, Teicoplanin, Telavancin, Tetracycline, Tobramycin, Trimethoprim/Sulfamethoxazole, and Vancomycin. The MRSA strain AF1214-14 was used as the positive control, whereas the *S. aureus* strain ATCC 12600 was used as a negative control.

Study II on AmpC/ESBL *E. coli* utilised the Kirby-Bauer disk diffusion assay to determine the zones of inhibition against five antibiotics: ceftazidime (30µg), ceftazidime-clavulanate combination (30µg + 10µg), cefoxitin (30µg), cefotaxime (30µg), and cefotaxime-clavulanate combination (30µg + 10µg). These five disks were used for the double-disk synergy test (DDST), typically used to test for the production of Ampicillin or extended-spectrum beta-lactamases in all confirmed *E. coli* isolates (n = 75). In principle, an isolate was regarded as an AmpC producer if the isolate showed resistance towards third-generation cephalosporins [ceftazidime - 30µg], the difference in the zone of inhibition between the ceftazidime (30µg) and ceftazidime + clavulanate (30µg + 10µg) was less than 5mm, and the isolate must also be resistant to cefoxitin. However, ESBL producers were designated when they showed resistance towards cefotaxime-30µg, and the difference in the zone of inhibition between the cefotaxime (30µg) and cefotaxime + clavulanate (30µg + 10µg) was more than 5mm. We screened for resistance against fluoroquinolones (using ciprofloxacin 10µg) and a carbapenem (meropenem, 10µg) in addition to the DDST. All antibiotic disks (Neo-Sensitabs) were sourced from Rosco Diagnostics, Albertslund, Denmark. All measurements and interpretations were based on the epidemiological cut-off (ECOFF) values established by the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2024). The reference strain, *E. coli* ATCC 25922, was used as the negative control strain during all screening tests.

The third study also used the broth microdilution assay by utilising screening isolates against different pre-selected concentrations of important antibiotics as contained in the EUVENC Sensititre plates (Thermo Scientific, Vantaa, Finland). The antibiotics contained in the EUVENC plate were chloramphenicol, vancomycin, teicoplanin, gentamycin, ampicillin, erythromycin, ciprofloxacin, daptomycin, tetracycline, linezolid, and synercid (quinupristin/dalfopristin). The ATCC 8459 strain of *Enterococcus faecium* was used as a control strain for the phenotypic assays.

For studies I and III, the Sensititre plates were prepared using the Sensititre AIM™ Automated Inoculation Delivery System, which dispenses bacterial lysate into Sensititre plates. After inoculation, the Sensititre™ Vizion™ Digital MIC Viewing System was used to read all plates. Based on the 2024 epidemiological cut-off values (ECOFF) openly available at https://mic.eucast.org/search, all isolates (from the three studies) were classified as resistant or susceptible to each of the

antibiotics (EUCAST, 2024). Isolates were characterised phenotypically as pan-susceptible when they did not express phenotypic resistance to any of the tested antibiotics. An isolate was designated to be multidrug resistant (MDR) if it confers resistance to at least three classes of antibiotics and extensively drug-resistant (XDR) if it confers resistance to at least five different classes of antibiotics (Magiorakos et al., 2012).

4.4 DNA Extraction

We extracted the total genomic material for each of the studies using the commercially available DNeasy blood and tissue Kit (Qiagen, Hilden, Germany) in a QIACUBE Connect (Qiagen, Hilden, Germany) following the manufacturer's instructions. Bacterial colonies were grown in Laurea Bartoni broth (Oxoid, Basingstoke, UK) at 37°C for 16–18 hours on a shaking platform. An extra step in the extraction of Gram-positive bacteria (studies I and III) involved extra treatment of the bacterial lysate with lysozyme (10 µL/mL) to pre-lyse the exopolysaccharides on the bacterial cell wall, which allows for better DNA yield. A 50µL-purified DNA was obtained for all DNA extractions, immediately placed on dry ice, and stored for downstream usage. The purified DNA was quantified with a Qubit Fluorometer 4.0 (Invitrogen, Singapore) and a DeNovix Nanodrop machine (DeNovix Inc., Delaware, USA).

4.4.1 *spa* typing

The MRSA isolates obtained in study I were spa typed prior to whole genome sequencing. The PCR amplification was done using the primers:

spa-1113f: 5′-TAA AGA CGA TCC TTC GGT GAG C-3′
spa-1514r: 5′-CAG CAG TAG TGC CGT TTG CTT-3′

Each reaction mix was a 25 µL reaction that contained DNA templates (1–2µl; 0.5 µl of both forward and reverse primers (10 µM µl); 0.5 µl of dNTP mix (10 mM); 2.5µl of 10× PCR buffer; 1.5 µl of $MgCl_2$ (25 mM); 0.5µl of Taq DNA polymerase, and nuclease-free water were used to top the reaction to 25µl. The PCR cycling conditions were an initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 seconds, followed by annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. A final extension was done for 6 minutes prior to holding the PCR amplicon at 4°C before electrophoresis. Each PCR amplicon was separated on a 1.5% agarose gel, stained with ethidium bromide, and viewed with Alpha Image viewer.

The PCR amplicons were then sent for Sanger sequencing at the Finnish Institute of Molecular Medicine (FIMM). The obtained sequences were analysed using the open-access CGE tool (spaTyper version 1.0) (Bartels et al., 2014).

4.5 Whole-genome sequencing

4.5.1 Sequencing

All the MALDI-ToF confirmed isolates (*S. aureus* – 22; *E.coli* – 75; *E. faecium* – 17) were whole-genome sequenced for each study. Sequencing was performed with Illumina NovaSeq 6000 (outsourced to Novogene, Cambridge, United Kingdom) with 2 × 150 bp read length and targeted genomic coverage of 100×. Library preparation was performed with a NEBNext Ultra DNA Library Prep Kit for Illumina with 300 bp fragment length.

4.5.2 Bioinformatic analyses

4.5.2.1 Assembly

Raw reads were assembled using SKESA v.2.3.0 (Souvorov et al., 2018) and compared with Velvet v. 1.1.04 (Zerbino and Birney, 2008) in Ridom SeqSphere+ software v10.0.0 (Jünemann et al., 2013). All assembled genomes were screened and quality controlled with FastQC v0.1.1.7 (Babraham Institute, 2021) and adapter removal with Trimmomatic v0.36 (Bolger et al., 2014). Quality trimming was performed with an average quality of ≥30 and a window of 20 bases. Remapping and polishing were performed with the BWA-MEM mapping algorithm. Data analysis of the sequences was performed using graphical interface-based commercial bioinformatic pipeline tools.

4.5.2.2 Species confirmation and bacterial typing

Species confirmation was done with Mash Distance v. 2.1 (Ondov et al., 2016) tool in Ridom SeqSphere+ software (Ridom, Munster, Germany). The multilocus sequence typing (MLST) was determined with Ridom SeqSphere+ software v10.0.0 pipeline.

The *S. aureus* MLST scheme was used for assigning sequence types (STs) to MRSA isolates for study I. The MRSA isolates were also spa typed in Ridom Seqsphere+ (StaphType tool), and the spa types were compared with the Sanger sequencing results. The Staphylococcus Cassette Chromosome (SCC) mec elements were screened using the SCC elements from the CGE's SCCmecFinder version 1.2 tool (Kondo et al., 2007).

The Warwick Achtman MLST scheme was used for typing of all *E. coli* isolates (Study II). Novel *E. coli* STs were applied from PubMLST (Jolley et al., 2018). *E. coli* serotypes were determined with CGE SerotypeFinder (Joensen et al., 2015), and the sequenced *E. coli* isolates were typed into phylogroups using the Clermont Phylotyper tool v.1.4.0 (Clermont et al., 2019). We screened afterwards for the transmissible locus of stress tolerance (tLST) variants by blasting the tLST variants (tLST1 and tLST2) against the 73 ESBL-*E. coli* genomes using Blastn (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi). The tLST ORFs screened were obtained from Zhang and Yang (2022). The presence of class 1 integrons was

screened using Integrall v. 1.2 (Moura et al., 2009). We screened for IS26, an insertion sequence element that has been reported to be prevalent in environmental samples, using the online Blast tool of ISFinder v.2.0.

The VR*E. faecium* isolates were typed for study III using the PubMLST *E. faecium* scheme (Jolley et al., 2018).

4.5.2.3 Antibiotic resistance genes, virulence factors, and plasmid replicons

We detected antibiotic and stress resistance genes for the three studies, using the NCBI AMRFinderPlus v. 3.2.3 tool (Feldgarden et al., 2019) within the Ridom SeqSphere+ software pipeline. The resistance genes were compared with the output of ResFinder 4.5.0 (CGE web server, DTU, Denmark) (Bortolaia et al., 2020). The stress resistance genes included those that are associated with resistance to heavy metals, disinfectants, and acids in certain isolates.

The virulence factors for the three isolates (MRSA, ESBL-*E. coli*, and VR*E. faecium*) were determined using the Virulence Factor Database (VFDB) (Chen et al., 2016) within the Ridom SeqSphere+ software pipeline. The Plasmid replicons were determined from assembled genomes with PlasmidFinder 2.1 (Camacho et al., 2009; Carattoli et al., 2014) with an identity threshold of 95% and a minimum length of 60% (Study III).

4.5.2.4 Phylogenetic analysis

The phylogenetic relatedness of the isolates was based on the CgMLST. All three studies assessed genomic comparison within wastewater isolates and with human surveillance isolates (studies II and III). I generated a minimum spanning tree for all studies showing the allelic differences between the core genomes of the isolates.

The Core genome multilocus sequence typing (cgMLST) comparison was constructed for Study I based on the *S. aureus* core genome consisting of 1861 targets. I used a cluster alert threshold of 10 to establish relatedness amongst the isolates.

I conducted the cgMLST for study II against 2513 targets for gene-by-gene allele calling to investigate the genetic relatedness of the assembled genomes. A cluster alert distance of 10 allele differences and a cluster alert quality threshold of at least 95% good cgMLST targets were used to detect closely related isolates. We further compared the wastewater ST131 isolates (n = 18) with clinical ST131 isolates (n = 21) reported by Kurittu et al. (2022) to identify genomic clusters of ST131 infections. ST131 is an internationally hypervirulent and successful clade that has been reported to be associated with human and animal clinical cases. A cut-off of 10 allelic differences was used to establish relatedness (clusters) among isolates.

The raw reads from the wastewater VR*E. faecium* isolates for Study III were analysed with RIDOM SeqSphere+ v.10.0.5 to perform a cgMLST analysis in which the WW isolates (n = 17) were compared with human surveillance VR*E. faecium*

isolates from national VRE surveillance (2016–2023, n = 754). Raw reads from the WW and human isolates were de novo assembled using the Velvet algorithm v. 1.1.04, and the public cgMLST scheme for *E. faecium* was used with default parameters.

4.6 Data analysis

For the three studies, qualitative data were presented as proportions and frequencies. The differences in the isolation rates of each pathogen between the ten WWTPs were tested using logistic regression with Bonferroni correction and adjusted for population size using SPSS v.29®. For study II, the chi-square analysis was used to test for associations between the relative abundance of plasmids and ARGs in *E. coli* isolates. The Sankey diagram was computed in Power BI® (Microsoft Corporation, USA). The ARG diversity map was generated using the 'geocoding' function in Ridom SeqSphere+. Finally, I used Cochrane's Q test to evaluate the association between the occurrence of the MDR pathogens during the eight sampling periods.

5 Results

5.1 Identification of targeted AMR pathogens (I-III)

Isolation rate of the bacterial pathogens

Study I showed significant differences exist in the isolation rates for methicillin-resistant *Staphylococcus aureus* (MRSA) between the direct plating of wastewater and pre-enriched samples. The directly plated samples revealed an isolation rate of 11.3% (n=9/80), whereas the pre-enriched samples had a higher isolation rate of 27.5% (n=22/80). Study II revealed a high isolation rate (97.5%, n=75/77) of *E. coli* on CHROMAgar ESBL plates, with presumptive isolates being obtained in all samples except two (samples 28 and 53). The isolation rate for VR *E. faecium was 22% (n=17/77)* for study III (Figure 5, Table 9).

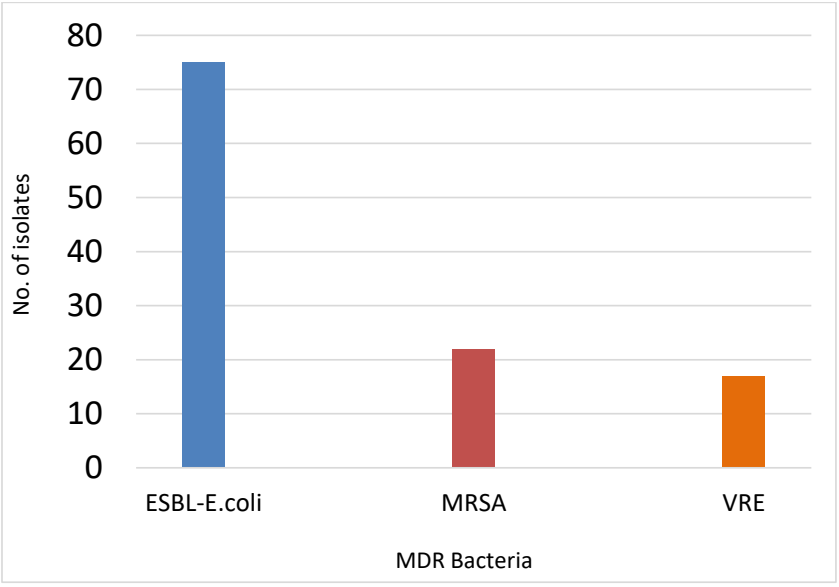


Figure 5. Isolation rates of targeted MDR pathogens in municipal wastewater. One colony was selected per sample. MRSA isolates (n=22/80), AmpC/ESBL-producing *E. coli* (n=75/77), and VR*E. faecium* (n=17/77).

Table 9. Occurrence of presumptive Methicillin Resistant *S. aureus (MRSA)*, AmpC/ESBL-producing *Escherichia coli*, and Vancomycin-resistant *Enterococcus faecium* in municipal wastewater across the ten wastewater treatment plants (2021-2022).

WWTP	No. of wastewater samples	No of positive samples		
		Study I MRSA (%)	Study II ESBL-*E. coli* (%)	Study III VR *E. faecium* (%)
Espoo	8	2 (25)	8 (100)	0 (0)
Helsinki	8	2 (25)	8 (100)	2 (25)
Kuopio	7	0 (0)	5 (71.4)	1(14.3)
Lappeenranta	8	3 (37.5)	8 (100)	0 (0)
Oulu	7	2 (28.6)	7 (100)	3 (43)
Pietarsaari	8	3 (37.5)	8(100)	1 (12.5)
Rovaniemi	7	3 (43)	7(100)	0 (0)
Seinäjoki	8	1 (12.5)	6 (75)	1 (12.5)
Tampere	8	3 (37.5)	8 (100)	2 (25)
Turku	8	3 (37.5)	8 (100)	6 (75)
Total	77	22 (27.5)	75 (95.7)	17 (22)

* - Study I (MRSA) was based on 80 samples (8 samples from each of the ten cities).

Statistically significant differences exist in the occurrences of the three pathogens among the wastewater samples collected. No significant differences exist between the cities in the occurrence of AmpC/ESBL *E. coli* ($p = 0.921$) and MRSA ($p = 0.754$), respectively. However, a significant difference in the VR *E. faecium* isolation rate ($p = 0.005$) with more isolates was obtained in Turku (n=6/8) than in other cities. No differences were observed in the temporal spread of the three MDR pathogens (MRSA, $p = 0.176$; AmpC/ESBL- *E. coli*, $p = 0.582$; and VRE, $p = 0.710$) (Figure 6).

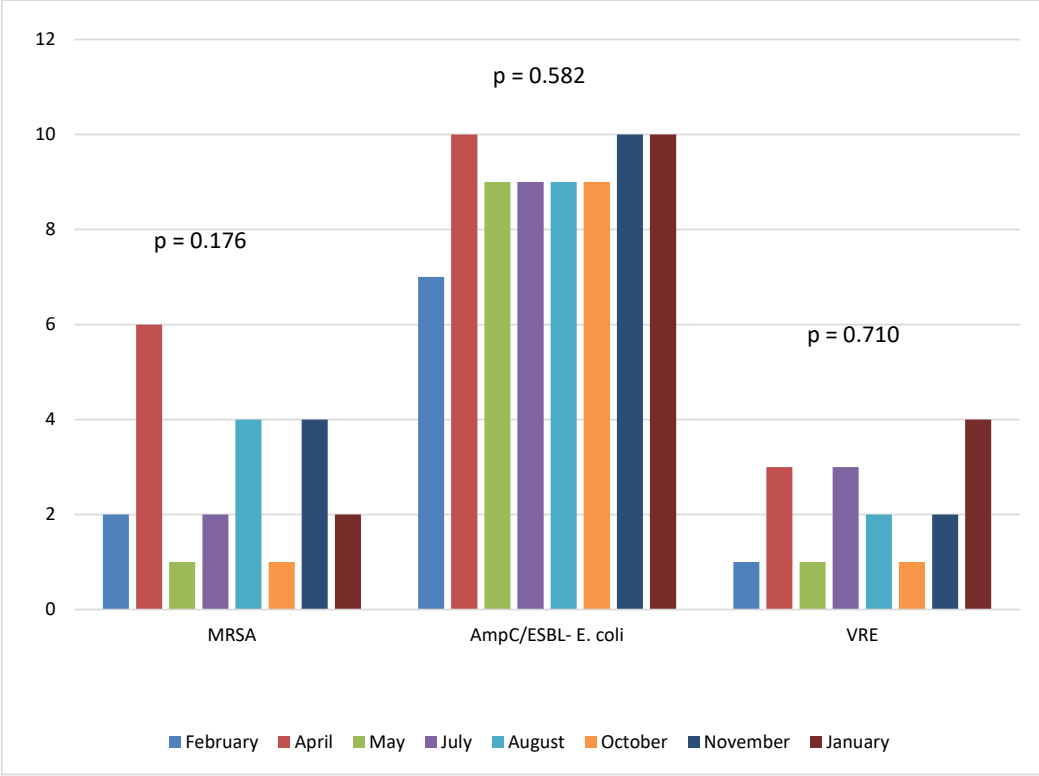


Figure 6. Temporal distribution of MDR pathogens during the eight sampling periods. The p-value was calculated using Cochrane's Q test.

5.2 Study I – Molecular characterisation of methicillin-resistant *S. aureus*

The highest MRSA occurrence in wastewater was during was in April 2021. The phenotypic antimicrobial susceptibility assay revealed that the WW MRSA isolates were multidrug resistant: Altogether 20 isolates (91%) were resistant to clindamycin, tetracycline, and daptomycin. There was a similar high resistance to fusidic acid (86.2%, n=19) and erythromycin (82%, n=18). Two isolates (9.1%) expressed phenotypic resistance to telavancin (a glycopeptide antibiotic) and ceftaroline (a fifth-generation cephalosporin), respectively.

MRSA *spa* types

There was 100% agreement in the *spa* types obtained from Sanger sequencing of isolates and those obtained after WGS of the isolates. Both *spa* typing tools revealed that the 22 isolates belonged to eight different *spa* types (Table 10). Two *spa* types

-- *t*304 and *t*008 -- were the most common among the isolates. They were both found in seven isolates (31.8%). In addition, *t*011 and *t*034, two *spa* types belonging to the CC398 complex, were detected in wastewater samples. No spa type was predominant in any of the cities because the four isolates from Rovaniemi belonged to three spa types: *t*008 (n=2), *t*127 (n=1), and *t*026 (n=1). All isolates harboured the staphylococcal cassette chromosome *mec* (SCCmec) type IVa(2B). Our findings revealed that WW surveillance could detect most of the clinically prevalent spa types reported by the THL. WW surveillance additionally correctly predicted the burden of the top four *spa* types in circulation.

Table 10. Comparison of *spa* typing of Methicillin-Resistant *S. aureus* isolated from wastewater in Finland against the 2021 Infectious Diseases Report (FINRES).

MLST	Spa type	CC	Number of isolates (%)	2021 Infectious Diseases Report
ST8	*t*008	CC8	31.8% (n=7)	11%
ST6	*t*304	CC5	31.8% (n=7)	10%
ST1	*t*127	CC1	9.1% (n=2)	5%
ST45	*t*026	CC45	9.1% (n=2)	<1%
ST9068	*t*172	-	4.6% (n=1)	5%
ST398	*t*011	CC398	4.6% (n=1)	<1%
ST398	*t*034	CC398	4.6% (n=1)	<1%
ST6	*t*5526	CC5	4.6% (n=1)	<1%

CC-Clonal complex. Adopted from Study I.

Genotypic features of MRSA isolates

Genotyping of the MRSA isolates revealed that six of the MRSA isolates (AA31, AA47, AA49, AA57, AA64, and AA72) harboured the *LukF-PV* and *lukS-PV* genes, which code the virulence factor-Panton–Valentine leukocidin (PVL), a two-component toxin that lyses phagocytic leucocytes. The PVL was detected in *t*008 isolates (USA 300 clone). These six PVL+ isolates additionally harboured the Arginine Catabolic Mobile Element (ACME), which plays a role in bacterial virulence and transmission and contains an arginine deiminase (arc) pathway and an oligopeptide permease (opp-3) system. This suggests that the isolates belonged to the USA300 clone (PVL and ACME positive CA-MRSA). Other virulence-associated genes detected included several other toxin genes (hla, hlgA, hlgB, hlgC, sea), host immune genes (*sak* and *scn),* and exoenzyme genes (*aur, spl*A, *spl*B, *spl*E). Further genotyping of the isolate revealed that the isolates belonged to five known STs, with 36.4% (n=8/22) and 31.8% (n=7/22) of the isolates belonging to ST6 and ST8, respectively. Two isolates each belonged to ST1, ST45, and ST398, respectively, whereas an isolate (AA66) was assigned to ST9068 by PubMLST.

Antibiotic resistance genes and plasmid replicons

A high concordance exists between the isolates' phenotypic and genotypic antibiotic resistance patterns. As expected in MRSA, the *mec*A gene was found in all 22 isolates. Two tetracycline resistance genes (*tet*K and *tet*M) were detected, with tet38 (which encodes an efflux pump) found in 18 isolates (81.8%). Resistance to beta-lactam antibiotics was conferred by *bla*Z in 54.5% (n=12) of the isolates. Three resistance genes confer resistance to aminoglycosides (*ant*(9)-Ia, *ant*(6)-Ia, and *aph*(3')-IIIa (Figure 2). The *sat*4 gene, a plasmid-mediated streptothricin acetyltransferase that confers resistance to Streptothricin (a nucleoside antibiotic), was detected in 13.6% (n=3/22) of the isolates. Resistance determinants to macrolide antibiotics (*mph*(C) / *msr*(A - erythromycin) and phosphonic antibiotics (*fos*B -Fosfomycin) were also detected in the seven isolates that belonged to spa type *t*008. Another macrolide resistance gene (*erm*(C) was detected in two isolates that belonged to spa type *t*127. A single isolate (spa type *t*034) harboured the *Inu*(B) / *Isa*(E) that confers resistance to clindamycin (Figure 7).
In terms of plasmid replicons, five variants of plasmids were detected. The Inc18 plasmid was the most abundant as it was detected in 72.7% (n=16/22) of the isolates. Other plasmid replicons detected were the rep_trans and repA_N, which were detected in 45.4% (n=10/22) and 40.9% (n=9/22) of the isolates, respectively. The rep3 and repL plasmids were detected in 31.8% (n=7) and 4.5% (n=1), respectively. Ten isolates harboured at least three plasmid replicons, and no plasmid replicons were detected in four isolates (ST6/*t*304).

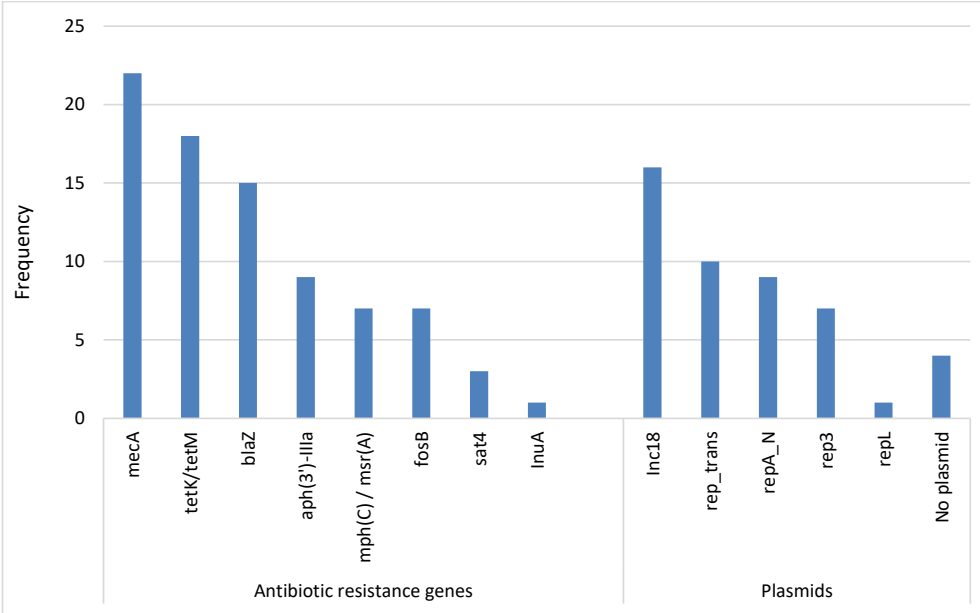


Figure 7. Antibiotic resistance genes and plasmids in wastewater MRSA isolates from Finland (n=22). Adopted from Study I.

Phylogenetic relatedness of wastewater MRSA isolates

The core genome MLST (based on 1604 of the 1861 genes) of the 22 isolates revealed that isolates aggregated into six clusters with some isolates having no allelic differences (Figure 8). The minimum spanning tree showed that five isolates from four different cities (Helsinki, Pietarsaari, Tampere, and Turku) belonging to the same ST6/*t*304 had 0 allelic differences and belonged to CT 12405. With <8 allelic differences, isolates from Espoo, Rovaniemi, Lappeenranta, and Tampere clustered together (ST8/*t*008/CT1925) and were therefore genomically indistinguishable. Two closely related and indistinguishable isolates clustered in Rovaniemi and Lappeenranta (ST1 and ST45). This indicated the clonal spread of these strains across some cities in Finland.

The minimum spanning tree, when compared to 122 open-access ST6 human MRSA sequences retrieved online, showed that WW sequences clustered with mecA isolates and t304 isolates (Figure 9). This indicates that wastewater surveillance, even without WGS, could reliably detect the circulating spa types and methicillin-resistant strains in the population. Figure 10 also compares the wastewater ST8/t008 (USA 300 clones) with some human open-access ST8/t008 (USA 300 clones) MRSA sequences (n=251). The minimum spanning tree obtained from the cgMLST also revealed that although the WW PVL+ were genomically diverse from human isolates, there was clustering of PVL+ isolates from PVL-negative isolates.

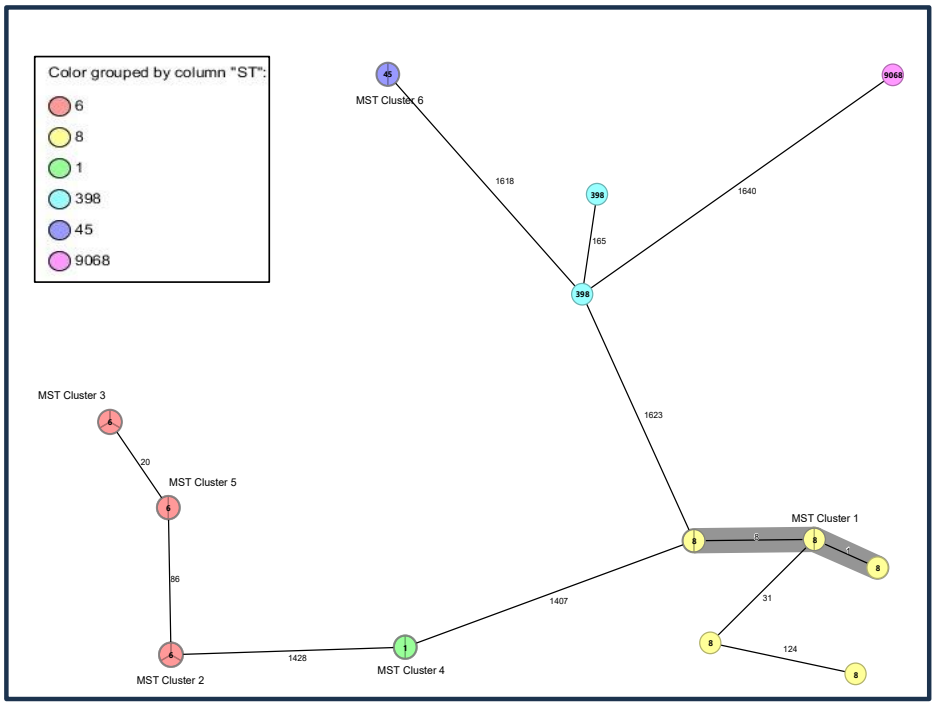


Figure 8. Core genome MLST of the isolates showing clustering of MRSA isolates (n=22). All samples were collected between February 2021 and January 2022. The numbers represent the allelic differences between isolates. The isolates were denoted with their sequence type (ST). Adopted from Study I.

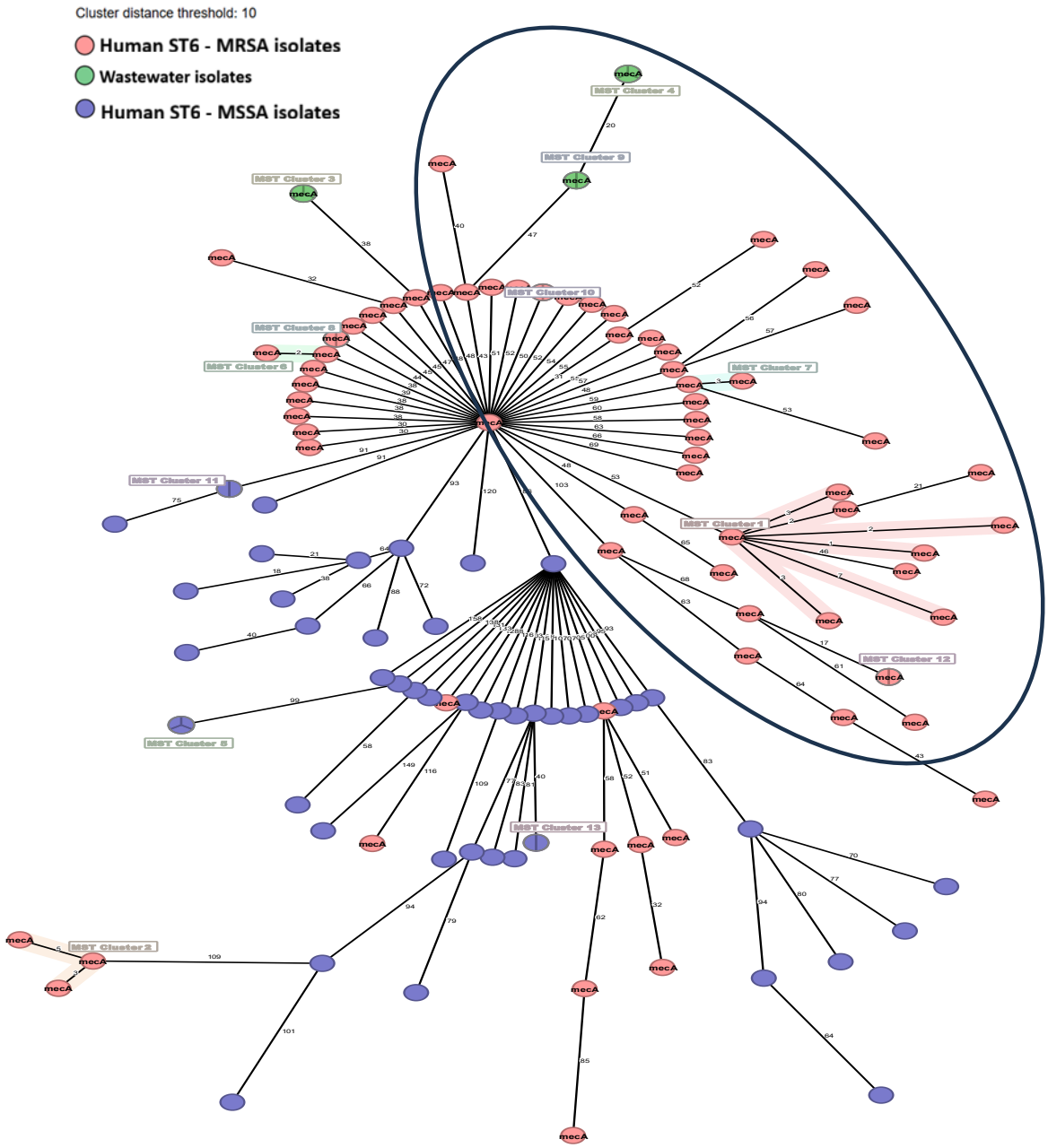


Figure 9. Core genome MLST of wastewater ST6 isolates (n=8) and open-access human ST6 MRSA isolates (n=122) showing clustering of *mec*A isolates (MRSA) vs methicillin susceptible *S. aureus*. The open-access isolates were obtained from human clinical and surveillance studies from eight countries (the United Kingdom, Germany, Singapore, Denmark, Pakistan, Australia, the USA, and China). MRSA – Methicillin-resistant *S. aureus;* MSSA – Methicillin-sensitive *S. aureus*.

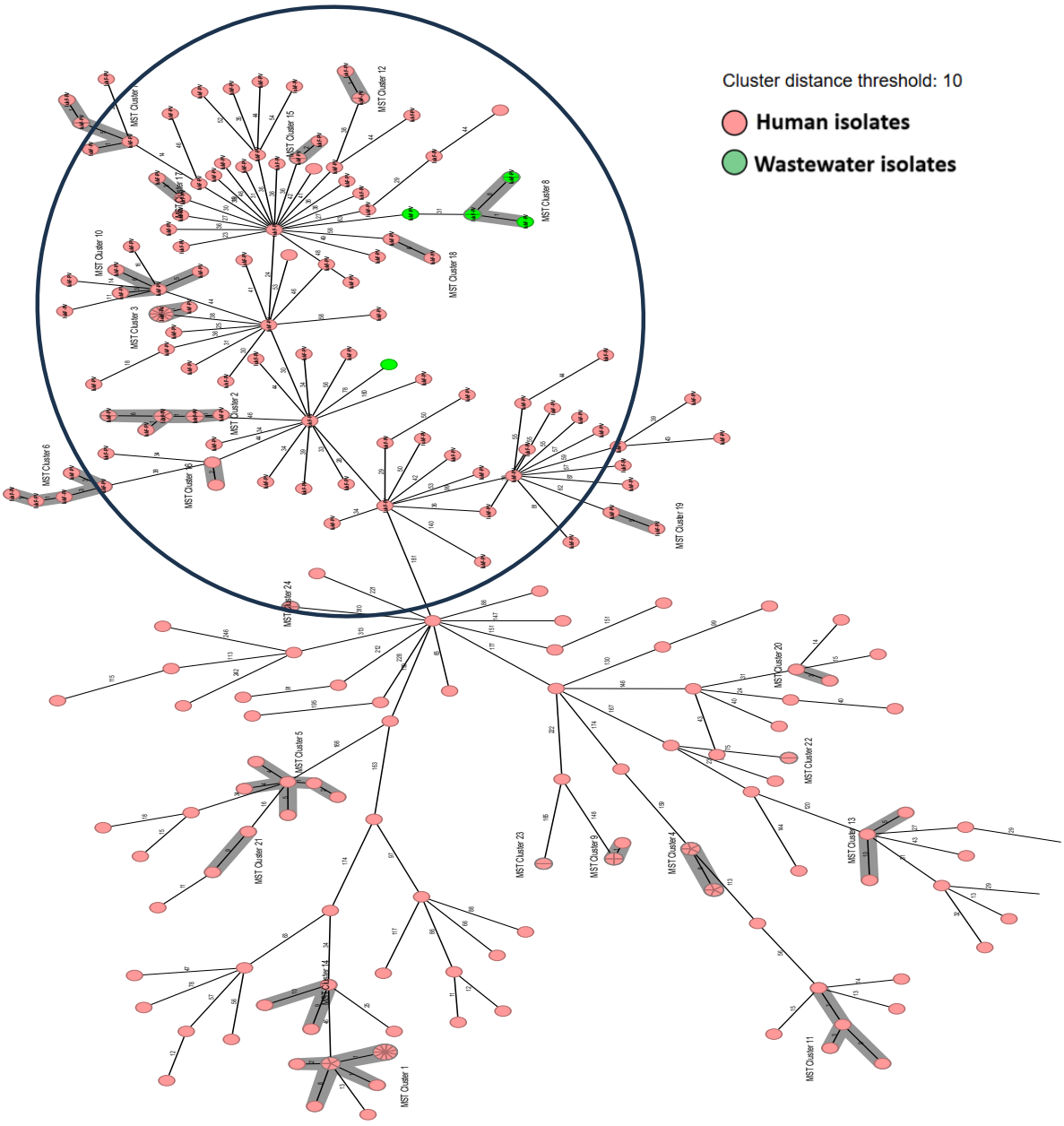


Figure 10. Minimum Spanning Tree based on Core genome MLST of the wastewater ST8 isolates (n=7) and open-access human ST8 MRSA isolates (n=251) (USA300 clone) showed clustering of most PVL+ MRSA isolates vs others (PVL- MRSA). The open-access isolates were obtained from human clinical and surveillance studies from five European countries (the United Kingdom, Germany, Denmark, Sweden, and Norway). The digits indicate the allelic differences between the genomes (indicating genetic relatedness). The open access sequences could be accessed from these accession numbers: PRJNA765573, PRJNA658260, PRJNA690682, PRJNA704240, PRJEB41130.

5.3 Study II - Wastewater surveillance for AmpC/ESBL-producing *Escherichia coli*

5.3.1 Phenotypic AmpC/ESBL-producing *Escherichia coli*

The second study used the double-disk synergy assay to test the 75 presumptive *E. coli* isolates. The assay revealed that 86.7% (n = 65/75) of the isolates were true ESBL-producing isolates, and two isolates were AmpC producers (2.7%, n=2/75). Phenotypic resistance to ciprofloxacin was expressed by 82.7% (n = 62/75) of the isolates. Of the 75 sequenced isolates, two sequences were excluded because they did not pass the quality control checks.

Genotypic profile of wastewater AmpC/ESBL-producing *Escherichia coli*

The following phylogroups were detected among the 73 sequenced isolates: A (n = 15), B1 (n = 3), B2 (n = 31), C (n = 2), D (n = 16), and F (n = 6). There were significant differences in the presence of class 1 integrons (*intI1* gene), the number of plasmid replicons, and the diversity and abundance of antibiotic, disinfectant, and stress-resistance genes across the phylogroups ($p < 0.05$). Our dataset further revealed that 43.8% (n = 32/73) of the isolates harboured the *IntI1* gene, especially among isolates belonging to phylogroup A (Table 11). The isolates belonged to 30 different STs, with eighteen isolates (24.7%) belonging to the globally disseminated and hypervirulent ST131 clone. Approximately 11% (n=7/73) of the isolates belonged to ST69. The other isolates (n = 44) belonged to other STs, as shown in Table 12.

The IS*26* element was detected in most (91.8%, n=67/73) of the genomes screened. In terms of plasmid replicons, all three isolates belonging to phylogroup B1 had the highest number of plasmid replicons, whereas those belonging to phylogroup B2 had the least number of plasmid replicons (41.9%, n = 13/31). The *qacE/qacEΔ1/qacL* gene was not detected in any of the five isolates belonging to phylogroup B1 (n = 3) or phylogroup C (n = 2). The two tLST variants (tLST1 and tLST2) were detected in 5.5% (n=4) of the 73 genomes. Analysis of the 73 isolates revealed a high molecular diversity of the isolates, with most isolates belonging to serotype O25:H4 (15%, n = 11/73) and O16:H5 (8.2%, n = 6/73). Table 12 shows the diversity of serotypes. Four isolates harboured both tLST1 variants, and tLST1 had complete ORFs; a few ORFs were missing in tLST2.

Table 11. Distribution of genomic features among AmpC/ESBL-producing *E. coli* (n = 73).

Escherichia coli phylogroup (No of isolates)		Genomic feature							
		Intl1	tLST	IS*26*	Plasmid replicons (1 or more)	Point mutations	3 or more ARGs	*qacE/qacEΔ1*	*ymgB*
Phylogroups	A (n = 15)	11(73.3)	3 (20)	15 (100)	11 (73.3)	15 (100)	14(93.3)	9 (60)	13(86.7)
	B1 (n = 3)	0 (0)	0 (0)	3 (100)	3 (100)	1 (33.3)	2 (66.7)	0 (0)	3 (100)
	B2 (n = 31)	13(41.9)	1(3.2)	29(93.5)	13 (41.9)	31 (100)	18(58.1)	8(25.8)	31 (100)
	C (n = 2)	0 (0)	0 (0)	1 (50)	1 (50)	1 (50)	0 (0)	0 (0)	2 (100)
	D (n = 16)	4(25)	0 (0)	14(87.5)	11 (68.8)	6 (37.5)	8 (50)	4 (25)	16 (100)
	F (n = 6)	4(66.7)	0(0)	5 (83.3)	4 (66.7)	5 (83.3)	6 (100)	2(33.3)	6 (100)

Phylogroup is a classification system for *E. coli* that is used to understand how different *E. coli* strains are related to each other, predict their potential to cause disease, and study their distribution in various hosts and environments. Phylogrouping divides strains into distinct genetic lineages, typically labelled A, B1, B2, and D, with other groups like C, E, F, and G also identified (Clermont et al., 2019). Adopted from Study II.

Table 12. Genotypic features of ESBL-producing *Escherichia coli* isolated from municipal wastewater in Finland.

City of Isolation	Isolate ID	ST	Serotype	Plasmids	No. of plasmids	Phylogroup	Class 1 Integron
Espoo	21V-01	1434	H14:O18ab	IncFIA(HI1), IncHI1A, IncHI1B(R27), IncM1, IncQ1.	5	A	IntI1
Espoo	21V-11	1193	O75:H5	IncFIA, IncB/O/K/Z, ColpEC648, Col156, Col(BS512), IncFIB(AP001918)	6	B2	None
Espoo	21V-21	2619	O1:H7	Col156, IncFIB(AP001918), IncFII(29)	3	B2	None
Espoo	21V-31	648	O1:H6	Col(BS512), ColpEC648, IncFIA, IncFIB(AP001918), IncFII(pRSB107), IncY	6	F	IntI1
Espoo	21V-41	405	O102:H6	IncFIB(AP001918), IncFIA, IncFII	3	D	IntI1
Espoo	21V-51	405	O102:H6	Col8282, ColpEC648	2	D	None
Espoo	21V-61	131	O25:H4	Col156, IncFIB(AP001918), IncFIA, IncFII(pRSB107)	4	B2	IntI1
Espoo	21V-71	1178	O130:H26	None	0	A	IntI1
Helsinki	21V-02	410	H21:O8	Col(BS512), IncFIB(K)(pCAV1099-114), IncFIB(pQil), IncFII(K), IncI1-I(Alpha), IncY	6	C	None
Helsinki	21V-12	636	O21:H7	Col440II	1	B2	IntI1
Helsinki	21V-22	80	O75:H7	IncFIB(AP001918), IncFII(29)	2	B2	None
Helsinki	21V-32	167	O101:H9	Col(BS512), ColpEC648, IncFIA, IncFIB(AP001918), IncFII	5	A	IntI1
Helsinki	21V-42	69	H18	IncFII(pEH01), col156	2	D	None
Helsinki	21V-52	38	O86:H18	Col156, IncFIB(AP001918), IncFII	3	D	None
Helsinki	21V-62	131	O16:H5	Col156, IncFIB(AP001918), IncFII	3	B2	IntI1
Helsinki	21V-72	69	O17/44:H18	IncFII, IncX4	2	D	None
Kuopio	21V-13	131	O16:H5	Col156, IncFIA, IncFII(pRSB107), IncFIB(AP001918)	3	B2	None
Kuopio	21V-23	131	O25:H4	IncFIA, IncFII(pRSB107), IncFIB(AP001918)	3	B2	IntI1

Kuopio	21V-33	1485	O83:H42	ColpVC, IncFIA, IncFIB(AP001918), IncFIC(FII), IncI1-I(Alpha), IncN2	6	F	IntI1
Kuopio	21V-63	38	O86:H18	None	0	D	None
Kuopio	21V-73	354	O45:H6	IncFIA, IncFIB(pB171), IncI(Gamma)	3	F	None
Lappeenranta	21V-04	38	H18:O7	None	0	D	None
Lappeenranta	21V-14	69	O15:H18	IncFII(pRSB107), Col156, IncX4	3	D	None
Lappeenranta	21V-24	88	H17	None	0	C	None
Lappeenranta	21V-34	69	O17/44:H18	IncFII	1	D	None
Lappeenranta	21V-44	10	O101:H10	None	0	A	IntI1
Lappeenranta	21V-54	399	O9:H12	IncFIB(K), IncFII, IncHI2, IncHI2A, repA(pKOX)	5	A	IntI1
Lappeenranta	21V-64	131	O25:H4	Col156, IncB/O/K/Z, IncFIA, IncFIB(AP001918), IncFII(pRSB107)	5	B2	None
Lappeenranta	21V-74	361	O9:H30	None	0	A	None
Oulu	21V-15	1486	O12:H20	IncFIA(HI1), IncR	2	A	IntI1
Oulu	21V-25	336	O20:H16	IncFIB(AP001918)	1	B1	None
Oulu	21V-35	450	O25	Col156, IncFIB(AP001918), IncFII(29)	3	A	IntI1
Oulu	21V-45	4684	O9:H34	Col156, IncFIB(AP001918), IncFii, IncI1-I(Alpha)	4	B1	None
Oulu	21V-55	1193	O75:H5	Col(BS512), Col156, IncFIB(AP001918), IncFIA, IncQ1	5	B2	IntI1
Oulu	21V-65	38	O86:H18	None	0	D	IntI1
Oulu	21V-75	1178	O130:H26	IncB/O/K/Z	1	A	IntI1
Pietarsaari	21V-06	5375	H15:O166	IncI1-I(Alpha), Col(MP18), Col156	3	D	None
Pietarsaari	21V-16	404	O75:H5	Col156, IncB/O/K/Z, IncFIB(AP001918), IncFII(29)	4	B2	IntI1
Pietarsaari	21V-26	28	O84:H7	IncFIB(AP001918), IncFII	2	B2	None
Pietarsaari	21V-36	131	O25:H4	IncFIA, IncFIB(AP001918), IncFII(pRSB107), IncX4	4	B2	IntI1

Pietarsaari	21V-46	69	H4	Col156, IncFIB(AP001918), IncI1-I(Alpha), IncFII	4	D	IntI1
Pietarsaari	21V-56	131	O25:H4	IncFIA, IncFII(pRSB107), IncFIB(AP001918)	3	B2	None
Pietarsaari	21V-66	69	H18	IncFII	1	D	None
Pietarsaari	21V-76	357	O13:H4	IncFII, IncI1-I(Alpha), IncX4	3	B2	None
Rovaniemi	21V-17	773	O11:H52	Col(BS512), IncFIA, IncFIB(AP001918), IncFII(pRSB107)	4	A	IntI1
Rovaniemi	21V-27	405	O102:H6	None	0	D	None
Rovaniemi	21V-37	131	O16:H5	Col156, IncFIA, IncFIB(AP001918),IncFII(pRSB107)	4	B2	IntI1
Rovaniemi	21V-47	131	O16:H5	IncFIA, IncFIB(AP001918), IncFII(pRSB107)	3	**B2**	IntI1
Rovaniemi	21V-57	131	O25:H4	IncFIB(AP001918), IncFII(pRSB107), IncFIA	3	B2	None
Rovaniemi	21V-67	131	O25:H4	Col156, IncFIA, IncFIB(AP001918), IncFII(pRSB107)	4	B2	IntI1
Rovaniemi	21V-77	4040	O1:H15	None	0	D	None
Seinäjoki	21V-08	10	O101:H9	Col440I, IncI1-I(Alpha), IncFIB(AP001918), IncFIB(K)	4	A	IntI1
Seinäjoki	21V-18	131	O25:H4	Col(BS512), Col156, IncFIA, IncFIB(AP001918), IncFII	4	B2	IntI1
Seinäjoki	21V-48	131	O25:H4	Col156, IncFIA, IncFIB(AP001918), IncFII(pRSB107)	4	B2	IntI1
Seinäjoki	21V-58	135	O83:H1	IncFII, IncX4	2	B2	None
Seinäjoki	21V-68	131	O25:H4	Col156, IncFIA, IncFIB(AP001918), IncFII(pRSB107)	4	B2	None
Seinäjoki	21V-78	10	O29:H10	IncFIB(AP001918), IncFIB(K)	2	A	None
Tampere	21V-09	1722	O1:H25	None	0	F	IntI1

Tampere	21V-19	131	O107:H5	IncFIB(AP001918), IncFII(29), IncI1-I(Alpha)	3	B2	None
Tampere	21V-29	398	O64:H4	IncI1-I(Alpha)	1	A	None
Tampere	21V-39	4456	O83:H4	IncFIB(AP001918), IncFIC(FII), IncX10	3	B2	None
Tampere	21V-49	1193	O75:H5	Col(BS512), Col156, IncFIA, IncFIB(AP001918)	4	B2	None
Tampere	21V-59	69	O15:H6	IncFIA, IncFIB(AP001918), IncFIC(FII), IncHI2A, IncHI2	5	D	IntI1
Tampere	21V-69	131	O25:H4	Col156, IncFIA, IncFIB(AP001918), IncFII(pRSB107)	4	B2	None
Tampere	21V-79	1193	O75:H5	Col156, IncFIA, IncFIB(AP001918)	3	B2	None
Turku	21V-10	1722	O1:H25	None	0	F	IntI1
Turku	21V-20	1431	O8:H30	IncFIB(pLF82-PhagePlasmid), IncFII	2	B1	None
Turku	21V-30	131	O16:H5	Col156, IncFIB(AP001918), IncFIA, IncFII(pRSB107)	4	B2	IntI1
Turku	21V-40	131	O16:H5	IncFIB(AP001918), Col156, IncFII(29)	3	B2	None
Turku	21V-50	401	O153:H25	incR	1	A	IntI1
Turku	21V-60	998	O2:H6	Col156, IncFIB(AP001918), IncFII, IncI1-I(Alpha), IncQ1	5	B2	None
Turku	21V-70	998	O2:H6	Col156, ColpEC648, IncB/O/K/Z, IncFIA, IncFIB(AP001918), IncFII(pRSB107),	6	B2	None
Turku	21V-80	457	O11:H6	IncFIB(AP001918), IncI1-I(Alpha), IncX1	3	F	None

The ST was based on the Warwick scheme. Adopted from Study II.

Diversity of resistance determinants in AmpC/ESBL-producing *Escherichia coli*

The CTX-M ESBL genes were detected in 84% (n = 63/73) of the isolates concurrently with the *bla*$_{\text{TEM-1}}$ (31.5%, n = 23/73) and *bla*$_{\text{OXA-1}}$ (9.6%, n = 7/73) genes that confer AmpC resistance. Two isolates (AE22 and AE71) harboured only the AmpC gene, whereas eight isolates were non-AmpC/ESBL isolates that harboured other resistance mechanisms. The ESBL genes detected were *bla*$_{\text{CTX-M-15}}$ (46.6%, n = 34/73), *bla*$_{\text{CTX-M-27}}$ (16.4%, n = 12/73), and three isolates that each harboured the *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-55}}$. Two isolates (AE02 and AE33) harboured the carbapenemase resistance gene *bla*$_{\text{KPC-2}}$ and the *bla*$_{\text{NDM-1}}$ gene, respectively. The *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, and *bla*$_{\text{SHV-12}}$ were each detected in a single isolate (Figure 11).

The *mcr-1.1* that confers resistance to a last resort antibiotic – colistin – was detected in only one isolate (AE59). Other clinically important antibiotic resistance determinants detected in the isolates include *aac*(6')-*Ib-cr5* (16.4%, n = 12/73) and *aad*A1 (9.6%, n = 7/73). Moreover, a combination of *aad*A5 / *aph*(3'')-Ib / *aph*(6)-Id was detected in 11% (n = 8/73) of the isolates, and fluoroquinolone resistance determinants were detected in 82.2% (n = 60/73) of the isolates. The plasmid-mediated quinolone resistance gene, *qnr*S1, was detected in 23.3% (n = 17/73) of the isolates, whereas chromosomal point mutations that could result in fluoroquinolone resistance were detected in 80% of the isolates (n = 59/73). These mutations include the *gyrA*_S83L (50.7%), *gyrA*_D87N (35.6%, n = 26/73), *parC*_S80I (37%, n = 27/73), *parC*_E84V (15%, n = 11/73), and the *parE*_I529L (21.9%, n = 16/73). Other ARGs detected include the *sul*1 (28.8%, n = 21/73), *sul*2 (31.5%, n = 23/73), *tet*(A) (26%, n = 19/73), *tet*(B) (13.7%, n = 10/73), *dfr*A14 (12.3%, n = 9/73), *dfr*A17 (20.5%, n = 15/73), and *mph*A genes (30.1%, n = 22/73). Others point to mutations that could confer resistance to chloramphenicol and rifampin [*marR*_S3N (12.4%, n = 9/73)], and fosfomycin [*uhpT*_E350Q (16.4%, n = 12/73), and *ptsI*_V25I / *uhpT*_E350Q (24.7%, n = 18/73)].

Several genes related to stress, disinfectant, and heavy metals were also detected in the isolates. The isolates harboured the *ymgB* gene (97.3%, n = 71/73), *qacE/qacEΔ1* gene (28.8%, n = 21/73), and a single isolate (AE59) harboured the *qacL* gene. There was a high abundance of multidrug efflux pump genes such as *acrF* (97.3%, n = 71/73), *emrD/E* gene (79.4%, n = 58/73), and *mdtM* (71.2%, n = 52/73). The acid shock protein gene (*asr*) was also detected in six (8.2%) of the isolates. These six isolates (AE04, AE06, AE08, AE09, AE24, and AE25) also harboured genes that confer resistance to arsenic (*ars*C, *ars*D, *arsR*), a heavy metal. Other heavy metal genes detected include those that confer resistance to mercury (11%, n = 8/73), silver (6.8%, n = 5/73), copper (4.1%, n = 3/73), and tellurium (4.1%, n = 3/73). Most of the isolates harboured one or two of these heavy metal resistance genes, while AE50 and AE54 harboured resistance to mercury, copper, silver, and tellurium.

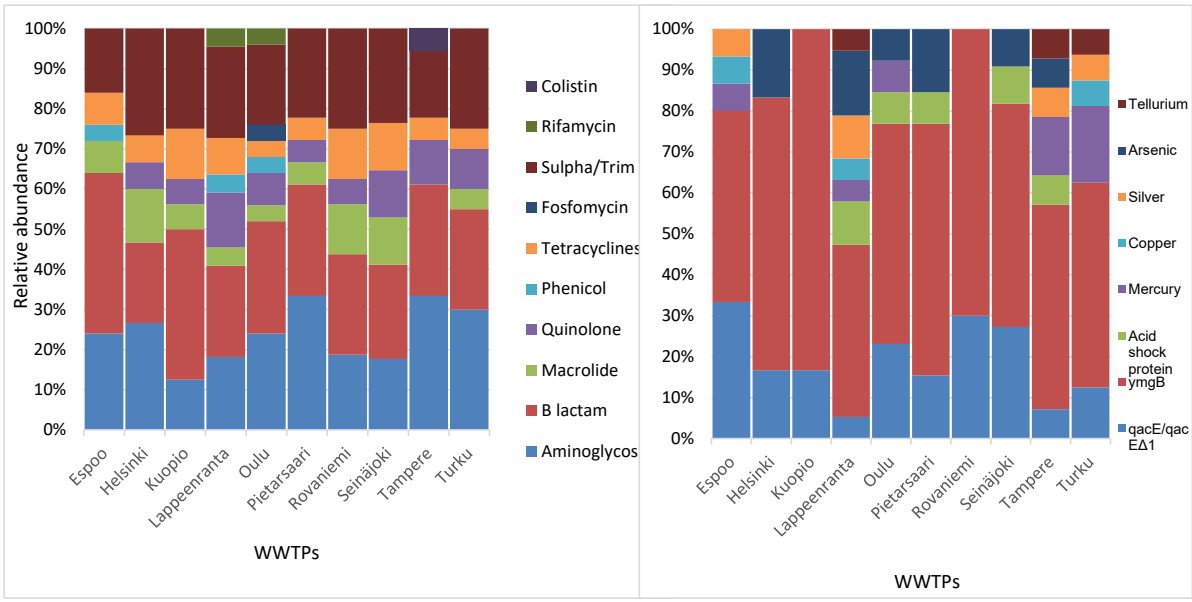
Figure 11. Antibiotic, disinfectant, and stress resistance genes in wastewater AmpC/ESBL-producing *Escherichia coli* (n=73). Adopted from study II.

Spatial variations in acquired resistance genes

Our findings revealed no significant differences in the diversity of acquired antimicrobial resistance genes across the ten WWTPs, despite more resistance genes being detected in samples from Oulu and Lappeenranta (Figure 12a). However, there were significant differences in the diversity and abundance of the stress resistance genes, with Kuopio and Rovaniemi having the least diversity (only *ymg*B and *qac*E/*qac*EΔ1). Lappeenranta had the highest diversity of stress resistance genes, with isolates from these cities harbouring resistance determinants such as those that confer resistance to copper (*pcoE, pcoS,pcoR, pcoD, pcoC, pcoB, pcoA*), mercury (*merC, merT, merR, merP*), silver (*silE, silP*), arsenic (*arsC, arsD, arsR*), and tellurium (*terD, terW, terZ*), in addition to *asr*, *ymg*B, and *qac*E (Figure 12b). There were also significant differences in plasmid diversity and relative abundance ($p < 0.05$) across the WWTPs, with an average of 1.8 plasmid replicons per bacterial isolate in samples from Lappeenranta, compared to the 3.6 plasmids per isolate from Espoo. There was no association between the presence of plasmid replicons and the diversity of ARGs in wastewater samples from each WWTP ($p = 0.307$).

Phylogenetic relatedness of AmpC/ESBL-producing *Escherichia coli*

The cgMLST, with only two genomic clusters, revealed high genomic diversity among the 73 AmpC/ESBL *E. coli* (Fig. 13). The ST131 isolates (n=18), ST 69 (n=7), as well as the four isolates, each of which were of ST38 and ST1193, all revealed high genomic diversity (Figure 13). The first cluster was isolated from Espoo: AE71 - A/ST1178/O130:H26-*bla*$_{\text{CTX-M-15}}$ and Oulu: AE75 - A/ST1178/O130:H26-*bla*$_{\text{TEM-1}}$. The second MST cluster comprised two isolates from Western Finland (Tampere and Turku) with no allelic differences and typed as F/ST1722/O1:H25-*bla*$_{\text{CTX-M-15}}$. Our analysis revealed four clusters from the human ST131 isolates when the wastewater ST131 isolates were compared with human surveillance ST131 isolates from Finland. There was, however, no genomic cluster between clinical and wastewater isolates, buttressing the hyperclonality of the ST131 sublineages (Fig. 14). There were at least 12 allelic differences in the core genomes of the closest isolates between the clinical and wastewater isolates (D29 and AE56) with both isolates typed as phylogroup B2/ST131/O25:H4--*bla*$_{\text{CTX-M-27}}$.



a. b.

Figure 12a. Relative abundance of antibiotic resistance genes per antimicrobial class in cultured AmpC/ESBL–producing *E. coli* isolates obtained from the 10 wastewater treatment plants (WWTPs) in Finland. (Sulfa/Trim - Sulfamethoxazole/Trimethoprim). b. Relative abundance of the metal and stress resistance genes in cultured AmpC/ESBL–producing *E. coli* isolates obtained from the sampled WWTPs. Adopted from study II.

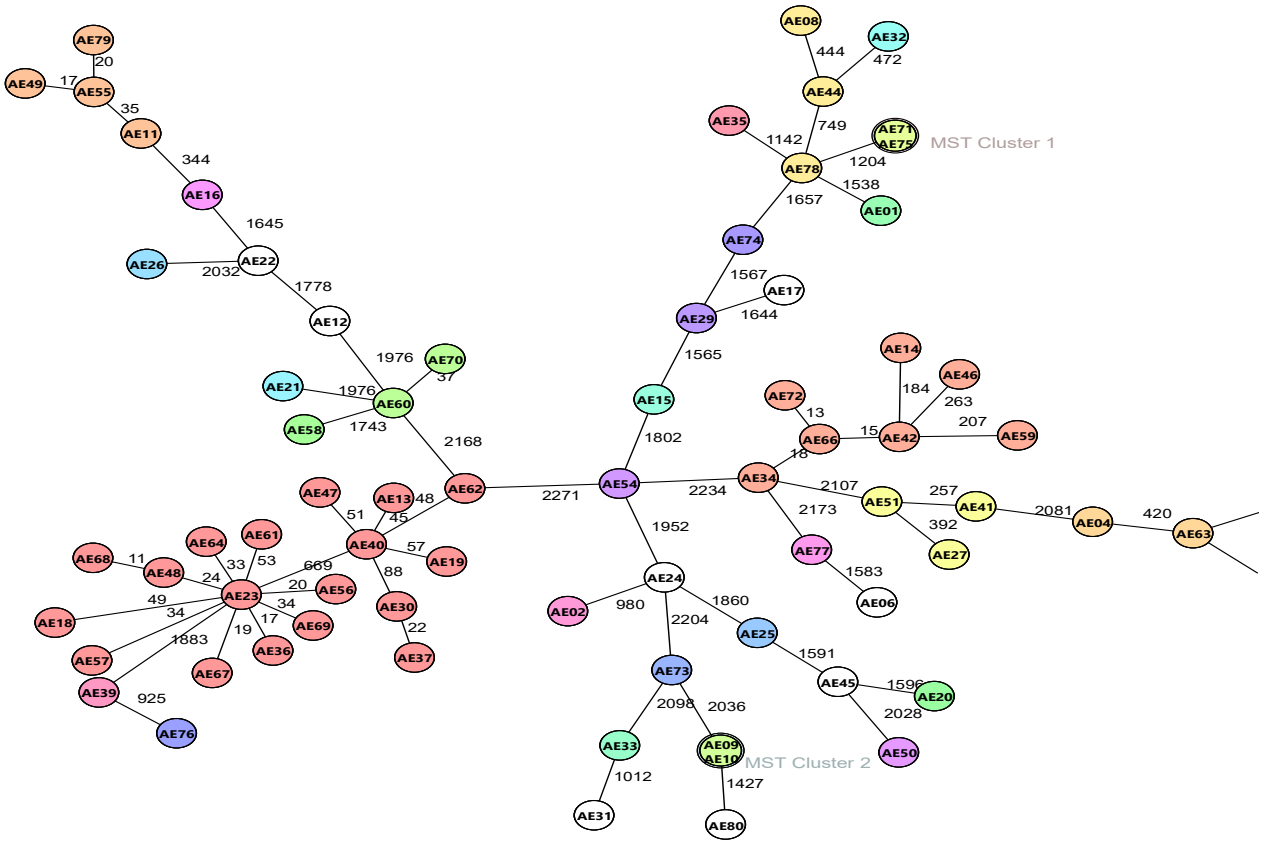


Figure 13. Minimum Spanning Tree based on core genome MLST of wastewater ESBL-producing *E. coli* isolates (n = 73). The Ridom SeqSphere+ MST was based on 2513 genes using a cluster distance threshold of 10. The digits indicate the allelic differences between the genomes (indicating genetic relatedness). Adopted from study II.

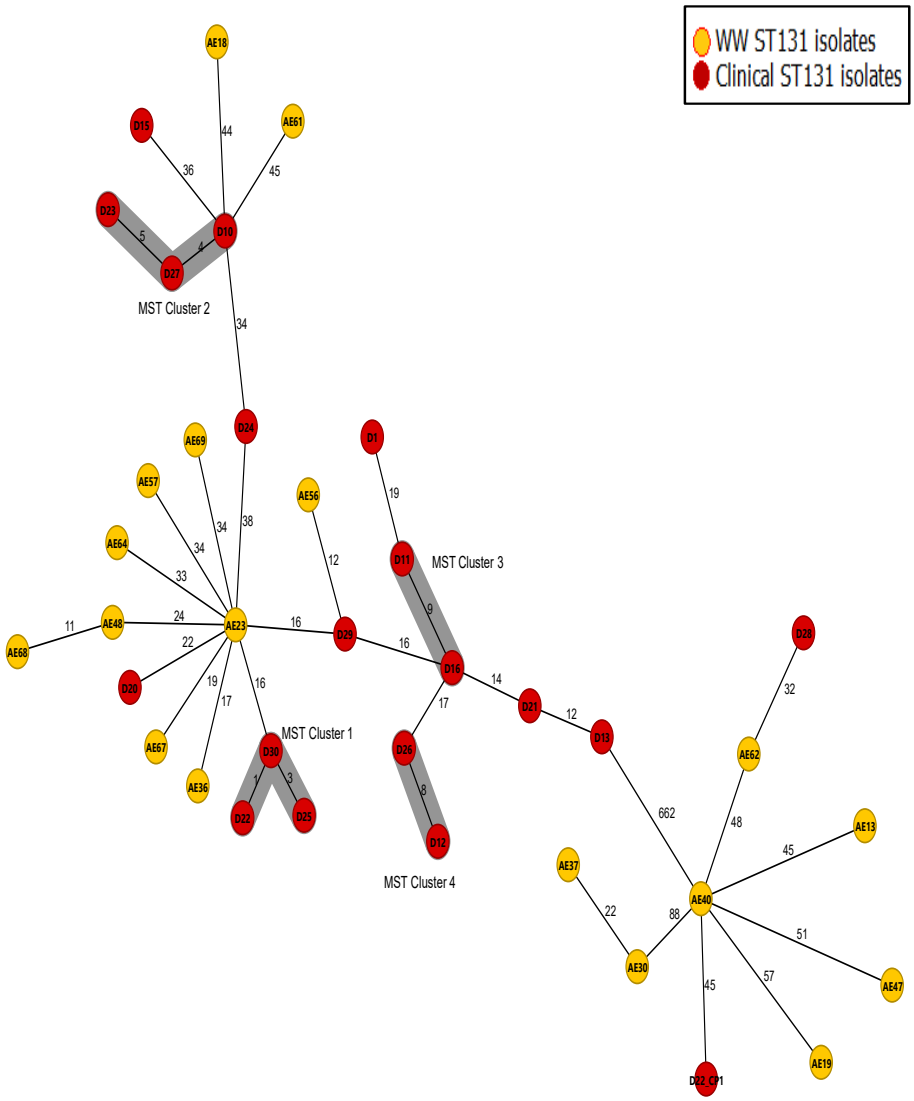


Figure 14. Minimum spanning tree (MST) from the core genome MLST of 39 ST131 isolates from wastewater isolates (n = 18) from study II and clinical isolates (n = 21) from Kurittu et al. (2022) in Finland. WW- wastewater. The MST was based on 2513 genes using a cluster distance threshold of 10. The small digits indicate the allelic differences between the genomes (indicating genetic relatedness). Adopted from study II.

5.4 Study III – Comparative core genome MLST of vancomycin-resistant *Enterococcus faecium*

The third study aimed to characterise another important public health pathogen. Apart from the intended target, VR*E. faecium* (22%, n = 17/77), three other *Enterococcus* strains were detected in the untreated municipal sewer system. These include *E. avium* (n = 2), *E. faecalis* (n = 2) and *E. gallinarum* (n = 3). No significant difference existed in the isolation rate of VR *E. faecium* across the ten WWTPs, although more isolates were retrieved from Turku (n = 6/8) (p = 0.471), and no VR *E. faecium* were isolated from the sewage collected from the Southern region of Espoo or the Northern region of Rovaniemi. The phenotypic antimicrobial susceptibility testing (AST) revealed that all isolates were resistant to ampicillin, teicoplanin, vancomycin, erythromycin, and ciprofloxacin, and had significant resistance to gentamycin (Figure 15). All isolates were, however, susceptible to chloramphenicol, daptomycin, and linezolid.

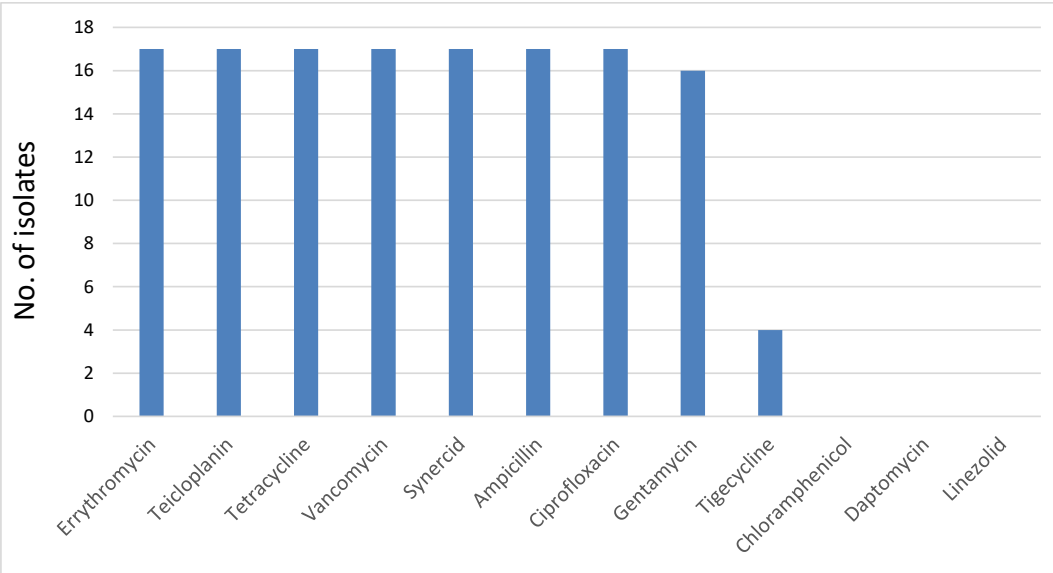


Figure 15. Phenotypic antimicrobial susceptibility testing of VR *E. faecium*.

Table 13. Genotypic profiles of Vancomycin-resistant *Enterococcus faecium* (n=17).

City	Isolate ID	ST	CT	ARGs	Plasmids
Helsinki	VR32	80	2046	aac(6')-I/aph(3')-IIIa, aac(6')-I, *vanA* / vanH-A / vanR / vanX-A / vanY-A / vanZ-A, erm(B) / msr(C), eat(A)	repUS15
Helsinki	VR62	117	5630	aac(6')-Iz, aac(6')-Ie, aph(2")-Ia, aph(3')-IIIa, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, erm(B) / msr(C), eat(A), dfrF	rep2, rep11a, rep18b, repUS12, repUS15
Helsinki	VR72	80	3302	aac(6')-I, aph(3')-IIIa, *vanA* / vanH-A / vanR / vanS / vanX-A / vanZ-A, msr(C), erm(B), erm(T), eat(A), tet(M), dfrG	rep2, rep14a, rep18b, rep18a, repUS1, repUS12, repUS15, repUS43
Kuopio	VR73	80	1470	aac(6')-I / aph(3')-IIIa, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, msr(C), eat(A), sat4	rep2, rep11a, rep17, rep18b, repUS15
Oulu	VR15	80	2099	aac(6')-I, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, msr(C), eat(A), dfrG	repUS12, repUS15, repUS43, rep17, rep18a, rep18b
Oulu	VR55	80	6196	aac(6')-I / aph(3')-IIIa, ant(6)-Ia, *vanB* / vanH-B / vanR / vanS / vanW / vanX-B / vanY-B, msr(C), eat(A), dfrG	rep2, rep18a, repUS12, repUS15, repUS43
Oulu	VR75	80	2099	aac(6')-I, aac(6')-Ie / aph(2")-Ia / aph(3')-IIIa, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, msr(C), eat(A), sat4, dfrG	rep17, rep18a, rep18b, repUS12, repUS15, repUS43,
Piertasaari	VR46	80	?	aac(6')-I, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, erm(B) /erm(T), msr(C), eat(A), tet(M)	rep2, rep11a, rep14a, rep17, repUS12, repUS15, repUS43
Seinajoki	VR38	78	?	aac(6')-I / aph(3')-IIIa, *vanA* / vanH-A / vanR / vanS / vanX-A / vanZ-A, erm(B) / msr(C), eat(A)	repUS1, repUS12, rep14b repUS15, rep2, rep11a, rep14a, , rep18a, rep18b
Tampere	VR19	787	1044	aac(6')-I, aph(3')-IIIa, ant(6)-Ia, *vanB* / vanH-B / vanR / vanS / vanW / vanX-B / vanY-B, erm(B) / msr(C), eat(A), sat4, tet(M), dfrG	rep2, rep11a, rep14a, rep17, rep18b, repUS15, repUS43
Tampere	VR69	80	2262	aac(6')-I, *vanA* / *vanB* / vanH-A / vanH-B / vanR, vanR / vanS, vanS / vanW / vanX-A / vanX-B / vanY-A / vanY-B / vanZ-A, erm(T) / msr(C), eat(A), tet(M)	rep2, rep17, repUS1, repUS15, repUS12, repUS43
Turku	VR10	80	2046	aph(3')-IIIa, aac(6')-I, *vanA* / vanH-A / vanR / vanX-A / vanY-A / vanZ-A, erm(B) / msr(C), eat(A)	repUS15, rep18b, rep29

City	Isolate ID	ST	CT	ARGs	Plasmids
Turku	VR20	787	1044	aac(6')-I / aph(3')-IIIaaac(6')-Iaac(6')-I / aph(3')-IIIaaac(6')-Iaac(6')-I / aph(3')-IIIaaac(6')-Iaac(6')-I / ant(6)-Iaaac(6')-I*vanB* / vanH-B / vanR / vanS / vanW / vanX-B / vanY-B, erm(B) / msr(C), eat(A), sat4, tet(M), dfrG	rep2, rep11a, rep14a, rep17, rep18b, repUS15, repUS43
Turku	VR30	80	2515	aac(6')-Ie, aph(2'')-Ia, aph(3')-IIIa, aac(6')-I, aac(6')-Ie, *vanA* / vanH-A / vanR / vanX-A / vanY-A / vanZ-A, msr(C), eat(A), tet(M), dfrG	repUS12, rep18b, rep18a, repUS15, repUS43, rep17
Turku	VR40	612	1026	aac(6')-I, *vanA* / vanH-A / vanR / vanS / vanX-A / vanY-A / vanZ-A, erm(T) / msr(C), eat(A), dfrG	repUS1, repUS12, rep14, repUS15, repUS43, rep18a
Turku	VR50	80	2046	aph(3')-IIIa, aac(6')-I, *vanA* / vanH-A / vanR / vanX-A / vanY-A / vanZ-A, erm(B) / msr(C), eat(A)	repUS15, rep18b, rep29
Turku	VR80	80	2046	aph(3')-IIIa, aac(6')-I, *vanA* / vanH-A / vanR / vanX-A / vanY-A / vanZ-A, erm(B) / msr(C), eat(A)	repUS15

Genotypic profile of wastewater vancomycin-resistant *E. faecium* isolates

The 17 VR *E. faecium* isolates belonged to the CC17 clonal complex, an ancestral clone of the hospital-associated clade A1 (Lee et. al., 2019). Of these, 12 isolates belonged to the epidemic-prone ST80 variant. Other STs were 787 (n = 2), 78 (n = 1), 117 (n = 1), and 612 (n = 1). Most of the isolates harboured the *vanA* gene (76.5%), while three isolates harboured the *vanB* gene (17.6%), and sample VR69 harboured both the *vanA* and *vanB* genes. There was a wide diversity of ARGs, with the aminoglycoside resistance gene (*aac*(6')-I), macrolide resistance gene (*msr*(C)), *eat*(A), and *van* gene clusters being conserved in all the WW isolates (Table 13). Resistance to β-lactam and fluoroquinolone was mediated amongst most of the isolates by chromosomal point mutations in the *pbp5* and *gyr*A p.S82Y and *par*C p.S80I genes, respectively.

Core genome MLST of human and wastewater vancomycin-resistant *E. faecium*

The cgMLST revealed that WW contained some of the most prevalent STs of VR *E. faecium* in circulation in Finland. Our study demonstrated the clonal spread of VR *E. faecium* in Finland, with three clusters detected among the WW isolates. Two of these clusters were among ST80 strains, and the third was among ST787 strains (Figure 16a). WW isolates formed eight clusters when compared with human isolates. Sample VR40 (ST612, CT1026 from Turku) clustered with 16 human isolates, with one to ten allelic differences between the isolates. The closest isolate was from the well-being services county of Southwest Finland, where the city of Turku is located (Figure 16b). The two ST787 isolates retrieved from Southwest Finland and Pirkanmaa were identical and clustered with 9 human isolates that were mostly obtained from the Pirkanmaa region (Fig. 16c). VR62-ST117/CT5630 and

VR72-ST80/CT3302 (two of the three samples from the Helsinki region) and VR38-ST78/CT8498 did not cluster with any human isolates, suggesting possible introduction through international (trans-border) travel.

The only WW ST80:CT1470 strain was genomically indistinguishable from several human samples obtained from the same region (Kuopio or North Savo) and had only a few (0 to 6) allelic differences when compared with 44 other human isolates (Fig. 16d). The four WW ST80:CT2046 strains isolated from Turku and Helsinki similarly clustered with 100 human isolates from Southwest Finland (Figure 16e) with a maximum of 3 allelic differences. The three sequences from Oulu (ST80:CT2099/6196) clustered with human isolates obtained in 2020 from the same region (Wellbeing Services County of North Ostrobothnia) (Figure 16f). The only isolate (VR69-ST80/CT2262) that harboured both the *vanA* and *vanB* genes was related and clustered with human isolates that harboured either *vanA* or *vanB* but not both from Kymenlaakso, North Savo, South Karelia, and Päijät-Häme wellbeing counties (Figure 16g). The WW sample VR30 (obtained from Turku in May 2021) was identical to one human sample that was isolated in 2019 in the well-being services county of Southwest Finland. Another isolate, VR46 (obtained from Pietarsaari in August 2021), was identical to one human sample (no allelic difference) that was isolated in 2021 in the well-being services county of Ostrobothnia.

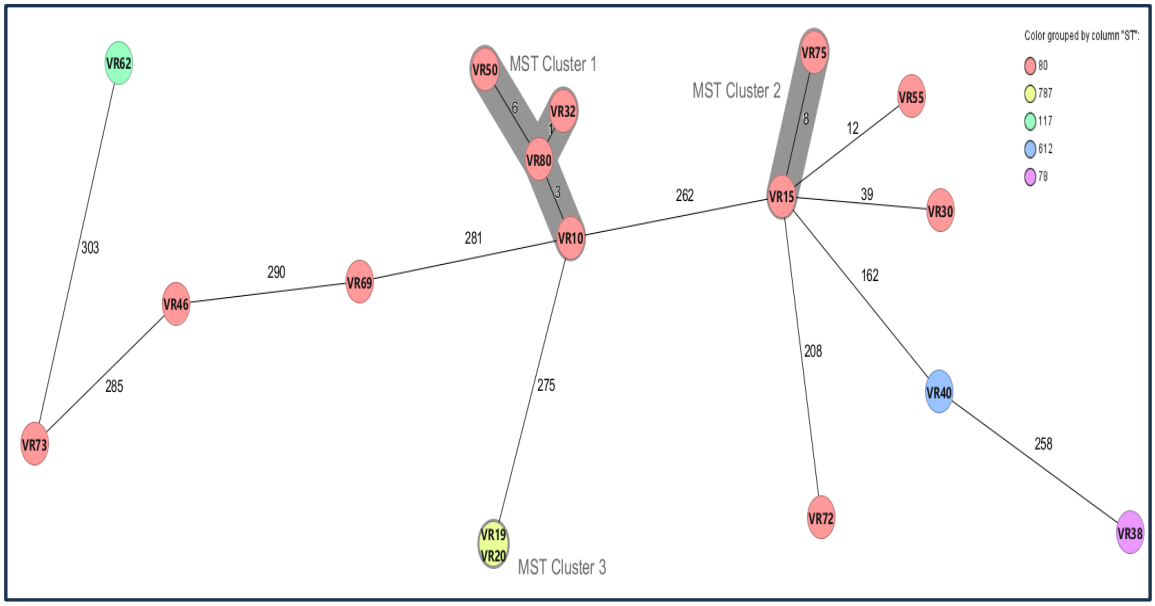


Figure 16a. Minimum spanning tree showing the core genome MLST of wastewater vancomycin-resistant *Enterococcus faecium* (n = 17). The RIDOM SeqSphere+ MST was based on 1260 genes using a cluster distance threshold of 10. The numbers indicate the allelic differences between the genomes. Adopted from study III.

Figure 16b-g. Minimum spanning tree showing the relatedness of wastewater vancomycin-resistant *Enterococcus faecium* to human isolates across Finland. The RIDOM SeqSphere+ MST was based on 1260 genes using a cluster distance threshold of 10. CT- Cluster type 7; ST – Sequence type. The red digits indicate the allelic differences between the genomes (indicating genetic relatedness). Adopted from study III.

6 Discussion

6.1 Strengths and Limitations of the Culture-based Approach to Wastewater AMR Surveillance

The findings of the three independent studies supported the utility of Wastewater-based surveillance (WBS) as a tool to evaluate the population carriage (either in infections or asymptomatically) of drug-resistant pathogens – *S. aureus, E. coli, and E. faecium*. Culture-based approaches remain the gold standard for confirming viable bacteria and their antimicrobial resistance (AMR) traits. We can perform phenotypic antimicrobial susceptibility testing (AST), which provides clinically relevant resistance profiles, by isolating priority pathogens from wastewater (Flach et al., 2021). This phenotypic confirmation is critical for linking environmental surveillance data to clinical outcomes, especially for high-risk clones such as the ST131 hypervirulent *E. coli*.

Another strength of the culture-based approach is the recovery of viable isolates that can be preserved and subjected to downstream analyses such as whole-genome sequencing (WGS), plasmid characterisation, or virulence testing (Hendriksen et al., 2019). This allows for detailed molecular epidemiology studies, strain typing, and linkage to clinical infections. Unlike purely molecular methods, culture-based surveillance thus generates a living repository of resistant bacteria for future research and reference. Some culture-based protocols, such as selective plating on CHROMagar, are relatively inexpensive and globally standardised, making them accessible to resource-limited settings where molecular approaches may not be feasible (WHO, 2021). This cost-effectiveness enhances comparability across sites and supports the One Health approach by integrating human, animal, and environmental antimicrobial resistance (AMR) surveillance. This can be useful in guiding antimicrobial AB stewardship programs in all One Health sectors and guiding antibiotic prescription policies in the future.

A number of limitations may restrict its usefulness as an early warning system, even if there is a strong association between the culture-based WBS of the three pathogens and the clinical bacterial resistance report in Finland. Firstly, we discovered that the pre-enrichment process greatly improves the detection sensitivity, even if a low degree of infection in a community may impact the bacterial concentration and make it extremely challenging to isolate. Secondly, it may be challenging and inconclusive to analyse wastewater monitoring data collected in

towns without municipal sewer systems. Thirdly, it is challenging to make direct comparisons between wastewater and human prevalence. This restriction might result from the challenge of identifying the origin of certain molecular characteristics (plasmids, bacteriophages, and ARGs) present in bacterial genomes. Another limitation for Study three involving VREfm was the small number of VREfm isolates. Moreover, a limitation of most single-cell genomics is that only one colony was selected from each sample, despite the many distinct colonies that were obtained from the samples. This limitation could be mitigated by selective enrichment with metagenomics. Another limitation is that culture-based approaches only detect organisms that can grow under laboratory conditions and may miss low-abundance resistance genes, novel variants, or horizontal gene transfer events occurring in the microbial community. Hence, culture methods are less suited for broad resistome monitoring. Finally, the approach is less efficient for near-real-time surveillance and public health decision making, especially during outbreaks, because it currently takes days to culture these pathogens and conduct AST.

Despite these limitations, culture-based wastewater surveillance could help overcome known limitations of clinical surveillance, such as low population coverage, high cost, testing and reporting delays. More research is needed to ensure standardisation of protocols and data interpretation standards.

6.2 Wastewater surveillance of multidrug-resistant bacterial pathogens (I, II, III)

The three studies revealed the high concordance between the WW isolates and those from human clinical or surveillance isolates. The culture-based approach is generally more compatible and similar in protocol to the traditional clinical human AMR surveillance in Finland. Matching MRSA spa types were obtained for Study I from WW surveillance and the Infectious Diseases Register in Finland (THL, 2022). The study also showed the clonal dissemination of MRSA in Finland. I could not do comparative whole genome phylogenomics of wastewater and human isolates for Study II, because screening and clinical ESBL-producing *E. coli* are not sequenced by the THL. Hence, I restricted the concordance to ST131, which was obtained by Kurittu et al. (2022). The cgMLST of ST131 also supported the notion that WBS enables the identification of isolates that are similar to those in circulation with wastewater and human isolates having as few as 12 allelic differences. My third study also revealed that WW surveillance could detect the same proportions of *vanA*/B, ST, and circulating strains as the human samples. The culture-based WBS approach identified three of the most common VREfm outbreak-associated STs in Finland (ST80, ST612, and ST787). The epidemic-prone ST80 strains have been reported as the most prevalent ST in Sweden (Fang et al., 2021), Denmark (Pinholt et al., 2019), Germany (Werner et al., 20202), Ireland (Egan et al., 2022), and across the globe. ST612 is not globally distributed because only a few studies have reported this ST (Suzuki et al., 2014; van Kleef-van Koeveringe et al., 2024), and ST787 has also been reported in Ireland. Two of the three WW isolates that did not cluster with any human isolates were from Helsinki and were more likely to be newly introduced into the country or could be an emerging strain (Arcilla et al., 2020). Hence, they should be monitored over the next few years.

The studies revealed a higher isolation rate for *E. coli* and lower rates for *S. aureus* and *E. faecium*. The phenotypic AST profile revealed that most of the isolates were MDR. For study I, the AST findings were contrary to the findings of the 2020 FINRES report, which reported that only 2.4% of the 2,176 MRSA isolated from clinical cases (blood and pus specimens) were MDR (THL, 2023). For studies II and III, although MDR, all of the reported resistance profiles in human samples were also observed in the WW isolates. These discrepancies may be explained by the various sampling sizes, sampling types (clinical versus wastewater), and the distinctions between hospital-based and population samples. Additionally, the development and spread of ARGs and ARBs in wastewater, particularly through horizontal gene transfer (HGT), are subject to continuous selection pressure from antibiotics and other co-selection variables in wastewater (Chau et al., 2022).

The core genome MLST revealed that subtyping at a higher granularity based on assembled contigs could enhance surveillance of priority pathogens and increase data comparability across geographical and temporal scales, which is essential for the identification and analysis of both domestic and international outbreaks (Pires et al., 2024). The cgMLST for Study I clustered the 22 MRSA isolates into five clusters with ST6/t304 and ST8/t008 dominating, indicating the clonal spread of these STs in Finland. The cgMLST indicated the diverse nature of WW isolates when compared to open-access human ST6/t304 and ST8/t008 strains. cgMLST revealed high genetic diversity and the existence of multiple successful lineages of ESBL-producing *E. coli* in circulation in Finland. The value of cgMLST was also evident when the MST revealed that some of the human ST131 isolates were related to WW isolates. For study III, cgMLST revealed that WW VREfm isolates clustered closely with human VREfm from most of the wellbeing service counties in Finland, with some isolates displaying no allelic differences. These findings were the basis for proposing WBS as a sensitive population-level surveillance tool that can be used in conjunction with other methods to detect and track these highly contagious and widely distributed clades and variants of priority pathogens.

Antibiotic Resistance Genes

Across the three studies, the phenotypic AST profile revealed that most of the isolates were MDR. For study I, the AST findings were contrary to the findings of the 2020 FINRES report, which reported that only 2.4% of the 2,176 MRSA isolated from clinical cases (blood and pus specimens) were MDR (THL, 2023). For studies II and III, although MDR, all of the reported resistance profiles in human samples were also observed in the WW isolates. These discrepancies may be explained by the various sampling sizes, sampling types (clinical versus wastewater), and the distinctions between hospital-based and population samples. Additionally, the development and spread of ARGs and ARBs in wastewater, particularly through HGT, are subject to continuous selection pressure from antibiotics and other co-selection variables in wastewater (Chau et al., 2022).

Study I showed that while the tet38 gene, a chromosomally encoded efflux transporter of *S. aureus*, also functions as an efflux transporter of fosfomycin, seven MRSA isolates (spa type t008 only) included the *fos*B gene, a resistance determinant for fosfomycin (Truong-Bolduc et al., 2018). The high prevalence of *aph*(3')-IIIa, the aminoglycoside resistance determinant, was consistent with

previous research (Khosravi et al., 2017). The *mec*A gene, which conferred resistance to methicillin, penicillin, and other beta-lactam antibiotics, was present in all isolates (Lade et al., 2022). Apart from the mecA gene, *bla*$_{Z}$ was primarily responsible for the resistance determinants to beta-lactam antibiotics (only a small number of isolates possessed the *bla*$_{I}$, *bla*$_{PC1}$, and *bla*$_{R1}$ genes). Additionally, a variety of other resistance mechanisms, such as target alteration, enzymatic drug inactivation, and reduced antibiotic uptake or efflux, may be responsible for this type of phenotypic resistance to antibiotics (Matsuoka et al., 2003). For example, the bacterial ribosome's alteration by erm-gene-encoded 23S rRNA methyltransferase has been linked to macrolide resistance determinants in MRSA (Matsuoka et al., 2003). However, the macrolide resistance determinants [*mph*(C), *msr*(A) and *erm*(C)] that could provide cross-resistance to lincosamides were present in nine isolates. The *tet*38 gene, a chromosomally encoded efflux transporter of *S. aureus*, also functions as an efflux transporter of fosfomycin, despite the fact that seven MRSA isolates (spa type t008 only) included the *fos*B gene, a fosfomycin resistance determinant (Truong-Bolduc et al., 2018). The high prevalence of *aph*(3')-IIIa, the aminoglycoside resistance determinant, was consistent with previous research (Ardic et al, 2006; Khosravi et al., 2017).

In my study II, 82.7% of our isolates exhibited phenotypic resistance to ciprofloxacin, consistent with 70–80% of the human samples examined in Finland between 2008 and 2019 (Ilmavirta et al., 2023). Fluoroquinolone prescription rates are modest in Finland (22 per 1000 adults annually; Koukkari et al., 2021), although fluoroquinolone resistance is common (Ilmavirta et al., 2023; Kurittu et al., 2022). This may be since the genes that encode the synthesis of ESBL are frequently found on the same mobile genetic element as genes that confer resistance to aminoglycosides and fluoroquinolones (Ilmavirta et al., 2023; Kurittu et al., 2022; Holmes et al., 2016). My results corroborate studies of changing beta-lactamase gene diversity and prevalence (Clarke et al., 2024; Tiwari et al., 2022). WGS showed a higher prevalence of the *bla*$_{CTX-M-15}$ gene in contrast to the findings of Kurittu et al. (2022), which found a higher occurrence of *bla*$_{CTX-M-27}$ in clinical isolates from Finland. According to a related study in an Israeli hospital context, ST131-*bla*$_{CTX-M-27}$-*E. coli* has been shown to have a greater transmission rate than ST131-*bla*$_{CTX-M-15}$-*E. coli* (Adler et al., 2012). These investigations lend credence to the idea that the most prevalent strains in human samples are evolving and that *bla*$_{CTX-M-27}$ has emerged as a rival to *bla*$_{CTX-M-15}$. The rise of blaCTX-M-55 as the most prevalent *bla* enzyme type in China significantly increases the variety and competition (Zeng et al., 2021). Fluoroquinolone-resistant extra-intestinal pathogenic *E. coli* in Vietnam and Qatar have been shown to co-occur with isolates of *aac*(6')-Ib-cr5, *bla*$_{OXA-1}$, and *bla*$_{CTX-M-15}$-ST1193 (Nguyen et al., 2021; Perez-Lopez et al., 2020). Colistin, the antibiotic of last resort, was resistant in only one isolate (*mcr-1.1*). This might be due to Finland's extremely low rate of antibiotic use (Koukkari et al., 2021). The index report in 2018 (Gröndahl-Yli-Hannuksela et al., 2018) and subsequently in 2023 in hospital wastewater (Markkanen et al., 2023) could support the low burden of clinical colistin resistance in Finland.

Sequenced isolates from hospital wastewater similarly have only a modest load of *bla*$_{KPC}$ and *bla*$_{NDM}$ genes (Majlander et al., 2021; Markkanen et al., 2023) and the Viikinmäki WWTP in Helsinki (Tiwari et al., 2022). These isolates, in addition to ARGs, also harboured several stress (acid, disinfectant, and heavy metal) resistance

genes. Studies have shown that the occurrence of heavy metal resistance genes (HMRGs) was significantly associated with the presence of disinfectant- or antibiotic-resistance genes (Seiler & Berendonk, 2012; Yang et al., 2020). Four isolates also had the transmissible locus of stress tolerance (tLST), a genomic island in *E. coli* that confers resistance to multiple stresses, including extreme heat and chlorine. Lastly, among phylogroup A isolates, the number of ARGs and tLST ORFs was negatively correlated, with more ARGs found in isolates that were tLST-negative (Zhang and Yang, 2022). Additionally, resistance determinants against heavy metals, particularly copper, silver, and mercury, were present in tLST-positive genomes.

My study III also revealed that wastewater-sourced isolates are highly resistant to ampicillin, gentamicins, erythromycin, teicoplanin, ciprofloxacin, synercid, and tigecycline, in concordance with the report of Oravcova et al., 2017. The resistance to aminoglycosides could be due to an innate resistance provided by the presence of cell walls in *Enterococcus spp.* (Lee et al., 2019). The high resistance to macrolides and Synercid (quinupristin/dalfopristin) could be due to the carriage of two (*msr*C and *eat*A) of the 26 known ABC-F family efflux pumps in all WW isolates (Ahmed & Baptiste, 2022; Murina et al., 2019). Studies that evaluated over 100,000 *E. faecalis* and *E. faecium* phenotypes from the EARS-NET, in agreement with our findings, reported a low prevalence of linezolid and daptomycin resistance (Ayobami et al., 2020; Markwart et al., 2021). The repertoire of resistance genes was similar to those previously reported (Gouliouris et al., 2019; Hricová et al., 2021; Radisic et al., 2024; Sanderson et al., 2019). One of these studies detected 28 antibiotic resistance genes in the hospital-adapted clade, of which 23 were represented in bloodstream, hospital sewage, and municipal WW isolates (Gouliouris et al., 2019).

Data from the metagenomic analysis of the Global Sewage Surveillance Project revealed that Enterococcus was assigned the largest number of ARGs and mainly shared these with other known Gram-positive species (Leekitcharoenphon et al., 2019; Munk et al., 2022). This largely confirms observations from the human isolates and suggests that the current choice of *Escherichia coli* and Enterococcus as indicator species for AMR surveillance is appropriate (Munk et al., 2022). Our findings also revealed that vancomycin resistance was mediated by more *vanA* and fewer *vanB* genes. Our findings were highly concordant with the THL reports, according to which more *vanA* than *vanB* VREfm isolates were in circulation during 2021–2022 (THL, 2024). The Aminoglycoside resistance gene (*aac*(6')-I) and macrolide resistance gene (*msr*(C)) were other detected ARGs. B-lactam and fluoroquinolone resistance was mediated by chromosomal point mutations in the pbp5 and *gyr*A p.S82Y and *par*C p.S80I genes, respectively. Studies have shown over the years that antimicrobial use (AMU) selects for certain AMR determinants (Van De Sande-Bruinsma et al., 2008). Hence, there was a positive association between antibiotic consumption (tonnes per year) and the diversity of ARGs detected in municipal wastewater in Finland. Even though wastewater treatment facilities drastically lower the microbial diversity of ARBs and ARGs, they do not totally eradicate all of these microorganisms, which could endanger the environment downstream (Jia et al., 2020; Mao et al., 2021; Wang and Chen, 2022), or they may even amplify certain genes through horizontal gene transfer (Karkman et al., 2018).

6.3 Public Health Implications and Policy Recommendations

The global emergence and spread of MDR bacterial pathogens have raised serious public health concerns, especially in terms of morbidity, mortality, social, and economic burden. WBS has been used to track population carriage of priority infectious pathogens, including the polio virus, SARS-CoV-2, RSV, and chemical agents such as illicit drug use as part of the WastPan project in Finland. WBS for AMR could have the following public health implications: Firstly, it allows for assessing the AMR burden at the community level, which could provide a comprehensive, unbiased picture of the burden and diversity of AMR genes circulating in a community without the need to test thousands of individuals. Secondly, it supports the tracking of resistance trends and the emergence of novel resistant determinants and serves, in a way, as an early warning tool. The outbreak or epidemic alert obtained from WBS could trigger targeted investigations and interventions before it becomes a widespread clinical problem. Thirdly, WBS could help to evaluate the impact of interventions such as antibiotic stewardship programs, which are aimed at reducing inappropriate use of antibiotics. Fourthly, WBS could have significant public health implications, because it enables us to identify disease hotspots (e.g., specific neighbourhoods, hospitals, nursing homes, or agricultural runoff points) and could, for example, enforce the disinfection of highly contaminated industrial wastewater before disposing of such wastewater into municipal wastewater systems. Finally, WBS could be crucial for determining disease 'X' that could be responsible for the next pandemic. It generally enables us to better understand the resistome and microbiome using the "One Health" approach. The public health utility of WBS has been significantly demonstrated for non-AMR targets in its ability to track variants of concern of the SARS-CoV-2 and community vaccine-derived polioviruses. WBS has been a key tool used by WHO and national public health institutes to track the virus (and its variants) in the global war to eradicate polio.

It is essential, regarding policy recommendations, to:

1. Establish standardised methods, indicators, and data linkage across sectors.

Governments should mandate and adopt harmonised protocols for sampling (locations, frequency), laboratory analyses (culture-based, molecular methods, metagenomics), and quality assurance to make WBS actionable for AMR surveillance. They should define priority targets (e.g., WHO priority pathogens, ARGs of high clinical relevance, mobile genetic elements) so that WBE data are clinically meaningful and comparable across regions. Moreover, data from WBE must be systematically linked with clinical AMR surveillance, animal/food sector monitoring, and environmental data to allow calibration: e.g., comparing wastewater ARG abundances with rates of resistant infections, antibiotic usage data, and information about agricultural runoff. This helps translate environmental signals into public health risk.

2. Invest in infrastructure, capacity building, and sustainable funding, especially in low- and middle-income settings (LMICs).

Policy frameworks should include funding for upgrading wastewater treatment and sampling infrastructure, equipping laboratories, training personnel, and setting up data systems. LMICs often lack centralised sewer systems or have long delays due to shipping, degradation, or lack of local expertise. Policies should promote "in-country" capacity so that sample collection, processing and sequencing (or other analysis) can be performed locally, ensuring timely and reliable results (Tiwari et al., 2025; Truyens et al., 2025). Long-term planning and budget allocation (not only pilot projects) are required to maintain continuity, ensure data representativeness over time, and allow for trend analysis and early warning.

3. Create governance, ethical, and regulatory frameworks, including data sharing and privacy.

WBE captures population-level data; policies should therefore define who owns and shares results, how privacy concerns are handled, and how results may be used (e.g., for public health interventions, regulation of effluents). Regulatory frameworks should also set environmental discharge standards (limits) for AMR markers in wastewater and effluents, especially from healthcare facilities and industrial/agricultural sources. Integration across One Health sectors (human health, animal health, environment) must be built formally (e.g., via inter-agency committees or legislation). Finally, public transparency and community engagement should be included to build trust and ensure that WBE is used for protecting health rather than for unintended punitive or discriminatory interventions.

6.4 Future Directions

6.4.1 Integration within the One Health approach

Wastewater, by its very nature, is a confluence point for human, animal, and environmental waste, making it an ideal matrix for a holistic One Health approach. The future of WBS within a One Health approach is not merely about testing more wastewater samples. It is also about intentionally designing an interconnected bio-surveillance network that reflects the interconnectedness of human, animal, and environmental health. Leveraging wastewater as a unified diagnostic fluid allows us to gain an unparalleled, real-time understanding of the health threats that emerge at these interfaces, ultimately leading to a more proactive, predictive, and resilient global health system.

The following key future directions could be useful in attaining the public health utility of WBS.

1. **Developing Integrated Sentinel Surveillance Networks:** This will involve the creation of purpose-built WBS networks that strategically sample from interconnected catchments from multiple sources. This will include samples from existing municipal WWTPs, wastewater runoff from concentrated animal feeding operations (CAFOs) for pigs, poultry, and cattle, slaughterhouses, and processing plants. Drainage water from farms that use antibiotics and manure as fertiliser, effluent from large veterinary clinics or hospitals, groundwater sources near agricultural or livestock areas, and around wildlife refuges or areas with high

wildlife density, is also used to monitor for zoonotic spillover. The goal is to move from isolated studies to coordinated monitoring. This would allow officials to trace the flow and evolution of a pathogen or an ARG from its source, into a community, and eventually into a broader environment.

2. **Standardising Methods for Cross-Domain Comparison:** This will involve a major thrust towards international and cross-disciplinary methodological standardisation. For instance, emphasis must be placed on uniform sample collection, concentration, and diagnostic and analytical methodologies. The future requires developing One Health-optimised protocols that ensure data from any unit, a city's WWTP, and a river are directly comparable. Standardisation enables us to conduct data normalisation (e.g., using pepper mild mottle virus (PMMoV) or crAssphage for human faecal normalisation, and specific markers for animal faecal normalisation). The goal is to create a unified "language" for WBS data, enabling true integration and meaningful meta-analyses across the three One Health domains.

3. **Advanced Bioinformatics and Data Integration Platforms:** The importance of a uniform and comparable analytical pipeline cannot be overemphasised. The pipeline should be easy to use, incorporating metadata such as infection rates, livestock disease reports, antibiotic usage, water quality metrics, temperature, and rainfall in making inferences. Thereafter, Artificial Intelligence and Machine Learning models will be crucial to identify patterns, predict outbreaks, and pinpoint sources of AMR emergence or zoonotic spillover events. The future goal will be to move from data collection to predictive analytics.

4. **Targeted Surveillance at the Human-Animal-Environment Interface:** This should involve using WBS to actively monitor high-risk interfaces where spillover events are most likely to occur, such as wet markets and livestock auction sites, peri-urban areas, and wildlife migration routes. The goal will be to transform WBS into an early-warning radar system for pandemics, capable of detecting a potential zoonotic threat before it achieves efficient human-to-human transmission.

5. **Addressing Ethical and Governance Frameworks:** This involves the proactive development of international ethical guidelines and governance models for One Health WBS. This must be respected and be based on the principles of privacy, data sovereignty, and transparent and equitable data sharing between human health, agricultural, and environmental agencies, both nationally and internationally. It is also essential to define clear protocols for which agency is responsible for investigating and acting upon a signal detected in a shared environment. The goal is to build trust and cooperation between all sectors (public health, agriculture, and environment) and the public, which is essential for the system to function effectively and ethically.

6.4.2 Wastewater-based surveillance in resource-limited settings and animals

The future of sewage surveillance in resource-limited settings is one of immense potential, requiring context-specific, innovative, and equitable approaches. Unlike

high-income countries with extensive sewer networks, these regions must adapt WBS to their unique infrastructure, priorities, and constraints. The term wastewater and environmental surveillance (WES) is most appropriate and more frequently used in these settings. The future lies in developing decentralised, affordable, and integrated systems that address the most pressing local public health needs, from polio eradication to antimicrobial resistance (AMR).

The future directions could include:

1. **Adaptive Sampling Methodologies:** This involves a shift from sampling a centralised plant to a distributed network of high-density/high-risk hubs such as open drain networks, septic and sanitation trucks, and specific hotspots such as hospitals, large schools, universities, and prisons as sentinel sites for outbreak detection. Additionally, the development and usage of low-cost, robust, battery-free, and affordable passive samplers (e.g., Moore swabs, tampons, or filter-filled containers), which can be deployed in open drains or water bodies for hours or days to concentrate pathogens, is essential to eliminate the need for expensive automatic samplers.

2. Building In-Country Capacity and Laboratory Networks: This involves improving the ability for in-country analysis of samples rather than sending samples abroad for analysis. This will help establish sustainable, tiered laboratory networks within regions and countries. The Hub-and-Spoke model can be used for the short-term, where basic sample processing (filtration, concentration) can be done in local district labs (the "spokes"), and more complex genetic analysis (qPCR, sequencing) is done at a central national or regional reference lab (the "hub"). Additionally, it is vital to leverage existing platforms such as the WHO polio surveillance sentinel sites and sentinel labs, as well as the HIV/TB molecular testing labs. Finally, it is crucial to invest heavily in training a new generation of local public health professionals - engineers, lab technicians, bioinformaticians, and epidemiologists - in wastewater-based epidemiology (WBE).

3. Field-Deployable Analytical Techniques: This involves the development and validation of low-cost, rapid, and field-deployable diagnostic tools. These include LAMP-based Assays over traditional qPCR. LAMP is cheaper, faster, more robust and can be performed with portable, battery-operated devices, enabling near-point-of-care wastewater testing. Microfluidic and paper-based diagnostics for novel diagnostic assays that can also detect multiple pathogens or AMR genes from small, crude wastewater samples without complex lab infrastructure should be encouraged. It is also essential to develop targeted multiplex panels that could simultaneously detect the top 10-20 priority pathogens or ARGs for that country.

4. **Data for Action:** This involves focusing on generating data that directly informs in-country public health action and policy. This enables easy integration with clinical surveillance, because wastewater data must not exist in a vacuum. There must also be a clear framework to transparently communicate protocols, results, and feedback(s) to laboratory staff, policymakers, healthcare workers, and the public, without stigma, especially when a hotspot is identified.

5. **Addressing Ethical and Logistical Challenges:** This involves proactively tackling the unique ethical and practical hurdles in each country, with special focus on establishing ethical guidelines for sampling in informal settlements to avoid community stigmatisation. Regarding logistical challenges, it is essential to demonstrate in-country that WBS can be more cost-effective than large-scale individual testing, especially for tracking public health emergencies like typhoid, cholera, and AMR, to secure sustained government and donor funding.

6.4.3 Sewage surveillance for animal wastewater

The application of WBS for animal disease surveillance is a rapidly emerging frontier with tremendous potential to reshape veterinary public health, biosecurity, and our understanding of disease dynamics within animal populations. The core principle remains the same as for human health: monitoring effluents from facilities where animals are congregated. The future lies in rapidly moving from proof-of-concept studies to integrated, operational systems that provide actionable intelligence. This involves the deliberate creation of a network of sentinel sampling sites across high-value animal hotspots. This will help to rapidly detect transboundary, notifiable, and epidemic-prone pathogens like Foot-and-Mouth Disease virus (FMDv), African Swine Fever virus (ASFv), Avian Influenza (AI), or Bovine Respiratory Disease complex before clinical signs appear, enabling pre-emptive isolation and treatment. It could also assist in herd health management and in evaluating on-farm biosecurity status by verifying the pathogen-free status of a herd for trade purposes. It could also help identify the impact and association of animal microbiota with animal nutrition indices and feed conversion rates in domesticated animals. Just like in human health, it could also help evaluate chemical and medicinal bye – by-products (metabolites) in the animal wastewater. Innovative investment models are needed for developing diagnostic advancements, especially through cost-effective, rapid, and pathogen-specific assays for high-consequence animal diseases. It is also essential to establish data analytics and decision support systems that transform raw animal wastewater data into actionable insights for farmers, veterinarians, and policymakers. We recommend creating user-friendly dashboards for farmers and vets to monitor their herd's health via wastewater data to promote the acceptability of WBS data. Improving the widespread adoption of wastewater data, demonstrating return on investment, as well as establishing clear protocols for data ownership and privacy, could help to institutionalise animal wastewater systems. Finally, we are proposing that, in the near future, regulatory authorities such as the WOAH should define and accept how WBS data can be used for official disease status declarations and trade agreements. We can create a healthier, more secure, and more sustainable future for animal agriculture and global public health by building integrated, efficient, and ethically sound wastewater surveillance systems.

7 Conclusions

1. Genomic wastewater-based surveillance (WBS) detected the same methicillin-resistant *Staphylococcus aureus* (MRSA) sequence types and spa types from wastewater treatment plants (WWTP) and human clinical / screening cases in Finland (Study I).
2. There was significant clustering of wastewater and human isolates with a higher prevalence of MRSA ST6 and ST8 (Study I), *E. coli* ST131 (Study II) and *E. faecium* ST80 (Study III).
3. Genomic WBS has the potential to provide complementary data and detect circulating strains of Extended Spectrum Beta Lactamase (ESBL)-producing *E. coli*, MRSA, and *VRE.fm* (Studies I, II, III).
4. Priority WBS antimicrobial resistance (AMR) pathogens could be used as a supplementary tool for epidemiological inference, assist public health authorities in assessing the evolution and epidemiology of these infections, and identify local drivers and risk factors (Studies I, II, III).
5. Future directions require a systems change that incorporates WBS in routine bio-surveillance with harmonised methods within One Health sectors.

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