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Extended-Spectrum Beta-Lactamase-producing *Enterobacteriaceae* through the lens of One Health in the Whole Genome Sequencing Era

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ACADEMIC DISSERTATION

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Not all those who wander are lost. - J.R.R. Tolkien

ABSTRACT

Antimicrobial resistance (AMR) is recognized as one of the most urgent global public health threats facing humanity today. The increasing number of deaths related to bacterial infections untreatable with antibiotics demands new and robust methods for studying and controlling the spread of AMR. Antimicrobial resistance, an ancient phenomenon at its core, has been affected by the discovery and profuse use of antibiotics in different sectors. Antibiotic use has been a key driver behind the successful spread of new resistance determinants in the microbial world.

The complex nature behind the epidemiology of AMR stems from versatile transmission mechanisms employed by microorganisms and mobile genetic elements (MGEs), and the interwoven web of potential transmission routes between humans, animals, and the environment. Horizontal transmission of resistance genes via MGEs, especially plasmids, has been essential in the successful dissemination of resistance genes globally. A One Health approach is required to understand the complexity of AMR.

Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBL/AmpC) and carbapenemases are among the most critical resistant pathogens globally, as these threaten the effectiveness of commonly used beta-lactam antibiotics used widely in modern medicine. ESBL-producing *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella pneumoniae*, are leading causes of both hospital-associated and community-acquired antibiotic-resistant infections. The localization of genes encoding for extended-spectrum beta-lactamases on plasmids enables transfer even between bacteria of different species. Advances in whole genome sequencing (WGS) have aided the discovery of new resistance determinants, made surveillance more accurate and rapid, and offered new powerful tools to interpret the epidemiology and transmission routes of bacteria and MGEs.

The aim of this thesis was to study the occurrence and epidemiology of ESBL/AmpC-producing *Enterobacteriaceae* in different sources in Finland including broiler production, food products, migratory birds, and human clinical samples. Using WGS, bacterial strains were characterized and compared to bacterial strains and plasmids from global databases to discover potential successful plasmids and resistance genes and bacterial sequence types. Furthermore, ESBL-producing *E. coli* from human clinical isolates from Finland were characterized to assess the similarity between strains of different human and non-human origins and assess potential transmission sources.

Poultry and broiler meat have been recognized as a reservoir for ESBL/AmpC-producing *E. coli* worldwide. In Finland, antibiotics have not been used in broiler production for over a decade, but nevertheless ESBL/AmpC-producing bacteria have been discovered in the production chain. To study the occurrence and transmission routes of ESBL/AmpC-producing *E. coli* in the broiler production pyramid, samples were taken from different stages of the

production pyramid. ESBL/AmpC-producing *E. coli* was detected in 26.7% of parent level birds, but ESBL/AmpC-producing *E. coli* was absent from egg surfaces after an incubation period at a hatchery, and *E. coli* was very rare in hatchlings (2.2%). The findings indicate the transmission routes of these bacteria in the production pyramid are a combination of horizontal and vertical routes, rather than strictly vertical.

The global nature of AMR was further studied by sampling migratory birds and imported food products. Fecal samples were collected from barnacle geese (*Branta leucopsis*) on two occasions in the southern part of Finland. Plasmids considered as internationally successful were recognized from barnacle geese feces and food products. Furthermore, a rare multireplicon plasmid was identified from *E. coli* from barnacle geese, indicating the adaptive nature of plasmids harboring resistance genes. ESBL/AmpC-producing *E. coli* was found in 4.5% of the barnacle goose fecal samples, mirroring the limitedly studied prevalence in the asymptomatic human population in Finland. Investigating the occurrence of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* in global food products demonstrated raw broiler meat as a potential source for these resistant bacteria. ESBL genes, such as $bla_{\text{CTX-M-15}}$, were recognized in certain food products together with human-associated *K. pneumoniae* multilocus sequence types, indicating a possible human-related source of transmission.

Study of ESBL-producing *E. coli* isolates from human patients in Finland with WGS identified $bla_{\text{CTX-M-27}}$ as the most common ESBL gene. This demonstrates the spread of globally successful subclade ST131-C1-M27 and supports the notion of a shift in the most dominant CTX-M enzymes in humans. Core genome multilocus sequence typing and comparison of the isolates suggested human-derived ESBL-producing *E. coli* isolates are distinct from ESBL/AmpC-producing *E. coli* isolates obtained from animal, food, and environmental sources in Finland. The ever-evolving global health pandemic of AMR demands for a combined effort of different sectors and continuous monitoring, in which WGS has proved to be invaluable.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals:

I Oikarainen, P. E., Pohjola, L. K., Pietola, E. S., and Heikinheimo, A. (2019). Direct vertical transmission of ESBL/pAmpC-producing *Escherichia coli* limited in poultry production pyramid. Veterinary Microbiology 231, 100–106. doi:10.1016/j.vetmic.2019.03.001.

II Kurittu, P., Khakipoor, B., Brouwer, M. S. M., and Heikinheimo, A. (2021). Plasmids conferring resistance to extended-spectrum beta-lactamases including a rare IncN+IncR multireplicon carrying $bla_{CTX\text{-}M\text{-}1}$ in *Escherichia coli* recovered from migrating barnacle geese (*Branta leucopsis*). Open Research Europe 1, 46. doi:10.12688/openreseurope.13529.1.

III Kurittu, P., Khakipoor, B., Aarnio, M., Nykäsenoja, S., Brouwer, M., Myllyniemi, A.-L., et al. (2021). Plasmid-borne and chromosomal ESBL/AmpC Genes in *Escherichia coli* and *Klebsiella pneumoniae* in global food products. Frontiers in Microbiology 12, 125. doi:10.3389/fmicb.2021.592291.

IV Kurittu, P., Khakipoor, B., Jalava, J., Karhukorpi, J., and Heikinheimo, A. (2022). Whole-genome sequencing of extended-spectrum beta-lactamase-producing *Escherichia coli* from human infections in Finland revealed isolates belonging to internationally successful ST131-C1-M27 subclade but distinct from non-human sources. Frontiers in Microbiology 12, 4142. doi:10.3389/fmicb.2021.789280.

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ABBREVIATIONS

AGISAR	Advisory Group on Integrated Surveillance of Antimicrobial Resistance
AMR	Antimicrobial resistance
AmpC	Ambler Class C beta-lactamase
ATCC	American Type Culture Collection
bla	Beta-lactamase gene
bp	Base pair
BPW	Buffered peptone water
BLAST	Basic Local Alignment Search Tool
CAZ	Ceftazidime
CC	Clonal complex
CDS	Coding sequence
CFO	Cefoxitin
CFU	Colony-forming unit
cgMLST	Core-genome multilocus sequence typing
CI	Confidence interval
CLA	Clavulanic acid
CTX	Cefotaxime-hydrolyzing β-lactamase isolated in Munich
CTX-M	Cefotaximase from Munich
DNA	Deoxyribonucleic acid
ECDC	European Centre of Disease Control
EFSA	European Food Safety Agency
ENA	European Nucleotide Archive
ESBL	Extended-spectrum beta-lactamase
ESKAPE	*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FEP	Cefepime
GAP-AMR	Global Action Plan on Antimicrobial Resistance
GES	Guiana-extended spectrum
HGT	Horizontal gene transfer
IMP	Imipenemase
Inc	Incompatibility
KPC	*Klebsiella pneumoniae* carbapenemase
OHHLEP	One Health High Level Expert Panel
MALDI-TOF	Matrix-assisted laser desorption ionization–time of flight mass spectrometry
MDR	Multidrug resistant
MGE	Mobile genetic element

MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MRP	Meropenem
NAP	National Action Plan
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NDM	New Delhi metallo-beta-lactamase
NGS	Next-generation sequencing
OXA	Oxacillinase
pAmpC	Plasmidic AmpC
PCR	Polymerase chain reaction
PER	Pseudomonas extended resistant
PBRT	PCR-based replicon typing
pMLST	Plasmid multilocus sequence typing
RST	Replicon sequence typing
SBS	Sequencing by synthesis
SHV	Beta-lactamase named after sulphydryl variable
SNP	Single-nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
ST	Sequence type
TEM	Beta-lactamase named after Temoniera
VEB	Vietnam extended-spectrum beta-lactamase
VIM	Verona integron-encoded metallo-beta-lactamase
WOAH	World Organisation for Animal Health
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug resistant

1 INTRODUCTION

Antimicrobial resistance (AMR) is considered one of the leading global public health threats worldwide. Infections with antibiotic-resistant bacteria cause increased mortality and morbidity, prolonged hospital stays and increased health-care costs (Ray et al., 2018). The burden of AMR is divided unequally, often affecting low-income countries with the most severe impact (Murray et al., 2022).

Gram-negative bacteria belonging to the family *Enterobacteriaceae*, especially *Escherichia coli* and *Klebsiella pneumoniae*, are the most prevalent in bacterial infections untreatable with beta-lactam antibiotics due to the production of extended-spectrum beta-lactamases (Brolund, 2014). *E. coli* and *K. pneumoniae* are found in the intestinal tract of humans and animals and are common causative bacteria in hospital-associated as well as community-acquired infections (Devi et al., 2020; Murray et al., 2022).

Extended-spectrum beta-lactamases include the ESBL and AmpC type enzymes, as well as carbapenemases, conferring resistance to extended-spectrum beta-lactams and in addition carbapenems, often used as the last resort antibiotics to treat severe infections in humans (EFSA, 2013; Castanheira et al., 2021). Extended-spectrum beta-lactams, such as third- and fourth-generation cephalosporins, are classified as the most critical antimicrobials for human medicine (WHO, 2019). The past few decades have seen a rapid increase in AMR worldwide, highly attributable to epidemic plasmids enabling the horizontal gene transfer of resistance determinants between bacteria in different environments (Carattoli, 2013; Mathers et al., 2015; Rozwandowicz et al., 2018).

ESBL/AmpC-producing *Enterobacteriaceae* have been isolated from many animal, food, and environmental sources such as wildlife and wastewaters, raising concerns for the potential contribution of these sources to human infections (Bréchet et al., 2014; Huijbers et al., 2015; FAO and WHO, 2019; Koutsoumanis et al., 2021). Especially poultry and poultry meat have been recognized as a reservoir for ESBL/AmpC-producing *E. coli*, and studies have found genetic similarities in resistance genes and plasmids from poultry-derived and human isolates (de Been et al., 2014; Huijbers et al., 2014; Roer et al., 2019). The successful spread of ESBL/AmpC-producing *Enterobacteriaceae* is also considered at least partly attributable to international travel (van der Bij and Pitout, 2012; Holmes et al., 2016; Woerther et al., 2017). ESBL/AmpC-producing and carbapenem-resistant *Enterobacteriaceae* have been detected in imported food products (Hasman et al., 2015; Zurfluh et al., 2015b, 2015a; Boss et al., 2016; Janecko et al., 2016; Ellis-Iversen et al., 2019). Vast differences are seen in the use of antimicrobials in food-producing animals between different countries (EMA, 2021). In addition to people and goods crossing country borders, wildlife such as migratory birds may roam long distances between different continents,

potentially disseminating AMR (Bonnedahl and Järhult, 2014; Dolejska and Papagiannitsis, 2018).

The last few decades have seen advances in whole genome sequencing (WGS) based methods, enabling accurate determination of study of genetic similarities between bacterial isolates and plasmids originating from different sources. WGS has proven an important tool for AMR detection and surveillance (Duggett et al., 2020). Dissemination of antimicrobial resistance genes, often via successful epidemic plasmids, presents a complex issue that demands continuous efforts to track and study the evolving field of AMR.

2 REVIEW OF THE LITERATURE

2.1 Antimicrobial resistance in a One Health context

Less than 20 years after the discovery of penicillin, Sir Alexander Fleming warned about the dangers of antimicrobial resistance in his Nobel acceptance speech in 1945 (Fleming, 1945):

"... It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body. The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant..."

The notion that selective pressure mediated by antimicrobial concentration in an organism's environment affects microbial populations holds true, even if antimicrobial resistance genes have been found to predate the use of antibiotics, proving that the phenomenon is ancient and occurs naturally in the environment (Dcosta et al., 2011). Nevertheless, the use of antimicrobials has accelerated the emergence and spread of antimicrobial resistance (AMR), both in human and animal populations (Holmes et al., 2016), often by the acquisition of acquired resistance determinants. Acquired AMR mechanisms are often encoded on genetic elements outside the chromosome, such as plasmids, and transmitted via horizontal gene transfer (HGT) (Carattoli, 2013; Peterson and Kaur, 2018). Examples of acquired resistance include plasmid-encoded genes for efflux pumps and enzymes that are able to degrade antibiotic molecules rendering them ineffective, or modify the target for an antibiotic, escaping the effect (Peterson and Kaur, 2018). Intrinsic mechanisms of bacteria for curbing antimicrobial effects are often encoded on the chromosome and include natural characteristics of the organism, such as permeability barriers (Fajardo et al., 2008).

Although an ancient phenomenon at its core, the last couple of decades have seen a rapid increase in AMR, and the World Health Organization (WHO) has declared AMR as one of the top ten global public health threats facing

humanity (WHO, 2021a). In 2017, the WHO published a priority list of bacteria for which new antibiotics are urgently needed (WHO, 2017). Classified among the highest, most critical priorities are carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as well as carbapenem-resistant and extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*. Similarly, the WHO has compiled a list for critically important antimicrobials for human medicine, which are to be saved for the treatment of human infections, to preserve their efficacy (WHO, 2019). Third-, fourth- and fifth-generation cephalosporins are at the top of the list.

Bacteria can confer resistance to extended-spectrum beta-lactams and carbapenems via multiple different mechanisms, including efflux pumps, reduced permeability, and inactivation by enzymes termed extended-spectrum β-lactamases (Sawa et al., 2020). Resistance to important antimicrobials hinders or prevents the treatment of bacterial infections and results in increased mortality, morbidity, health-care costs and significant economic losses for national economies. In addition to antimicrobial use, the spread of AMR is accelerated by factors such as poor sanitary conditions in both humans and animals, inadequate infection and disease prevention measures, and lack of education and enforcement of legislation (Holmes et al., 2016; Hendriksen et al., 2019). Human population increase triggers the demand for animal-source nutrition, especially LMICs, leading to increased antimicrobial use in food-producing animals (van Boeckel et al., 2015, 2019). Globally, food-producing animals receive the bulk of antimicrobials used, but large differences are seen between countries. Different countries have different legislations and recommendations. In the European Union, the use of antimicrobials as growth promoters in animals is prohibited, and in the United States the extra-label use of fluoroquinolones and third-generation cephalosporins in animals is restricted. Furthermore, antimicrobials have to be prescribed by a veterinarian in many countries (McEwen and Collignon, 2018).

Just as the prevalence of AMR and customs surrounding antimicrobial treatment in animals differ between countries, the burden of AMR is also not divided equally among continents and countries. On the contrary, low-income countries (LMIC) with low resources are most burdened with AMR (van Boeckel et al., 2019; Murray et al., 2022). A study using statistical modeling has predicted that there were 4.95 million deaths associated with bacterial AMR in 2019, from which 1.27 million were attributable to bacterial AMR. The highest death rate attributable to AMR was estimated to occur in western sub-Saharan Africa (27.3 deaths per 100 000) and the lowest in Australasia (6.5 deaths per 100 000) (Murray et al., 2022). It has been estimated that in the European Union/European Economic Area (EU/EEA) alone, more than 670 000 infections each year are caused by bacteria resistant to antimicrobials and 33 000 deaths are directly attributed to resistant infections (WHO, 2022). Surveillance data for many regions globally is lacking, due to factors such as poor documentation, inadequate regulations and their implementation and enforcement (Kuralayanapalya et al., 2013).

The need for robust and multisectoral surveillance programs has been noted and emphasized on an international level, and the United Nations with its agencies promotes a global, holistic, One Health approach to tackling the problem. WHO Member States have adopted a Global Action Plan on AMR (GAP-AMR), which requires member states to develop National Action Plans (NAPs) for combating AMR implementing a One Health approach. Furthermore, the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR) and the WHO Surveillance Prevention and Control of AMR have developed a protocol named 'Tricycle' for the global surveillance of extended-spectrum beta-lactamase-producing *Escherichia coli* from humans, animals, and the environment (WHO, 2021c). The protocol offers instruction on developing a multisectoral approach for AMR surveillance using minimal resources and aims to increase multisectoral collaboration. Collaboration between different sectors and financial resources such as laboratories, personnel, and finances are needed for effective surveillance. AMR determinants are often found on zoonotic bacteria able to colonize or infect both animals and humans and can move bi-directionally between both hosts. The environment, including surface waters, sewage, wildlife, and soil, among others, may also serve as reservoirs for resistant pathogens.

The idea of a multisectoral approach can be dated back hundreds of years. In ancient history, both people and animals have been cared for by the same healers and priests (Schwabe, 1964; Zinsstag et al., 2005). Although human and animal medicine studies ultimately diverged in the academic world, the rekindling of these two professions have been seen in the past centuries, such as in the 18th century when Claude Bourgelat, the founder of the first veterinary school in Lyon, France, suggested to include human clinical training in the curriculum, an idea that was nonetheless met with criticism (Zinsstag et al., 2005).

In the 20th century, Calvin Schwabe, the founder of the Department of Epidemiology and Preventive Medicine at the University of California Davis School of Veterinary Medicine, introduced the term 'One Medicine', the concept which has been known as 'One Health' from the first decade of the 21st century onwards (Schwabe, 1964; Atlas, 2012). Schwabe is considered the pioneer of veterinary epidemiology and known for combining techniques from human disease tracking in the study of animal diseases (Atlas, 2012).

Recently, the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH), the United Nations Environment Programme (UNEP) and the World Health Organization (WHO) have formed the One Health High Level Expert Panel (OHHLEP), which has defined One Health as follows (WHO, 2021b):

"*One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems.*

It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and inter-dependent.

The approach mobilizes multiple sectors, disciplines and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development."

A One Health approach can be understood as the collaboration between different sectors and professionals to work together for a common goal, the optimal health of people, animals, and the environment (McEwen and Collignon, 2018). As AMR involves all these sectors, a One Health approach for research, prevention and control measures is warranted, and the implementation can be seen in the surveillance programs at the national and international level.

2.2 Extended-spectrum beta-lactamases

2.2.1 A brief history

Antibiotics belonging to the beta-lactam class are among the most used antibiotics worldwide (ECDC, 2021; Fisher et al., 2005; Bush and Bradford, 2016; Browne et al., 2021). Following the discovery of benzylpenicillin by Alexander Fleming in the 1920s (Fleming, 1929), a substantial amount of new penicillin derivates and related antibiotics representing cephalosporins, carbapenems, monobactams, and cephamycins have been added to the beta-lactam repertoire (Worthington and Melander, 2013; Bush and Bradford, 2016). The armament race between beta-lactam antibiotics and resistant bacteria has been in place since the very beginning of antibiotic discovery. Even before the wide adoption of penicillin to treat infections, the first beta-lactamase was observed in *E. coli* (Abraham and Chain, 1940). Many Gram-negative bacteria are naturally resistant to beta-lactams through chromosomally coded enzymes, which have been hypothesized to be of penicillin-binding protein (PBP) origin, originally formed to protect bacteria from beta-lactams produced by soil bacteria in the environment (Ghuysen, 1991; Bradford, 2001). However, due to the rapid spread of resistant determinants by mobile genetic elements, it is the plasmid-mediated antimicrobial resistance that is considered a more serious plight.

In the 1960s, the first plasmid-mediated beta-lactamase from *E. coli*, termed 'TEM-1' due to its origin from a patient named Temoniera, was discovered in Greece (Datta and Kontomichalou, 1965; Med, 1984). SHV-1 (standing for *sulphydryl variable*), or PIT-2 (for the researcher 'Pitton' who described the enzyme in 1972), was also among the first beta-lactams discovered in Gram-negative bacteria, (Pitton, 1972; Bush, 2018). SHV-1 was originally found in *K. pneumoniae* encoded in the chromosome but has been identified mostly from plasmids in *E. coli* (Bradford, 2001). As new beta-lactams were introduced, new beta-lactamases emerged, reasserting the role of selective pressure and overuse of antimicrobials as drivers behind AMR. In the 1980s, oxyimino-cephalosporins were widely used for serious bacterial infections, which was

followed by the discovery of the first extended-spectrum beta-lactamase (ESBL), SHV-2, from clinical *Klebsiella* and *Serratia marcescens* isolates in Germany in 1983 (Knothe et al., 1983; Bradford, 2001). Compared to SHV-1, this enzyme was found to have a single amino acid substitution, glycine replaced by serine at position 238 (Barthélémy et al., 1988). The third-generation cephalosporins, such as cefotaxime and ceftazidime, were originally introduced in the 1980s to treat infections caused by ampicillin hydrolyzing TEM-1 and SHV-1-producing *E. coli* and *K. pneumoniae*, but also to counteract the spread of beta-lactam resistance to other pathogens, such as *Haemophilus influenzae* and *Neisseria gonorrhoeae* (Paterson and Bonomo, 2005; Tooke et al., 2019).

Originally described with various differing names, CTX-M type extended-spectrum beta-lactamases emerged in different parts of the world in the mid 1980s (Bonnet, 2004). The name CTX-M was used in a German study, which deemed the enzyme *cefotaximase from Munich* (Bauernfeind et al., 1990). CTX, referring to cefotaximase, stems from the enzymes' higher level of hydrolytic activity against cefotaxime than against ceftazidime (Jacoby, 1994; Bonnet, 2004). Phylogenetic analyses suggest enzymes in the CTX-M family are derived from chromosomal *bla* genes from *Kluyvera* spp. from which they were incorporated into mobile genetic elements (Cantón et al., 2008). Replacing TEM and SHV, CTX-M type beta-lactamases have become the most dominant enzyme type worldwide since the early 2000s and have been isolated from multiple sources, including both nosocomial environments and communities, companion and food-producing animals, food products, and the environment (Castanheira et al., 2021). Furthermore, CTX-M have been detected in many members within the order Enterobacterales, *Pseudomonas aeruginosa,* and *Acinetobacter spp*., (Castanheira et al., 2021), but the family *Enterobacteriaceae* remains the most conjoined with these enzymes (Cantón et al., 2012b). CTX-M-15 has been recognized as the dominant enzyme type from human-derived isolates in most parts of the world, whereas CTX-M-9 variant, CTX-M-14, is prevalent in Asia and Spain, and CTX-M-2 is still prevalent in South America (Bevan et al., 2017). In China, also CTX-M-55 is increasingly detected in both animals and humans, suggesting an animal origin for human occurrence (Zhang et al., 2014; Bevan et al., 2017). Starting in the late 2000s in Japan, CTX–M–27 has recently been noted to gain a foothold in rivaling the globally dominant CTX–M–15 in many parts of the world, and has been found from human, animal, food, and environmental sources in Europe, North America, and Asia (Bevan et al., 2017).

Even before the discoveries of the TEM, SHV, and CTX-M families, the first beta-lactamase was actually found from an *E. coli* in 1940 (Abraham and Chain, 1940). Termed AmpC later, these enzyme's genes were originally located in the chromosome of Gram-negative bacteria, but have become in large part plasmid-mediated since the 1980s, speeding up global dissemination (Doi and Paterson, 2007). In addition to TEM, SHV, and CTX-M, several additional ESBL families have been described, including unrelated GES (Guiana-extended spectrum) detected mostly in *P. aeruginosa*, PER (Pseudomonas extended resistant) more prevalent in *P. aeruginosa* and *A.*

baumannii than Enterobacterales, and VEB (Vietnam extended-spectrum β-lactamase) (Bradford, 2001; Castanheira et al., 2021).

Carbapenemases are extended-spectrum beta-lactamases with the broadest hydrolysis spectrum, hydrolyzing beta-lactams including carbapenems (Queenan and Bush, 2007; Cantón et al., 2012a). The first carbapenemase in *Enterobacteriaceae* (NmcA) was identified in 1993 (Naas and Nordmann, 1994), and the first plasmid-mediated carbapenemase, KPC (for *Klebsiella pneumoniae* carbapanemase) in 2001 (Yigit et al., 2001). Since then, KPC-producing strains have been identified globally from *Enterobacteriaceae*, with emerging new carbapenemase families, including NDM (New Delhi metallo-beta-lactamase), IMP (imipenemase), and VIM (Verona integron-encoded metallo-beta-lactamase), each with distinct geographical predilections (Queenan and Bush, 2007; Cantón et al., 2012a; Cui et al., 2019).

2.2.2 Definition of extended-spectrum beta-lactamases

Extended-spectrum beta-lactamases include enzymes referred to as ESBLs (for extended-spectrum beta-lactamases), AmpC beta-lactamases and carbapenems. Although there is no final consensus for the exact definition, ESBLs are commonly defined as beta-lactamases capable of hydrolyzing the penicillins, first-, second-, third-, and fourth-generation cephalosporins, and monobactam (aztreonam), but usually not cephamycins or carbapenems. In addition, they are inhibited by beta-lactamase inhibitors such as clavulanic acid, and traditional ESBLs are also inhibited by other older inhibitors sulbactam and tazobactam, as well as newer inhibitors (e.g., avibactam, vaborbactam, and relebactam) (Paterson and Bonomo, 2005; Castanheira et al., 2021). It has been proposed that any enzyme hydrolyzing oxyimino-beta-lactams could be considered an ESBL, but to be classified as an ESBL, an enzyme must be inhibited by clavulanic acid (Castanheira et al., 2021). Newer fifth-generation cephalosporins, such as ceftaroline targeted especially towards methicillin-resistant *Staphylococcus aureus* infections, differ in their activity against different ESBL- and AmpC-producing bacterial organisms (Duplessis and Crum-Cianflone, 2011; Corcione et al., 2021). Genes coding for ESBLs often reside in mobile genetic elements, such as plasmids (Rozwandowicz et al., 2018; Castanheira et al., 2021).

AmpC type beta-lactamases usually confer resistance to penicillins, second- and third-generation cephalosporins including beta-lactam inhibitor combinations, and cephamycins, but usually not to fourth-generation cephalosporins and carbapenems. Resistance conferred by AmpC type beta-lactamases can be due to an inducible, chromosomal resistance triggered by the presence of beta-lactam compounds (especially in species such as *Enterobacter cloacae*, *Serratia marcescens*, *Citrobacter freundii*, and *Pseudomonas aeruginosa*), mutations in *ampC* regulatory genes (in species such as *E. coli*, *Shigella* spp., and *Acinetobacter baumannii*), or the presence of plasmid-mediated *ampC* genes (in species such as *K. pneumoniae*, *E. coli*, and *Salmonella* spp.) (Tamma et al., 2019). Plasmidic AmpCs are referred to as pAmpC.

Carbapenems are antibiotics used to treat serious infections in humans, which makes the increasing carbapenem resistance a worrisome trend, since carbapenemases hydrolyze all beta-lactams including the carbapenems and frequently aztreonam (EFSA, 2013).

Several phenotypic tests based on the synergy between third-generation cephalosporins and clavulanate have been developed for the detection of extended-spectrum beta-lactamase-producers. These include the double-disk synergy test, combination disk method, and ESBL Etest (Drieux et al., 2008). Guidelines often recommend a two-step method where susceptibility is screened for with an indicator cephalosporin and subsequently non-susceptible isolates are further examined to confirm ESBL/AmpC-production with a synergy test (Drieux et al., 2008).

2.2.3 Classification

In addition to the patchwork-like nomenclature of beta-lactamases, their classification can also be viewed as multifaceted. Two schemes are commonly used: the Ambler classification scheme and the Bush-Jacoby-Medeiros classification system (Ambler et al., 1991; Bush et al., 1995; Paterson and Bonomo, 2005).

In the Bush-Jacoby-Medeiros classification scheme beta-lactamases are divided into four main groups and multiple subgroups based on their functional similarities of substrate and inhibitor profiles (Bush and Jacoby, 2010). In the Ambler scheme, beta-lactamases are divided into four major classes (A, B, C, and D) based on their amino acid similarity and also their hydrolytic mechanism. Classes A, C, and D represent serine beta-lactamases, whereas enzymes in class B represent metallo-beta-lactamases. Serine beta-lactamases use serine at the enzyme’s active site, whereas class B enzymes use metal zinc ions. Prominent and widely disseminated enzyme families in class A include TEM, SHV, CTX-M and KPC, NDM, and VIM in class B, and CMY in class C (Tooke et al., 2019). Ambler class C beta-lactamases, also known as AmpC cephalosporinases, have a similar structure to class A beta-lactamases, with the exception that class C enzymes are better adapted for larger side chains of cephalosporins (Jacoby, 2009). Class D beta-lactamases include oxacillinase (OXA) beta-lactamases, with particular concern addressed towards enzymes conferring carbapenem resistance, such as OXA-48 (Tooke et al., 2019). Interestingly, modern phylogenetic analyses have showed that OXA-type beta-lactamases were already present in plasmids millions of years ago (Barlow and Hall, 2002).

2.2.4 Mode of action

Beta-lactamases confer resistance to beta-lactam antibiotics by hydrolyzing the beta-lactam ring found in these compounds (Bush, 2018). As discussed above, beta-lactamases can be divided into two groups based on this hydrolysis process. Hydrolysis is achieved either by formation of an acyl enzyme with an

active-site serine, or by one or two zinc ions in the active site enabling the hydrolytic reaction (Bush, 2018).

The -CO-NH structure of the beta-lactam ring mimics the alanyl-alanine dimer involved in the cross-linkage of building the peptidoglycan pillar structure on bacterial cell walls (Tooke et al., 2019). Formation of peptidoglycan transpeptide cross-links is inhibited, since the beta-lactam ring reacts with penicillin-binging proteins (PBPs), resulting in PBP acylation and death of the bacterial cell. PBPs, vital for the peptidoglycan cross-linking process of bacteria, and beta-lactamases have, in fact, been found to share similar structures, suggesting PBPs are the precursors of beta-lactamases, or that they have co-evolved (Bush, 2018; Tooke et al., 2019; Sawa et al., 2020).

2.3 *Enterobacteriaceae* as a reservoir for extended-spectrum beta-lactamases

The family *Enterobacteriaceae* is the bacterial family that is mostly associated with the production of extended-spectrum beta-lactamases, and species *E. coli* and *K. pneumoniae* from this family are the most important and prevalent in AMR infections and their spread (Brolund, 2014). *K. pneumoniae* belongs to a bacterial group termed ESKAPE (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) comprising major causative agents for serious bacterial infections in hospital settings worldwide with growing numbers of multidrug resistance and virulence (Rice, 2008; Mulani et al., 2019).

E. coli has been identified as one of the leading pathogens for human deaths associated with AMR, and the leading pathogen for most deaths attributable to AMR in 2019 (Murray et al., 2022). Furthermore, third-generation cephalosporin-resistant *E. coli* was calculated to attribute to 50 000 – 100 000 deaths globally in 2019 (Murray et al., 2022). Following closely behind, carbapenem-resistant *K pneumoniae*, and third-generation cephalosporin-resistant *K pneumoniae* were also each estimated to attribute to 50 000 – 100 000 deaths (Murray et al., 2022).

Infections caused by ESBL-producing *Enterobacteriaceae* depend on the host bacteria and its pathogenicity. Infections caused by bacteria harboring antimicrobial resistance genes are not more virulent per se, than susceptible strains, but resistant infections are more complicated to treat with a limited antibiotic arsenal, or in the case of pandrug-resistant bacteria, untreatable. An infection caused by ESBL/AmpC-producing *E. coli* or *K. pneumoniae* is typically preceded by intestinal colonization (Doi et al., 2017).

2.3.1 *Escherichia coli*

Discovery

In 1885, a German pediatrician Theodor Escherich described *Bacterium coli commune*, a bacterium population inhabiting the lower part of neonate intestinal tract (Escherich, 1885; Hacker and Blum-Oehler, 2007; Méric et al., 2016). Discovered during his work on neonatal dysentery, this organism, later termed *Escherichia coli*, has been pivotal in the field of microbiology, and central to multiple Nobel Price-awarded studies (Hacker and Blum-Oehler, 2007; Dunne et al., 2017). A highly versatile organism, *E. coli* has been harnessed as a workhorse in the laboratory, contributing to studies from fundamental microbiology to applications including genetic engineering and biotechnology, pharmaceuticals, evolution, and physiology (Blount, 2015; Kneifel and Forsythe, 2017).

Common properties

Escherichia coli are Gram-negative, lactose-fermenting, non-sporulating, facultative anaerobes residing in the gastrointestinal tract of warm-blooded animals and reptiles, found especially in the caecum and the colon of the large intestine (Tenaillon et al., 2010). Despite its commensal role in the microbiota of the gut, *E. coli* doubles as an opportunistic pathogen (Denamur et al., 2021), and pathogenic strains exist as well (Kaper et al., 2004). Outnumbered by anaerobes with a ratio of 100:1 to 10,000:1, *E. coli* still represents the most common aerobic bacterium found in the gastrointestinal tract with a prevalence of over 90% in the human population (Tenaillon et al., 2010). One gram of feces contains between 10^7 to 10^9 colony-forming units (CFU).

E. coli are typically rod shaped with a size of 2.0 – 6.0 µm in length and 1.1 – 1.5 µm in width, with motility attributed to peritrichous flagella (Percival and Williams, 2013). *E. coli* are taxonomically classified in the class *Gammaproteobacteria*, order *Enterobacterales* and family *Enterobacteriaceae* (Liu, 2019). The genome size varies from 4.2 to 6.0 Mbp, comprising a circular chromosome and typically from two to four plasmids per strain (Denamur et al., 2021). Due to recurrent DNA deletions and acquisitions, the gene count differs from 3,900 to 5,800 genes, with approximately 2,000 genes constituting the conserved core genome (Vila et al., 2016; Denamur et al., 2021).

Due to its natural habitat, *E. coli* is used as an indicator organism for fecal contamination (Tenaillon et al., 2010), although some strains have been found to survive and reproduce in extraintestinal environments such as soil and freshwaters (Jang et al., 2017; Abram et al., 2021).

Typing

Several techniques can be applied to study *E. coli* population genetics (Tenaillon et al., 2010), including methods based on the phenotype or genotype (Fratamico et al., 2016). Serotyping, a traditional phenotype-based

typing method, is based on approximately 186 different O-polysaccharide antigens, 53 flagellar H-antigens, and over 60 capsular K-antigens, resulting in numerous different serotypes (Kauffman, 1947; Tenaillon et al., 2010; Fratamico et al., 2016). However, defining the serotype based on O and H antigens became the custom due to laboratories' differing resources to type K antigens (Denamur et al., 2021). Another phenotype-based tool for studying the population structure, multilocus enzyme electrophoresis (MLEE), was developed in the 1980s (Selander and Levin, 1980), followed by a genotypic method, multilocus sequence typing (MLST), in the 1990s (Maiden et al., 1998; Enright and Spratt, 1999). Based on determining the alleles for seven or eight housekeeping gene loci (Table 1), three different MLST schemes exist for *E. coli* (Clermont et al., 2015). Developed for pathogenic *E. coli*, the *Ec*MLST database is curated by the STEC Center at Michigan State University (Qi et al., 2004), while the more commonly used Warwick scheme developed by Mark Achtman is hosted at the Warwick Medical School (Coventry, UK) (Wirth et al., 2006). Exhibiting the highest nucleotide diversity (Clermont et al.), the Pasteur scheme developed by Sylvain Brisse and Erick Denamur is hosted at the Pasteur Institute (Paris, France) (Jaureguy et al., 2008).

Developments in especially genetic-based typing and subtyping methods occur constantly, and at least 17 different methods are used to type *E. coli*, being variably adopted by different geographic locations and laboratories (Rivas et al., 2015). In addition to the above discussed methods, pulsed field gel electrophoresis (PFGE) is a commonly used tool for various foodborne pathogens and is considered the golden standard for typing (Rivas et al., 2015). Another common typing method is multiple-locus variable-number tandem repeats analysis (MLVA), which has become increasing popular due to high-throughput, high-resolution and excellent reproducibility (Rivas et al., 2015). Many other typing tools exists, such as broadly-available PCR methods, including a method using enterobacterial repetitive intergenic consensus (ERIC) sequences as primer binding sites for PCR amplification, but with varying reproducibility and discriminatory power (Rivas et al., 2015; Fratamico et al., 2016).

Furthermore*, E. coli* can be divided into different phylogenetic groups, each with differing metabolic characteristics and virulence and antimicrobial resistance gene profiles (Abram et al., 2021). This division originally consisted of four groups (A, B1, B2, and D) based on triplex PCR, a technique introduced in 2000, which uses genetic markers *chuaA*, *yjaA,* and a DNA fragment TspE4.C2 representing a putative lipase esterase gene (Clermont et al., 2000; Gordon et al., 2008; Tenaillon et al., 2010). Later, more phylogroups (C, F, and E) and subdivisions have been introduced and previous groupings restructured (Abram et al., 2021).

Molecular typing methods have largely been replaced or complimented by newer sequence-based technologies, such as MLST (Maiden et al., 1998; Denamur et al., 2021). With whole genome sequencing, traditional typing methods can be incorporated into *in silico* typing of strains, such as O:H typing and *fimH* allele typing. WGS enables a deeper resolution to traditional typing methods, since instead of using only seven to eight housekeeping genes, MLST

can be defined for a strain's core genome or whole genome, depending on the relatedness of the analyzed isolates (Denamur et al., 2021). Furthermore, single nucleotide polymorphisms (SNPs) can be determined from sequence data, which allows for inferring information on the evolutionary history and phylogeny of the isolates (Denamur et al., 2021).

Table 1. Different multilocus sequence typing schemes developed for *Escherichia coli*. Adapted from EcMLST (http://shigatox.net/ecmlst/cgi-bin/scheme), Wirth et al., 2006, and Institut Pasteur (https://bigsdb.pasteur.fr/ecoli/).

***Ec*MLST**	**Warwick scheme**	**Pasteur scheme**
Gene (gene product)	Gene (gene product)	Gene (gene product)
aspC (aspartate aminotransferase)	*adk* (adenylate kinase)	*dinB* (DNA polymerase)
clpX (caseinolytic protease)	*fumC* (fumarate hydratase)	*trpA* (tryptophan synthase subunit A)
fadD (acyl-CoA synthetase)	*gyrB* (DNA gyrase)	*trpB* (tryptophan synthase subunit B)
icdA (isocitrate dehydrogenase)	*icd* (isocitrate dehydrogenase)	*icdA* (isocitrate dehydrogenase)
mdh (malate dehydrogenase)	*mdh* (malate dehydrogenase)	*polB* (polymerase PolII)
lysP (lysine-specific permease)	*purA* (adenylosuccinate dehydrogenase)	*putP* (proline permease)
uidA (beta-glucuronidase)	*recA* (ATP/GTP binding motif)	*uidA* (beta-glucuronidase)
		pabB (p-aminobenzoate synthase)

Pathogenic Escherichia coli

Although largely found as a commensal member of the intestinal microbiota, non-pathogenic *E. coli* have the potential to cause opportunistic infections. Pathogenic strains, on the other hand, are divided into separate pathotypes, or pathovars, based on factors such as adhesion and colonization mechanisms and toxin production (Liu, 2019). Pathogenic *E. coli* strains are responsible for

morbidity and mortality worldwide (Croxen et al., 2013) and the main infections caused by pathogenic *E. coli* in humans include gastroenteric and urinary infections, septicaemia, and central nervous system infections.

Pathogenic *E. coli* strains are able to cause disease either extraintestinally, known as extraintestinal pathogenic *E.* coli (ExPEC), or intestinally, collectively known as intestinal pathogenic *E. coli* (InPEC) or diarrheagenic *E. coli* (DEC) (Robins-Browne et al., 2016). Pathotypes within InPEC/DEC include enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli (*EIEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), diffusely-adherent *E. coli* (DAEC), and adherent-invasive *E. coli* (AIEC) (Nataro and Kaper, 1998; Kaper et al., 2004; Croxen et al., 2013; Robins-Browne et al., 2016; Liu, 2019). In human ExPEC infections, *E. coli* strains most often belong to phylogroups B2 and D (Clermont et al., 2013; Micenková et al., 2016).

Avian pathogenic *E. coli* (APEC) causes morbidity, mortality, and economic losses in the poultry industry sector worldwide. This avian-specific ExPEC pathotype has been found to share genetic similarities with human ExPEC strains, including high similarities in virulence and antibiotic resistance gene profiles (Smith et al., 2007; Mitchell et al., 2015).

Food products and food-producing animals, including poultry, cattle, and pigs, as well as companion animals have been found to carry ExPEC strains, potentially enabling zoonotic transmission routes of these pathogenic *E. coli*, either through direct contact with animals or through the food chain via contaminated food products (Vincent et al., 2010; Nordstrom et al., 2013; Mitchell et al., 2015; Singer, 2015; Fratamico et al., 2016). However, the documentation of transmission routes is difficult due to the vast diversity of ExPEC strains and possible sources, both within human-related sectors (i.e., community, hospitals) and non-human sources, including livestock, companion animals, sewage, and the environment (Manges and Johnson, 2012; Singer, 2015). *E. coli* is disseminated not only by agricultural practices, such as manure and sludge application, and cattle feces, but also by anthropogenic sources from wastewaters from communities and hospitals (Martak et al., 2020). Although wastewater is filtered through wastewater treatment plants in developed countries, *E. coli*, including antimicrobial resistance genes, are present in outflows and thus disseminate in water environments and soils (Bréchet et al., 2014; Martak et al., 2020).

Infections caused by ExPEC strains are often opportunistic, but factors, such as systemic immunocompromise, can be recognized as predisposing factors (Robins-Browne et al., 2016). Infections caused by ExPEC include urinary tracts infections (uropathogenic *E. coli*, UPEC), peritonitis, pneumonia, meningitis (neonatal meningitis-associated *E. coli*, NMEC; meningitis-associated *E. coli*, MAEC), and septicaemia (sepsis-associated *E. coli*, SEPEC) (Robins-Browne et al., 2016; Liu, 2019). Urinary tract infections may become complicated and involve haemolytic-uremic syndrome (HUS) (Liu, 2019).

ExPEC strains possess factors which can improve their adhesion and colonization ability, for example, type I fimbriae and different adhesins (Pitout, 2012). The InPEC/DEC strains harbor specific virulence markers, which are used as defining factors, such as shiga toxins or *eae,* gene coding for adhesion proteins (Robins-Browne et al., 2016). Capsules composed of acidic polysaccharides are important for virulence in pathogenic *E. coli*, offering protection from environmental stress factors including immune responses from the host (Azurmendi et al., 2020). Hydrophobic fimbriae allow *E. coli* to adhere to different host and organ sites, contributing to the pathogenic properties of the organism (Percival and Williams, 2013). *fimH*, involved in the coding for fimbriae (Klemm and Christiansen, 1987), can be used in *fimH* subtyping of *E. coli* clonal groups, which is particularly useful for human-pathogenic *E. coli* belonging to the clonal group ST131 with multiple subgroups carrying the *fimH30* allele and showcasing multidrug resistance (Roer et al., 2017).

Emerging in 2008 on three continents, *E. coli* belonging to the ExPEC pathotype and sequence type ST131 has been recognized as the dominant clonal group (or clonal complex, CC131) among ExPEC isolates in humans globally (Nicolas-Chanoine et al., 2014). This clonal lineage is often associated with multidrug resistance, conferring resistance to fluoroquinolones, as wells as beta-lactams via ESBL production (Nicolas-Chanoine et al., 2014). Other frequent STs in human ExPEC infections include ST10, ST38, ST69, ST73, and ST405 (Nicolas-Chanoine et al., 2014; Micenková et al., 2016). Regarding ESBL-producing *E. coli* strains, especially ST10 and ST88 have been found from food and food-producing animals in multiple countries (Day et al., 2019). ST10 from both human and food sources have been identified, although with varying serotypes (Day et al., 2019).

Although different STs commonly dominate in animal hosts, ST131 is occasionally isolated from animal sources. ST131 *E. coli* from extraintestinal infections from dogs and cats have been found to be closely related to human derived ST131 *E. coli* isolates, indicating there is a bi-directional transfer of these pathogens, or more likely, human spillover to animal hosts (Kidsley et al., 2020). Among humans, ST131 *E. coli* spreads mainly between patients via the feco-oral route and contaminated food (Platell et al., 2011). Along with representing the dominant high-risk ExPEC lineage, ST131 *E. coli* has been an important contributor behind global multidrug resistance and $bla_{\text{CTX-M}}$ genes (Doi et al., 2017). Three major lineages or clades for ST131 exists: clades A, B and C (Matsumura et al., 2017). Since the 2000s, clade C has become globally dominant (Pitout and DeVinney, 2017). Two subclades of clade C, possessing *fimH*30 allele and termed H30R/C2 and H30Rx/C2, have in large part been responsible for the global dissemination of ST131 *E. coli* and ESBL genes harbored by these strains (Kondratyeva et al., 2020). Clade C2 is characterized by the presence of $bla_{\text{CTX-M-15}}$ and plasmids of the IncF family (more specifically [F2:A1B-]) have been associated with this clade, while $bla_{\text{CTX-M27}}$ has been recently recognized as a subclade of C1, termed C1-M27 (Matsumura et al., 2016; Decano and Downing, 2019; Kondratyeva et al., 2020). Strains belonging to the C1-M27 clade harbor $bla_{\text{CTX−M−27}}$, possess *fimH* allele 30 and

a prophage-like genomic island named M27PP1, with or without another prophage-like region, M27PP2 (Matsumura et al., 2016).

CC131 belonging to clade A usually correspond with *fimH*41, whereas those withing clade B coincide with *fimH*22 (Matsumura et al., 2017). Previous studies have indicated that clade C has evolved from clade B in the 1980s (ben Zakour et al., 2016; Stoesser et al., 2016; Pitout and DeVinney, 2017). Earlier, clades A and B have been considered to be generally susceptible to antibiotics and harbor less antimicrobial resistance (Pitout and Finn, 2020). However, in a recent Norwegian study clade A was found to be dominant among bloodstream infections and increased non-susceptibility to fluoroquinoles and acquisition of bla_{CTX} genes was observed (Gladstone et al., 2021).

Detection

E. coli has been intensively studied in humans, animals, and the environment because of its pathogenic potential, abundance, and suitability to double as an indicator organism for antimicrobial resistance and fecal contamination. Methods vary, but among the commonly used media for detection include MacConkey agar.

MacConkey agar, originally developed by Alfred T. MacConkey (1861–1931), was the first solid differential media. The idea of the media was born in the late 1890s when MacConkey was working as part of a group at the University of Liverpool commissioned to develop best practices for treatment of sewage (Smith, 2019). To protect the public from harmful waterborne diseases, drinking water had to be assessed for gram-negative bacteria, indicative of feces contamination. Cultivation of *E. coli* is based on mimicking the gastrointestinal tract, the natural habitat of the species. Later, some modifications were made to the formula by Albert Grunbaum and Edward Hume (Smith, 2019).

MacConkey is used for the isolation and differentiation of Gram-negative bacteria, with crystal violet and bile salts in its composition inhibiting the growth of gram-positive organisms. A fermentable carbohydrate, lactose monohydrate, is used to differentiate lactose fermenters from non-lactose fermenting species. Lactose-fermenting strains, such as *E. coli*, grow pink on the media, as acid produced by the process lowers the pH of the medium and causes a color change in the dye (Figure 1). A pH lower than 6.8 will cause neutral red, a pH indicator in the medium, to turn red. Opaque pink bile precipitation is additionally formed around *E. coli* colonies. Other composites of the medium include pancreatic digest of gelatin and peptones for bacterial growth, sodium chloride for osmotic balance, and agar for solidifying the medium (Jung and Hoilat, 2020).

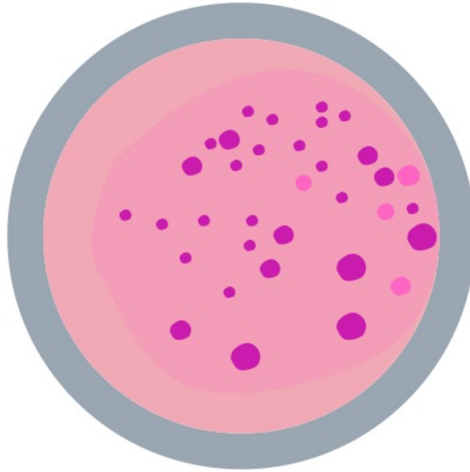


Figure 1. Representation of typical *Escherichia coli* colonies growing on a selective MacConkey medium, with typical 'disco-pink' color, round shape, and the slightly opaque pink 'veil' surrounding bacterial growth.

2.3.2 *Klebsiella pneumoniae*

Discovery and common properties

Carl Friedlander first described *Klebsiella pneumoniae* in 1882, a bacteria isolated by Edwin Klebs in 1875 from the lungs of a patient who had suffered and died from pneumonia (Friedlaender, 1882). In addition to humans, *Klebsiella* species are found in the environment, including surface waters, plants, soil, and animals (Bagley, 1985).

The genus *Klebsiella* belong to the family *Enterobacteriaceae* and are Gram-negative, rod-shaped, lactose-fermenting, facultative anaerobic, and nonmotile (Podschun and Ullmann, 1998). Taxonomy relating to *K. pneumoniae* has been updated in the recent years, after WGS-based studies have demonstrated that many isolates identified as *K. pneumoniae* are closely related species, sharing 95 - 96 % average nucleotide identity with *K. pneumoniae sensu stricto* (Wyres et al., 2020). Named as *K. pneumoniae* species complex, this group consists of seven members: *K. pneumoniae* sensu stricto, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *variicola*, *K. variicola* subsp. *tropica*, '*K. quasivariicola*', and *K. africana* (Wyres et al., 2020). The members of the *K. pneumoniae* complex differ from other *Klebsiella* species, sharing only 90% average nucleotide identity. *K. pneumoniae sensu stricto* comprises approximately 85% of clinical isolates identified as *K. pneumoniae* (Holt et al., 2015; Wyres et al., 2020), and for that reason *K. pneumoniae* will be the focus of following chapters.

The majority of *K. pneumoniae* isolates have a polysaccharide capsule covering the bacterial cell (Struve and Krogfelt, 2003). Like *E. coli*, *K. pneumoniae* produces pink colonies on MacConkey agar due to fermenting lactose (Jung and Hoilat, 2020), but no bile precipitation is observed around the colonies (Smith, 2019) and colonies appear wet and mucoid due to capsule production (Jung and Hoilat, 2020). *K. pneumoniae* is well known for the ability to produce biofilms, which are extracellular matrixes of bacterial communities merged together with proteins, exopolysaccharides, DNA, and lipopeptides (Donlan, 2002). Biofilms protect bacteria from antimicrobial molecules and allow attachment to many living and non-living surfaces, such as equipment in hospitals (Vuotto et al., 2014).

The genome size for *K. pneumoniae* is typically 5 – 6 Mbp, encoding approximately 5 000 – 6 000 genes, from which 1 700 genes are considered the conserved core genome and the rest the variable accessory genome (Holt et al., 2015). The pan-genome, or the sum of core and accessory genes, is very diverse among *K. pneumoniae*, although most accessory genes are present in only approximately less than 10% of the genomes (Holt et al., 2015). Genomic analyses suggest these accessory genes are shared with other *Klebsiella* species, other Enterobacterales or even with bacterial species from other orders (Holt et al., 2015). The accessory genome is considered to divide *K. pneumoniae* strains into opportunistic, hypervirulent, and multidrug-resistant strains (Martin and Bachman, 2018). Contrary to other potential pathogens such as *E. coli*, *K. pneumoniae* is ubiquitous in the environment, and no human-specific lineages have been clearly distinguished from the strains detected in the environment or animals, highlighting the zoonotic nature of this pathogen (Struve and Krogfelt, 2004).

Typing

Several different methods have been developed and used for the characterization as well as outbreak detection of *K. pneumoniae,* but MLST represents the most commonly used method. Phenotypic methods include phage typing, bacteriocin typing, and serotyping of the capsular antigen (Hansen et al., 2002; Brisse et al., 2004; Diancourt et al., 2005). Other molecular methods include repetitive sequence-based PCR and pulsed-field gel electrophoresis (Chen et al., 2014). MLST divides *K. pneumoniae* into distinct STs based on allelic variations in seven housekeeping genes (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) (Diancourt et al., 2005). Previously, three distinct phylogroups of *K. pneumoniae* (KpI, KpII, and KpIII) were defined (Brisse et al., 2004; Holt et al., 2015), but more recently, these phylogroups have been reassigned as distinct species belonging to the *Klebsiella pneumoniae* species complex, together with additional four members, totaling at seven phylogroups currently (KpI - KpVII) (Wyres et al., 2020).

Infections caused by K. pneumoniae

Infections caused by *K. pneumoniae* are often described in immunocompromised individuals, neonates, or the elderly. *K. pneumoniae* is

responsible for infections both in healthcare settings as well as in the community worldwide, and infections caused by *K. pneumoniae* include urinary tract and bloodstream infections, pneumoniae, sepsis, pyogenic liver abscesses, and meningitis and can be life-threatening (Paczosa and Mecsas, 2016). *K. pneumoniae* is among the leading causes for hospital-acquired infections in the United States (Magill et al., 2014).

Virulence factors contributing to pathogenicity and ability to cause invasive disease include siderophores, fimbrial adhesins, specific polysaccharide capsule serotypes, and genes encoding for hypermucoidy, named *rmpA* (Struve and Krogfelt, 2004; Holt et al., 2015). The ability of *K. pneumoniae* to adhere to catheters and form biofilms is associated with catheter-associated urinary tract infections in humans (Schroll et al., 2010). Invasive infections caused by hypermucoid *K. pneumoniae* have been reported in many animals, including animals in the wild, such as sea lions suffering from pleuritis and pneumoniae (Jang et al., 2010) and multisystemic abscesses in African green monkeys (Twenhafel et al., 2008). *K. pneumoniae* is also a common cause for clinical bovine mastitis (Podder et al., 2014). Hypervirulent strains have been described, first in East Asia and soon after worldwide, and have been defined as strains able to cause usually more severe infections in healthy individuals, often in the community (Harada and Doia, 2018; Chang et al., 2021). Common properties for hypervirulent *K. pneumoniae* strains separating these strains from the so-called 'classical *K. pneumoniae*' include hypermucoviscosity and hypervirulence, ability to cause disease at more unusual sites including pyogenic liver abscesses, pneumonia, meningitis, necrotizing fasciitis, and endophthalmitis (Zhu et al., 2021; Dong et al., 2022). Virulence factors found in hypervirulent *K. pneumoniae* include factors enhancing survival and infection, most importantly relating to capsule formation and siderophores, but also including lipopolysaccharide, fimbriae, and outer membrane proteins (Zhu et al., 2021; Dong et al., 2022). Furthermore, unlike classical *K. pneumoniae*, hypervirulent strains can cause metastatic infections (Zhu et al., 2021).

K. pneumoniae colonization rates vary between continents, with studies showing a rate of 18.8 – 87.7% in Asia and 5 – 35% in Western countries in humans (Gorrie et al., 2017; Russo and Marr, 2019; Chang et al., 2021). Furthermore, colonization with *K. pneumoniae* has been identified as a risk factor for subsequent infection, and infections most often originate from a patient's own intestinal microbiota (Gorrie et al., 2017; Chang et al., 2021). *K. pneumoniae* has also been identified from a diverse selection of food products, including poultry and pig meat, milk, and infant formula milk powder, seafood, as well as from surfaces in meat-packing factories (Hu et al., 2021b).

MDR *K. pneumoniae* has become increasingly prevalent in the last two decades, conferring resistance to extended-spectrum beta-lactams and carbapenems in addition to many other antimicrobial classes such as aminoglycosides and fluoroquinolones (Navon-Venezia et al., 2017). Previously, hypervirulent and MDR *K. pneumoniae* were believed to originate from non-overlapping lineages, as antimicrobial resistance genes were noted to be acquired by classical *K. pneumoniae* strains. However, more recently,

MDR hypervirulent clones with simultaneous hypervirulence and multidrug resistance have been observed (Dong et al., 2022). In the case of infection with MDR or extensively drug resistant (XDR) *K. pneumoniae*, only very selected antimicrobial treatment options are available. It is a concern that plasmid-mediated resistance to colistin, a last-line antibiotic, has gained prevalence in these MDR and XDR *K. pneumoniae* strains (Navon-Venezia et al., 2017; Bengoechea and Sa Pessoa, 2019). In addition, *K. pneumoniae* has been more successful than *E. coli* in acquiring genes encoding for carbapenemases, including KPC, NDM, VIM, IMP, and OXA (Chang et al., 2021).

2.3.3 Epidemiology and prevalence of extended-spectrum beta-lactamases

The epidemiology pertaining to resistance mediated by extended-spectrum beta-lactamases is multi-factored, as dissemination occurs in many environments, such as communities, hospitals, food production plants, and farms. The rate of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* have increased in the past two decades around the world, although with uneven distributions between continents and even countries within one continent (Doi et al., 2017). Since the 1980s, there has been a shift from TEM and SHV family ESBLs to the CTX-M family, a change from hospital-confined-only to community-related transmission, and investigations behind attributable sources for infections with ESBL-producing bacteria (Doi et al., 2017). Furthermore, the 'jump' of AmpC beta-lactamases from the chromosome to plasmids has assisted their spread, making the AmpC type CMY-2 prevalent in poultry sources (Jacoby, 2009; Bush, 2018). The success of the global dissemination of extended-spectrum-beta-lactamases is in large part attributed to plasmids and the horizontal gene transfer (HGT) of resistance genes (Bush, 2018).

Drivers of ESBL/AmpC-mediated resistance

The prevalence of ESBL/AmpC-producing organisms varies widely between countries and continents, as has been documented for ESBL-producing *E. coli*, with its prevalence in humans and livestock is considerably higher in Southeast Asia, Africa, and South America than in Europe (Kuralayanapalya et al., 2013; Woerther et al., 2013; Bezabih et al., 2021). Large differences are also seen within Europe, with a lower prevalence in Northern Europe and the Netherlands, compared to Southern and Eastern Europe (EFSA and ECDC, 2021).

Drivers behind the dissemination and increase of antimicrobial resistance worldwide include the use and overuse of antimicrobials in humans as well as in animals, global traffic of people, goods, and animals, and inappropriate hygiene measures and unsatisfactory sanitary conditions (Castanheira et al., 2021). International travel, especially to South and Southeast Asia, Africa and South America, have been identified as risk factors for intestinal colonization with multidrug-resistant Gram-negative bacteria (Woerther et al., 2017; Lääveri et al., 2018; Kantele et al., 2021).

Colonized humans have been found to have a high concentration of ESBL-producing *E. coli* in their feces (10^2–10^8 cfu/g) (Girlich et al., 2014), similar to animals (10^3–10^7 cfu/g fecal concentration) (Horton et al., 2011; Hansen et al., 2013), underlining the risk for feco-oral transmission and heightened probability for dissemination of AMR, especially in unsanitary conditions (Bezabih et al., 2021). The infrastructure for sanitation, wastewater treatment, and access to clean water differs among regions, possibly explaining the travel-related AMR dissemination. After travel to India, 86% of Swiss travelers and over 90% of Danish travelers were reported to be colonized with ESBL producing *E. coli* (Kuenzli et al., 2014; Dall et al., 2019). Contracting traveler's diarrhea and medication with antimicrobials during travel have been identified as risk factors for the colonization with ESBL-producing *Enterobacteriaceae* (Kantele et al., 2015).

In addition to international travel of people and the controlled movement of companion and food-producing animals, wild animals cross country borders without surveillance. Migratory birds, potentially carrying resistant microorganisms gathered from a variety of sources including anthropogenic waste, travel vast distances across continents (Bonnedahl and Järhult, 2014; Dolejska and Papagiannitsis, 2018). Within-flock dissemination of ESBL-producing *E. coli* has been noted to be effective in wild birds (Sandegren et al., 2018). Large flocks of wild birds feed and defecate on crop fields, as well as in densely human-populated areas such as recreational parks (Elmberg et al., 2017). Furthermore, crops contaminated with fecal matter can pose a risk for transmission of resistant bacteria to food-producing animals. Waterfowls, such as barnacle geese (*Branta leucopsis*), have seen a rise in population size during recent years, leading to thousands of birds migrating through different parts of the European continent each year, some also breeding in newly established locations like Finland (Jensen, G.H., Madsen, J., Nagy, S., 2018).

Differences in the prevalence of AMR between countries is most likely in part, albeit importantly, attributed to the remarkably differing customs of using antimicrobials. Antimicrobial sales for veterinary use in 2020 ranged from 2.3 to 393.9 mg/PCU (population correction unit), while the median for all participating countries was 51.9 mg/PCU (European Medicines Agency (EMA), 2021). 'Hot spots' for AMR in animals have been identified in China and India, while emerging hot spots are predicted to be located in Kenya and Brazil (van Boeckel et al., 2019). The consumption of antimicrobials is predicted to increase, especially in low- and middle-income countries, with the demand for increased animal-source protein production and human population growth (van Boeckel et al., 2015; Schar et al., 2018). The prevalence of resistance towards antimicrobials in food-producing animals has been shown to be affected by antimicrobial use, and to correlate to a higher resistance prevalence in *E. coli* in pigs, poultry, and cattle (Chantziaras et al., 2014; Hoelzer et al., 2017). Antimicrobial use in animals is also proven to have an affect on AMR prevalence in human populations (ECDC et al., 2017; Tang et al., 2017).

Wildlife and environment

Some animals may act as sentinels for environmental pollution with resistant pathogens. Gulls have been noted to carry ESBL genes most likely acquired from human sources, such as landfills, which pinpoints the important fact that the direction of transmission of antimicrobial resistant pathogens and microorganisms can move both ways. In Sweden, gulls were found to have a significantly higher occurrence of ESBL-producing *E. coli* compared with Swedish community carriers (17% in gulls versus 5% in humans) (Atterby et al., 2017). Although clonal transmission between these sources was not largely evidenced, genetic similarities were nevertheless observed, including the same plasmid-ESBL-gene-combinations, such as InF and IncI1 plasmids harboring $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ (Atterby et al., 2017). These combinations were also observed in surface waters, indicating surface waters as potential transmission points. As ESBL/AmpC-genes are harbored by enterobacteria, it is not surprising that reservoirs for these bacteria have been identified in water-related environments such as sewage, sludge, and soil (Talukdar et al., 2013; Huijbers et al., 2015; Berendes et al., 2020).

Food-producing animals and food production

Using contaminated irrigation water in agriculture may serve as a transmission route of resistant bacteria into the food chain (Reinthaler et al., 2010). Cross-contamination at food-producing facilities is another possible dissemination route (FAO and WHO, 2019), as is usage of animal-derived manure (Hartmann et al., 2012). Concurrent exposure to feed and environment contaminated with resistant bacteria and antimicrobial administration may lead to the amplification of resistant bacteria in animal intestines (Witte, 2000; Marshall and Levy, 2011; FAO and WHO, 2019).

In addition to livestock, the production of seafood in unsanitary conditions poses an increased risk for the dissemination of AMR (Boss et al., 2016). Coastal waters contaminated with fecal matter and post-harvest contamination in landing centers and food markets serve as major contamination points for seafood (Singh et al., 2020). In India, nearly 72% of tested fresh seafood products at a food market were found to confer resistance to extended-spectrum beta-lactamases (Singh et al., 2020). Aquaculture may subsequently be a source for resistant bacteria for fruits and vegetables through the use of irrigation water affected by seafood production (Done et al., 2015). Lack of sanitation has also been observed to affect global AMR gene abundance and diversity in untreated sewage samples collected globally in various countries, and AMR gene abundance has also been found to strongly correlate with socio-economic, health, and environmental factors (Hendriksen et al., 2019).

Concerning food products, poultry meat has been recognized as an important reservoir for ESBL/AmpC-producing *E. coli*, with historically high prevalence rates (Ewers et al., 2012; EFSA and ECDC, 2021). ESBL/AmpC-producing *E. coli* is thought to spread within poultry production both through a vertical (from parent birds to offspring) and horizontal (via environmental sources)

transmission routes. These bacteria have been isolated from all levels of the poultry production, from grandparent flock to hatchlings, as well as from the production environment and feed (Dierikx et al., 2013; Gazal et al., 2021). The poultry production structure is pyramid-like, where the top level consists of breeding stock, or pedigree animals, produced by only a few companies dominating the global market. The pedigree animals are kept at facilities with a high biosecurity level and available details regarding the production are limited (European Commission, 2016). Pedigree animals are the basis for the next generation of animals, a process including three steps: great-grandparent to grandparent level, and to parent stock level from which day-old chicks to be raised as commercial broilers are produced (European Commission, 2016).

Although extended-spectrum cephalosporin use in poultry has been restricted in the EU since 2012 (European Commission, 2012), ESBL/AmpC-producing *E. coli* have been found at all levels of the broiler production pyramid in EU member states, including grandparent and parent stock (Dierikx et al., 2013), hatcheries, commercial broiler fattening farms (Daehre et al., 2018), slaughterhouses and broiler meat (Voets et al., 2013; von Tippelskirch et al., 2018; EFSA and ECDC, 2021). ESBL/AmpC-producing *E. coli* has been found in the poultry sector even in countries with overall limited antimicrobial use in livestock (Agersø et al., 2014; Mo et al., 2014). The spread of ESBL/AmpC-producing *E. coli* has been proposed to occur via four major pathways along the poultry production chain, including vertical transmission route from parent birds to offspring, horizontal transmission within hatcheries and within broiler breeding farms, between farms or from the environment (Dame-Korevaar et al., 2019). In Finland, broiler parent birds are usually imported as day-old hatchlings, from which linings of the transportation boxes are examined for ESBL/AmpC-producing *E. coli* (FINRES-Vet, 2021).

Specific monitoring is driven by EU legislation, which resulted in the finding in 2018 of ESBL/AmpC-producing *E. coli* in 48.3% of broilers from EU member states (EFSA and ECDC, 2021). That same year, the number for broilers in Finland was 13.1%, but in 2020, a dramatic decrease to 0.3% was observed (FINRES-Vet, 2021).

CTX-M-1 has been found to be a common ESBL in poultry and broiler meat in many countries such as the Netherlands, and it is often found from human samples, indicating poultry-sources may be an important reservoir for CTX-M-1-producing *E. coli* infections or colonization in humans (Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Doi et al., 2017). Later research has identified *bla*$_{\text{CTX-M-1}}$-harboring plasmids, IncI1 in particular, as the common denominator among human and poultry samples (de Been et al., 2014; Day et al., 2019). CMY-2 is another commonly found enzyme in poultry sources, and there have been reports of a potential zoonotic transmission route of ST131 *E. coli* with an IncI1 plasmid harboring *bla*$_{\text{CMY-2}}$ from a broiler source resulting in human bloodstream infection (Roer et al., 2019). Previous studies have, indeed, suggested that genes encoding for ESBLs are transmitted between different sources via plasmids. In Sweden, 5% of the healthy population were found to carry *bla*-harboring plasmids overlapping with plasmid replicon

types obtained from *E. coli* from broiler meat and poultry (Börjesson et al., 2016).

Companion animals

ESBL-producing *E. coli* among companion animals has also been shown to differ between continents, with the lowest prevalence in Oceania (0.63% in dogs and 0% in cats) and highest in Africa (16.56% in dogs and 16.82% in cats), resulting in a global prevalence of 6.87% for dogs and 5.04% for cats (Salgado-Caxito et al., 2021). STs and beta-lactamase genes have been found to be diverse among dogs and cats in all continents, but the most commonly identified genes include *bla*$_{CTX-M-15}$ and *bla*$_{SHV-12}$ on sequence types ST38 and ST131 (Salgado-Caxito et al., 2021).

Humans

In a healthcare setting, ESBL-producing *Enterobacteriaceae* can transmit from patient to patient, and via healthcare workers acting as involuntary vectors. The rate of transmission depends on the species in question, which differ in virulence factors affecting traits such as adhesion. ESBL-producing *K. pneumoniae* from colonized patients has been shown to have a higher transmission rate (2-fold higher) compared to patients with ESBL-producing *E. coli* (Hilty et al., 2012).

Based on a literature review, the global intestinal carriage rate of ESBL-producing *E. coli* among healthy individuals in the community has increased 8-fold in the past two decades, from 2.6% (95% confidence interval [CI] 2.9 – 51.3%) in the early 2000s to 21.1% (95% CI 15.8 – 27.0%) in 2015 – 2018 (Bezabih et al., 2021). During the whole examination period, regional differences were observed that are in line with the current trend, with the highest carriage rate in South-East Asia (27%; 95% CI 2.9 – 51.3%) and the lowest in Europe (6.0%; 95% CI 4.6 – 7.5%) (Bezabih et al., 2021).

In the EU/EEA, AMR surveillance is monitored and reported on a yearly basis. In 2020, 2.9% and 6.5% of *E. coli isolates* from urinary specimens from women and men, respectively, were ESBL-producers in Finland (Finres, 2021). From invasive *E. coli* and *K. pneumoniae* isolates in Finland in 2020, 7.2% of both bacteria, respectively, were resistant to third-generation cephalosporins, while carbapenem resistance was extremely low, 0.0% and 0.1%, respectively (WHO, 2022). Third-generation cephalosporin resistance in invasive *E. coli* was equal to or above 50% in five out of 40 reporting countries in Europe, while carbapenem resistance above 1% was reported in six countries. A majority of countries (18 out of 40, mostly in southern and eastern Europe) reported 50% or more of invasive *K. pneumoniae* isolates to be third-generation cephalosporin-resistant, while carbapenem resistance was observed to be equal or above 25% in 12 countries, and equal to or above 50% in six out of 41 reporting countries (WHO, 2022).

2.4 Plasmids and the spread of extended-spectrum beta-lactamases

In addition to vertical transfer of an organism's genes from a parent to offspring, prokaryotes possess the ability to transfer genes horizontally, referred to as horizontal gene transfer (HGT) (Lederberg, 1946; Rankin et al., 2011). This transfer of genes occurs independently of bacterial reproduction, making the transfer of genes rapid between and within genomes. HGT is facilitated by mobile genetic elements (MGE), a term to describe a wide variety of different genomic sequences, including plasmids, prophages, transposons, insertion sequences, pathogenicity islands, and restriction and modification systems (Partridge et al., 2018; Vale et al., 2022). The mobilome, representing the mobile elements in a bacterial genome, are important for bacterial evolution and adaptation since they can adjust their copy number, change their insertion location, generate new gene functions, and modify gene expression (Vale et al., 2022). Regarding the global increase of AMR in clinically relevant *Enterobacteriaceae*, plasmids are at the core of this phenomenon. Antimicrobial resistance genes located on plasmids have been able to transfer among bacteria of even different species, genera, and kingdoms, offering a competitive benefit to bacterial populations exposed to antimicrobial treatment. The success of a particular plasmid type as a mediator of AMR has depended on the host range, conjugative properties as well as the efficiency of plasmid conjugation (Thomas and Nielsen, 2005; Carattoli, 2013). Successful plasmids often confer resistance to several different classes of antibiotics, giving the bacterial cell an advantage in environments where several antimicrobials are present simultaneously (Carattoli, 2013).

Plasmid structure

Plasmids are extra-chromosomal, double-stranded DNA molecules capable of self-replication and often of circular form (Carattoli, 2009). Originally, the term plasmid was used to describe any extra-chromosomal elements, as described by Joshua Lederberg in 1952 (Lederberg, 1952). Plasmids possess systems for their replication and copy number control and can acquire other MGEs such as transposons and insertion sequences for the mobilization of virulence and AMR genes (Carattoli, 2013). Addiction systems harbored by many plasmids are composed of toxin-antitoxin factors, that ensure the stable inheritance of plasmids during cell division, as plasmid-free daughter cells are killed (Hayes, 2003). This system ensures plasmid survival in bacterial populations even without the selective pressure (i.e., presence of antimicrobials in the environment). Plasmids, with an evolutionary history and continuous lineages of their own, are considered living organisms (Carattoli, 2013). In addition to their natural role in the microbial world, plasmids have been harnessed as cloning vectors in the laboratory, making them important tools in molecular epidemiology (Partridge et al., 2018).

The plasmid genome is considered to consist of a stable ‘backbone’ and a variable accessory gene content. Core genes comprising the backbone include

those encoding for replication, maintenance, conjugative and mobilization functions, the main tool kit for plasmid survival (Thomas, 2002). The basic structure of all plasmids consists of *ori*, the origin of replication, and the initiation gene *rep*, which is usually co-located with *ori* (Partridge et al., 2018). The replication can be triggered by an RNA transcript, but the binding of Rep (the initiation protein encoded by *rep*) to DNA repeat sequences (iterons) is more common. Although the initiation of replication is controlled by plasmids themselves, they usually make use of the bacterial hosts' replication machinery for the actual DNA synthesis. This exploitation of the host's replication system is one factor in determining the host range of plasmids, as some plasmids are more specialized to the replication proteins of specific bacterial taxa (Partridge et al., 2018). Multireplicon plasmids, with multiple replication regions in their genome, exist as well, and are particularly common in IncF type plasmids (Rozwandowicz et al., 2018). Some plasmids may also form multireplicon cointegrates, such as IncR plasmids with IncN or IncA/C plasmids (Drieux et al., 2013; Papagiannitsis et al., 2013). The multireplicon status of a plasmid may be cumbersome due to increased fitness cost to the host, but on the other hand, might increase the plasmid's host range (Villa et al., 2010; Partridge et al., 2018).

Plasmids are inherited to daughter bacterial cells during cell division, but also via horizontal transmission. Genes involved in horizontal transfer, or *tra* genes, in conjugative, self-transmissible plasmids increase the size of the plasmid backbone. Conjugative plasmids in Gram-negative bacteria possess *tra* genes encoding for mating pair formation proteins (MPF) acting as a type IV secretion system (T4SS) pore during conjugation and forming a conjugative pilus, and DNA transfer replication (DTR) proteins, such as relaxase nicking the origin of transfer (*oriT*) in the exported DNA strand (Partridge et al., 2018). MPF proteins can benefit mobilizable, nonconjugative plasmids with limited DTR proteins (or *mob*), enabling their horizontal transfer. The accessory regions of a plasmid genome usually consist of genes coding for functions benefiting the bacterial host, such as increased virulence via virulence genes or ability to withstand antimicrobials via resistance genes, as well mobile elements such as insertion sequences, transposons and integrons, involved in the virulence and resistance gene integration to the plasmid genome (Partridge et al., 2018). Often plasmids conferring multidrug resistance are often large (over 50 kb), self-conjugative and control their rate of replication by controlling their copy number.

The potential benefits offered to bacterial cells via plasmid-encoded traits such as antimicrobial resistance, often come with a burden referred to as fitness cost (San Millan and MacLean, 2017). Knowledge gaps exist in understanding all the mechanisms underlying fitness cost, but specific resistance genes on multiresistance plasmids have been shown to inflict a burden on the host cell, while certain genes are cost-free when inspected individually (Rajer and Sandegren, 2022). It has been shown that a plasmid-borne $bla_{\text{CTX-M-15}}$ reduces the fitness of the bacterial host slightly via impacting the growth rate of the cell (Rajer and Sandegren, 2022). This effect is conferred by a beta-lactamase signal peptide SME-1 which exports the beta-lactamase enzyme into to the periplasm, where the enzyme hydrolyzes beta-lactam antibiotic molecules

protecting the bacterial cell (Kadonaga et al., 1984; Lingappa et al., 1984). Therefore, the negative impact on bacterial fitness is thought to occur due to the reduction in the normal transfer of other proteins to the periplasmic space (Rajer and Sandegren, 2022). Overall, however, the fitness cost afflicted by low-copy-number resistance plasmids is often considered minor or even neutral (Vogwill and Maclean, 2015; Rajer and Sandegren, 2022).

Classification

Several alternative schemes for plasmid typing exist. Proposed in 1971 by Hedges and Datta, the first classification system separated plasmids based on their incompatibility, or the phenomenon that closely related plasmids cannot be maintained in the same cell due to common replication controls (Datta and Hedges, 1971; Novick, 1987). Currently at least 27 incompatibility groups, or Inc groups, exist for *Enterobacteriaceae* that are recognized by the Plasmid Section of the National Collection of Type Cultures (London, United Kingdom) (Carattoli, 2009). These include major plasmid families occurring in *Enterobacteriaceae* (HI2, HI1, I1, X, L/M, N, FIA, FIB, FIC, FII, W, Y, P, A/C, T, K, B/O) as well as six variants for IncF (FII to VII) and three variants for IncI (I1, Iγ, I2) (Carattoli, 2009; Rozwandowicz et al., 2018). The incompatibility testing based on conjugation experiments was followed by a hybridization scheme (Couturier et al., 1988), and subsequently PCR-based replicon typing (PBRT) and a method based on sequencing. PBRT and in silico typing with PlasmidFinder (Carattoli et al., 2014) incorporate the classical Inc groupings. In addition, typing scheme based on conjugative and mobilization relaxase genes, MOB typing, exists (Francia et al., 2004). Some plasmids can be further differentiated with plasmid multilocus sequence typing (pMLST) (Carattoli et al., 2014). For IncF plasmids often forming multireplicons, a replicon typing scheme (RST) was developed to differentiate these plasmids with a FAB formula (Villa et al., 2010). FIA replicons are typed based on their *repA* gene encoding the replication protein, FIB replicons are typed based on the *repB* gene, and FII replicons are typed based on their *copA* gene. FII replicon is sometimes replaced by FIC (Villa et al., 2010)

Incompatibility groups in Enterobacteriaceae harboring extended-spectrum beta-lactamases

Plasmids isolated from human, animal, and environmental sources show large regional and source-dependent differences. In Europe, IncI plasmids on *E. coli* strains in animals are typical, whereas in Asia the most prevalent plasmid type is IncF, and in North and South America IncA/C. IncF type plasmids are dominant in human sources, but similar to animals in Europe, IncI type plasmids are recovered from human sources in Europe (Rozwandowicz et al., 2018). Plasmids that are frequently recovered from multiple sources and regions globally are termed ‘epidemic’ plasmids, and these include IncF, IncI, IncA/C, and IncH (Carattoli, 2009; Rozwandowicz et al., 2018). The plasmid types that have been relevant in the research of this thesis are described briefly below.

IncF

The successful dissemination of class A beta-lactamases is attributed to their typical localization to conjugative plasmids (Palzkill, 2018). The globally successful *bla*$_{CTX-M-15}$ gene is usually located on plasmids of the IncF group, especially IncFII in ST131 and ST405 *E. coli* (Naseer and Sundsfjord, 2011; Nicolas-Chanoine et al., 2014). IncF, named so after *fertility factor*, was the first conjugative plasmid identified in bacteria (Partridge et al., 2018). IncF plasmids exhibit a low-copy-number and vary in size from tens of kilobases (kb) to a couple hundred kb (Carattoli, 2009). IncF plasmids are confined to *Enterobacteriaceae* and are the most frequently recovered plasmid type from human and animal *E. coli* especially in Asia (Rozwandowicz et al., 2018). In addition to ESBLs, IncF plasmids have been found to often harbor genes conferring resistance to carbapenemases, aminoglycosides, and fluoroquinolones. IncF harboring *bla*$_{CTX-M-1}$ has been isolated from animal sources while *bla*$_{TEM-1}$ mostly from human sources (Rozwandowicz et al., 2018).

IncI

Incompatibility groups I1 (Iα), Iγ, B/O, K, and Z are similar in their serologies and morphologies of their pili (Praszkier et al., 1991), and are thus grouped together into I-complex plasmids (Partridge et al., 2018; Rozwandowicz et al., 2018). Plasmids of the IncI type are conjugative, low-copy-number, narrow host range, and vary in size from 50 to 250 kb (Garcillán-Barcia et al., 2011). From the variants within this group (I1/IncIα, I-γ, and I2/IncIδ), IncI1 and I-γ are similar, but differ in their Inc RNA sequences as well as the the stability structure of the plasmid genome, which is conserved in IncI1 but non-existent in I-γ (Takahashi et al., 2011). The I-complex plasmids share a shufflon region with a gene (*rci*) encoding a recombinase, which allows for recombination of different *pilV* genes at the shufflon-specific *sfx* sites, affecting recipient specificity (Komano et al., 1994; Gyohda et al., 2006).

IncI plasmids have been detected especially in *E. coli* from animal sources, especially poultry in Europe, carrying *bla*$_{CTX-M-1}$ or *bla*$_{CMY-2}$ (Johnson et al., 2011; Partridge et al., 2018; Rozwandowicz et al., 2018). IncI harboring *bla*$_{CTX-M-1}$ is often identified from *E. coli* representing ST10, ST58 ST117, and ST131 (Leverstein-van Hall et al., 2011; Wang et al., 2014). ST10 *E. coli* is also associated with IncI plasmids harboring *bla*$_{TEM-52}$ in livestock sources (Leverstein-van Hall et al., 2011). The genes *bla*$_{CTX-M-55}$ and *bla*$_{KPC-3}$ have been described in IncI2 plasmids (Chen et al., 2013; Lv et al., 2013; Tijet et al., 2014), and worrisomely, *mcr* genes conferring resistance to colistin have been found from *E. coli* originating from human and animal sources in countries in the Americas and Asia, respectively, carried by IncI2 plasmids (Tijet et al., 2017; Yang et al., 2017).

IncN

IncN plasmids are relatively small (30 - 70 kb), conjugative plasmids with a broad-host-range, and often colocalized with IncF type plasmids (Szmolka et

al., 2011; Rozwandowicz et al., 2018). The conjugation region in IncN plasmids is divided into two parts separated by *fipA* involved in fertility inhibition and *nuc*, a gene encoding for a nuclease (Partridge et al., 2018). The conjugation regions include genes encoding for entry exclusion functions and pilus formation, as well as *oriT*, the origin of transfer, from which conjugal transfer of plasmid DNA is initiated (Coupland et al., 1987). *E. coli* carrying IncN ST1 often harbors $bla_{CTX\text{-}M\text{-}1}$ and has been described in animal, human and environmental sources in Europe (Dolejska et al., 2013), and the combination of IncN-$bla_{CTX\text{-}M\text{-}1}$ has been detected in humans, animals and the environment in Denmark, Italy, Greece, and the Czech Republic (Moodley and Guardabassi, 2009; Bortolaia et al., 2010a, 2010b). Other ESBL-encoding genes that have been described in IncN plasmids include $bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}32}$, $bla_{CTX\text{-}M\text{-}40}$, and $bla_{CTX\text{-}M\text{-}65}$ (Carattoli, 2009), while $bla_{VIM\text{-}1}$ has been described in human patients in southern European countries in IncN plasmids from *K. pneumoniae* among other *Enterobacteriaceae* (Giakkoupi et al., 2003; Loli et al., 2006; Aschbacher et al., 2011; García-Fernández et al., 2011). IncN plasmids have been found to be prevalent plasmid types in APEC strains and avian commensal *E. coli* but were not found from human commensal *E. coli* in a study conducted in the USA (Carattoli, 2009).

IncX

IncX plasmids are small, narrow-host-range plasmids with a size of 30 – 50 kb (Garcillán-Barcia et al., 2011). They are divided into six subtypes, X1 to X6, and have been shown to have three *ori* regions (Rozwandowicz et al., 2018). IncX plasmids have been described from *Salmonella* and *E. coli*, often harboring resistance genes conferring resistance to extended-spectrum beta-lactams or carbapenems (bla_{KPC} and bla_{NDM}) (Pál et al., 2017; Espinal et al., 2018) and quinolones, tetracyclines, trimethoprim and even colistin (*mcr-1* and *mcr-2*) (Hasman et al., 2015; Falgenhauer et al., 2016; Xavier et al., 2016).

IncR, IncY

Some of the more rarely detected plasmids include IncR and IncY. IncR plasmids are broad-host-range and their size varies between 40 – 160 kb (Garcillán-Barcia et al., 2011). Even though research suggests they are not conjugative due to the absence of *tra* genes and relaxase (Compain et al., 2014), these plasmids are most likely mobilizable (Bielak et al., 2011). Furthermore, IncR plasmids have been shown to form multireplicon cointegrates with plasmids of IncN, $IncFII_K$, and IncA/C types (Compain et al., 2014; Guo et al., 2016; Partridge et al., 2018). IncR plasmids have been described in *K. pneumoniae*, and to a lesser extent in *Enterobacter cloacae* and *E. coli*, harboring genes conferring resistance to carbapenems (bla_{NDM}, bla_{KPC}, and bla_{VIM}) as well third-generation cephalosporins ($bla_{CTX\text{-}M\text{-}15}$) (Carattoli, 2013).

IncY are considered prophages replicating as autonomous plasmids, with a size of 90 – 100 kb and low copy numbers (Rozwandowicz et al., 2018). IncY harboring *mcr-1* has been described in *E. coli* from a pig farm in China (Zhang

et al., 2017). It has also been identified with *bla*$_{CTX-M-15}$ in *K. pneumoniae* from a patient in South Korea (Shin and Ko, 2015).

2.5 Whole genome sequencing

2.5.1 Events leading to whole genome sequencing

Until the 1980s, traditional culture-based methods were widely in place for studying microorganisms, but the advances in DNA-based analysis methods and the realization of the abundance of unculturable bacteria via traditional methods led to the increasing use of sequencing in the study of the microbial world (Giani et al., 2020; Ciuffreda et al., 2021).

In 1977 the first DNA genome, a 5386 basepairs (bp) long bacteriophage named φX174, was sequenced by Frederick Sanger and his research team (Sanger et al., 1977a) using a method developed in 1975 by Sanger and Coulson, called the 'plus and minus' method (Sanger and Coulson, 1975). This method was already used to determine two short regions of the φX174 bacteriophage in 1975 (Sanger and Coulson, 1975; Sanger, 1988). Events leading to this point, the sequencing of a complete DNA genome and the development of sequencing methods, can be dated back to 1953 when the double helix structure of DNA was discovered (Franklin and Gosling, 1953; Watson and Crick, 1953), the amino acid position studies in proteins, particularly in insulin, were conducted (Sanger and Thompson, 1953a, 1953b), and the sequencing of RNA molecules succeeded (Holley et al., 1965). The first DNA molecule was sequenced in 1968, being 12 bases long and belonging to a bacteriophage named λ (Wu and Kaiser, 1968).

In 1977, the same year as the first DNA genome was sequenced, two breakthroughs followed in the sequencing world. First, a method by Maxam and Gilbert (Maxam and Gilbert, 1977) utilizing radiolabeled DNA treated with chemicals, proved superior to the 'plus and minus' technique and was widely used for many years (Sanger, 1988; Heather and Chain, 2016; Giani et al., 2020), and as such could be referred to as the beginning of first-generation of DNA sequencing (Heather and Chain, 2016).

But it was the development of a 'chain-termination' or dideoxy technique, later referenced to as 'Sanger sequencing', that ultimately advanced sequencing technology in the years to come (Sanger et al., 1977b; Heather and Chain, 2016). Sanger sequencing proved more popular due to further technical improvements and better usability compared to the chemical method and remained the most popular sequencing technique for multiple decades (Giani et al., 2020), and is still used alongside next-generation sequencing technologies, especially when the interest is in a specific DNA region and a fragment size of 500 – 1000 bp is sufficient. One of the advances was the introduction of a 'shotgun' method, where short, cloned fragments are produced by cloning with bacterial vectors and then sequenced in parallel, speeding up the process (Staden, 1979). A shotgun protocol employing a M13

phage vector was published two years later (Messing et al., 1981) and subsequently multiple whole-genome projects were completed (Giani et al., 2020).

Further technical developments followed in the 1980s and 1990s, accelerated by the Human Genome Project and the goal of achieving better throughput with lower costs, which gave way to 'next-generation' or 'second-generation' sequencing techniques introduced in the early 2000s (Giani et al., 2020; Hu et al., 2021a). Owing to massively parallel sequencing, next-generation sequencing (NGS) was able to provide substantially more data output in a shorter time compared to the conventional capillary electrophoresis-based Sanger sequencing (Heather and Chain, 2016; Hu et al., 2021a).

Alongside these short-read NGS technologies a new wave of sequencing technologies have arisen, also termed 'third-generation' sequencing, utilizing longer reads to overcome issues such as resolving genome-wide repeats (Hu et al., 2021a). Massive advances have been seen in the study of genomics during the last decades, from labor-intensive and time-consuming methods in laboratories to fast, high-throughput pipelines able to produce multiple gigabases of data in a single run. NCBI GenBank, database for genomic sequences, was founded in 1982, and ever since the number of bases in the database has doubled approximately every 18 months (GenBank and WGS Statistics).

As of November 12th, 2021, 26 264 *E. coli* genomes are deposited in NCBI Genome, the database for complete genomes (NCBI, 2021a). A search in the NCBI Nucleotide database, containing all the sequence data from GenBank, EMBL, and DDBJ, the members of the International Nucleotide Sequence Databases Collaboration (INSDC), yields a result of 9 971 538, with '*Escherichia coli*' as the search term (NCBI, 2021b).

The next-generation short-read and third-generation long-read technologies are discussed in more detail in the next two sections.

2.5.2 Short-read sequencing

The first *E. coli* (*E. coli* K-12) was sequenced in sections over a period of almost six years combining different sequencing strategies, including a Janus M13 shotgun strategy, and published in 1997 (Blattner et al., 1997).

The first largely commercially successful next-generation sequencing technique is considered pyrosequencing (Heather and Chain, 2016), making use of measuring pyrophosphate generated by the DNA polymerization reaction with fluorescence instead of visualizing radio- or fluorescently-labelled dideoxynucleotides with electrophoresis on polyacrylamide gel used in the first-generation methods (Nyrén and Lundin, 1985; Hyman, 1988; Ronaghi et al., 1996). The pyrosequencing technology was later commercialized by 454 Life Sciences (later purchased by Roche) (Giani et al., 2020), which brought the first NGS DNA sequencer, called GS20, to the

market in 2003 (Margulies et al., 2005). In this technique, DNA molecules are clonally amplified via a bead-based method using an emulsion PCR (Ronaghi et al., 1996).

A company called Solexa (later acquired by Illumina) developed another sequencing by synthesis (SBS) technology, which is capable of paired-end sequencing, i.e., sequencing the DNA fragment from both ends, producing high quality sequence data (Mitra et al., 2003; Giani et al., 2020; Hu et al., 2021a). Illumina NGS is based on fluorescent-labeled reversible terminator technology, which first incorporates bridge amplification PCR to produce clusters of DNA libraries (Hu et al., 2021a) to produce a stronger signal for base calling accuracy (Giani et al., 2020). In each sequencing cycle, a fluorescently labeled reversible terminator-bound deoxynucleotide is added and the produced fluorescent signal is imaged. Newer Illumina instruments, such as NovaSeq 6000, use a technology called 2-channel SBS to achieve faster sequencing times (Illumina, 2021a). Instead of the original four images, only two fluorescent dyes and two images per cycle are used to simplify nucleotide detection. DNA clusters are imaged using red and green wavelength filter bands, and subsequently red or green clusters are recorded as cytosine and thymine bases, respectively, clusters seen as both red and green are recorded as adenine bases, and unlabeled clusters as guanine bases (Illumina, 2021a).

Many NGS technologies share the same basic principles with Sanger sequencing, such as the usage of DNA polymerase to add fluorescent nucleotides to a template strand. However, NGS is massively parallel, meaning it can sequence millions of fragments during one run, compared to just one fragment sequenced at a time by Sanger sequencing. In general, NGS approaches involve DNA fragmentation, adding of adapters, surface attachment (either beads such as in ThermoFischer Ion Torrent, or flow cells like in Illumina platforms), and amplification. The NGS workflow begins first with acquiring purified nucleic acid of the sample to be studied. Then library preparation, sequencing, and data analysis follow (Illumina, 2021a).

In sequencing, a library consists of a collection of DNA or RNA fragments representing either the whole genome or a specific target region, depending on the research question (Pereira et al., 2020). To prepare a library, nucleic acid is fragmented and then sequence adapters are added prior to enrichment. A good quality library ideally contains an equal amount of all fragments (Aird et al., 2011). Adapters are short sequences containing the full complement of sequencing primer hybridization sites (Illumina, 2021b). A step combining DNA fragmentation and adding of adapters has also been developed, reducing the library preparation time (Illumina, 2021c).

Illumina, offering multiple different sequencing systems, is the most popular NGS provider (Goodwin et al., 2016) with over three billion USD in revenue in 2020 (Illumina, 2021f). Some earlier developed sequencing platforms have discontinued (Goodwin et al., 2016; Giani et al., 2020), but other NGS companies currently provide short-read sequencing including, for example, the Ion Torrent platform by ThermoFisher Scientific, which is based on detecting changes in pH during the sequencing process (Rothberg et al., 2011).

The probability that a base is called incorrectly in the sequencing process is measured by a quality score by a phred-like algorithm (Ewing et al., 1998), termed as a Q score, which is defined as:

$$Q = -10\log_{10}(e)$$

Where 'e' represents the estimated probability of a base being called incorrectly. For example, a quality score of 30 (Q30) represents an error rate of 1 in 1000 bases, which corresponds to a base call accuracy of 99.9. The majority of bases sequenced with Illumina technology represent Q30 or higher (Illumina, 2021d) offering the most accurate base-by-base sequencing technology currently available. The error rate of 0.1% is mostly attributed to substitution errors, and only rarely insertions or deletions (Hu et al., 2021a).

In Illumina sequencing, the read length produced is determined by the reagents used in sequencing cycles, as one base is sequenced per cycle. A 300-cycle kit produces read lengths of 2 x 150 bp for a paired-end run (Illumina, 2021e).

2.5.3 Long-read sequencing

Achieving complete, high-quality assemblies is often difficult especially for plasmids, which contain many repeated sequences complicating the assembly graphs and resulting in fragmented assemblies (Arredondo-Alonso et al., 2017; Lemon et al., 2017). Differentiating plasmid genomes from the chromosome is also challenging with fragmented, short-read data. Long-read sequencing has been shown to recover even complete plasmid sequences of over 90 kb in a single read, omitting the need for *de novo* assembly afterwards, granting that the accuracy of the reads is high (Li et al., 2018). Furthermore, long-read sequencing technologies can use native nucleic acids, omitting the use of amplification steps and bias introduced by the PCR process.

Two prominent long-read sequencing technologies currently exist: Single Molecule, Real-Time (SMRT) sequencing commercialized by Pacific Biosciences (PacBio) since 2011, and Nanopore sequencing commercialized by Oxford Nanopore Technologies (ONT) since 2005 (Giani et al., 2020). Both technologies are based on single molecule sequencing, although with differing principles. In addition, synthetic approaches also exist. These techniques produce synthetic long reads by reconstructing clusters of short reads marked with the same barcode (Liu et al., 2021).

The original *E. coli* strain described in 1885 by Theodor Escherich was sequenced over 130 years after its discovery in 2017 with PacBio SMRT sequencing (Dunne et al., 2017). During SMRT library preparation, special hairpin-shaped adapters are ligated to both ends of double-stranded DNA to produce a circular template (Travers et al., 2010). Next, a primer and a polymerase are added to the library. The sequencing instrument consists of a

"SMRT Cell" that contains millions of tiny wells, called zero-mode waveguides. Light is emitted and recorded in real time as polymerase adds labeled nucleotides to a single template immobilized in a zero-mode waveguide well (PacBio, 2020; Levene et al., 2003; Hu et al., 2021a). Reads produces by SMRT sequencing are typically 10–20 kbp in length but reads longer than 50 kbp are possible (Rhoads and Au, 2015; Giani et al., 2020).
A recently invented technique within SMRT sequencing named circular consensus sequencing can generate highly accurate (>99.8%) long reads with an average length of 13.5 kb, also called high-fidelity reads (Wenger et al., 2019). This technique allows for accurate detection of single nucleotide variants also from hard-to-sequence areas of the genome and matches the accuracy of short-read technologies and Sanger sequencing (Wenger et al., 2019). Even longer reads, averaging 10–25 kb with over 99.5% accuracy, can be achieved with a HiFi sequencing protocol producing highly accurate long reads (Hon et al., 2020).

The idea of nanopore sequencing was already sketched as an idea in a notebook in 1989, although the first commercial MinION devices by ONT were not released until 2014 (Deamer et al., 2016). Nanopore sequencing is a technique based on an electrically resistant membrane with bioengineered α-hemolysin protein pores with an inner diameter of 1 nm (nanopores). Single-stranded DNA or RNA passes through a nanopore, disrupting the current. This change in current is then translated to a nucleic acid sequence via basecalling algorithms in real time (Deamer et al., 2016; Jain et al., 2016; Ciuffreda et al., 2021). Basecalling refers to the computational process of interpreting signals produced during sequencing into the corresponding base sequence. The nanopore-containing membranes are incorporated into devices called flow cells. The MinION flow cell contains 512 channels, each connecting to four pores, totaling 2048 nanopores per one flow cell (Ip et al., 2015). The channels are controlled with an application specific integrated circuit (Lu et al., 2016). The MinION device, weighing only 90 g, can be used with a portable computer, enabling true in-field usage. Other sequencing platforms offered by ONT include the benchtop GridION and PromethION, which can incorporate a larger number of flow cells, thus producing even higher data output. An adaptor device named 'Flongle' with 126 channels was also introduced in 2019, targeted for smaller scale experiments (Lu et al., 2016). The smallest sequencer to date, SmidgION, is still in development and is planned to be optimized for pairing with a smartphone (Oxford Nanopore Technologies, 2021b). To maximize flow cell usage, several samples can be pooled together for sequencing by adding barcode adapters in the library preparation process (Ciuffreda et al., 2021). These multiplexed samples are then demultiplexed after sequencing with command-line tools to bin data generated from each sample together again.

Library preparation consists of shearing of the DNA, an optional step to repair damaged DNA, end-repair of DNA fragments and addition of an adenine base to 3' ends of the fragments, adapter ligation, and purification (Lu et al., 2016). Depending on the chosen library preparation strategy, the 'template' and 'complement' strands can be sequenced either as individual strands or immediately one after another, the latter method increasing the read accuracy

(Lu et al., 2016). Sequencing the double-stranded DNA fragments multiple times to achieve a consensus sequence, such as in ‘circular consensus sequencing’ employed by PacBio, has been shown to improve read quality (Wang et al., 2021). In a library preparation method called ‘2D’, used in the early versions of nanopore sequencing, a motor protein first guides the ‘template’ strand through the nanopore, followed by the ‘complement’ strand which is ligated to the template with a hairpin adapter. In 1D library preparation, each strand is ligated with an adapter and sequenced one by one. Another strategy, termed $1D^2$, uses special adapters ligated to each strand to increase the probability of the same nanopore sequencing the complement strand immediately after the template strand, generating similar consensus sequences as in the 2D strategy (Wang et al., 2021).

ONT sequencing offers the longest read lengths, averaging at 23 kilobases (kb) (Deschamps et al., 2018) and with a current published record of 2.3 megabases (Mb) (Payne et al., 2019) or even > 4 Mb reported on the ONT webpage (Oxford Nanopore Technologies, 2021a). Compared to SMRT sequencing, in which the longevity of the polymerase is the limiting factor regarding read length, in nanopore sequencing the limitation is mostly attributed to the ability to provide high-molecular weight DNA to the pore (Amarasinghe et al., 2020). Although improvements regarding the production of very high-molecular DNA have been made, this has an adverse effect on the sequencing yield (Jain et al., 2018).

The accuracy of long-read sequencing has also seen improvements in recent years, with a <1% raw base-called error rate for SMRT (Wenger et al., 2019) and <5% for ONT sequencers (Jain et al., 2018) shown. Compared to short-read NGS technologies, however, the error rate is relatively high. To improve accuracy and remove errors, error correction and polishing of are commonly used before and after assembly (Wang et al., 2021). The errors could be due to abnormal current fluctuations or misinterpretation of the electrical signals, which could be corrected by further improvements in the basecalling process (Rang et al., 2018). Although GC bias is considered to affect mostly short-read technologies, it has been shown that reads with high GC regions have a higher error rate compared to low-GC reads also in nanopore sequencing (Delahaye and Nicolas, 2021).

The benefits of long-read sequencing over short-read approaches include genome assemblies with better quality due to lower GC and low-complexity region bias (Rhoads and Au, 2015; Giordano et al., 2017), which provides a more robust coverage of the whole genome (Roberts et al., 2013; Díaz-Viraqué et al., 2019). The benefits of long-read sequencing technologies can be compared to the completion of a jigsaw puzzle: the larger the size of a single piece, the easier it is to complete the puzzle correctly. Similarly, longer reads spanning over a longer piece of the genome contain distinguishable overlap with other reads, enabling the more accurate recognition of the correct nucleotide order, genome-wide repeats, and structural variants. Different sequencing technologies are summarized in Figure 2.

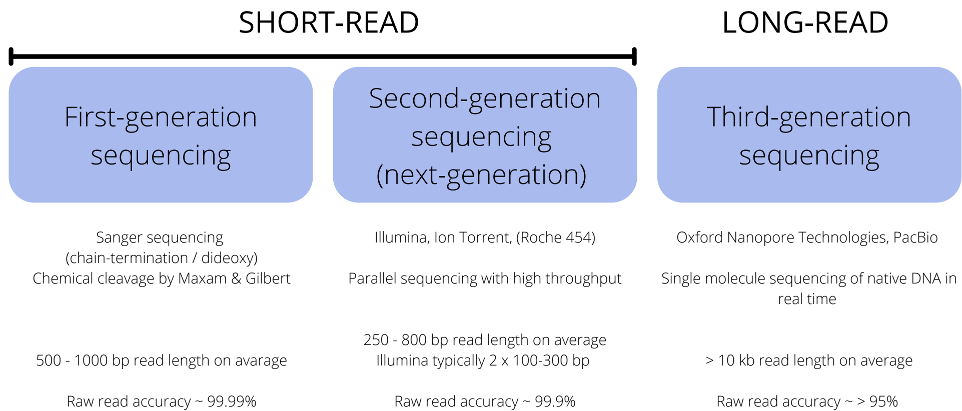


Figure 2. Different sequencing technologies with average read lengths and accuracies.

2.5.4 The hybrid approach

Certain genome regions, especially on plasmids, contain many repetitive regions, which are hard to reconstruct using short-read sequencing technologies alone (de Toro et al., 2014; Arredondo-Alonso et al., 2017). To evaluate the possible risk of AMR spread via horizontal gene transfer it is crucial to determine the location (chromosome/MGE) of AMR genes correctly. Furthermore, AMR genes and virulence genes acquired by HGT are often flanked with repetitive insertion sequences, complicating the correct assembly from short-read-only data with multiple contigs and gaps (Ashton et al., 2015). Long-read sequencing technologies are, however, able to resolve even the difficult-to-assemble repetitive regions, but often fail to provide the same accuracy as the short-read approaches (Wick et al., 2017a; Berbers et al., 2020), which makes combining these two techniques highly promising. This hybrid approach, the combination of short-read and long-read technologies, has been shown to reconstruct genomes, such as plasmids harboring AMR genes, successfully and accurately (Ashton et al., 2015; Sović et al., 2016; George et al., 2017; Wick et al., 2017a) (Figure 3).

Different hybrid assembly strategies have been developed to combine raw reads produced by these two technologies (Chen et al., 2020), such as a tool named Unicycler (Wick et al., 2017b) which has been shown to achieve contiguous genomes. The Unicycler pipeline produces an assembly graph from short-read data and subsequently employs long reads to build bridges, resolving repetitive regions and achieving a complete genome assembly (Wick et al., 2017b) generating fully closed genomes and outperforming other hybrid assemblers (de Maio et al., 2019). Long reads provide the information for the structure of the genome, while short reads correct errors present in the long reads.

A hybrid approach to error correction is currently outperforming the long-read-only approach (Zhang et al., 2020), although improvements in long-read sequencing accuracy and error correction tools are expected to simplify the sequencing pipeline in the future (Amarasinghe et al., 2020).

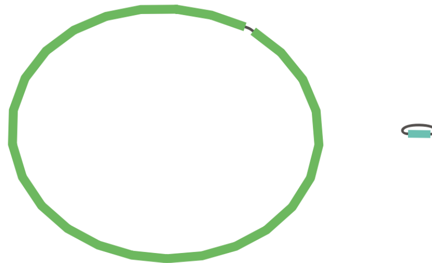


Figure 3. Representation created with Bandage (Wick et al., 2015) of an *Escherichia coli* genome from Illumina reads combined with Nanopore reads assembled with a hybrid approach using Unicycler v 0.4.8 (Wick et al., 2017b). The hybrid assembly succeeded in generating a complete circularized chromosome (large circle) and a plasmid (small circle).

2.5.5 Sequence data analysis

After acquiring basecalled raw reads from sequencing platforms, quality control to remove poor-quality data and adapter sequences is the important next step. Removal of sequencing artifacts is crucial for accurate deductions from WGS data, such as SNP detection (Oakeson et al., 2017). Tools such as Trimmomatic (Bolger et al., 2014) are used for technical sequence removal quality filtering, i.e., to remove reads below a certain threshold length determined for the analysis.

Several different assembly approaches exist to build a representation of the genome from fragmented raw reads produced by the sequencing platforms. A genome assembly consists of continuous sequences, 'contigs', which are generated by aligning overlapping sequencing reads (Segerman, 2020). On average, each raw read covers a specific location of the genome multiple times, thus resulting in overlaps that are used for building the contigs with different algorithms. For example, a coverage (also known as depth), of 100x equals to each base in the genome being sequenced 100 times on average. Higher coverages usually amount to a higher confidence in base calling. After successful assembly, a continuously evolving and expanding array of bioinformatic tools exist for further analysis, such as antimicrobial resistance and virulence gene detection, in silico multilocus sequence typing, annotation and plasmid replicon detection. Different databases are continuously updated to include new gene variants. The preferable database and bioinformatic tool chosen depend on the research question.

3 AIMS OF THE STUDY

The main aim of this thesis was to determine the molecular epidemiology of ESBL/AmpC-producing *E. coli* between animal, human and environmental interface in Finland. Whole genome sequencing was employed to assess the genetic connections between different sources and study plasmids carrying beta-lactamase resistance genes. In addition, the prevalence and transmission of susceptible *E. coli* in the broiler production environment was assessed. Imported food products were studied for both ESBL/AmpC-producing *E. coli* and *K. pneumoniae*. The final study of the thesis (Study IV) compiled all isolates together and compared them to ESBL-producing *E. coli* obtained from infection samples from human patients in Finland.

Specific aims of the studies included in the thesis were as follows:

1. To study the prevalence and transmission routes of ESBL/AmpC-producing *E. coli* and *E. coli* without antibiotic selection in the broiler production pyramid by sampling parent birds, egg surfaces, chicks, and the production environment (I)

2. To study the effect of a competitive exclusion product on the presence of ESBL/AmpC-producing *E. coli* on egg surfaces after the incubation period (I)

3. To study the occurrence of ESBL/AmpC-producing *E. coli* in migrating barnacle geese in Finland and identify resistance and virulence genes and plasmids harboring beta-lactamase genes (II)

4. To compare resistance genes and plasmids identified in ESBL/AmpC-producing *E. coli* from migrating barnacle geese to plasmids of international origin to determine the possible dissemination of successful plasmids harboring beta-lactamase genes (II)

5. To study imported food products of wide origin for the presence of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* and determine potential epidemic plasmid types harboring beta-lactamase genes (III)

6. To apply WGS to determine beta-lactamase genes identified from ESBL-producing *E. coli* infections in humans in Finland and to compare human isolates to ESBL/AmpC-producing *E. coli* isolates obtained from non-human sources in Finland and from international sources submitted to public repositories (IV)

4 MATERIALS AND METHODS

4.1 Sample collection

A summary of samples collected in studies I–IV is presented in Table 2.

Table 2. A summary of samples collected in studies I–IV.

Study	Source	Year	Country	Reference
I	Parent broiler caecum (n=450) Egg surface (n=450) Broiler chick (n=422) Production environment (n=20)	2017	Finland	Study I
II	Barnacle goose feces (n=200)	2017 and 2018	Finland	Study II
III	Imported food (n=200) Raw broiler meat subsamples (n=40)	2018	Country of collection: Finland Origin of samples specified in Appendix I & II	Study III
IV	Human clinical isolates (n=30)	2018 – 2020	Finland	Study IV

4.1.1 Poultry production

For Study I, samples were collected from poultry production and included samples from the cloaca of parent broilers (n=450), the production environment (n=20), egg surfaces (n=450), and broiler chicks (n=422). Samples originated from a single laying house hall comprising approximately 4000 birds. The cloacal samples from parent broilers, environmental samples, and eggs were collected during one sampling day. Sterile culture swabs (Copan Transystem, Copan Diagnostics, Italy) were used to obtain cloacal samples of the parent broilers and the environmental samples, for which the swabs were moistened with sterile buffered peptone water (BPW) (Oxoid, Basingstoke, Hampshire, UK). For the eggs, altogether 450 freshly laid eggs originating

from the same laying house hall were collected for further sampling. Of these 450 eggs, surface samples were obtained during the same sampling day for 300 out of the 450 eggs by immerging in 20 ml of sterile BPW. After sampling, 150 of these sampled eggs were additionally dipped into competitive exclusion solution product, Broilact (Orion Corp., Orion Pharma, Espoo, Finland). The unsampled eggs (n=150) were left untreated and unsampled at the laying house. All swab and BPW samples were transported to the laboratory for further analysis.

Subsequently, all the 450 eggs were transported to the hatchery according to normal production procedure, including disinfection measures on the arrival to the hatchery. After the egg incubation period (17 days), all fertilized eggs (n=428) were transported from the hatchery to the laboratory for further studies. The egg surfaces were re-sampled with the same procedure by dipping in BPW, and now the previously unsampled eggs were included for sampling. Subsequently, the eggs were cut open and the chick intestines and yolk sac sampled from all the eggs from which an embryo was identified (n=422).

4.1.2 Barnacle goose

For Study II, 200 barnacle goose droppings were collected during fall 2017 (n=100) and spring 2018 (n=100) from the same urban park area in Helsinki, Finland. Fresh droppings were collected into individual plastic bags and transported to the laboratory within one hour for further analysis.

4.1.3 Imported food

During fall 2018, 200 imported food products for Study III were purchased from nine different grocery stores located in the Helsinki region. The products were divided into four different categories: vegetables (n=60), fruits and berries (n=50), meat products (n=60), and fish and seafood products (n=30). Raw, frozen, cooked, and ready-to-eat products were included. Ten out of the 60 meat products consisted of raw broiler meat packages, each from the same batch number and weighing 2 kg. The samples were transported to the laboratory immediately after purchasing and stored at +4 °C until further analysis was started within 24 hours.

4.1.4 Human clinical samples

For Study IV, phenotypically ESBL-producing *E. coli* isolates (n=30) were obtained from the Eastern Finland Laboratory Centre Joint Authority Enterprise (ISLAB) during spring 2020. The isolates were part of the health district's routine procedures and collected from clinical specimens between 2018 and 2020. The isolates were re-cultivated from stored samples and transported to the University of Helsinki with Copan M40 Transystem sterile transport swabs (Copan Transystem, Copan Diagnostics, Italy).

4.2 Isolation of ESBL/AmpC-producing *E. coli* and *K. pneumoniae*

Primary enrichment (I-III)

For Study I, the samples were enriched by immersing culture swabs in 9 ml of sterile BPW (Oxoid, Basingstoke, Hampshire, United Kingdom) for parent broiler cloacal samples and the production environment samples. For the egg surface samples, the 20 ml BPW used for dipping of the eggs was incubated. The chick intestine and yolk samples were additionally immersed in 9 ml of sterile BPW for the enrichment.

For Study I, the occurrence and transmission of *E. coli* cultured without antibiotic supplement was also studied. To achieve this, a subset of the parent broiler cloacal samples (n=30), chick intestine and yolk samples (n=90), egg surface samples (n=30) were plated from the enrichments onto MacConkey agar plates without antibiotic supplement and incubated identically to other plates as described for Study I.

For Study II, 1 ± 0.1 g of the feces samples were weighed and inserted into a test tube with 9 ml sterile BWP for enrichment.

For Study III, a 25 g dissection of each food sample was obtained and homogenized (Stomacher 400 laboratory blender, Seward, United Kingdom) with 225 ml of sterile BWP. The food samples were dissected from multiple different sites of the product to achieve more extensive representation of each product. The ten 2 kg raw broiler meat packages were further subsampled to obtain a better understanding of ESBL/AmpC-producing bacteria present in a single product of high ESBL/AmpC risk status. Four additional subsamples were obtained from each package, totaling 40 subsamples.

All the enrichments were incubated at 37 °C overnight.

Selective isolation (I-III)

After incubation, a loopful (10 μl) of the enrichments were plated on MacConkey agar (Lab M, Lancashire, United Kingdom; Scharlau Chemie s.a., Sentmenat, Spain for Study I; Oxoid, Basingstoke, Hampshire, United Kingdom for Studies II–III) supplemented with 1 mg/ml cefotaxime.

The plates were initially incubated at 44 °C for 18 – 22 h. For Study III, an additional MacConkey plate with cefotaxime supplement was prepared with a loopful (10 μl) of the enrichment and incubated at 37 °C to improve the detection of *Enterobacteriaceae* listed as highest priority by WHO (WHO, 2017), such as *Salmonella* spp.

Subsequently, a typical *E. coli* colony was selected from each plate with growth and re-streaked on MacConkey agar plate supplemented with cefotaxime 1

mg/ml and incubated at 37 °C for 18 – 22 h in Study I. For studies II and III, one colony from each morphologically different bacterial growth was selected.

The outline for isolating ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* in the studies is described in Figure 4 and described in more detail in sections 4.2.1 to 4.2.3.

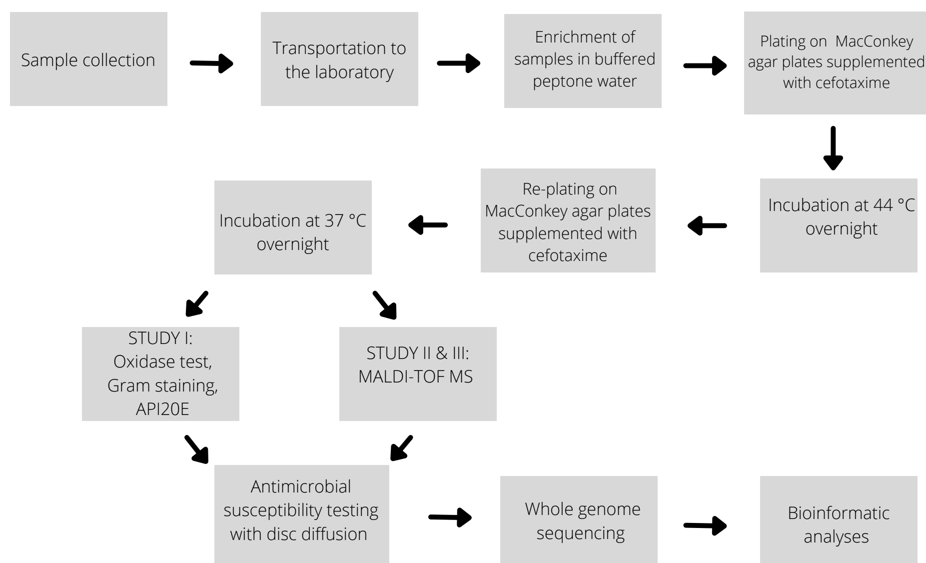


Figure 4. Outline for isolating ESBL/AmpC-producing *Escherichia coli* and/or *Klebsiella pneumoniae* in the studies.

4.2.1 Phenotypic tests (I)

For Study I, a typical colony from MacConkey agar plates from each sample was confirmed as *E. coli* by using an oxidase test (Thermo Fischer Scientific Inc., Waltham, MA, USA), Gram staining and an API20E biochemical test (Biomérieux, Marcy-l'Etoile, France).

4.2.2 Species determination with MALDI-TOF (II, III)

After achieving a pure bacterial culture, the isolates from Studies II and III were streaked onto a bovine blood agar plate and incubated at 37°C overnight for bacterial species determination with a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) based Bruker Biotyper (Bruker Daltonics). A score value of 2.0 – 3.0 was considered high-confidence and was set as the criteria for species determination. For Study II, all isolates identified as *E. coli*, and for Study III, all isolates identified as *E. coli* and *K. pneumoniae* were stored at -70 °C for further characterization.

4.2.3 Antimicrobial susceptibility testing (I-III)

To determine ESBL, AmpC, ESBL+AmpC, or carbapenemase phenotype, antimicrobial susceptibility testing was performed according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST, 2017). Figure 5 depicts the disks used for Studies I, II, and III.

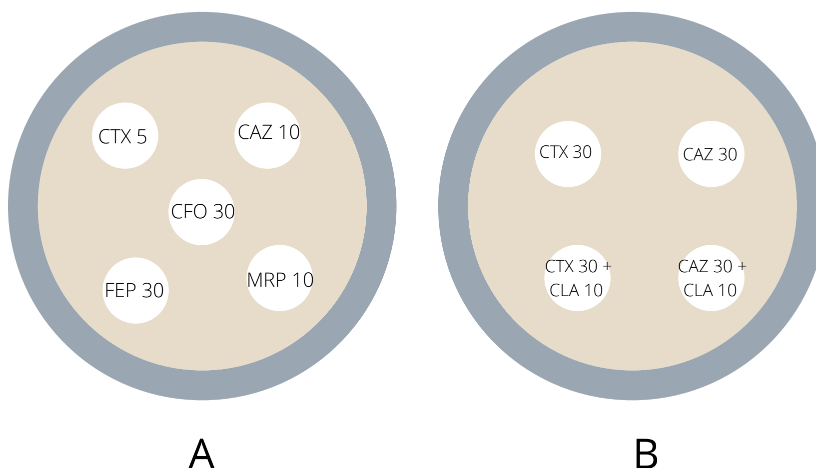


Figure 5. Antibiotic disks used in the studies. **(A)** CTX 5 = cefotaxime 5 µg, CAZ 10 = ceftazidime 10 µg, CFO 30 = cefoxitin 30 µg, FEP 30 = cefepime 30 µg, MRP 10 = meropenem 10 µg. **(B)** Disks used in the combination disk diffusion test: CTX 30 = cefotaxime 30 µg, CTX 30 + CLA 10 = cefotaxime 30 µg + clavulanic acid 10 µg, CAZ 30 = ceftazidime 30 µg, CAZ 30 + CLA 10 = ceftazidime 30 µg + clavulanic acid 10 µg. FEP 30 was used in Studies II and III, all other disks were used in all Studies I – III.

For Study I, susceptibility to third-generation cephalosporins was tested with ceftazidime (10 µg) (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark) and cefotaxime (5 µg) (Oxoid Ltd, Basingstoke, Hampshire, UK), to cephamycin with cefoxitin (30 µg), and to carbapenem with meropenem (10 µg) (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). Synergism between third-generation cephalosporins and clavulanic acid was tested with a combination disk diffusion test with cefotaxime + clavulanic acid 30 µg + 10 µg and ceftazidime + clavulanic acid 30 µg + 10 µg (Neo-Sensitabs, Rosco Diagnostica A/S, Taastrup, Denmark). For Studies II and III, fourth-generation cephalosporin, cefepime (30 µg) (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark), was additionally included in the analysis. The epidemiological cut-off values were used as a reference (MIC EUCAST). *E. coli* ATCC 25922 was included as a quality control.

In addition to resistance to third-generation cephalosporins (cefotaxime and/or ceftazidime), resistance to cephamycin (cefoxitin) and <5 mm difference in inhibition zones in the combination disk diffusion test were used as criteria for AmpC production, whereas ESBL production was evidenced by resistance to third-generation cephalosporins and ≥ 5 mm difference in the

combination disk diffusion test. Resistance to third-generation cephalosporins, resistance to cephamycin and ≥ 5 mm difference in the combination disk diffusion test were interpreted as a combined ESBL + AmpC phenotype. Additionally, susceptibility to fourth-generation cephalosporin (cefepime) was interpreted as a characteristic for AmpC production, whereas resistance to cefepime was interpreted as an indicator for ESBL production.

For Study IV, antimicrobial susceptibility testing was performed at Eastern Finland Laboratory Centre Joint Authority Enterprise (ISLAB). For specimens other than blood and urine susceptibility testing was performed using the disk diffusion method according to EUCAST standards with third-generation cephalosporins (cefpodoxime, ceftazidime, and ceftriaxone), together with amoxicillin-clavulanic acid (Oxoid, Basingstoke, Hampshire, UK). Combination disk diffusion test was performed with the same antibiotic disks as for Studies I – III, and additionally an AmpC disk test (Mast Group Ltd., Bootle, UK) was used for presumptive ESBL-producing *E. coli* isolates. Susceptibility testing for urine samples was performed with a Vitek 2 AST-N385 card (bioMeriéux, Marcy-L'Etoile, France), and for blood samples with both Vitek 2 and disk diffusion method according to EUCAST standards.

4.3 Whole genome sequencing

Two different sequencing strategies were used in the studies included in this thesis. Short-read sequencing was employed in all studies, and long-read sequencing and subsequent hybrid assembly approach was included in Study II and III to study plasmid replicons in more depth. The following chapters describe the isolate selection and sequencing strategies in more depth.

4.3.1 Selection of isolates for sequencing

For Study I, a representative collection of *E. coli* isolates (n = 23) was chosen for whole genome sequencing. Chosen isolates consisted of isolates from parent birds (8 resistant isolates, 4 isolates without antibiotic selection), outer eggshell samples (4 resistant isolates, 4 isolates without antibiotic selection), the environmental sample (1 resistant isolate), and chick intestines (2 isolates without antibiotic selection).

For Study II, all phenotypically ESBL/AmpC-producing *E. coli* isolates (n=9) were subjected to WGS with Illumina to study the presence of AMR and virulence genes and plasmid replicons, as well as to assess the multilocus ST. To study the complete sequences and to identify plasmid replicons carrying *bla* genes, all ESBL/AmpC-producing *E. coli* isolates (n=9) were additionally long-read sequenced.

For Study III, from all food samples positive for ESBL/AmpC-producing *E. coli* or *K. pneumoniae,* a collection of isolates was chosen for WGS analysis in order to study the presence of AMR, virulence genes, and plasmid replicons, as well as to assess the multilocus sequence type (MLST). If applicable, a

representative from each ESBL/AmpC enzyme type category (ESBL, AmpC, or ESBL together with AmpC) and bacterial species (*E. coli* or *K. pneumoniae)* was chosen from each positive food sample, excluding subsamples. Consequently, from one to three isolates were chosen for whole genome sequencing with Illumina platform from each positive sample. Subsequently, seven short-read sequenced *E. coli* isolates were chosen for long-read sequencing to study beta-lactamase harboring plasmids in more depth. Isolates were chosen from short-read sequenced isolates to represent a wide selection of different beta-lactamases, MLST types, and plasmid replicons.

For Study IV, all ESBL-producing *E. coli* isolates obtained from human infections (n=30) were subjected to WGS.

4.3.2 DNA extraction (I-IV)

Bacterial DNA was extracted and purified with a PureLink Genomic DNA Mini Kit (Invitrogen by Thermo Fischer Scientific, Carlsbad, CA, USA) according to manufacturer's instructions. The assessment of DNA quality was carried out using a NanoDrop ND-1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and DNA quantity was measured using a Qubit 2.0 fluorometer (Invitrogen, Life Technologies, Carlsbad, CA, USA). An optical density of 1.8 – 2.0 at 260/280 nm was set as a threshold.

For Study II and III a concentration of ≥ 50 ng/µl was set as a threshold and for Study IV a concentration of ≥ 10 ng/µl with a minimum amount of 0.2 µg were set as thresholds.

4.3.3 Short-read sequencing (I-IV)

For Studies I – III, sequencing was performed with an Illumina Novaseq 6000 (Center for Genomics and Transcriptomics, Tübingen, Germany) with paired-end reads, 100× coverage and 2× 100 bp read length, and a minimal phred quality score of 30. Library preparation was performed with an Illumina Nextera XT. For Study IV, sequencing was performed with an Illumina Novaseq 6000 (Novogene, Cambridge, UK) with 100 × coverage and 2 × 150 bp read length.

4.3.4 Long-read sequencing (II, III)

To study the isolates in more depth and to identify plasmid replicons carrying resistance genes, all ESBL/AmpC-producing *E. coli* isolates (n=9) of Study II and a selection (n=7) of *E. coli* isolates from Study III were additionally long-read sequenced. For Study III, isolates were chosen from short-read sequenced isolates to represent a wide selection of different *bla* genes, MLST types, and plasmid replicons.

DNA extraction and purification were performed as described above. For Study II, DNA extracts from all isolates were multiplexed in a random order with either SQK-LSK 108 or SQK-LSK 109 ligation sequence kit (Oxford

Nanopore Technologies, Oxford, UK), depending on the availability of the respective flow cells, as described in more detail in the following sentences. DNA extracts from four geese isolates (H11, H21, H29, and H163) were multiplexed using the SQK-LSK108 ligation sequence kit (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's protocol. Libraries were loaded onto FLO-MIN106D R9.4.1 MinION flow cells (Oxford Nanopore Technologies, Oxford, UK) used with the MinION Mk1B sequencing device and sequenced with MinKNOW software v19.06.8 for 48 h. For five isolates (H5, H58, H68B, H98, and H193) and all the isolates from Study III, DNA extracts from three or two isolates at a time were multiplexed using the SQK-LSK109 ligation sequence kit (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer's protocol. Libraries were loaded onto FLO-FLG001 R9.4.1 Flongle flow cells (Oxford Nanopore Technologies, Oxford, UK) used with the MinION Mk1B sequencing device and sequenced with MinKNOW software v 19.06.8 for 20 – 24 h.

4.4 Data analyses

Analysis of sequence data was performed both with freely available bioinformatic tools, as well as with commercial bioinformatic pipelines. Both graphical user interface-based tools and command line-based solutions were employed. These are described in more detail in the following chapters and a summary of the tools used is depicted in Figure 6.

	STUDY I	STUDY II	STUDY III	STUDY IV
SEQUENCING PLATFORM	Illumina Novaseq 6000	Illumina Novaseq 6000 Oxford Nanopore	Illumina Novaseq 6000 Oxford Nanopore	Illumina Novaseq 6000
ASSEMBLY	SPAdes v 3.9	Unicycler v 0.4.9b	SPAdes v 3.9 Unicycler v 0.4.8	SKESA v 2.3.0
SPECIES CONFIRMATION	KmerFinder v 2.5	KmerFinder v 3.1	KmerFinder v 3.1	Mash Distance v 2.1
MULTILOCUS SEQUENCE TYPE	MLST v 1.8	MLST v 2.0	MLST v 2.0	MLST v 2.0 PubMLST
SEROTYPE & FIMH TYPE	-	-	-	SerotypeFinder v 2.0 FimTyper v 1.0
AMR GENES	KmerResistance v 2.2	ResFinder v 4.1	ResFinder v 3.1 + v 3.2	NCBI AMRFinderPlus v 3.2.3 ResFinder v 4.1
VIRULENCE GENES	VirulenceFinder v 1.5	VirulenceFinder v 2.0	VirulenceFinder v 2.0	VirulenceFinder v 2.0 VFDB
GENOMIC COMPARISON OF BACTERIAL ISOLATES	CSI Phylogeny v 1.4	-	-	cgMLST-based MST BRIG v 0.95
PLASMID REPLICONS	PlasmidFinder v 1.3	PlasmidFinder v 2.1	PlasmidFinder v 2.1	PlasmidFinder v 2.0
PLASMID TYPING	-	pMLST v 2.0	pMLST v 2.0	pMLST v 2.0
ANNOTATION OF PLASMIDS	-	Prokka v 1.13	Prokka v 1.13 MobileElementFinder v 1.0.3	-
PLASMID COMPARISON & VISUALIZATION	-	BRIG v 0.95, BacCompare GrapeTree v 1.5, SnapGene	BRIG v 0.95 EasyFig v 2.2.2	-

Figure 6. Summary of bioinformatic tools used for whole genome sequence data analysis in Studies I – IV.

4.4.1 Genotypic characterization of bacterial isolates (I-IV)

Genotypic characterization of the sequenced isolates followed the same pattern in principle for all studies. After quality control of sequence data, the raw reads were assembled, and different bioinformatic tools were used to detect traits and genes of interest.

4.4.1.1 Assembly

For Study I and short-read-only sequenced isolates of Study III, raw reads were assembled with SPAdes v 3.9 (Nurk et al., 2013b).

For Study II and long-read-sequenced isolates of Study III, Oxford Nanopore FAST5 read files were basecalled using Guppy v 3.4.1 (Oxford Nanopore Technologies, Oxford, UK) with FASTQ output and demultiplexed with Qcat v 1.1.0 (Oxford Nanopore Technologies, Oxford, UK). Quality trimming was performed with BBDuk (BBTools v 38.71, Joint Genome Institute, USA) using a QTRIM value of seven. Hybrid assembly of Illumina and Nanopore sequences was performed with Unicycler v 0.4.9b for Study II, and v 0.4.8 for Study III (Wick et al., 2017b) set at default values.

For Study IV, raw reads were assembled within Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) (Jünemann et al., 2013) pipeline with SKESA v 2.3.0 (Souvorov et al., 2018) together with quality control with FastQC v 0.11.7 (Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data) and adapter trimming with Trimmomatic v 0.36 (Bolger et al., 2014).

4.4.1.2 Species confirmation

Species identification was confirmed *in vitro* with KmerFinder v 2.5 (Hasman et al., 2014; Larsen et al., 2014) using short-read assembled contigs for Study I. KmerFinder v 3.1 (Hasman et al., 2014; Larsen et al., 2014; Clausen et al., 2018) was used for hybrid assembled contigs for Study II, and for short-read assembled contigs for Study III. The web-based tool is provided by the Center for Genomic Epidemiology (CGE) (DTU, Denmark) available at http://www.genomicepidemiology.org.

For Study IV, Mash Distance v 2.1 (Ondov et al., 2016) within Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) (Jünemann et al., 2013) pipeline was used for species confirmation.

4.4.1.3 Multilocus sequence typing in vitro

Multilocus sequence type (MLST) was determined with assembled contigs using MLST v 1.8 for Study I and MLST v 2.0 (Larsen et al., 2012) provided by CGE (DTU, Denmark) for Studies II, III, and IV. *E. coli* scheme 1 (Wirth et al., 2006) within the analysis tool was selected.

For Study IV, the sequence type was firstly determined with *E. coli* MLST Warwick v 1.0 and *E. coli* MLST Pasteur v 1.0 based on the PubMLST database (Jolley et al., 2018) within the Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) pipeline (Jünemann et al., 2013).

4.4.1.4 Serotyping and fimH typing (IV)

FimTyper v 1.0 (Roer et al., 2017) was used to identify the *fimH* allele for isolates obtained from ESBL-producing *E. coli* infections from Finnish patients in Study IV. SerotypeFinder v 2.0 (Joensen et al., 2015) was used to confirm the serotype for the same isolates. Both tools are web-based and provided by CGE (DTU, Denmark).

4.4.1.5 Detection of virulence and antimicrobial resistance genes

For Study I, VirulenceFinder v 1.5 and for Studies II and III VirulenceFinder v 2.0 (Joensen et al., 2014) was used to determine virulence genes from assembled contigs from short-read sequence data with an identity percentage threshold of 90% and a minimum length of 60%. For Study III, VirulenceFinder v 2.0 was additionally used to identify resistance genes residing on plasmid contigs from hybrid-assembled data.

Virulence factor database (VFDB) (Chen et al., 2016) from within Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) pipeline (Jünemann et al., 2013) was first used to determine virulence genes for *E. coli* isolates in Study IV, and VirulenceFinder v 2.0 (Joensen et al., 2014) was then used to confirm the results.

For Study I, KmerResistance v 2.2 (Clausen et al., 2016) was used for identifying antimicrobial resistance genes with unassembled raw reads, with a 70% identity threshold and depth correlation of 10%.

Acquired resistance genes for isolates in Study II were determined with ResFinder v 4.1 (Zankari et al., 2012; Carattoli et al., 2014) using hybrid-assembled FASTA files with a 90% identity threshold and minimum lenght of 60%. Because acquired beta-lactamase genes were not detected for isolate H68B in Study II, ResFinder v 4.1 was additionally used for detecting chromosomal point mutations for this isolate.

Using default values for identity and coverage, ResFinder v 3.1 and v 3.2 (Zankari et al., 2012; Carattoli et al., 2014) were used for determining acquired resistance genes in short-read assembled contigs and hybrid-assembled contigs, respectively, for isolates in Study III.

For Study IV, NCBI AMRFinderPlus v 3.2.3 (Feldgarden et al., 2019) from within Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) pipeline (Jünemann et al., 2013) was used first to detect resistance genes, and ResFinder v 4.1 ((Camacho et al., 2009; Zankari et al., 2017; Bortolaia et al., 2020) was used for confirming the results.

4.4.1.6 Phylogenetic analysis (I)

For Study I, CSI Phylogeny v 1.4 (Kaas et al., 2014) available at the CGE (DTU, Denmark) platform was used with default values to assess the single nucleotide polymorphism (SNP) differences between dominating ST types. One isolate of each ST group was used as the reference genome for both analyses.

4.4.1.7 cgMLST-based genomic comparison of bacterial isolates (IV)

All 30 ESBL-producing *E. coli* isolates obtained from ISLAB were first compared among each other with core genome multilocus sequence typing (cgMLST) targeting 2520 genes in Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) (Jünemann et al., 2013) and results were visualized with a minimum spanning tree (MST) within the software.

Isolates were then compared to available ESBL/AmpC-producing *E. coli* sequences isolated from various sources in Finland between 2012 and 2018. A total of 67 previously sequenced isolates were included, from sequences

originating from the current study and public databases (Appendix III), and sources were comprised of broiler meat (n=5) (Päivärinta et al., 2020), broiler caecum (n=5) (Päivärinta et al., 2020), broiler parent caecal samples (n=8) (Study I), broiler egg surfaces (n=4) (Study I) and broiler production environment (n=1) (Study I), imported food products (n=16) (Study III), barnacle geese (n=9) (Study II), wastewater (n=1) (unpublished), cattle (n=1) (Päivärinta et al., 2016), veterinarians (n=9) (Verkola et al., 2019), and healthy adults (n=8) (Gröndahl-Yli-Hannuksela et al., 2020). cgMLST-based MST including 2520 gene targets was constructed with Ridom SeqSphere+ software v 7.0.4 (Ridom GmbH, Germany) (Jünemann et al., 2013).

4.4.1.8 Detection of international clades (IV)

ESBL-producing *E. coli* isolates from human infections obtained from ISLAB were compared with BLASTn to publicly available reference strain KUN5781 (GenBank accession: LC209430) (Matsumura et al., 2016) to determine the presence of C1-M27 clade-specific prophage-like regions M27PP1 and M27PP2. Results were visualized with BRIG v 0.95 (Alikhan et al., 2011) for isolates with matching regions.

4.4.2 Plasmid analysis (I-IV)

Plasmid replicons carried by the studied isolates were determined with PlasmidFinder (Carattoli et al., 2014), v 1.3 for Study I, v 2.1 for Studies II and III, and v 2.0 for Study IV. An identity threshold of 95% and a minimum length of 60% were set for all studies. Plasmid STs were determined for beta-lactamase harboring plasmid replicons with pMLST v 2.0 for replicon types with available typing schemes (Carattoli et al., 2014) for Studies II, III, and IV.

4.4.2.1 Annotation (II, III)

For Studies II and III, the hybrid-assembled plasmid sequences were annotated with Prokka v 1.13 (Seemann, 2014) and manually curated with BLASTn/BLASTp. Mobile genetic elements were additionally searched for with MobileElementFinder v 1.0.3 (Johansson et al., 2021) for Study III.

4.4.2.2 Genomic comparison and visualization (II, III)

For both Studies II and III, plasmid sequences were compared with previously published plasmids and structures were visualized using different freely available tools.

For Study II, BLAST Ring Image Generator (BRIG) v 0.95 (Alikhan et al., 2011) was used to compare and visualize plasmids to their respective publicly available reference plasmids, and for Study III, Easyfig v 2.2.2 (Sullivan et al., 2011) was employed for the same purpose.

In both studies, BLASTn search in National Center for Biotechnology Information (NCBI) database was used to compare plasmid sequences to

publicly available plasmids isolated from various sources with available metadata. These comparisons were visualized with BRIG v 0.95 (Alikhan et al., 2011) for Study III and for Study II the studied plasmids were first compared using BacCompare (Liu et al., 2019) and the 20 best matches with available metadata for each studied incompatibility type (for IncY plasmids only six similar, previously published plasmids with available metadata were found) were used to build a core genome MLST (cgMLST) based tree with 95% occurrence for discriminatory loci in BacCompare. The minimum spanning tree was visualized using GrapeTree v 1.5 (Zhou et al., 2018). Information on included previously published plasmids with available metadata from NCBI GenBank are provided in Appendix IV for Study II.

Additionally, a rarely detected multireplicon type plasmid, named pZPK-H11, from Study II was visualized with SnapGene software (from Insightful Science, available at https://www.snapgene.com).

4.4.3 Statistical analysis (I)

Fisher's exact test was used to compare the prevalence of *E. coli* without antibiotic selection in the three different groups of egg surface and embryo samples for Study I. Analysis was carried out with SPSS version 24 (IBM, New York, NY, USA). P-values < 0.05 were regarded as significant.

4.4.4 Deposition of sequence data (II-IV)

Raw reads for Studies II, III, and IV have been deposited at EMBL-EBI European Nucleotide Archive (ENA) under accession numbers PRJEB42655, PRJEB377791 and PRJEB47797, respectively. Accession numbers for sequenced isolates are provided in Appendix V.

4.4.5 Ethical approval (I)

For Study I, an approval for the study plan was obtained from the Ethical Review Board in the Humanities and Social and Behavioral Sciences and Viikki Campus Research Ethics Committee from the University of Helsinki. Other studies (II – IV) did not concern animal or human subjects and therefore no ethical approval was needed.

5 RESULTS

5.1 Identification of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* isolates (I–III)

5.1.1 Poultry production

For Study I, ESBL/AmpC-producing *E. coli* was detected in 120 (26.7%) broiler parent birds, 4 (1.3%) outer eggshells prior to the incubation period, and 1 (5%) broiler production environmental sample. After the incubation period none of the embryo intestine samples or eggshell surfaces were positive for ESBL/AmpC-producing *E. coli*.

5.1.1.1 Identification of E. coli *without antibiotic supplement*

For Study I, presence of non-ESBL/AmpC-producing *E. coli* was investigated with plating on MacConkey agar plates without antibiotic supplement. *E. coli* was found in 25 out of 30 (83.3%) sampled broiler parent birds, 13 out of 20 (65%) sampled outer eggshells prior to the egg incubation period at the hatchery and in two out of 90 (2.2%) sampled broiler embryo samples. None of the outer eggshells after the egg incubation period were positive for *E. coli*. Results are depicted in Table 3.

Table 3. Occurrence of *Escherichia coli* without antibiotic selection in broiler production.

Sampling	No. of positive samples / total no. of collected samples (%)
Parent birds	25/30 (83.3)
Egg surface before egg incubation period, total	13/20 (65)
Group 1	6/10 (60)
Group 2	7/10 (70)
Group 3	Not determined
Egg surface after egg incubation period, total	0/30 (0.0)
Group 1	0/10 (0)
Group 2	0/10 (0)
Group 3	0/10 (0)
Embryo intestines, total	2/90 (2.2)
Group 1	1/30 (3.3)
Group 2	1/30 (3.3)
Group 3	0/30 (0.0)

5.1.2 Barnacle goose

For Study II, 98 out of 200 barnacle goose feces samples (49%) were positive for bacterial growth on MacConkey agar plates supplemented with cefotaxime (1 mg/l). Of these, 55 (56%) samples were collected in fall 2017 and 43 (44%) in spring 2018. From these, nine (4.5%) samples were identified as *E. coli*. Seven (78%) of these originated from fecal samples collected on the first sampling period in fall 2017 and two (22%) from the second sampling period in spring 2018. All *E. coli* samples originated from fresh, wet droppings.

5.1.3 Imported food

5.1.3.1 Main samples

For Study III, 14 out of 200 (7%) imported food samples were positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* (Table 4). ESBL/AmpC-producing *E. coli* was found in 2 out of 60 (3%) vegetable samples, more specifically from two coriander samples originating from Malaysia from the same batch purchased from the same store. ESBL/AmpC-producing *K. pneumoniae* was found in 1 out of 60 (1%) of vegetable samples, from a chili pepper originating from Malaysia purchased from the same store as the coriander samples.

ESBL/AmpC-producing *E. coli* was found in 10 out of 60 (17%) of meat samples in Study III, all of Lithuanian raw broiler meat origin from the same batch and same store. One of these broiler meat samples was additionally positive for ESBL/AmpC-producing *K. pneumoniae*. A frozen turkey meat sample of Polish origin was positive for ESBL/AmpC-producing *K. pneumoniae*, meaning altogether 2 out of the 60 (3%) sampled meat products were positive for ESBL/AmpC-producing *K. pneumoniae*. Positive samples originating from food products are presented in Figure 7.

Table 4. Occurrence of ESBL/AmpC-producing producing *Escherichia coli* and/or *Klebsiella pneumoniae* in imported food samples.

Food category	No. of samples analyzed	No. of samples positive for ESBL/AmpC *E. coli* and/or *K. pneumoniae* (%) [a]	No. of samples positive for ESBL/AmpC *E. coli* (%)	No. of samples positive for ESBL/AmpC *K. pneumoniae* (%)
Vegetables	60	3 (5)	2 (3)	1 (2)
Fruits & berries	50	0 (0)	0 (0)	0 (0)
Meat	60	11 (18)	10 (17)	2 (3)
Seafood	30	0 (0)	0 (0)	0 (0)
Total	200	14 (7)	12 (6)	3 (2)

[a] Based on phenotypic tests (EFSA, 2011; EUCAST, 2017) and species identification with matrix-assisted laser desorption ionization–time of flight mass spectrometry, including results with a score value of 2.0 – 3.0.

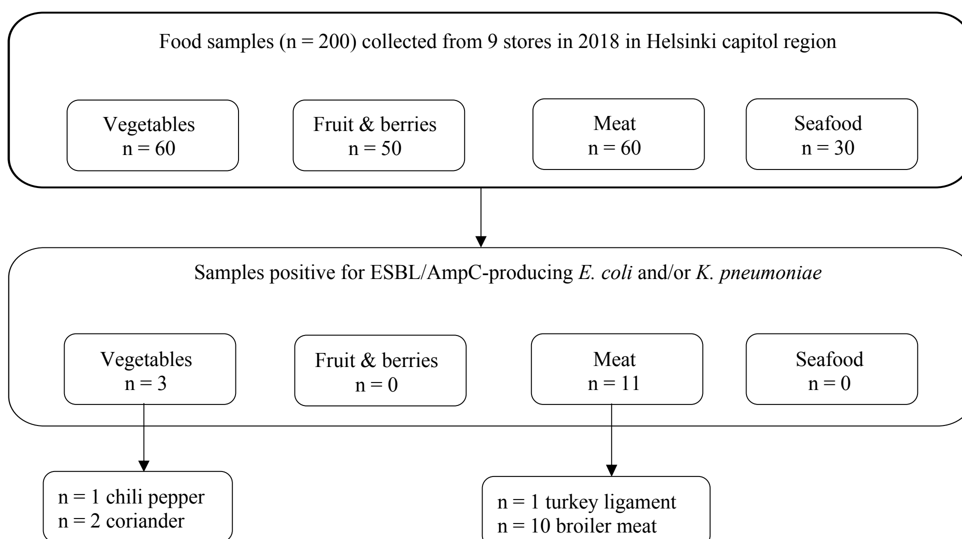


Figure 7. Food samples positive for ESBL/AmpC-producing *Escherichia coli* and/or *Klebsiella pneumoniae* based on phenotypic tests (EFSA, 2011; EUCAST, 2017) and species identification with matrix- assisted laser desorption ionization–time of flight mass spectrometry, including results with a score value of 2.0 – 3.0.

5.1.3.2 Subsamples

From broiler meat subsamples in Study III, 36 out of 40 (90%) were positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* (Table 5).

Table 5. Occurrence of ESBL/AmpC-producing *Escherichia coli* and/or *Klebsiella pneumoniae* in subsamples of imported broiler meat.

	No. of samples analyzed	**No. of samples positive for ESBL/AmpC *E. coli* and/or *K. pneumoniae* (%)** [a]	**No. of samples positive for ESBL/AmpC *E. coli* (%)**	**No. of samples positive for ESBL/AmpC *K. pneumoniae* (%)**
Subsamples	40	36 (90)	35 (89)	4 (10)

[a] Based on phenotypic tests (EFSA, 2011; EUCAST, 2017) and species identification with matrix-assisted laser desorption ionization–time of flight mass spectrometry, including results with a score value of 2.0 – 3.0.

5.2 MALDI-TOF (II, III)

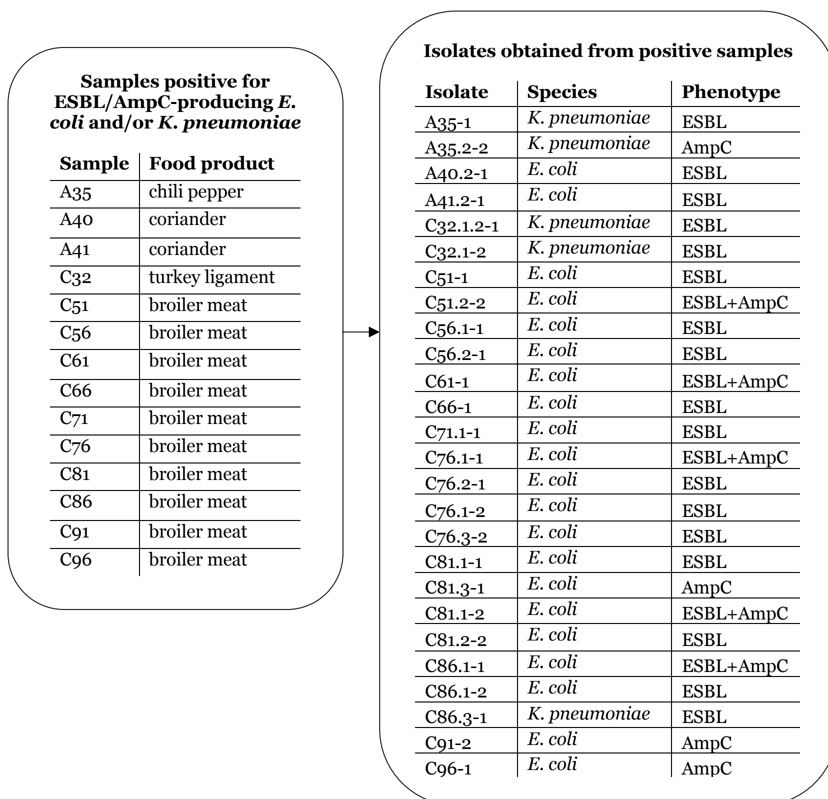
5.2.1 Barnacle goose

For Study II, one colony from each sample positive for bacterial growth on MacConkey agas plates supplemented with cefotaxime (n=98) was selected for species identification with MALDI-TOF MS. Nine (4.5%) samples were identified as *E. coli*. Other identified species included *Enterococcus* spp. (n=31) (*E. faecium*, *E. mundtii*, *E. gallinarum*, *E. casseliflavus*, and *E. faecalis*), *Bacillus oleronius* (n=3), *Bacillus pumilus (n=2),* and *Rummeliibacillus pycnus (n=2)*. For the rest of the samples, species could not be reliably identified with MALDI-TOF.

5.2.2 Imported food

5.2.2.1 Main samples

For Study III, 313 isolates were recovered from 152 out of 200 imported food samples, and originated from samples incubated at both 44 °C and 37 °C. These isolates were all subjected to bacterial species identification with MALDI-TOF MS. Information on samples and isolates positive for bacterial growth and ESBL/AmpC-producing *E. coli* and *K. pneumoniae* is provided in Figure 8. Altogether, 21 isolates were identified as *E. coli* and five as *K. pneumoniae*.



Samples positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae*

Sample	Food product
A35	chili pepper
A40	coriander
A41	coriander
C32	turkey ligament
C51	broiler meat
C56	broiler meat
C61	broiler meat
C66	broiler meat
C71	broiler meat
C76	broiler meat
C81	broiler meat
C86	broiler meat
C91	broiler meat
C96	broiler meat

Isolates obtained from positive samples

Isolate	Species	Phenotype
A35-1	*K. pneumoniae*	ESBL
A35.2-2	*K. pneumoniae*	AmpC
A40.2-1	*E. coli*	ESBL
A41.2-1	*E. coli*	ESBL
C32.1.2-1	*K. pneumoniae*	ESBL
C32.1-2	*K. pneumoniae*	ESBL
C51-1	*E. coli*	ESBL
C51.2-2	*E. coli*	ESBL+AmpC
C56.1-1	*E. coli*	ESBL
C56.2-1	*E. coli*	ESBL
C61-1	*E. coli*	ESBL+AmpC
C66-1	*E. coli*	ESBL
C71.1-1	*E. coli*	ESBL
C76.1-1	*E. coli*	ESBL+AmpC
C76.2-1	*E. coli*	ESBL
C76.1-2	*E. coli*	ESBL
C76.3-2	*E. coli*	ESBL
C81.1-1	*E. coli*	ESBL
C81.3-1	*E. coli*	AmpC
C81.1-2	*E. coli*	ESBL+AmpC
C81.2-2	*E. coli*	ESBL
C86.1-1	*E. coli*	ESBL+AmpC
C86.1-2	*E. coli*	ESBL
C86.3-1	*K. pneumoniae*	ESBL
C91-2	*E. coli*	AmpC
C96-1	*E. coli*	AmpC

Figure 8. Isolates positive for ESBL/AmpC-producing *E. coli* and/or *K. pneumoniae* from imported food samples.

5.2.2.2 Subsamples

Regarding broiler meat subsamples in Study III, 135 isolates were recovered from 40 subsamples. From these 135 isolates, 109 were identified with MALDI-TOF MS with a score value of 2.0 – 3.0. From these isolates, 86 were identified as *E. coli* and four as *K. pneumoniae*. For 23 isolates, species identification was impossible.

5.3 Antimicrobial susceptibility (I-III)

5.3.1 Poultry production

For Study I, all of the isolates subjected to antimicrobial susceptibility testing were resistant to third-generation cephalosporins (cefotaxime and ceftazidime) and second-generation cephamycin (cefoxitin). None of the isolates were resistant to carbapenem (meropenem). According to the combination disk diffusion test, out of the total 120 positive parent bird isolates 33 (27.5%) were phenotypically AmpC producers and 87 (72.5%) ESBL and AmpC producers. Four isolates (1.3%) out of 300 tested egg surfaces

prior to incubation were positive for ESBL/AmpC-producing *E. coli* and all were phenotypically ESBL + AmpC producers. The only environmental sample positive for ESBL/AmpC-producing *E. coli* was phenotypically an ESBL + AmpC producer. Phenotypic results are shown in Table 6.

Table 6. Occurrence of ESBL/AmpC-producing *Escherichia coli* in broiler production.

	No. of positive samples / total no. of collected samples (%) [a]			
Sampling	Total ESBL/AmpC	ESBL[b]	AmpC	ESBL + AmpC
Parent birds	120/450 (26.7)	0/120 (0.0)	33/120 (27.5)	87/120 (72.5)
Egg surface before incubation period, total	4/300 (1.3)	0/300 (0.0)	0/300 (0.0)	4/300 (1.3)
Group 1[c]	2/150 (1.3)	0/150 (0.0)	0/150 (0.0)	2/150 (1.3)
Group 2	2/150 (1.3)	0/150 (0.0)	0/150 (0.0)	2/150 (1.3)
Group 3	Not determined	Not determined	Not determined	Not determined
Egg surface after incubation period, total	0/428 (0.0)	0/428 (0.0)	0/428 (0.0)	0/428 (0.0)
Group 1	0/141 (0.0)	0/141 (0.0)	0/141 (0.0)	0/141 (0.0)
Group 2	0/144 (0.0)	0/144 (0.0)	0/144 (0.0)	0/144 (0.0)
Group 3	0/143 (0.0)	0/143 (0.0)	0/143 (0.0)	0/143 (0.0)
Embryo intestines, total	0/422 (0.0)	0/422 (0.0)	0/422 (0.0)	0/422 (0.0)
Group 1	0/138 (0.0)	0/138 (0.0)	0/138 (0.0)	0/138 (0.0)
Group 2	0/141 (0.0)	0/141 (0.0)	0/141 (0.0)	0/141 (0.0)
Group 3	0/143 (0.0)	0/143 (0.0)	0/143 (0.0)	0/143 (0.0)
Environmental samples	1/20 (5)	0/20 (0)	0/20 (0)	1/20 (5)

[a] Based on phenotypic tests (EFSA, 2011; EUCAST, 2017).

[b] ESBL = isolate expressed only ESBL phenotype; AmpC = isolate expressed only AmpC phenotype; ESBL + AmpC = isolate expressed both ESBL and AmpC phenotype.

[c] Group 1 = rinsing sample; Group 2 = rinsing sample followed by Broilact (Orion Corp., Orion Pharma, Espoo, Finland) treatment; Group 3 = no treatment before incubation, rinsing sample after incubation.

5.3.2 Barnacle goose

For Study II, all nine isolates subjected to antimicrobial susceptibility testing were resistant to third-generation cephalosporins (cefotaxime and ceftazidime). According to the combination disk diffusion test, four isolates were phenotypically ESBL producers, four isolates AmpC producers, and one isolate an ESBL + AmpC producer.

5.3.3 Imported food

5.3.3.1 Main samples

Altogether, 21 isolates from the main samples were identified as *E. coli*. From these, 13 (62%) were phenotypically ESBL producers, three (14%) AmpC producers, and five (24%) produced both AmpC and ESBL. From the five isolates from the main samples identified as *K. pneumoniae*, four were phenotypically ESBL producers and one an AmpC producer. All isolates were resistant to third-generation cephalosporin (cefotaxime and ceftazidime), except one *E. coli* isolate that was susceptible to ceftazidime but resistant to cefotaxime. None of the isolates were resistant to carbapenem (meropenem).

5.3.3.2 Subsamples

From the 86 isolates identified as *E. coli*, 43 (50%) were ESBL producers, 20 (23%) AmpC producers, and 23 (27%) produced both AmpC and ESBL. All four *K. pneumoniae* isolates were ESBL producers. All isolates from the subsamples were resistant to third-generation cephalosporin (cefotaxime and ceftazidime), except one *K. pneumoniae* isolate that was susceptible to ceftazidime but resistant to cefotaxime. None of the subsample isolates were resistant to carbapenem (meropenem).

5.4 Genomic characteristics of ESBL/AmpC-producing *E. coli* and *K. pneumoniae* (I-IV)

5.4.1 Poultry production

From all the ESBL/AmpC-producing isolates (n=13) subjected to WGS in Study I, two different MLST types were identified: ST2040 and ST429 (Table 7). The resistant isolates belonging to both STs proved to be closely related, as phylogenetic analysis showed 0 – 10 SNP differences within ST2040 isolates and 0 – 4 SNP differences within ST429 isolates.

The only beta-lactam resistance gene detected in the isolates was the AmpC type bla_{CMY-2}. Isolates belonging to ST429 carried the additional resistance genes *aac(3)-Vla* and ant(3”)-Ia (*aadA1*) (aminoglycoside resistance), *sul1* (sulfonamide resistance) and *tet*(A) (tetracycline resistance), whereas bla_{CMY-2} was the only resistance gene identified from ST2040 isolates.

All ST429 isolates carried virulence genes *gad*, *iroN*, *iss,* and *mchF*, and all ST2040 isolates harbored *cma*, *gad*, *Ipfa*, *iroN*, *iss,* and *tsh*.

Plasmid results are described in chapter *5.5. Plasmid analysis*.

Table 7. Genomic characteristics of ESBL/AmpC-producing *Escherichia coli* (n = 13) isolated from the broiler production chain.

Isolate[a]	Origin	MLST[b]	Resistance genes	Virulence genes	Phenotype[c]
1E61-R	Egg surface	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	ESBL + AmpC
1E74-R	Egg surface	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	ESBL + AmpC
2E151-R	Egg surface	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC
2E177-R	Egg surface	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	ESBL + AmpC
PS310-R	Parent bird	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	AmpC
PS61-R	Parent bird	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	AmpC
PS174-R	Parent bird	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	AmpC
PS371-R	Parent bird	ST2040	bla_{CMY-2}	*cma, gad, Ipfa, iroN, iss, iss, tsh*	AmpC
PS57-R	Parent bird	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC
PS184-R	Parent bird	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC
PS148-R	Parent bird	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC
PS378-R	Parent bird	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC
Y13-R	Environment	ST429	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*	*gad, iroN, iss, iss, mchF*	ESBL + AmpC

[a] R = Extended-spectrum beta-lactamase (ESBL) / AmpC (AmpC) producing *E. coli*; prefix 1 = Group 1, rinsing sample; prefix 2 = Group 2, rinsing sample followed by Broilact treatment.

[b] MLST = multilocus sequence type.

[c] Based on phenotypic tests (EFSA, 2011; EUCAST, 2017).

5.4.1.1 E. coli without antibiotic supplement

E. coli isolates obtained from cultivation without antibiotic supplement (n = 10) subjected to WGS consisted of seven different MLST types: ST106, ST428, ST88, ST906, ST1286, ST10, and ST453 (Table 8). Two of the four isolates originating from parent bird samples were positive for resistance genes in the sequence data analysis, one for the sulfonamide resistance gene *sul1* and the other for beta-lactam resistance gene bla_{TEM-1B}. Two embryo samples cultivated without antibiotic supplement were positive for *E. coli*. Of these, one was positive for a narrow-spectrum beta-lactamase bla_{TEM-1C} and tetracycline resistance gene *tet*(A).

Table 8. Genomic characteristics of *Escherichia coli* (n = 10) isolated without antibiotic supplement from the broiler production chain.

Isolate[a]	Origin	MLST[b]	Resistance genes	Virulence genes
1E5-S	Egg surface	ST88	–	*gad, iha, Ipfa, iss, mchB, mchC, mchF*
1E10-S	Egg surface	ST906	–	*gad, lpfA*
2E153-S	Egg surface	ST906	–	*gad, lpfA*
2E157-S	Egg surface	ST1286	–	*gad, iroN, iss, mchF, tsh*
PS2-S	Parent bird	ST10[c]	–	*gad, iha, iss*
PS16-S	Parent bird	ST1286	–	*gad, iroN, iss, mchF*
PS19-S	Parent bird	ST453	bla_{TEM-1B}	*gad, lpfA, iss*
PS30-S	Parent bird	ST10	*sul1*	*astA, astA, cba, cma, gad, ireA*
1C14-S	Chick intestine	ST106[c]	bla_{TEM-1C}, *tet*(A)	*air, astA, eilA, gad, IpfA, iroN, iss,mchB, mchC, mchF, mchF, mcmA*
2C151-S	Chick intestine	ST428	–	*gad, iroN, iss, iss, mchF, vat*

[a] S = *E. coli* without antibiotic selection; prefix 1 = Group 1, rinsing sample; prefix 2 = Group 2, rinsing sample followed by Broilact treatment.

[b] MLST = multilocus sequence type.

[c] One or more loci do not perfectly match any previously registered MLST allele.

5.4.2 Barnacle goose

All ESBL/AmpC-producing *E. coli* isolates (n = 9) from Study II were subjected to WGS with both short- and long-read technologies, and analysis of the data revealed altogether seven different STs, depicted in Table 9. Plasmid results are described in section *5.5. Plasmid analysis*.

All the isolates harbored resistance genes against multiple antibiotic classes, including beta-lactams, aminoglycosides, fluoroquinolones, macrolides, lincosamides, streptogramin B, phenicols, sulfonamides, tetracycline, and trimethoprim. No fosfomycin or rifampicin resistance genes were detected.

Isolate H68B was phenotypically an AmpC producer, but no beta-lactamase genes were identified in the WGS analysis. Isolate H21 was phenotypically an AmpC producer but harbored both the AmpC type bla_{CMY-2} and ESBL type bla_{TEM-32}.

The isolates harbored a wide variety of different virulence genes with 38 different genes found altogether. The increased serum survival gene *iss*, tellurium ion resistance gene *terC*, and glutamate decarboxylase gene *gad* were found in all isolates, and the long polar fimbriae gene *lpfA* in seven isolates. Enterobactin siderophore receptor gene *iroN* and temperature sensitive hemagglutinin gene *tsh* were both found in three isolates. Two of the isolates harbored the adhesin intimin coding *eae* gene, which is associated with enteropathogenic *E. coli* (Frankel et al., 1998; Müller et al., 2016), but no Shiga toxin coding genes were found.

Table 9. Genomic characteristics of ESBL/AmpC-producing *Escherichia coli* isolates from barnacle geese.

Isolate	MLST[a]	Resistance genes	Virulence genes	Phenotype[b]
H5	ST359	*bla*$_{CTX-M-1}$, *floR, mdf(A), aph(6)-Id, aph(3")-Ib, sul2, dfrA*	*astA, cea, cia, etsC, gad, hlyF, iroN, iss, iucC, iutA, lpfA, ompT, sitA, terC, traT, tsh*	ESBL
H11	ST58	*bla*$_{CTX-M-1}$, *qnrS1, aadA2b, lnu(F), mdf(A), aph(6)-Id, aph(3")-Ib, dfrA5, sul2, tet(A)*	*cea, cia, cvaC, etsC, fyuA, gad, hlyF, iroN, irp2, iss, iucC, iutA, lpfA, mchF, ompT, sitA, terC, traT, tsh*	ESBL
H21	ST115)	*bla*$_{CMY-2}$, *bla*$_{TEM-32}$, *mdf(A)*	*air, celb, chuA, cia, eilA, etsC, gad, hlyF, hra, iha, iss, iucC, iutA, kpsE, kpsMII_K5, mcbA, ompT, papC, sitA, terC, traT*	AmpC
H29	ST453	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *qnrS1, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, tet(A), dfrA1, mdf(A), aadA1*	*fyuA, gad, irp2, iss, kpsE, kpsMIII_K10, lpfA, papC, sitA, terC*	ESBL
H58	ST1594	*bla*$_{CMY-2}$, *mdf(A)*	*astA, celb, gad, gad, hra, iha, iss, iucC, iutA, kpsE, kpsMII, ompT, sitA, traT, terC*	AmpC
H68B	ST3580	Chromosomal point mutation in AmpC promoter, *mdf(A), tet(A)*	*cba, cma, etsC, gad, hlyF, iroN, iss, iucC, iutA, lpfA, ompT, sitA, terC, traT, tsh*	AmpC
H98	ST453	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *qnrS1, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, tet(A), dfrA1, mdf(A), aadA1*	*fyuA, gad, irp2, iss, kpsE, kpsMIII_K10, lpfA, papC, sitA, terC*	ESBL
H163	ST2178	*bla*$_{CMY-2}$, *mdf(A)*	*cia, cif, eae, espA, espF, espJ, gad, iss, lpfA, nleB, sepA, terC, tir*	AmpC
H193	ST2178	*bla*$_{CMY-2}$, *mdf(A)*	*cia, cif, eae, espA, espF, espJ, gad, iss, lpfA, nleB, sepA, terC, tir*	AmpC+ESBL

[a] MLST = multilocus sequence type
[b] Based on phenotypic tests (EFSA, 2011; EUCAST, 2017).

5.4.3 Imported food

The genomic characteristics of short-read sequenced 17 *E. coli* and four *K. pneumoniae* isolates in Study III are presented in Table 10. Plasmid results are described in section *5.5. Plasmid analysis*.

A total of 15 different STs were identified from the *E. coli* isolates and three different STs from the *K. pneumoniae* isolates.

All the isolates carried multiple different antimicrobial resistance genes toward critically important antimicrobials (WHO, 2019) including aminoglycosides, third- and fourth-generation cephalosporins, macrolides, and quinolones. All isolates harbored one or more *bla* genes. A majority (9/16) of the isolates originating from raw broiler meat were positive for the AmpC type *bla*$_{CMY-2}$. Other common resistance genes identified included genes

conferring resistance to, lincosamide, streptogramin B, phenicol, sulfonamide, tetracycline, and trimethoprim.

All *K. pneumoniae* isolates harbored plasmid-mediated quinolone resistance (PMQR) and fosfomycin resistance genes. Two of these isolates harbored gene *aac(6′)-Ib-cr*, known to confer resistance toward both aminoglycosides and fluoroquinolones (Frasson et al., 2011). Only five *E. coli* isolates were positive for PMQR genes and no fosfomycin resistance genes were identified in any *E. coli* isolates.

Multiple different virulence genes were identified in the isolates, including adhesin intimin coding *eae* gene from one broiler meat isolate, which is associated with enteropathogenic *E. coli* (Frankel et al., 1998; Müller et al., 2016).

Table 10. Genomic characteristics of ESBL/AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from imported food.

Isolate[a]	Product	Origin	Species	MLST[b]	Resistance genes	Virulence genes	Phenotype[c]
A35-1	Chili pepper	Malaysia	*K. pneumoniae*	ST307	*aac(3)-IIa, aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id,* $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}28}$, $bla_{TEM\text{-}1B}$, *oqxA, oqxB, qnrB1, fosA, catB3, sul2, tet*(A), *dfrA14*	N/A[d]	ESBL
A35.2-2	Chili pepper	Malaysia	*K. pneumoniae*	ST101	$bla_{DHA\text{-}1}$, $bla_{SHV\text{-}28}$, *oqxA, oqxB, qnrS1, fosA, sul1, tet*(A), *dfrA1*	N/A	AmpC
A40.2-1	Coriander	Malaysia	*E. coli*	ST155	*aph(3')-Ia, aph(3'')-Ib, aph(6)-Id,* $bla_{CTX\text{-}M\text{-}55}$, $bla_{TEM\text{-}1B}$, *mdf*(A), *floR, ARR-2, sul2, tet*(A), *dfrA14*	*cma, gad, IpfA*	ESBL
A41.2-1	Coriander	Malaysia	*E. coli*	ST479[e]	*aac(3)-IV, aadA5, aph(4)-Ia,* $bla_{CTX\text{-}M\text{-}65}$, *oqxA, oqxB, mdf*(A), *floR, sul1, sul2, tet*(A), *dfrA17*	*gad, IpfA*	ESBL
C32.1-2	Frozen turkey ligament	Poland	*K. pneumoniae*	ST307	*aac(3)-IIa, aac(6')-Ib-cr, aph(6)-Id, aph(3'')-Ib,* $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}28}$, $bla_{TEM\text{-}1B}$, *oqxA, oqxB, qnrB1, fosA, catB, sul2, tet*(A), *dfrA14*	N/A	ESBL
C51-1	Raw broiler meat	Lithuania	*E. coli*	ST189	*aadA1, aadA2,* $bla_{SHV\text{-}12}$, *mdf*(A), *cmlA1, sul3, tet*(A)	*astA, cif, eae, espA, espB, espF, espJ, gad, nleA, nleB, tir*	ESBL
C51.2-2	Raw broiler meat	Lithuania	*Escherichia fergusonii*[f]	ST8330	*aac(3)-IV, aadA1, ant(2'')-Ia, aph(3')-Ia, aph(3'')-Ib, aph(4)-Ia, aph(6)-Id,* $bla_{CMY\text{-}2}$, $bla_{TEM\text{-}1B}$, *catA1, floR, sul1, sul2, tet*(B)	*cma, gad, mchF*	ESBL+AmpC
C56.1-1	Raw broiler meat	Lithuania	*E. coli*	ST4994	$bla_{TEM\text{-}52C}$, *mdf*(A)	*air, astA, celb, eilA, gad, iha, iss*	ESBL

C61-1	Raw broiler meat	Lithuania	*E. coli*	ST1011	*aadA2, aph(3'')-Ib, aph(6)-Id, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *mdf*(A), *sul2, tet*(A), *dfrA12*	*cma, eilA, iroN, iss, mchF*	ESBL+AmpC
C66-1	Raw broiler meat	Lithuania	*E. coli*	ST423	*aadA1, aadA2, bla*$_{CARB-2}$, *bla*$_{SHV-12}$, *mdf*(A), *cmlA1, sul3, tet*(A), *dfrA16*	*gad, IpfA*	ESBL
C71.1-1	Raw broiler meat	Lithuania	*E. coli*	ST1485	*aadA1, aadA2, aph(3'')-Ib, aph(6)-Id, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *mdf*(A), *cmlA1, sul2, sul3, tet*(A), *dfrA14*	*air, eilA, gad, iha, iroN, IpfA, iss, mchF, mcmA, tsh*	ESBL
C76.1-1	Raw broiler meat	Lithuania	*E. coli*	ST201	*aadA1, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *qnrS1, mdf*(A), *cmlA1, sul3, tet*(A), *dfrA15*	*cma, gad, iroN, IpfA, iss*	ESBL+AmpC
C76.1-2	Raw broiler meat	Lithuania	*E. coli*	ST83[e]	*aadA1, aadA2, bla*$_{SHV-12}$, *mdf*(A), *cmlA1, sul3, tet*(A)	*astA, gad, iroN, iss, mchF, tsh*	ESBL
C81.1-1	Raw broiler meat	Lithuania	*E. coli*	ST38	*aadA5, bla*$_{CTX-M-1}$, *mdf*(A), *sul2, dfrA17*	*air, eilA, gad*	ESBL
C81.3-1	Raw broiler meat	Lithuania	*E. coli*	ST1638	*aadA1, aadA2, aph(3'')-Ib, aph(6)-Id, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *qnrB19, mdf*(A), *cmlA1, sul3, tet*(B), *dfrA8*	*gad, iss*	AmpC
C81.1-2	Raw broiler meat	Lithuania	*E. coli*	ST641	*aadA1, aadA2, bla*$_{CARB-2}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *qnrS1, mdf*(A), *cmlA1, sul3, tet*(A), *dfrA16*	*etpD, gad, IpfA, iss*	ESBL+AmpC
C86.1-1	Raw broiler meat	Lithuania	*E. coli*	ST641	*aadA1, aadA3, bla*$_{CARB-2}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *qnrS1, mdf*(A), *cmlA1, sul3, tet*(A), *dfrA16*	*etpD, gad, iss, IpfA*	ESBL+AmpC
C86.1-2	Raw broiler meat	Lithuania	*E. coli*	ST38	*aadA1, aadA5, aph(3'')-Ib, aph(6)-Id, bla*$_{CTX-M-1}$, *mdf*(A), *floR, sul1, sul2, tet*(A), *dfrA17*	*air, eilA, gad, ireA*	ESBL
C86.3-1	Raw broiler meat	Lithuania	*K. pneumoniae*	ST37	*aac(3)-IId, aph(3'')-Ib, aph(6)-Id, bla*$_{CTX-M-15}$, *bla*$_{SHV-81}$, *bla*$_{TEM-1B}$, *oqxA, oqxB, fosA, sul2, dfrA14*	N/A	ESBL
C91-2	Raw broiler meat	Lithuania	*E. coli*	ST117	*aadA1, aph(3'')-Ib, aph(6)-Id, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *mdf*(A), *sul1, sul2, tet*(A), *dfrA8*	*celb, gad, ireA, iroN, IpfA, iss, mchB, mchC, mchF, pic*	AmpC
C96-1	Raw broiler meat	Lithuania	*E. coli*	ST88	*aadA1, aph(3'')-Ib, aph(6)-Id, bla*$_{CMY-2}$, *bla*$_{TEM-1B}$, *mdf*(A), *mph*(B), *catA1, sul1, sul2, dfrA1, dfrA8*	*gad, iss, IpfA*	AmpC

[a] Suffix *-1* indicates first incubation temperature of 44 °C and *-2* of 37 °C.

[b] MLST = multilocus sequence type

[c] Based on phenotypic tests; tests (EFSA, 2011; EUCAST, 2017).

[d] N/A = not applicable for *K. pneumoniae*

[e] scheme *Escherichia coli* #1 used first, if sequence type (ST) unidentified then #2 (Jaureguy et al., 2008) used

[f] identified as *E. coli* with SpeciesFinder v 1.2 (Larsen et al., 2014)

5.4.4 Human clinical samples

The genomic characteristics of human clinical samples (n = 30) obtained from Eastern Finland healthcare district are depicted in Table 11. Plasmid results are described in chapter *5.5. Plasmid analysis*. Resistance genes other than *bla* and virulence genes are depicted in Appendix VI.

Most isolates (22 out of 30; 73%) were of sequence type ST131. Nineteen of these isolates represented serotype O25:H4 and *fimH*30 allele, whereas three isolates represented serotype O16:H5 and *fimH*41 allele. Non-ST131 isolates (n=8) represented a total of eight different sequence types.

A majority of the isolates (21 out of 30; 70%) harbored multiple antimicrobial resistance genes toward different antibiotic classes, such as beta-lactams, aminoglycosides, tetracycline, sulfonamides, macrolides, and trimethoprim. All isolates were positive for a beta-lactamase gene, with $bla_{CTX-M-27}$ (n=14; 47%) being the most frequent, followed by $bla_{CTX-M-15}$ (n=10; 33%). Other *bla* genes detected consisted of $bla_{CTX-M-55}$ (n=2), $bla_{CTX-M-14}$ (n=1), $bla_{CTX-M-3}$ (n=1), bla_{SHV-12} (n=1), and bla_{TEM-52} (n=1). Thirteen of the $bla_{CTX-M-27}$-positive isolates were of sequence type ST131, and one of ST38. Most isolates positive for $bla_{CTX-M-15}$ were also of sequence type ST131 (n=9), with one isolate representing ST978.

Resistance genes against sulfonamides were identified in 60% of the isolates, trimethoprim in 53%, aminoglycoside is 67%, and tetracycline in 60%. Chromosomal quinolone resistance mutations in *gyrA*, *parC*, *parE*, or *marR* were also common, and were identified in 90% of the isolates. Chromosomal mutations in *ptsI* and *uhpt* associated with fosfomycin resistance were also frequent, being detected in 77% of the isolates. PMQR gene *aac(6')-Ib-cr* was detected in two isolates.

Isolates were furthermore abundant in virulence genes, harboring genes associated with extraintestinal pathogenic *E. coli* (ExPEC) (Johnson et al., 2003), such as P fimbrial adhesin *pap*, polysialic acid transport protein *kpsMII*, ferric aerobactin receptor *iutA*, and S and F1C fimbriae *sfa*. All except one isolate were positive for the previously described threshold of two or more of the five virulence genes (*pap, kps, iutA, sfa/foc, afa/dra*) defined as discriminatory for ExPEC classification (Johnson et al., 2003; Kanamori et al., 2017). No Shiga toxin (*stx*) genes were found.

Table 11. Genomic characteristics of ESBL-producing *Escherichia coli* isolates from human clinical samples obtained from Eastern Finland healthcare district.

Isolate	Specimen type	MLST[a]	Serotype	*fimH* type	*bla* gene(s)
D1	Urine	ST131	O25:H4	*fimH*30	$bla_{CTX-M-27}$
D2	Joint	ST978[b]	O4:H27	*fimH*2	$bla_{CTX-M-15}$

D3	Scrotum	ST38	O1:H15	*fimH*65	$\mathit{bla}_{\text{CTX-M-27}}$
D4	Maxillary sinus	ST1[c]	O18:H7[d]	*fimH*18	$\mathit{bla}_{\text{CTX-M-14}}$, $\mathit{bla}_{\text{TEM-1}}$
D5	Eye conjunctiva	ST1193	O75:H5	*fimH*64	$\mathit{bla}_{\text{CTX-M-55}}$
D6	Wound	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$, $\mathit{bla}_{\text{TEM-1}}$
D7	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$, $\mathit{bla}_{\text{OXA-1}}$
D8	Bile	ST131	O16:H5	*fimH*41	$\mathit{bla}_{\text{CTX-M-27}}$
D9	Abscess	ST131[b]	O16:H5	*fimH*41	$\mathit{bla}_{\text{CTX-M-15}}$, $\mathit{bla}_{\text{TEM-1}}$
D10	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$
D11	Lung (bronchoalveolar lavage)	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D12	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D13	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D14	Blood	ST162	O8:H19	*fimH*32	$\mathit{bla}_{\text{SHV-12}}$
D15	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$
D16	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D17	Abdominal cavity	ST537	O75:H5	*fimH*5	$\mathit{bla}_{\text{TEM-52}}$
D18	Urine	ST59	O1:H7	*fimH*41	$\mathit{bla}_{\text{CTX-M-55}}$, $\mathit{bla}_{\text{TEM-1}}$
D19	Urine	ST405	O2:H4	*fimH*56	$\mathit{bla}_{\text{CTX-M-3}}$
D20	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D21	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D22	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D23	Wound	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$
D24	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$, $\mathit{bla}_{\text{OXA-1}}$
D25	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D26	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D27	Blood	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-15}}$
D28	Blood	ST131	O16:H5	*fimH*41	$\mathit{bla}_{\text{CTX-M-15}}$, $\mathit{bla}_{\text{TEM-1}}$
D29	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$
D30	Urine	ST131	O25:H4	*fimH*30	$\mathit{bla}_{\text{CTX-M-27}}$

[a] MLST = multilocus sequence type

[b] MLST v 2.0 (Larsen et al., 2012) used to verify result (with Warwick scheme).

[c] Pasteur scheme used when sequence type not identified with Warwick scheme.

[d] SerotypeFinder v 2.0 (Joensen et al., 2015) used to verify result.

[e] Uncertain hit, ST cannot be trusted.

5.4.4.1 Genomic comparison of bacterial isolates

E. coli isolates obtained from the Eastern Finland healthcare district with cgMLST revealed clusters forming between isolates of sequence type ST131 (Figure 9). Clusters were defined as allele differences of 10 or less, based on a previous study considering single nucleotide polymorphism differences of ≤ 10 in *E. coli* to be indicative of the same origin (Schürch et al., 2018). ST131 isolates possessing $bla_{\text{CTX-M-27}}$ and *fimH*30 formed three clusters, whereas one cluster was observed among isolates representing ST131, fimH30, serotype O25:H4 and antimicrobial resistance genes *aadA2*, $bla_{\text{CTX-M-15}}$, *mph*(A), *sul1*, *tet*(A), *dfrA12*. Isolates belonging to different sequence types did not form clusters, as allele differences ranged from 640 to more than 2000.

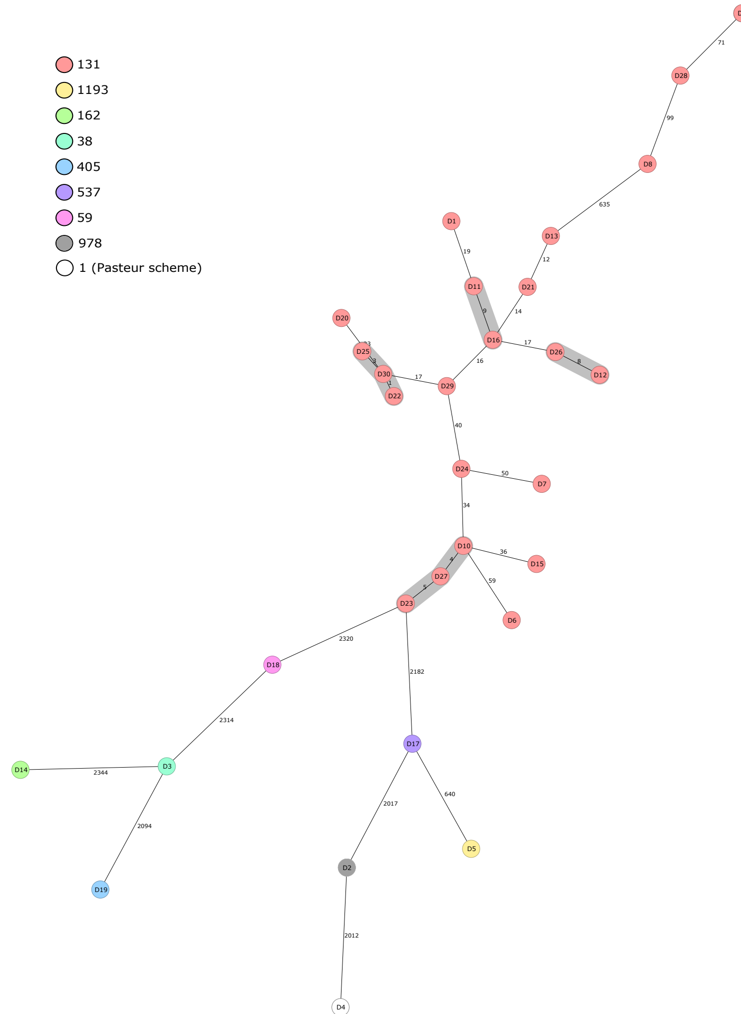


Figure 9. Minimum spanning tree of 30 human ESBL-producing *Escherichia coli* isolates obtained from patients in Eastern Finland during 2018 – 2020. Tree was calculated in Ridom SeqSphere+ with 2513 core genome multilocus sequence typing (cgMLST) targets and 7 *E. coli* MLST Warwick targets (pairwise ignoring missing values, logarithmic scale). Nodes are coloured according to sequence type. Number of allelic differences between isolates are indicated on the connecting lines. Clusters are defined as ≤ 10 allelic difference and shaded in grey.

Comparison of the 30 human ESBL-producing *E. coli* isolates obtained from the Eastern Finland healthcare district with previously sequenced ESBL/AmpC-producing *E. coli* isolates in Finland demonstrated no close connections between human and non-human sources (Figure 10). No close clusters were observed among human isolates either, although the closest distance of 24 allele differences was identified between an isolate from a healthy veterinarian and a clinical sample from ISLAB, both representing ST131-*bla*$_{CTX\text{-}M\text{-}27}$. Additionally, ST131-*bla*$_{CTX\text{-}M\text{-}15}$ isolates from two clinical samples from ISLAB and a healthy volunteer differed by only 40 and 44 alleles

and isolates from two healthy veterinarians and a healthy volunteer by 18 and 26 alleles.

Clusters were, however, observed among isolates within their respective studies from poultry production (Oikarainen et al., 2019), broiler meat (Päivärinta et al., 2020), and broiler caecum (Päivärinta et al., 2020) In addition, an isolate representing ST1594-bla_{CMY-2} from broiler caecum (Päivärinta et al., 2020) demonstrated no allele differences with an isolate originating from barnacle goose (Kurittu et al., 2021).

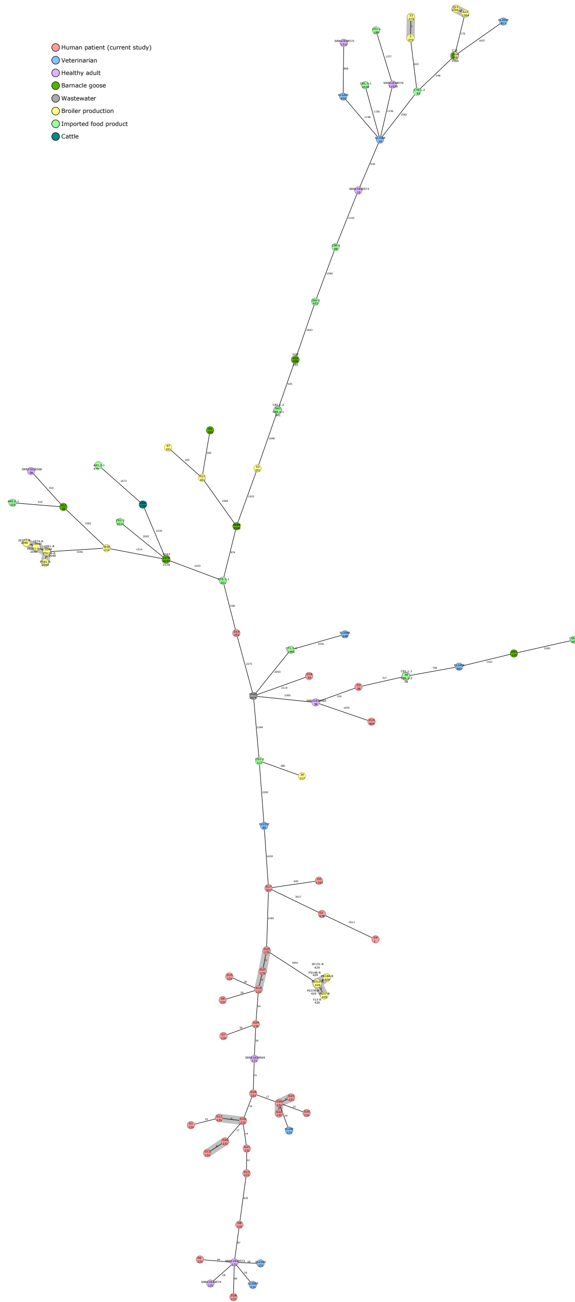


Figure 10. Minimum spanning tree of 97 ESBL/AmpC-producing *Escherichia coli* isolates calculated in Ridom SeqSphere+ with 2513 core genome multilocus sequence typing (cgMLST) targets and 7 *E. coli* MLST Warwick targets (pairwise ignoring missing values, logarithmic scale). Nodes are coloured according to isolation source. Sequence type is indicated under the isolate name. Number of allelic differences between isolates are indicated on the connecting lines. Clusters are defined as ≤ 10 allelic difference and shaded in grey.

5.4.4.2 *E. coli* ST131 C1-M27 subclade comparison

All of the 12 ESBL-producing *E. coli* isolates from ISLAB representing ST131 with $bla_{CTX\text{-}M\text{-}27}$ and *fimH*30 were positive for the C1-M27 clade-specific prophage-like 11,894-bp region M27PP1 and four isolates were additionally positive for the 19,352-bp prophage-like region M27PP2 (Matsumura et al., 2016) (Figure 11).

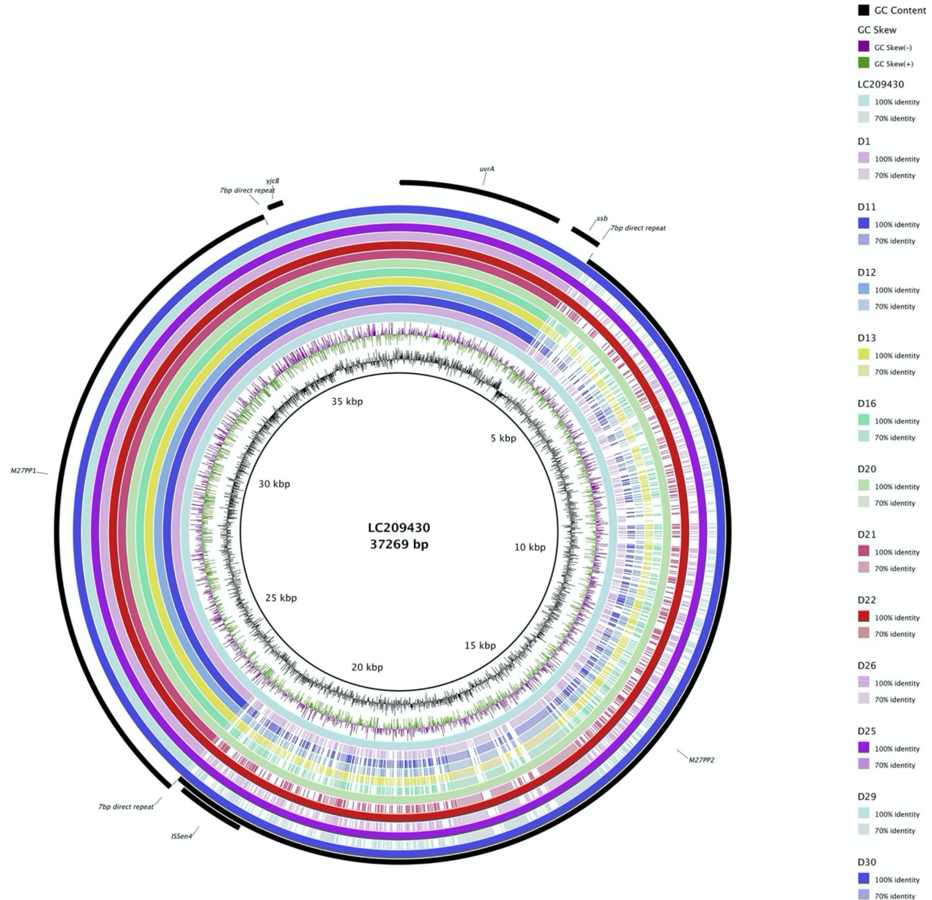


Figure 11. ST131 *Escherichia coli* C1-M27 clade-specific regions, prophage-like genomic islands M27PP1 and M27PP2, of KUN5781 (GenBank accession: LC209430) compared to CTX-M-27-producing ST131 *E. coli* with *fimH* 30 allele from the current study. GC content and GC skew are depicted on the inner map with distance scale and predicted coding sequences depicted on the outer ring.

5.5 Plasmid analysis (I-IV)

5.5.1 Broiler production

For Study I, plasmid replicons were identified from short-read sequence data. From two to five different plasmid replicons were detected in each isolate (Table 12). All of the ST429 isolates harbored plasmids belonging to families of IncB/O/K/Z, IncFIB, and IncFIC(FII), whereas four of the six ST2040 isolates harbored plasmids belonging to families of IncX1, ColpVC, IncFIB, and IncFIC(FII).

Table 12. Plasmids detected in *Escherichia coli* isolates obtained from poultry production.

Isolate [a]	Origin	MLST[b]	Plasmid replicons	Resistance genes
1E61-R	Egg surface	ST2040	ColpVC, IncFIB, IncFIC(FII), IncX1	bla_{CMY-2}
1E74-R	Egg surface	ST2040	ColpVC, IncFIB, IncFIC(FII), IncX1	bla_{CMY-2}
2E151-R	Egg surface	ST429	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
2E177-R	Egg surface	ST2040	ColpVC, IncFIB, IncFIC(FII), IncX1	bla_{CMY-2}
1E5-S	Egg surface	ST88	ColpVC, IncFII(pRSB107)	–
1E10-S	Egg surface	ST906	ColpVC, IncFII(29), p0111	–
2E153-S	Egg surface	ST906	ColpVC, p0111	–
2E157-S	Egg surface	ST1286	ColpVC, IncFIC(FII), IncFIB, IncFII(29)	–
PS2-S	Parent bird	ST10[c]	ColpVC, IncFII(29), p0111	–
PS16-S	Parent bird	ST1286	IncFIC(FII), IncFIB, IncFII(29)	–
PS19-S	Parent bird	ST453	IncFIB, IncI1, IncX1	bla_{TEM-1B}
PS30-S	Parent bird	ST10	IncFIB, IncFII(29), IncX1, p0111	*sul1*
PS310-R	Parent bird	ST429	IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
PS61-R	Parent bird	ST2040	ColpVC, IncFIB, IncFIC(FII),	bla_{CMY-2}
PS174-R	Parent bird	ST2040	ColpVC, IncFIB, IncFIC(FII), IncI2	bla_{CMY-2}
PS371-R	Parent bird	ST2040	ColpVC, IncFIB, IncFIC(FII), IncX1	bla_{CMY-2}
PS57-R	Parent bird	ST429	Col156, ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
PS184-R	Parent bird	ST429	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
PS148-R	Parent bird	ST429	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
PS378-R	Parent bird	ST429	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*
1C14-S	Chick intestine	ST106[c]	Col(MG828), ColpVC, IncFIB, IncFII, IncI1	bla_{TEM-1C}, *tet*(A)
2C151-S	Chick intestine	ST428	Col(MG828), ColpVC, IncFIB, IncFII,	–
Y13-R	Environment	ST429	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII)	*aac(3)-VIa*, *ant(3")-Ia*, bla_{CMY-2}, *tet*(A), *sul1*

[a] R = Extended-spectrum beta-lactamase (ESBL) / plasmidic AmpC (pAmpC) producing *E. coli*; S = *E. coli* without antibiotic selection; prefix 1 = Group 1, rinsing sample; prefix 2 = Group 2, rinsing sample followed by Broilact treatment.

[b] MLST = multilocus sequence type.

[c] One or more loci do not perfectly match any previously registered MLST allele.

5.5.2 Barnacle goose

The isolates in Study II carried from one to six plasmid replicons. Four different incompatibility groups were recognized with IncI1 type identified in three out of the nine isolates and being the most common. The results are summarized in Table 13.

All but one from the nine hybrid-sequenced isolates were found to harbor a plasmid replicon with a *bla* gene. The assembly succeeded in constructing all the replicons carrying a *bla* gene into one circular contig. The isolate without identifiable *bla*-gene-carrying plasmid (H68B) was found to be positive for a chromosomal point mutation in the AmpC promoter, and harbored multiple IncF type replicons.

Table 13. Plasmids identified in hybrid sequenced ESBL/AmpC-producing *Escherichia coli* isolates from barnacle geese.

Isolate	Number of total contigs	ESBL-plasmid name (replicon; pMLST)[a]	*bla* gene(s) on ESBL-plasmid	Other resistance genes on ESBL-plasmid	Virulence genes on ESBL-plasmid	Other plasmid replicons in isolate
H5	6	pZPK-H5 (IncI1; ST38, CC-3)	$bla_{\text{CTX-M-1}}$	*floR*	*cia*	IncFIB, IncFIC(FII), IncFII(29)
H11	11	pZPK-H11 (IncN+IncR; IncN: ST1)	$bla_{\text{CTX-M-1}}$	*qnrS1, aadA2b, lnu(F)*	–	IncFIB, IncFII, IncFII(pCoo), IncQ1
H21	9	pZPK-H21 (IncK)	$bla_{\text{CMY-2}}$, $bla_{\text{TEM-32}}$	–	*traT*	Col156, Col8282, IncFIB, IncFII, IncFII
H29	3	pZPK-H29 (IncY)	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$	*qnrS1, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, tet(A)*	–	–
H58	20	pZPK-H58 (IncK)	$bla_{\text{CMY-2}}$	–	*traT*	Col8282, ColpVC
H68B	3	–	Chromosomal point mutation in AmpC promoter	–	–	IncFIA, IncFIB, IncFIC(FII), IncFII(pHN7A8)
H98	3	pZPK-H98 (IncY)	$bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$	*qnrS1, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, tet(A)*	–	–
H163	9	pZPK-H163 (IncI1; ST23, CC-2)	$bla_{\text{CMY-2}}$	–	*cia*	ColpVC, IncFIB, IncFII

H193	4	pZPK-H193 (IncI1; ST23, CC-2)	*bla*$_{CMY-2}$	–	*cia*	ColpVC, IncFIB, IncFII

[a] CC = clonal complex; pMLST = plasmid multilocus sequence type.

5.5.2.1 Plasmid structures and genomic comparison

Plasmids were found to represent four different incompatibility groups: IncI1, IncK, IncY and a multireplicon IncN+IncR. The replicons were compared to previously published reference plasmids with BLASTn (Figure 12).

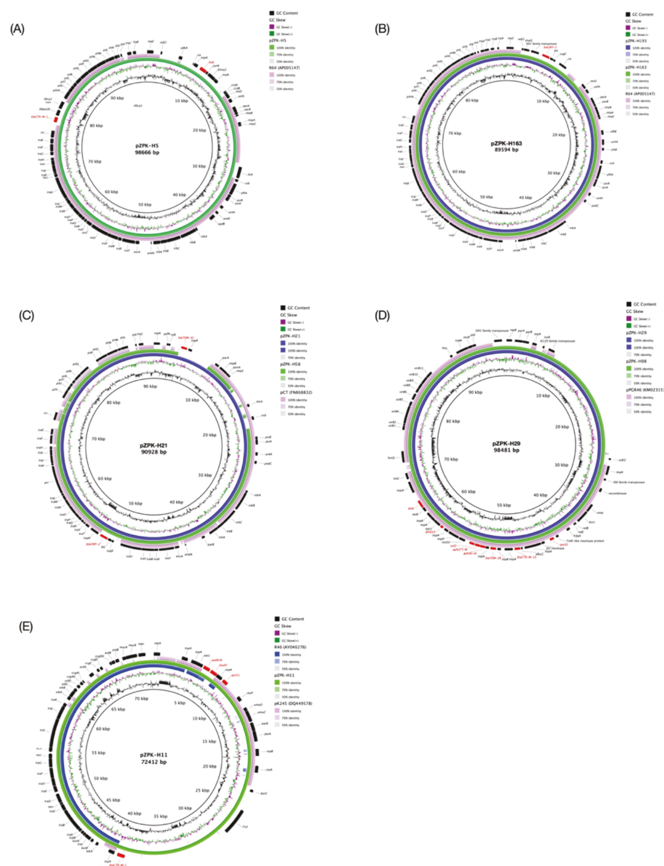


Figure 12. Circular comparison of the studied plasmids with previously published reference plasmids. GC content and GC skew are depicted in the inner map with distance scale. Predicted coding sequences of the plasmid named within the circle are depicted in the outer ring with antimicrobial resistance genes highlighted in red. (A) pZPK-H5 compared with IncI1 type reference R64 (GenBank accession: AP005147), (B) pZPK-H163 and pZPK-H193 compared with IncI1 type reference R64 (GenBank accession: AP005147), (C) pZPK-H21 and pZPK-H58 compared with IncK type reference pCT (GenBank accession: FN868832), (D) pZPK-H29 and pZPK-98 compared with IncY type reference pPGR46 (GenBank accession: KM023153), (E) pZPK-H11 compared with IncN type R46 (GenBank accession: AY046276) and IncR type pK245 (GenBank accession: DQ449578).

Three isolates harbored IncI1 type replicons with plasmid-encoded *bla*$_{CTX-M-1}$ or *bla*$_{CMY-2}$. Typical IncI plasmid backbones with maintenance and stability-related (*parAB*, *impCAB*, *ssb*, *psiAB*, *ardA*, *pndAC*), transfer-associated, shufflon, and pilus formation regions (Figure 12A–B) were identified in all IncI1 plasmids.

The two plasmids carrying *bla*$_{CMY-2}$ were found to be identical with BLASTn pairwise alignment, with 89.6 kb size, G+C content of 50.31% and predicted 101 predicted coding sequences (CDSs). The IncI1- *bla*$_{CTX-M-1}$ plasmid was 98.7 kb in size with a G+C content of 50.2% and 112 CDSs.

Comparison of the studied IncI1 plasmids to previously published plasmids from GenBank using BLASTn demonstrated similarity to a limited number of plasmids both of human and avian origin. A cgMLST-based minimum spanning tree built with 75 discriminatory loci (95% occurrence) is visualized in Figure 13.

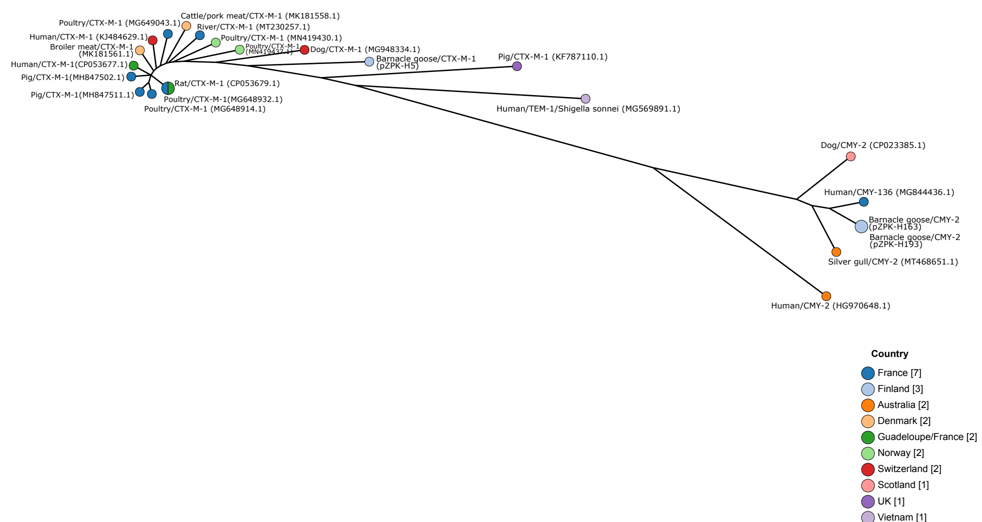


Figure 13. Minimum spanning tree based on core genome multilocus sequence typing of IncI1 type plasmids. Plasmids pZPK-H5, pZPK-H163, and pZPK-H193 compared with 20 previously published IncI1 plasmids from GenBank. Nodes are colored by the country of origin and node text includes information on the source, *bla* gene, and bacterial species if other than *Escherichia coli*. GenBank accession numbers are provided in parentheses.

IncK plasmids with *bla*$_{CMY-2}$ were identified in two isolates, with 88% coverage and 99.99% identity with BLASTn pairwise alignment. The size of the plasmids was 90.9 kb and 79.2 kb, G+C content 52.51% and 52.16%, and 108 and 92 CDSs were predicted, respectively. Like IncI1 plasmids, the IncK plasmids demonstrated typical I-complex plasmid backbones with maintenance and stability, conjugation, shufflon, and pilus formation regions (Figure 12C). Figure 14 displays a cgMLST-based minimum spanning tree with 64 discriminatory loci (95% occurrence) between the two IncK plasmids and the included previously sequenced plasmids.

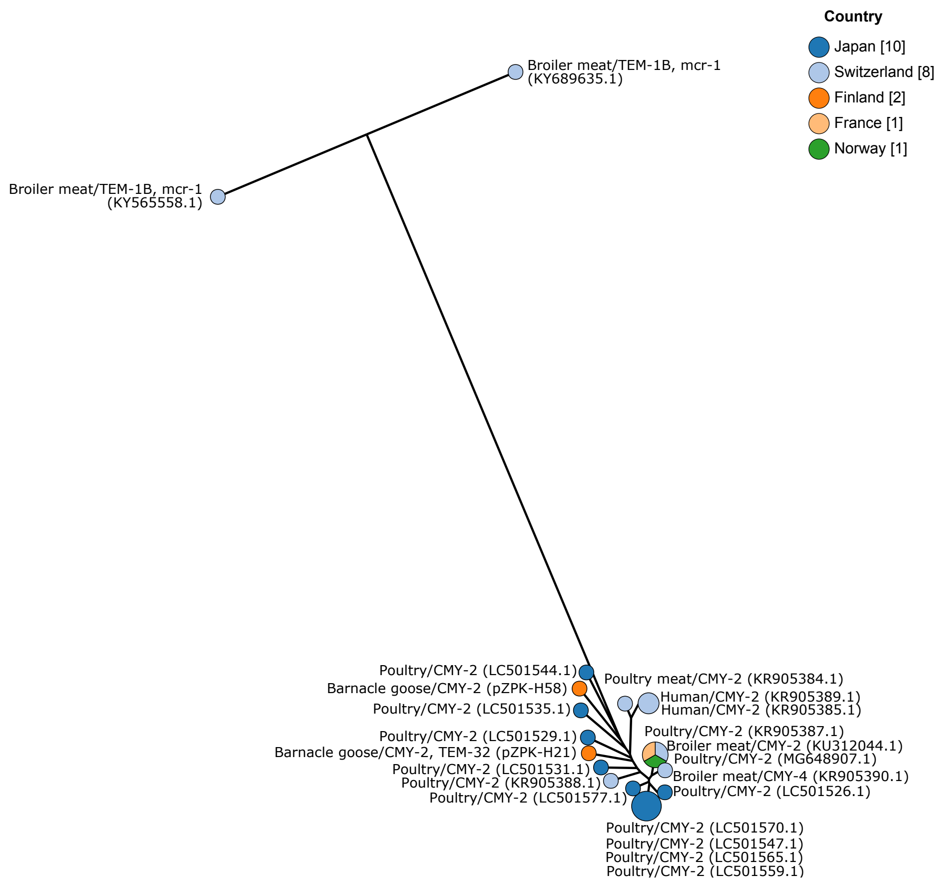


Figure 14. Minimum spanning tree based on core genome multilocus sequence typing of IncK type plasmids. Plasmids pZPK-H21 and pZPK-H58 compared with 20 previously published IncK or IncB/O/K/Z plasmids from GenBank. Nodes are colored by the country of origin and node text includes information on the source, *bla* gene, and possible additional *mcr-1*. GenBank accession numbers are provided in parentheses.

The IncY plasmids, both carrying *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM-1B}}$, from two isolates were highly similar with BLASTn pairwise alignment, with 100% coverage and 99.94% identity. Both also carried the resistance genes qnrS1, aph(6)-Id, aph(3")-Ib, sul2, dfrA14, and tet(A) and were 98.5 kb in size, had a G+C content of 51.13% and 98 or 99 predicted CDSs.

Only six previously published IncY plasmids with available metadata from the NCBI database were similar to pZPK-H29 and pZPK-H98 (Appendix IV). A cgMLST-based minimum spanning tree included 20 discriminatory loci (95% occurrence) and did not indicate clusters (Figure 15).

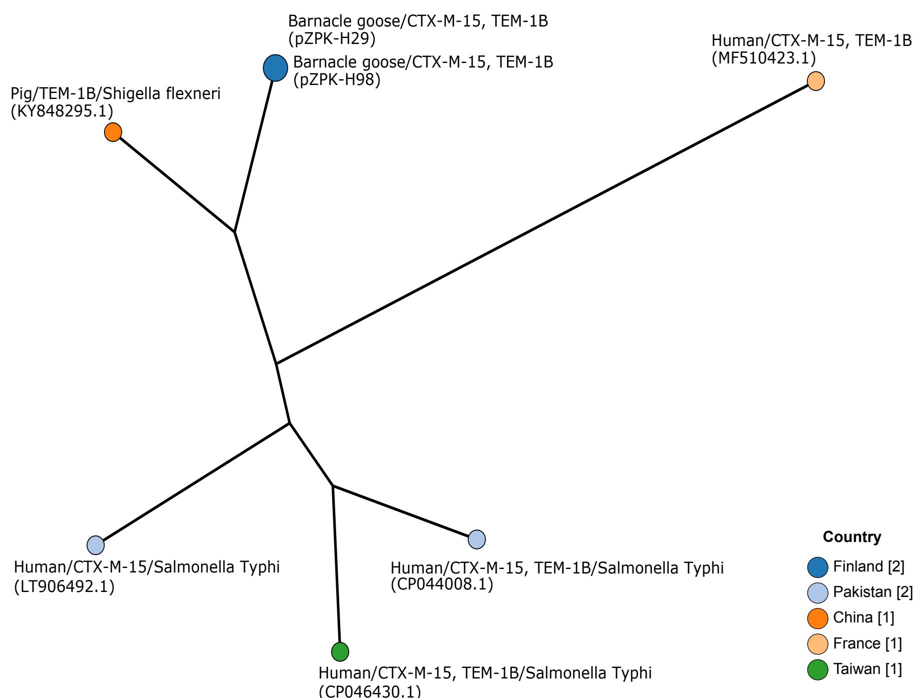


Figure 15. Minimum spanning tree based on core genome multilocus sequence typing of IncY type plasmids. Plasmids pZPK-H29 and pZPK-H98 compared with six previously published IncY plasmids from GenBank. Nodes are colored by the country of origin and node text includes information on the source, *bla* gene, and bacterial species if other than *Escherichia coli*. GenBank accession numbers are provided in parentheses.

A multireplicon comprising IncN and IncR plasmids was identified in one isolate. The multireplicon was 72.4 kb in size, had a G+C content of 50.91% and 78 predicted CDSs. The structure and identified genes are depicted in Figure 16.

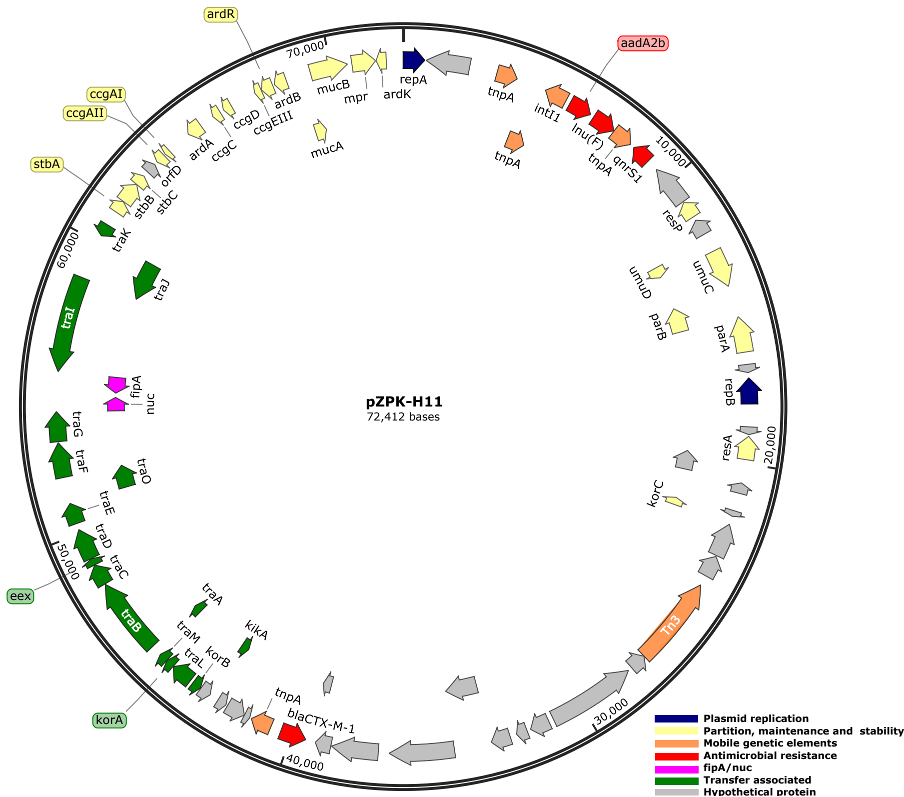


Figure 16. Genetic structure of multireplicon plasmid pZPK-H11. Predicted coding sequences and their orientation are represented by arrows colored based on the function of the gene product. Size of the plasmid is depicted on the outer circle.

The backbone was similar to IncN reference R46 (GenBank accession: AY046276), with replicon *repA*, stability-related *stbABC*, *mucA*, and *mucB* involved in mutagenesis enhancement, genes involved in plasmid DNA protection from type I restriction enzymes (*ccgC*, *ccgD*, *ccgAI*, and *ccgAII*), antirestriction-associated *ardA*, *ardB*, and *ardR*, and two transfer-associated *tra* loci ((Delver and Belogurov, 1997; Carattoli et al., 2010; Dolejska et al., 2013). IncR structures were *repB*, *parAB*, and *umuCD* and multimer resolvase, but toxin–antitoxin *vagDC* operon involved in plasmid maintenance and *retA* reverse transcriptase were not identified (Guo et al., 2016). Transcriptional regulator *korC* was identified outside IncN or IncR reference plasmids regions (Ludwiczak et al., 2013). *bla*$_{\text{CTX-M-1}}$ next to a mobile genetic element *tnpA* was outside of a multidrug resistance cassette containing *aadA2b*, *lnu(F)*, and *qnrS1*, which were located downstream of *repA*.

A cgMLST-based minimum spanning tree was built with only 34 discriminatory loci (95% occurrence) (Figure 17), as top hits from the NCBI database included mostly IncN replicons or multireplicons with IncF+IncF.

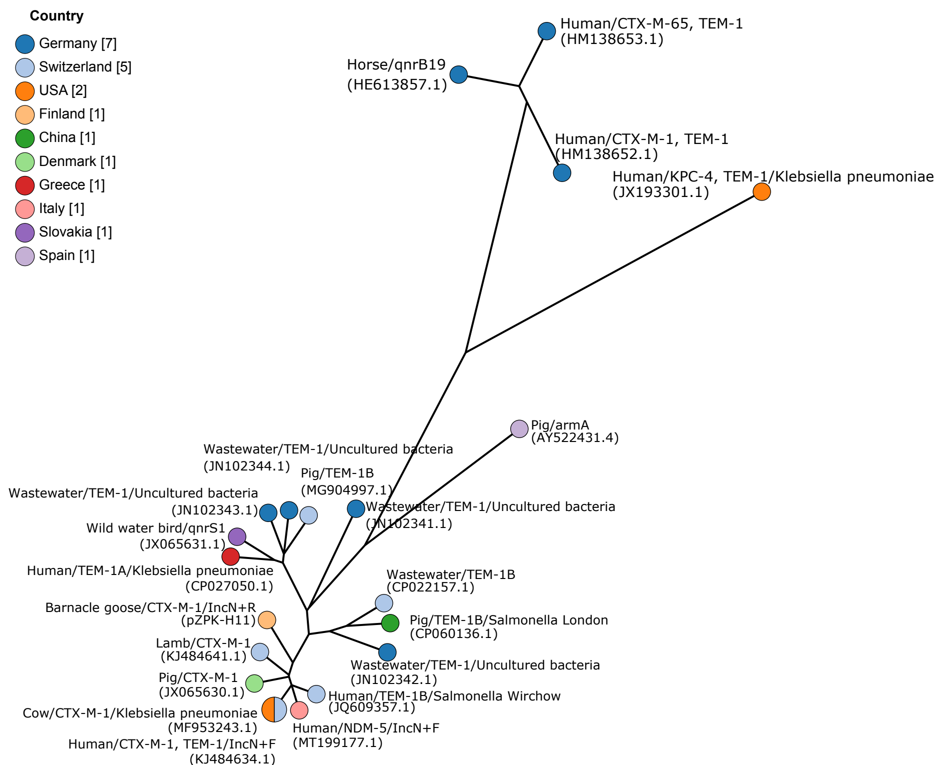


Figure 17. Minimum spanning tree based on core genome multilocus sequence typing of IncN+IncR multireplicon. Plasmid pZPK-H11 compared with 20 previously published plasmids from GenBank. Nodes are colored by the country of origin and node text includes information on the source, *bla* gene, or other resistance gene if the *bla* gene was not present, bacterial species if other than *Escherichia coli*, and replicon type if other than IncN. GenBank accession numbers are provided in parentheses.

5.5.3 Imported food

The short-read sequenced isolates (n=21) in Study III harbored from one to seven different plasmid replicons, averaging at three replicon types per isolate (Table 14). The most frequently found plasmids were IncFIB and IncI1-Iγ, detected in 11 and 10 isolates, respectively. IncI1-Iγ plasmids were detected in the sequenced broiler meat isolates.

Analysis of plasmid sequence types with pMLST v 2.0 (Carattoli et al., 2014) identified multiple different sequence types and clonal complexes for IncI1-Iγ plasmids, and variable FAB formulae for IncF replicons. The most common STs for IncI1-Iγ were ST12 and ST95-CC9. ST12 was identified together with bla_{CMY-2} and ST95-CC-9 together with bla_{SHV-12}, both in three different *E. coli* isolates with varying bacterial sequence types. Additionally, IncI1-Iγ ST3-CC3 was found in two ST38 *E. coli* isolates together with resistance gene $bla_{CTX-M-1}$.

Table 14. Plasmid replicons identified from short-read sequenced ESBL/AmpC-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates from imported food.

Isolate[a]	Species	MLST[b]	Plasmid replicons	pMLST / RST[c]	pMLST v 2.0 profile selected
A35-1	*K. pneumoniae*	ST307	IncFIB(K), IncFII(K)	[K7:A-:B-]	IncF RST
A35.2-2	*K. pneumoniae*	ST101	IncFIB(K), IncFII(pK91)	[K9:A-:B-]	IncF RST
A40.2-1	*E. coli*	ST155	IncFIB, IncFIC(FII)	[F18:A-:B1]FIC4[d]	IncF RST
A41.2-1	*E. coli*	ST479[e]	IncFIB, p0111	[F-:A-:B76]	IncF RST
C32.1-2	*K. pneumoniae*	ST307	IncFIB(K), IncFII(K)	[K7:A-:B-]	IncF RST
C51-1	*E. coli*	ST189	IncI1-I(Gamma)	ST95 (CC-9)	IncI1 MLST
C51.2-2	*Escherichia fergusonii*[f]	ST8330	ColpVC, IncB/O/K/Z, IncFIB, IncFII, IncI2, IncX1	[F54:A-:B1]	IncF RST
C56.1-1	*E. coli*	ST4994	Col156, Col8282, ColpVC, IncI1-I(Gamma), IncI2(Delta)	ST36 (CC-3)	IncI1 MLST
C61-1	*E. coli*	ST1011	IncFIB, IncFII, IncI1-I(Gamma)	ST2 (CC-2)	IncI1 MLST
C66-1	*E. coli*	ST423	IncFIB(pLF82), IncFII(pSE11), IncI1-I(Gamma)	ST95 (CC-9)	IncI1 MLST
C71.1-1	*E. coli*	ST1485	IncB/O/K/Z, IncFIA, IncFIB, IncFIC(FII), IncHI2, IncHI2A, p0111	[F18:A5:B1]FIC4[d]	IncF RST
C76.1-1	*E. coli*	ST201	IncFIB, IncFII(pCoo), IncI1-I(Gamma)	ST12	IncI1 MLST
C76.1-2	*E. coli*	ST83[e]	IncFIA, IncFIB, IncI1-I(Gamma)	ST95 (CC-9)	IncI1 MLST
C81.1-1	*E. coli*	ST38	Col156, IncFII(29), IncI1-I(Gamma)	ST3 (CC-3)	IncI1 MLST
C81.3-1	*E. coli*	ST1638	IncFIB, IncFIC(FII), IncFII(pHN7A8), IncI1-I(Gamma), IncX1, Col(pHAD28)	ST12	IncI1 MLST
C81.1-2	*E. coli*	ST641	IncFIB, IncFIB(pLF82), IncFII(pSE11), IncX1	[F72:A-:B-][g]	IncF RST
C86.1-1	*E. coli*	ST641	IncFIB, IncFIB(pLF82), IncFII(29), IncFII(pSE11), IncX1	[F29:A-:B-]	IncF RST
C86.1-2	*E. coli*	ST38	Col156, IncFII(29), IncI1-I(Gamma)	ST3 (CC-3)	IncI1 MLST
C86.3-1	*K. pneumoniae*	ST37	IncFIA(HI1), IncR	[F-:A13:B-]	IncF RST
C91-2	*E. coli*	ST117	ColpVC, IncB/O/K/Z, IncFIB, IncFIC(FII), IncFII(29)	[F18:A-:B1]FIC4; multiple perfect hits for IncFII: IncFII29, IncFII18	IncF RST
C96-1	*E. coli*	ST88	IncFII(29), IncI1-I(Gamma)	ST12	IncI1 MLST

[a] Suffix *-1* indicates first incubation temperature of 44 °C and *-2* of 37 °C.

[b] MLST = multilocus sequence type

[c] pMLST 2.0 for plasmid sequence type (ST) (CC = clonal complex; pMLST = plasmid multilocus sequence typing; RST = replicon sequence typing).

[d] pMLST tool recognizes IncFII as part of IncFIC template; no IncFII replicon present in the isolate.

[e] scheme *Escherichia coli* #1 used first, if sequence type (ST) unidentified then #2 (Jaureguy et al., 2008) used.

[f] identified as *E. coli* with SpeciesFinder v 1.2 (Larsen et al., 2014).

[g] PlasmidFinder with minimum values identified IncFII replicons with <95% identity which explains RST result with IncFII.

The two coriander and five broiler meat *E. coli* isolates additionally sequenced with long-read sequence technology revealed the location of resistance genes to either a plasmid or chromosome (Table 15). Beta-lactamase genes were located on IncF type plasmids in the coriander isolates and on IncI1-Iγ plasmids in four broiler meat isolates, all with different pMLST profiles. One broiler meat isolate (C86.1-1) harbored a chromosomal bla_{CMY-2} and a plasmid-encoded bla_{TEM-1B} on an IncX1 plasmid. This isolate additionally harbored IncF type plasmids, from which no *bla* genes were recovered, and therefore IncX1 from this isolate was selected for further analysis. Isolate C61-1 from broiler meat carried a plasmid-encoded bla_{CMY-2} together with chromosomal bla_{TEM-1B}. Four of the hybrid-sequenced isolates were found to carry additional plasmid-encoded resistance genes in addition to *bla* genes. Five isolates also possessed plasmid-encoded virulence genes.

Table 15. Hybrid sequenced *Escherichia coli* food isolates from imported food products and corresponding beta-lactamase gene-carrying plasmids.

Isolate[a]	ESBL-plasmid name (replicon; pMLST/RST)[b]	Plasmid size (bp)	*bla* gene	Other resistance genes on plasmid	Virulence genes on plasmid
A40.2-1	pZPK-A40.2-1 (IncFIB, IncFIC(FII); [F-:A-:B1] FIC-4)	120698	$bla_{CTX-M-55}$, bla_{TEM-1B}	*ARR-2, floR, dfrA14, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, tet(A), sul2*	*cma, cvaC, hlyF, iucC, iutA, ompT, sitA*
A41.2-1	pZPK-A41.2-1 (IncFIB, p0111; [F-:A-:B76])	153291	$bla_{CTX-M-65}$	*floR, oqxA, oqxB, dfrA17, aac(3)-IV, aadA5, aph(4)-Ia, sul1, sul2*	*papC*
C51-1	pZPK-C51-1 (IncI1-Iγ; 95 (CC-9))	121837	bla_{SHV-12}	*aadA1, aadA2b, tet(A), sul3, cmlA1*	*cib*
C56.1-1	pZPK-C56.1-1 (IncI1-Iγ; 36 (CC-3))	89504	$bla_{TEM-52C}$	-	-
C61-1	pZPK-C61-1 (IncI1-Iγ; 2 (CC-2))	97275	bla_{CMY-2}	-	*cia*
C81.1-1	pZPK-C81.1-1 (IncI1-Iγ; 3 (CC-3))	112374	$bla_{CTX-M-1}$	-	*cib*
C86.1-1	pZPK-C86.1-1_X1 (IncX1)	47686	bla_{TEM-1B}	*qnrS1*	

[a] Suffix *-1* indicates first incubation temperature of 44 °C and *-2* of 37 °C.

[b] pMLST 2.0 for plasmid sequence type (ST) (CC = clonal complex; pMLST = plasmid multilocus sequence typing; RST = replicon sequence typing).

5.5.3.1 Plasmid structures and genomic comparison

The seven *bla*-encoding plasmids identified from hybrid-sequenced isolates were compared to previously published reference plasmids according to their

respective incompatibility types, as well as to previously sequenced, similar plasmids identified from the NCBI database with a BLASTn search.

Plasmids belonging to the IncF group were found to be highly variable when compared to each other and a previously published, annotated IncF plasmid with FAB formula [F18:A-:B1:C4] (GenBank accession: MK878890.1) (Figure 18). The size of the IncF type plasmids identified were typical as multireplicon IncFIB/IncFIC named pZPK40.2-1 had a size of 120.7 kb, G+C content of 51.45% and 136 CDSs. The pZPK41.2-1 plasmid carried an IncFIB replicon together with p0111 with a size of 153.3 kb, G+C content of 49.93%, and 165 CDSs.

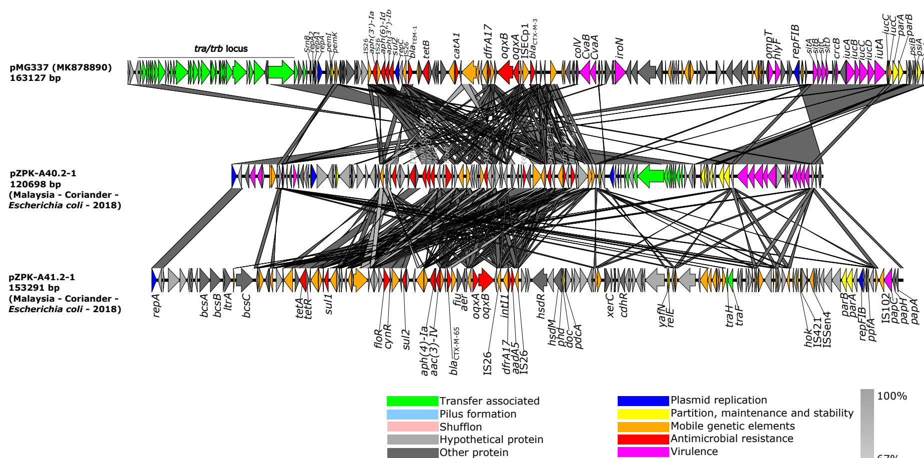


Figure 18. Linear comparison of IncFIB/IncFIC(FII) and IncFIB/p0111 plasmids identified in this study with previously published plasmids (GenBank accession number in parentheses for reference plasmid; for plasmids identified in this study the country of origin, source, bacterial species and year of isolation is provided in parentheses). Gray areas between plasmid sequences indicate the percentage of nucleotide sequence identity. The arrows represent coding sequences and their orientation and are colored based on their predicted function.

The IncX1 plasmid was 47.7 kb in size with a G+C content of 43.13% and 56 CDSs predicted. The plasmid had a typical IncX1 backbone with replication genes *pir* and *bis*, pilus associated genes *pilX* and genes involved in partition, stability, and conjugation (Johnson et al., 2012) (Figure 19).

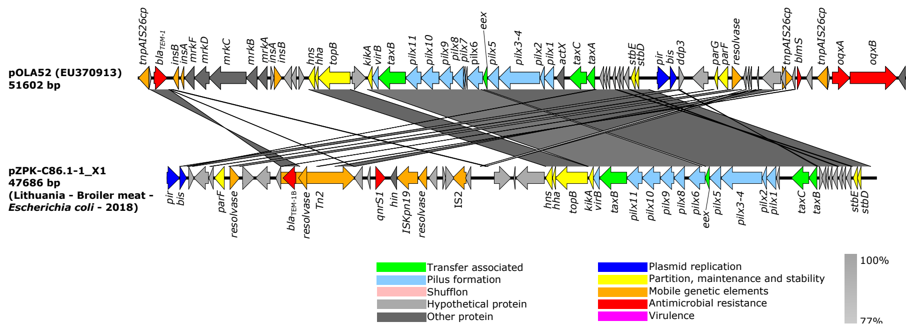


Figure 19. Linear comparison of IncX1 plasmid identified in this study with a previously published plasmid (GenBank accession number in parentheses for reference plasmid; for the plasmid identified in this study the country of origin, source, bacterial species and year of isolation is provided in parentheses). Gray areas between plasmid sequences indicate the percentage of nucleotide sequence identity. The arrows represent coding sequences and their orientation and are colored based on their predicted function.

The four IncI1-Iγ type plasmids had a size of 89.5 to 121.8 kb, G+C content of 50.24 to 51.65%, and 97 to 136 CDSs. Comparing plasmids to IncI1 type reference plasmid R64 (GenBank accession: AP005147.1) and IncIγ type reference plasmid R621a (GenBank accession: AP011954.1) indicated three of the four of the plasmids were slightly more similar to the IncIγ type R621a, which was chosen for comparison (Figure 20), but results are here referred to as IncI1-Iγ. Plasmids demonstrated usual IncI type backbones with conjugational, pilus formation and maintenance and stability regions, and *pndCA* addiction system was identified in all plasmids. In one plasmid (pZPK-C81.1-1) *bla* gene and IS5 were located near the shufflon region. Variability was observed in the accessory region regarding resistance genes and inserted elements.

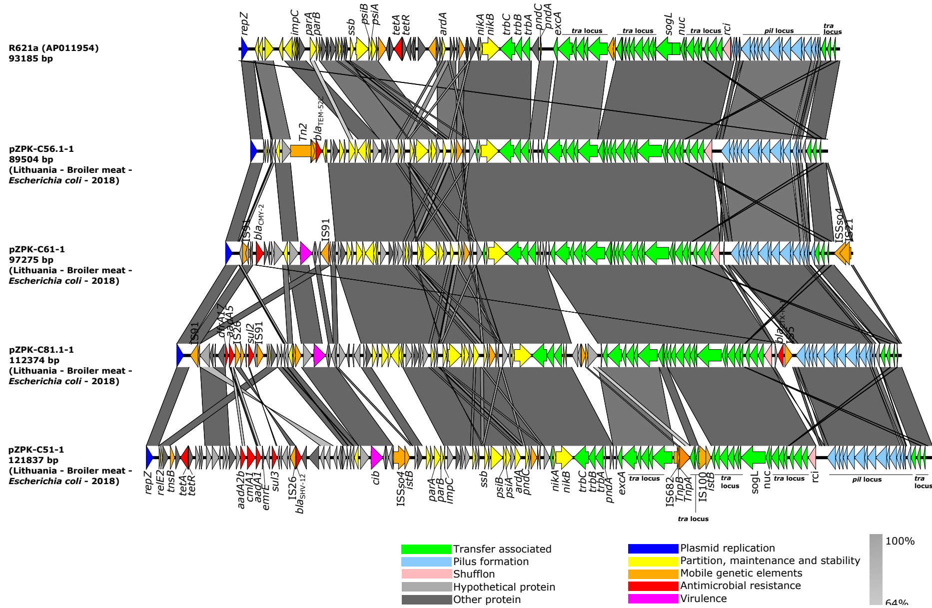


Figure 20. Linear comparison of IncI1-Iγ plasmids identified in this study with previously published plasmids (GenBank accession numbers in parentheses for reference plasmids; for plasmids identified in this study the country of origin, source, bacterial species and year of isolation is provided in parentheses). Gray areas between plasmid sequences indicate the percentage of nucleotide sequence identity. The arrows represent coding sequences and their orientation and are colored based on their predicted function.

Comparison with BLASTn to previously published plasmids in the NCBI database identified similar plasmids from mostly livestock and food sources, although human-derived isolates were included in the top hits also (Figure 21).

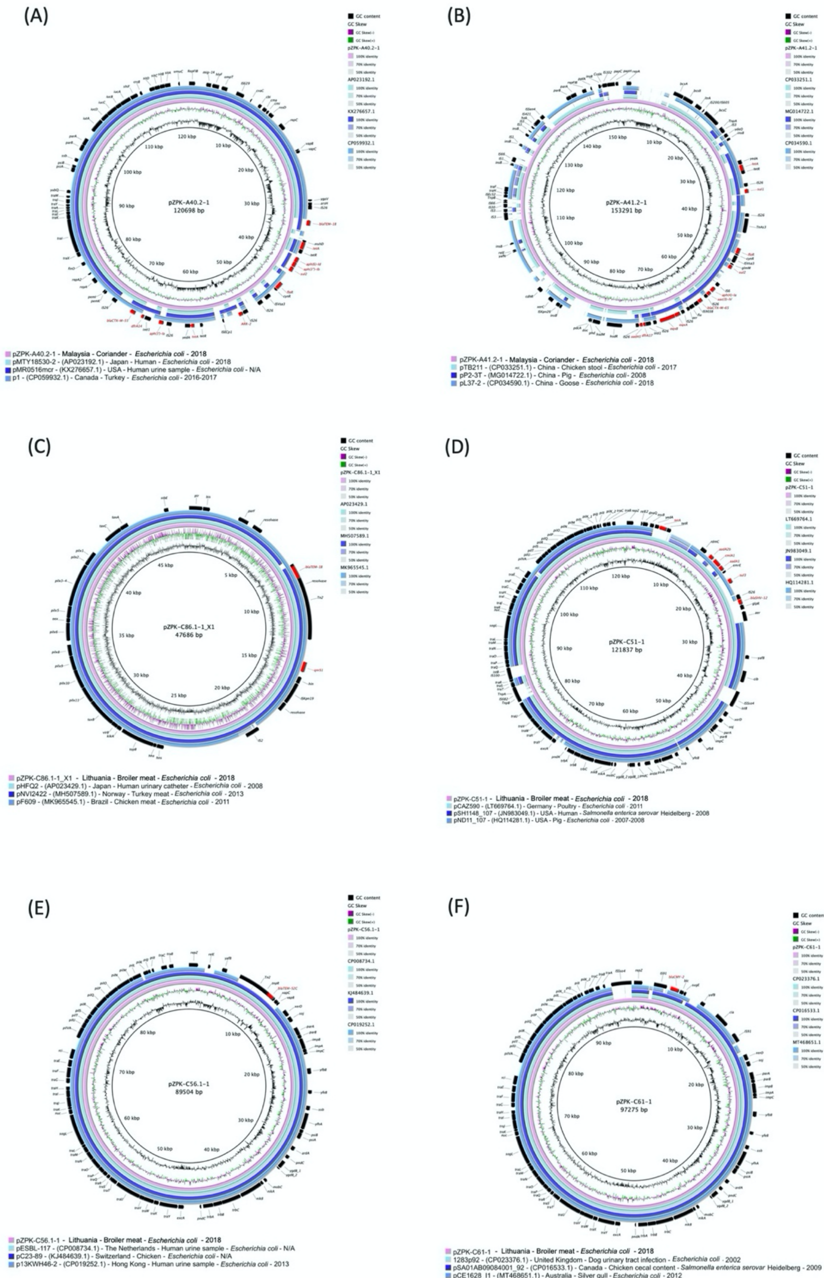


Figure 21. Circular comparisons of the studied plasmids to previously published similar plasmids (GenBank accession numbers are provided in parentheses in the figure legends after plasmid name, followed by country, source, bacterial species and year of isolation; N/A, not available). GC content and GC skew of the studied plasmids are depicted in the inner map with distance scale and the outer ring represents predicted coding sequences with antimicrobial resistance genes highlighted in red. The plasmids in this study included (A) pZPK-A40.2-1, (B) pZPK-A41.2-1, (C) pZPK-C86.1-1_X1, (D) pZPK-C51-1, (E) pZPK-C56.1-1, (F) pZPK-C61-1, and (G) pZPK-C81.1-1.

5.5.4 Human clinical samples

In Study IV, 29 out of 30 ESBL-producing *E. coli* isolates were found to carry at least one plasmid replicon (Table 16). Plasmids belonging to the IncF family were dominant, with IncFIB replicons appearing in 24, IncFIA in 21, and IncFII in 13 isolates. The most common FAB formulae based on the replicon sequence typing scheme were [F1:A2:B20] and [F-:A2:B20], appearing in 11 and four isolates, respectively. Col156 replicons were identified in 13 isolates, and IncI1 plasmids from five isolates, all with varying plasmid multilocus sequence types (pMLSTs).

Table 16. Plasmids identified from ESBL-producing *Escherichia coli* isolates from human clinical samples obtained from Eastern Finland healthcare district.

Isolate	Specimen type	MLST[a]	*bla* gene(s)	Plasmid replicon(s)	pMLST / RST
D1	Urine	ST131	$bla_{CTX-M-27}$	IncFIA, IncFIB, IncFIB(H89-PhagePlasmid), IncFII(pRSB107), IncX4	[F1:A2:B20]
D2	Joint	ST978[b]	$bla_{CTX-M-15}$	IncFIA, IncFIB, IncFII(pRSB107)	[F1:A1:B10]
D3	Scrotum	ST38	$bla_{CTX-M-27}$	IncFIA, IncFIB, IncFII(pRSB107), Col(BS512), Col156	[F1:A2:B20]
D4	Maxillary sinus	ST1[c]	$bla_{CTX-M-14}$, bla_{TEM-1}	IncFIB	[F46:A-:B20]
D5	Eye conjunctiva	ST1193	$bla_{CTX-M-55}$	IncB/O/K/Z, Col(BS512), Col(MG828)	-
D6	Wound	ST131	$bla_{CTX-M-15}$, bla_{TEM-1}	IncFIA, IncFIB, IncFII(pRSB107), Col156	[F1:A2:B20]
D7	Blood	ST131	$bla_{CTX-M-15}$, bla_{OXA-1}	No plasmids found	-
D8	Bile	ST131	$bla_{CTX-M-27}$	IncFIA, IncFIB, IncFII(pRSB107), IncX3, IncY, Col(BS512), Col156	[F1:A2:B20]
D9	Abscess	ST131[b]	$bla_{CTX-M-15}$, bla_{TEM-1}	IncFIB, IncFIB(H89-PhagePlasmid), IncFII(29), IncFII(pCoo)	[F29:A-:B10]
D10	Blood	ST131	$bla_{CTX-M-15}$	IncFIA, IncFIB, Col(BS512)	[F36:A1:B20][e]
D11	Lung (bronchoalveolar lavage)	ST131	$bla_{CTX-M-27}$	IncFIA, IncFIB, IncFII(pRSB107), Col156	[F1:A2:B20]
D12	Urine	ST131	$bla_{CTX-M-27}$	IncFIA, IncFIB, IncFII(pRSB107), IncI1	[F1:A2:B20]
D13	Urine	ST131	$bla_{CTX-M-27}$	IncB/O/K/Z, IncFIA, IncFIB, IncFII, IncFII(pRSB107), Col156, Col8282	[F84:A2:B20][e]
D14	Blood	ST162	bla_{SHV-12}	IncFIA, IncFIC(FII), IncI1, IncQ1	[F18:A6:B-][e] / ST26 (CC-2)

D15	Urine	ST131	*bla*$_{\text{CTX-M-15}}$	IncFII, IncI1	[F2:A-:B-] / ST173
D16	Urine	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, IncFII(pRSB107), Col156	[F1:A2:B20]
D17	Abdominal cavity	ST537	*bla*$_{\text{TEM-52}}$	IncI1	ST36 (CC-3)[e]
D18	Urine	ST59	*bla*$_{\text{CTX-M-55}}$, *bla*$_{\text{TEM-1}}$	IncFII(pCoo)	[F10:A-:B-]
D19	Urine	ST405	*bla*$_{\text{CTX-M-3}}$	IncFIB, IncFII(29), IncI1, Col(BS512), Col156, Col156, Col156	[F29:A-:B10] / ST57 (CC-5)
D20	Blood	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, IncFII(pRSB107), Col156	[F1:A2:B20]
D21	Blood	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, IncFII(pRSB107)	[F1:A2:B20][e]
D22	Urine	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, Col(pHAD28), Col156	[F-:A2:B20]
D23	Wound	ST131	*bla*$_{\text{CTX-M-15}}$	IncFIA, IncFIB, Col(BS512)	[F-:A1:B20][e]
D24	Urine	ST131	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{OXA-1}}$	IncFIA, IncFIB, IncX4, Col156	[F-:A2:B20]
D25	Blood	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB	[F-:A2:B20]
D26	Urine	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, IncFIB(H89-PhagePlasmid), IncFII(pRSB107), IncI1	[F1:A2:B20] / IncI1 unknown
D27	Blood	ST131	*bla*$_{\text{CTX-M-15}}$	IncFIA, IncFIB, Col(BS512)	[F22:A1:B20][e]
D28	Blood	ST131	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$	IncFIB, IncFII(29), Col156	[F29:A-:B10]
D29	Urine	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, IncFII(pRSB107), Col156	[F1:A2:B20]
D30	Urine	ST131	*bla*$_{\text{CTX-M-27}}$	IncFIA, IncFIB, Col(pHAD28), Col156	[F-:A2:B20]

[a] MLST = multilocus sequence type; pMLST 2.0 for plasmid sequence type (ST) (CC = clonal complex; pMLST = plasmid multilocus sequence typing; RST = replicon sequence typing).

[b] MLST v 2.0 (Larsen et al., 2012) used to verify result (with Warwick scheme).

[c] Pasteur scheme used when sequence type not identified with Warwick scheme.

[d] SerotypeFinder v 2.0 (Joensen et al., 2015) used to verify result.

[e] Uncertain hit, ST cannot be trusted.

5.6 Statistical analysis (I)

For Study I, Fisher's exact test (P = 1.000) found no significant difference between different groups of chick intestine samples (Groups 1, 2, and 3). There

was also no significant difference in the presence of *E. coli* found on egg surfaces from the tested Groups 1 and 2.

6 DISCUSSION

6.1 ESBL/AmpC-producing *E. coli* along the poultry production chain (I)

Vertical spread along the poultry production chain has been traditionally considered the main transmission route for ESBL/AmpC-producing bacteria. Recent years have seen studies with further evidence for horizontal transmission routes within the poultry sector, however (Dierikx et al., 2013; Projahn et al., 2017, 2018; Dame-Korevaar et al., 2019). The results of Study I support the findings that ESBL/AmpC-producing *E. coli* transfer along the broiler production chain is not strictly maintained by vertical transmission routes, but rather, a combination of vertical and horizontal transmission events.

In Study I, 26.7% of the sampled parent birds were found to carry ESBL/AmpC-producing *E. coli*, but only 1.3% of egg surfaces tested positive for ESBL/AmpC-producing *E. coli* soon after laying before routine disinfection protocols at the broiler parent farm. *E. coli* cultured without third-generation cephalosporin supplement was found from 65% of the egg surfaces soon after laying. However, after a three-week incubation period at the hatchery and routine disinfection procedures, none of the egg surfaces were positive for *E. coli*. Furthermore, only two (2.2%) broiler embryos were positive for *E. coli* after the incubation period at the hatchery. These findings indicate that *E. coli*, ESBL/AmpC-producing or not, does not transfer via the vertical route from parent birds to developing embryos routinely, nor does it penetrate the egg surface from environmental sources routinely. Two sequence types, ST2040 and ST429, with very few (<10) SNP differences within strains from each ST, were identified in ESBL/AmpC-producing *E. coli* from both the broiler parents and egg surfaces soon after lay. This demonstrates successful clonal spread of *E. coli* within the broiler parent farm, which does not, however, continue to the next production steps. In earlier studies, *E. coli* survived on egg surfaces even after disinfection, possibly hidden within eggshell pores (Mezhoud et al., 2016), although in reduced numbers (Cadirci, 2009; Projahn et al., 2017).

Regardless of these findings, the production level broilers have demonstrated ESBL/AmpC-producing *E. coli* in relatively high numbers in previous years compared to other tested production animals in Finland (FINRES-Vet, 2021.). This suggests that even with the use of an all-in-all-out method of rearing animals and diligent cleaning methods between batches, ESBL/AmpC-producing bacteria can proliferate at the broiler production level. Following the sampling year of Study I, the prevalence of ESBL/AmpC-producing *E. coli* in Finnish production level broilers at slaughter was 13.1% in 2018 (EFSA and ECDC, 2021). Of these, 11.4% were AmpC-producers. The prevalence of

ESBL/AmpC-producing *E. coli* outside of northern Europe has, however, been substantially higher in many European countries. The habit of using competitive exclusion (CE) products combined with antibiotic-free rearing of broilers in Finland has been suggested to contribute to the low prevalence compared with other regions. In Study I, the benefit of immersion of eggs to CE solution could not be assessed due to low *E. coli* occurrence in all egg groups, but previous studies demonstrated the added benefit of using CE products in hatchlings (Nuotio et al., 2013; Ceccarelli et al., 2017).

Interestingly, the prevalence of ESBL/AmpC-producing *E. coli* has seen a drastic change in Finland during the last couple of years. During routine national monitoring, only 0.3% of broilers at slaughter and broiler meat samples at retail, respectively, tested positive for ESBL/AmpC-producing *E. coli* in 2020 (FINRES-Vet, 2021.). Since 2018, none of the liners from transport boxes of imported poultry flocks for broiler meat production have tested positive for ESBL/AmpC-producing *E. coli*. This situation has also seen a significant change, since in 2016 39% and in 2017 22% of liners tested positive (FINRES-Vet, 2021.). Whether major modifications have been implemented in the pedigree or grandparent level of the broiler production pyramid is unknown, but these findings would suggest that after 2017, the ESBL/AmpC burden brought along with imported parent flocks has diminished. This resulted in a delayed clearance of ESBL/AmpC-producing *E. coli* and *bla*-harboring plasmids in the poultry production environment in Finland. A similar trend has been observed in the poultry sector in Sweden (Svensk Fågel, 2021), as well as in Norway and Denmark (EFSA and ECDC, 2021).

6.2 ESBL/AmpC-producing *E. coli* in migratory birds (II)

In Study II, ESBL/AmpC-producing *E. coli* was found in 4.5% of the sampled barnacle goose fecal samples. This is slightly less than has been observed for ESBL/AmpC-producing *E. coli/K. pneumoniae* in Finnish asymptomatic humans (6.3%) (Rintala et al., 2018). Earlier studies have found the prevalence of ESBL/AmpC-producing *E. coli* in wild birds to be higher, although it must be considered that these studies focused on different species and were conducted outside Finland. In a study examining mainly aquatic-associated bird species, ESBL/AmpC-producing *E. coli* was observed in 15.7% of the 21 bird species tested (Veldman et al., 2013), whereas in Spain 15%, in Germany 13.8% and in Mongolia 10.8% of wild birds tested were positive for ESBL/AmpC-producing *E. coli* (Guenther et al., 2011; Alcalá et al., 2016). Typical for avian sources, the most prevalent resistance gene identified in Study II was bla_{CMY-2}. $bla_{CTX-M-15}$, often found in humans, and $bla_{CTX-M-1}$, a gene common in food-producing animals, were also observed. Wild birds in Catalonia, Spain, have also been found to harbor bla_{CMY-2} and $bla_{CTX-M-15}$ (Darwich et al., 2019). Interestingly, this study also found the carbapenemase gene bla_{OXA-48} in a barn owl. In Study II, no carbapenemase-coding genes were observed. However, a limitation of Study II is the fact that the cefotaxime supplement used for selection of ESBL/AmpC-producing isolates does not detect isolates which solely produce OXA-48 (Martinez-Martinez et al., 2017).

The identified STs did not indicate overlap with the successful STs circulating in human *E. coli* infections.

Barnacle geese do not feed on landfills, contrary to gulls in many areas. Wild birds may acquire resistant bacteria from anthropogenic sources, which has been suspected in Sweden where $bla_{\text{CTX-M-15}}$- and $bla_{\text{CTX-M-14}}$-harboring *E. coli* isolates from wild gulls were found to be similar to human isolates as well as isolates from surface waters (Atterby et al., 2017). Wildlife has been recognized as potential reservoir for resistant bacteria (Carroll et al., 2015) and it has been estimated that above 60% of emerging infectious diseases since the 1940s have been of zoonotic origin (Jones et al., 2008). Although the transmission of resistance determinants and pathogens can be bi-directional, migratory birds are able to cross country borders and potentially disseminate novel resistance genes to new areas. In a Dutch modelling study, however, contact with wild birds was estimated to attribute to only 0.3% of community-acquired intestinal carriage of ESBL/AmpC-producing *E. coli* in humans (Mughini-Gras et al., 2019).

ExPEC and APEC associated serum survival gene *iss* (Tivendale et al., 2004; Johnson et al., 2008) was found from all the isolates in Study II. Furthermore, another APEC associated gene, the glutamate decarboxylase gene, *gad* (Azam et al., 2020), was frequent in the isolates. These findings demonstrate the potential risk of wildlife-derived *E. coli* to colibacillosis infections to other avian species as well. Based on the findings in Study II, barnacle geese carry epidemic plasmids harboring *bla* genes in Finland but the occurrence is limited.

6.3 ESBL/AmpC-producing *E. coli* and *K. pneumoniae* in global food products (III)

The food isolates sequenced in Study III demonstrated multidrug resistance, and resistance to critically important antimicrobials (WHO, 2019). Multiple different STs were identified, including STs previously described in human infections, such as ST37 *K. pneumoniae* in China (Zhu et al., 2015; Zhang et al., 2016; Xiao et al., 2017), ST307 *K. pneumoniae* in Italy, Colombia, and the UK (Villa et al., 2017), and ST38 *E. coli* in Bangladesh and the UK (Hasan et al., 2015; Day et al., 2019a). All of the tested imported raw broiler meat samples harbored ESBL/AmpC-producing *E. coli*, which supports the observation that broiler meat has been recognized as a major reservoir for ESBL/AmpC-producing *E. coli* (EFSA, 2011). Despite this observation, a decreasing trend has been noted in EU surveillance, as in 2020 31.5% (EFSA and ECDC, 2022) of studied broiler meat samples were positive for presumptive ESBL/AmpC-producing *E. coli* in specific monitoring when in 2018 the proportion was 39.8% (EFSA and ECDC, 2021).

Although the sample size was limited, ESBL/AmpC-producing *Enterobacteriaceae* were not recovered from any fruit or seafood products in Study III. Previous studies have obtained differing results, however, such as in Spain where 10.6% of sampled raw fish products and 19.4% of sushi were

found to harbor ESBL-producing *Enterobacteriaceae* (Vitas et al., 2018). In Vietnam, 62.5% of local fish products were positive for ESBL-producing *E. coli* (Le et al., 2015). These differences might stem from the limited sample size in Study III, and the fact that many imported seafood products are precooked. Cooked meat products were free of ESBL/AmpC-producing *Enterobacteriaceae* in Study III, which is similar to a finding in a study conducted in Spain (Vitas et al., 2018), but differs from a Chinese study, which found 6.7% of cooked meat products were positive for ESBL-producing *E. coli* (Jiang et al., 2014). Finding of enterobacteria after heating procedures in the food production chain is an indication of poor hygiene practices or potential cross contamination.

A very limited number of vegetable samples in Study III were positive for ESBL/AmpC-producing *Enterobacteriaceae*, which follows the findings of a UK based study (Day et al., 2019a), whereas Zurfluh et al. found 25.4% of imported vegetable products to contain ESBL-producing *Enterobacteriaceae* in Switzerland (Zurfluh et al., 2015a). In this study the food products originated mostly from Asia, whereas samples in Study III were obtained from nearly all continents. A German study, however, found only a very limited amount (1 out of 399 vegetable samples) to carry cefotaxime-resistant *E. coli* (Kaesbohrer et al., 2019).

Resistance genes often associated with human infections or colonization were identified in Study III: $bla_{CTX-M-15}$, bla_{SHV-12}, and bla_{OXA-1} (Cantón et al., 2008; Livermore et al., 2019). Finding these genes from *E. coli* and *K. pneumoniae* from food products indicates potential human-derived contamination and underlines the importance of good hygiene especially in food-producing plants and along the food chain. Furthermore, a population-based modelling study in the Netherlands estimated food to account for 18.9% of carriage of ESBL/AmpC-producing *E. coli* in the community (Mughini-Gras et al., 2019), but food products are considered to be unlikely to contribute to invasive infections in humans (Day et al., 2019a). The results in Study III highlight the importance of good kitchen hygiene practices while handling raw poultry meat and also demonstrate the potential of vegetables to be contaminated with ESBL-producing enterobacteria.

6.4 Comparison of ESBL-producing *E. coli* isolates from Finnish patients and non-human sources (IV)

Genomic comparison with a cgMLST-based method found 30 ESBL-producing *E. coli* isolates from human clinical samples in Study IV to be genetically distinct from non-human sources isolated in Finland previously. Overall, the results of Study IV support the findings of recent modelling-based studies, which have concluded that humans are the main source for ESBL-producing *E. coli* human colonization and infection (Day et al., 2019; Mughini-Gras et al., 2019). Furthermore, a Swedish study found overlap in resistance genes and plasmids in ESBL/AmpC-producing *E. coli* isolates from humans, animals, and the environment, although no evidence for clonal transmission between these sources was evidenced (Börjesson et al., 2016).

Interestingly, most of the human clinical isolates in Study IV were found to represent ST131 and harbored *bla*$_{CTX-M-27}$, belonging to a recently discovered C1-M27 subclade. Strains belonging to this subclade were first reported in Japan but have recently been identified in increasing numbers around Europe, such as in fecal samples from children in France (Birgy et al., 2017), human clinical isolates in Germany (Ghosh et al., 2017), and hospitalized patients in Berlin, Geneva, Madrid, and Utrecht (Merino et al., 2018). Furthermore, *E. coli* strains belonging to this subclade were observed from oyster and mussel samples collected at near-shore-sites in Brazil, suggesting a possible introduction of this epidemic clone via maritime traffic, or wastewater effluents from animal or human sources, such as hospitals (Fernandes et al., 2020). It is noteworthy that ST131-*bla*$_{CTX-M-27}$-*E. coli* has been observed to transfer at higher rates compared to ST131-*bla*$_{CTX-M-15}$-*E. coli* in an Israeli hospital setting (Adler et al., 2012). The findings in Study IV support the recent finding of CTX-M-27 arising as a new dominant enzyme type in human clinical samples beside CXT-M-15. Furthermore, the results support previous findings that the majority of subclade C1-M27 strains only possess one of the prophage-like regions, M27PP1, without M27PP2 (Matsumura et al., 2016; Decano and Downing, 2019).

Two of the human isolates with sequence types ST1193 and ST59 in Study IV possessed *bla*$_{CTX-M-55}$, which was also identified from ST155 *E. coli* from one food sample in Study III (coriander from Malaysia), as well as from ST58 *E. coli* from a healthy human fecal sample in Finland previously (Gröndahl-Yli-Hannuksela et al., 2020). ST1193 is a dominant ST among UPEC isolates in female patients in China (Zeng et al., 2021) and due to chromosomal quinolone resistance genes, such as *gyrA*, *parC* and *parE* is frequently detected in fluoroquinolone-resistant *E. coli* isolates from urinary tract infections in the United States (Tchesnokova et al., 2019). ST1193 *E. coli* in Study IV was obtained from an eye conjunctive sample from a human patient, and harbored chromosomal quinolone resistance genes (*gyrA*, *marR*, *parC*, *parE*) in addition to *bla*$_{CTX-M-55}$. ST1193, along with ST131, is considered to have human-origin if found in animals, because of their less frequent occurrence in animal sources (Kidsley et al., 2020). Although *bla*$_{CTX-M-27}$ was not observed in Study IV from non-human samples, ST131 *E. coli* strains belonging to C1-M27 subclade have been found from pig isolates in the UK and companion animals in France previously (Melo et al., 2019; Duggett et al., 2021).

MDR was frequent in the human clinical samples in Study IV. Trimethoprim resistance genes *dfrA17* and *dfrA12* were frequent, and these genes have been associated with UPEC isolates previously (Lee et al., 2001). Other frequently observed resistance genes in the MDR human clinical isolates included those conferring resistance to tetracycline, aminoglycoside, sulfonamide as well as chromosomal resistance genes conferring resistance to quinolones. These findings indicate ESBL-producing *E. coli* strains causing infections often carry multiple resistance genes, thus complicating antimicrobial treatment.

6.5 Role of plasmids in dissemination of *bla* genes (I-IV)

Although long-read sequencing was not employed in Study I, plasmid replicons were detected from short-read sequence data. ESBL/AmpC resistance genes have been suggested to disseminate in the poultry production chain via plasmids (Dame-Korevaar et al., 2017). IncX1, previously linked to $bla_{\mathrm{SHV-12}}$ and $bla_{\mathrm{TEM-52}}$ in broilers (Huijbers et al., 2014), was detected in ST2040 *E. coli* isolates in Study I. ST429 *E. coli* isolates were found to carry plasmid replicons belonging to the I-complex (IncB/O/K/Z) and IncF type (IncFIB and IncFIC). The broiler-associated $bla_{\mathrm{CMY-2}}$ has been identified from IncB/O/K/Z and IncFIA/FIB replicons in *E. coli* in poultry previously (Touzain et al., 2018). An *E. coli* strain harboring IncF and IncI1 type replicons and resistance genes *tet*(A) and $bla_{\mathrm{TEM-1C}}$ was recovered from one hatchling sample, demonstrating that *E. coli* strains along with plasmids can in some instances penetrate the broiler egg and developing embryo.

Study II identified *bla* genes on four different plasmid replicon types in *E. coli* from barnacle geese. The plasmids identified were IncI1 and IncK, the rare IncY, and the multireplicon IncN+IncR. IncI1 plasmids were found to harbor $bla_{\mathrm{CMY-2}}$ and $bla_{\mathrm{CTX-M-1}}$, which is a common finding among this plasmid type in poultry sources (Leverstein-van Hall et al., 2011; Accogli et al., 2013) and have also been found from other wild avian species in Florida (Poirel et al., 2012). In Study II $bla_{\mathrm{CMY-2}}$ was also identified from IncK type plasmid. This combination has previously been found mainly in different animal sources in Europe (Rozwandowicz et al., 2018).

In Study III, IncI1-Iγ plasmid was recovered from four broiler meat isolates, all harboring a different bla gene, thus demonstrating the diversity and versatility of plasmids recovered even from samples originating from the same batch. Comparison to plasmids from public databases demonstrated the highest similarity with plasmids obtained from livestock samples, but similarities to plasmids from companion animal sources were observed as well. IncI1-Iγ with $bla_{\mathrm{CMY-2}}$ was highly similar to a plasmid from a canine urinary tract infection sample in the UK (GenBank accession: CP023376.1), and another IncI1-Iγ with $bla_{\mathrm{CTX-M-1}}$ was similar to a $bla_{\mathrm{CTX-M-1}}$-harboring plasmid from an *E. coli* from a dog in Switzerland (GenBank accession: MG948334.1). These findings indicate a potential for plasmids to disseminate between food products, companion animals, and potentially their owners.

IncF type plasmids were recovered from two vegetable samples in Study III. Plasmid pZPK-A41.2-1 harbored *papC*, the P fimbriae encoding gene associated with UPEC (Yazdanpour et al., 2020). Plasmid pZPK-A40.2-1 was found to be similar to a human urine sample from the United States, which harbored the colistin resistance gene *mcr-1* (GenBank accession: KX276657.1). These findings indicate the potential of IncF plasmids acquiring new resistance genes and the potential to colonize the human urinary tract. The vegetable samples were of Asian origin, and the plasmids from the samples were found to harbor $bla_{\mathrm{CTX-M-55}}$ and $bla_{\mathrm{CTX-M-65}}$, both frequent *bla* genes identified from poultry sources in Asia (Park et al., 2019). IncF type

plasmids harboring *bla* genes are frequently identified in isolates from both humans and animals, especially in Asia (Rozwandowicz et al., 2018). In Study III, one sequenced broiler meat sample was positive for an IncX1 type plasmid, which was found to be highly similar to plasmid originating from un urinary catheter sample in Japan (GenBank accession: AP023429.1). Furthermore, the plasmid carried plasmid-mediated fluoroquinolone resistance gene *qnrS1*, which has been observed earlier in IncX plasmids (Dobiasova and Dolejska, 2016).

The anthropogenetic source of *bla*-harboring plasmids in wildlife can be speculated, especially regarding the finding of IncY harboring the human-associated $bla_{CTX\text{-}M\text{-}15}$ in *E. coli* from barnacle geese in Study II. This plasmid type has previously been detected in the environment and fish in Tanzania (Moremi et al., 2016), wastewater treatment plant in China (Jiang et al., 2019), and patient samples (Yasir et al., 2020). In Study II a very rare multireplicon IncN+IncR was observed, demonstrating that wild animals may act as melting pots for rare and new plasmid types to generate and transfer globally. Comparative analysis of the plasmids found in Study II to previously published plasmids showed high similarities to plasmids from other avian-derived samples for IncI1 and IncK. $bla_{CMY\text{-}2}$-harboring IncI1 plasmids were highly similar to an IncI1 plasmid from a human urinary tract sample in France (GenBank accession: MG844436.1), a clinical sample from Australia (GenBank accession: HG970648.1), and an Australian silver gull (GenBank accession: MT468651.1). The $bla_{CTX\text{-}M\text{-}1}$-harboring IncI1 plasmid in Study II was highly similar to a plasmid from a poultry source in France (GenBank accession: MG648932.1) and to a plasmid from a healthy human's sample from Switzerland (GenBank accession: KJ484629.1). These similarities indicate that even without the clonal spread of bacterial pathogens, plasmids may be able to disseminate between different sources.

Most human clinical isolates sequenced in Study IV were found to harbor plasmids belonging to the IncF family, which have been fundamental in the global spread of $bla_{CTX\text{-}M}$ genes (Bevan et al., 2017). Although only short-read sequencing was performed on these isolates, the ingredients for a typical IncF multireplicon were detected. Typing with in silico RST identified [F1:A2:B20] from 10 ST131 *E. coli* with either $bla_{CTX\text{-}M\text{-}27}$ (n=9) or $bla_{CTX\text{-}M\text{-}15}$ (n=1) and from one ST38 with $bla_{CTX\text{-}M\text{-}27}$, which is an identical plasmid-resistance gene combination identified from human clinical isolates in the United States (Mostafa et al., 2020). The results in Study IV indicate that multireplicon [F1:A2:B20] is associated with *E. coli* strains belonging to C1-M27 subclade. IncI1 type plasmid replicons were also frequent in the human clinical isolates in Study IV, and harbored a variety of ESBL genes (bla_{SHV-12}, $bla_{CTX-M-15}$, bla_{TEM-52}, $bla_{CTX-M-3}$, and $bla_{CTX-M-27}$) but $bla_{CTX-M-1}$, the gene often associated with food-producing animals such as poultry (Bevan et al., 2017; Rozwandowicz et al., 2018), was not identified from the human clinical isolates. The sample size in Study IV, however, was limited. In a Dutch study, IncI1 plasmid harboring $bla_{CTX\text{-}M\text{-}1}$ has been identified from humans as well as food-producing animals, suggesting potential transmission via the food chain (de Been et al., 2014). Further long-read sequencing for human isolates in Study IV and subsequent genomic comparisons would be required for an in-

depth comparison of plasmids identified in Studies I – IV. The cgMLST-based comparison of *E. coli* isolates obtained in Studies I–IV did not indicate close similarities between the bacterial strains from human and non-human sources, but plasmids belonging to the same families were identified, and this should be further investigated.

6.6 Future aspects

WGS-based methods have opened a door to a new era of comparative genomics where the complicated relationships between microorganisms, mobile genetic elements and hosts of human, animal, and environmental origin can be inspected in an in-depth manner. The epidemiological studies and source-tracking of AMR determinants are able now to consider the complex nature of all the parts involved in the dissemination of AMR.

However, challenges remain, including technical sequencing-related issues, subsequent sequence data analysis, and the fact that by cultivation methods only a fraction of the whole picture of the complex microbial world can be inspected, in a cross-cut manner. Detection of new or modified AMR determinants might be limited given that databases do not offer an impeccable source of comparable information.

Metagenomics offers a culture-independent method of studying genetic material and has been utilized already in global AMR surveillance via wastewater (Hendriksen et al., 2019). As the cost-effectiveness and the ability to detect MGEs and link these to bacterial hosts and resistance genes improves, metagenomics will provide a powerful tool to gain more insight into complex interactions behind AMR dissemination between humans, animals and the environment. The combined effort of different sectors in a One Health manner is required for the efficient study, surveillance, and prevention of the ever-evolving global health pandemic of AMR.

7 CONCLUSIONS

1. ESBL/AmpC-producing *E. coli* are not transmitted strictly by a vertical route in the broiler production pyramid, but rather a combination of vertical and horizontal transmission routes. Even in the absence of antibiotic washes, egg surfaces are free from *E. coli* after incubation period at the hatchery (I).

2. ESBL/AmpC-producing *E. coli* was detected in 4.5% of fecal samples obtained from barnacle geese. While the percentage is relatively low, migratory birds often feed and defecate near human housing, recreational parks, and waters, contributing to the load of AMR in the environment (II).

3. Wild migratory birds carry epidemic plasmids as well as rarely detected plasmids with genes encoding for frequently detected extended-spectrum beta-lactamases, highlighting the role of wildlife as vehicles for plasmid and AMR emergence and dissemination (II).

4. Imported raw broiler meat may act as a reservoir for ESBL/AmpC-producing *E. coli* and *K. pneumoniae*, with the potential for harboring multidrug resistance and plasmids identified as globally successful (III).

5. Sporadic findings of ESBL-producing *E. coli* and *K. pneumoniae* from vegetable samples highlights the importance of hygiene measures in food production and preparation, as well as demonstrates the potential cross-contamination risk of enterobacteria in unhygienic conditions (III).

6. ST131 *E. coli* harboring $bla_{\text{CTX-M-27}}$ was dominant in ESBL-producing *E. coli* isolates from human clinical samples in Finland. Isolates belonged to C1-M27 subclade, supporting the recent findings of the spread of *E. coli* clones belonging to this clade and the increasing prevalence of CTX-M-27 (IV).

7. ESBL-producing *E. coli* isolates from human clinical samples in Finland were distinct from ESBL/AmpC-producing *E. coli* isolates of non-human sources in Finland based on core genome multilocus sequence analysis. The results support whole genome sequencing-based methods as effective for detecting and surveilling of AMR trends (IV).

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APPENDIX I

Sampled products in Study III including information on country of origin, store of purchase and whether the product was fresh or frozen, and whether it originated from a same batch number with another sampled product (samples from the same batch are colored with the same color).

A = vegetable

Sample ID	Product	Fresh/frozen	Country of origin	Store of purchase
A1	Broccoli	Fresh	Spain	Store A
A2	Napolitana salad mix (rucola, mizuna, spinach)	Fresh	Italy	Store B
A3	Sugar pea	Fresh	Kenya	Store B
A4	Champignon	Fresh	Lithuania	Store B
A5	Snack carrot, frozen	Frozen	Belgium	Store B
A6	Spinach	Frozen	France	Store B
A7	Stir fry vegetable mix (carrot, zucchini, red pepper, asparagus bean, green onion)	Fresh	Unknown	Store B
A8	Stir fry vegetable mix (cabbage, carrot, red onion, sugar pea)	Fresh	Unknown	Store B
A9	Stir fry vegetables (red pepper, carrot, red onion, pak choi, sugar pea)	Fresh	Unknown	Store B
A10	Stir fry vegetable mix (champignon, pak choi, carrot, red onion, fennel)	Fresh	Unknown	Store B
A11	Stir fry vegetable mix (carrot, red cabbage, Chinese cabbage, kale)	Fresh	Unknown	Store B
A12	Stir fry vegetable mix (carrot, cabbage, red pepper, asparagus bean)	Fresh	Unknown	Store B
A13	Vegetable mix (carrot, zucchini, red pepper)	Fresh	Unknown	Store B
A14	Ginger (organic)	Fresh	China	Store B
A15	Asparagus	Fresh	Peru	Store B
A16	Lemon grass	Fresh	Thailand	Store B
A17	Green beans (haricot vert)	Fresh	Kenya	Store B
A18	Garlic	Fresh	China	Store B
A19	Sweet onion	Fresh	Peru	Store B
A20	Sweet potato	Fresh	Egypt	Store B
A21	Zucchini	Fresh	Spain	Store B
A22	Ginger	Fresh	China	Store B
A23	Dill (organic)	Frozen	Germany	Store C
A24	Brussels sprouts	Frozen	France	Store C
A25	Baby carrots	Frozen	Belgium	Store C
A26	Corn	Frozen	Belgium	Store C
A27	Champignon	Fresh	The Netherlands	Store C
A28	Rucola (organic)	Fresh	Italy	Store C
A29	Chili pepper mix	Fresh	Spain	Store C
A30	Plum tomato	Fresh	Spain	Store C
A31	Broccoli (organic)	Fresh	Italy	Store C
A32	Celery	Fresh	Germany	Store C
A33	Guichai flower	Fresh	Thailand	Store D
A34	Young okra	Fresh	Thailand	Store D
A35	Red chili pepper	Fresh	Malaysia	Store D

A36	Lemongrass	Fresh	Thailand	Store D
A37	Lemongrass	Fresh	Thailand	Store D
A38	Lemongrass	Fresh	Thailand	Store D
A39	Turkish paprika	Fresh	Turkey	Store D
A40	Coriander	Fresh	Malaysia	Store D
A41	Coriander	Fresh	Malaysia	Store D
A42	Spinach	Fresh	Unknown	Store D
A43	Ronfun natural fungus	Fresh	Unknown	Store D
A44	Ronfun natural fungus	Fresh	Unknown	Store D
A45	Sweet basil	Fresh	Laos	Store E
A46	Sweet basil	Fresh	Laos	Store E
A47	Thai parsley	Fresh	Laos	Store E
A48	Thai parsley	Fresh	Laos	Store E
A49	Holy basil	Fresh	Laos	Store E
A50	Holy basil	Fresh	Laos	Store E
A51	Stir fry vegetable mix (carrot, cabbage, red pepper, asparagus bean)	Fresh	Unknown	Store B
A52	Stir fry vegetable mix (carrot, cabbage, red pepper, asparagus bean)	Fresh	Unknown	Store B
A53	Stir fry vegetable mix (carrot, zucchini, red pepper, asparagus bean, green onion)	Fresh	Unknown	Store B
A54	Stir fry vegetable mix (carrot, zucchini, red pepper, asparagus bean, green onion)	Fresh	Unknown	Store B
A55	Stir fry vegetable mix (cabbage, carrot, red onion, sugar pea)	Fresh	Unknown	Store B
A56	Stir fry vegetable mix (cabbage, carrot, red onion, sugar pea)	Fresh	Unknown	Store B
A57	Stir fry vegetable mix (carrot, red cabbage, Chinese cabbage, kale)	Fresh	Unknown	Store B
A58	Stir fry vegetable mix (carrot, red cabbage, Chinese cabbage, kale)	Fresh	Unknown	Store B
A59	Stir fry vegetables (red pepper, carrot, red onion, pak choi, sugar pea)	Fresh	Unknown	Store B
A60	Stir fry vegetables (red pepper, carrot, red onion, pak choi, sugar pea)	Fresh	Unknown	Store B

B =fruit

Sample ID	Product	Fresh/frozen	Country of origin	Store of purchase
B1	Grape	Fresh	Brazil	Store A
B2	Cantaloupe	Fresh	Brazil	Store B
B3	Pineapple (smoothie mix)	Frozen	Costa Rica	Store B
B4	Mango (smoothie mix)	Frozen	India	Store B
B5	Raspberry	Fresh	Morocco	Store B
B6	Highbush blueberry	Fresh	South Africa	Store B
B7	Watermelon cut in pieces	Fresh	Costa Rica	Store B
B8	Pineapple cut in pieces	Fresh	Unknown	Store B
B9	Cape gooseberry	Fresh	Colombia	Store B
B10	Snack fruit mix (pineapple, European and North American cantaloupe)	Fresh	Unknown	Store B
B11	Snack fruit mix (grape, apple)	Fresh	Unknown	Store B
B12	Snack fruit mix (pineapple, European and North American cantaloupe, dark grape)	Fresh	Unknown	Store B
B13	Snack fruit mix (pineapple, cantaloupe, grape)	Fresh	Unknown	Store B
B14	Watermelon, cut	Fresh	Brazil	Store B
B15	Honeydew melon, cut	Fresh	Brazil	Store B

B16	Plum (organic)	Fresh	Spain	Store B
B17	Lime	Fresh	Mexico	Store B
B18	Date	Fresh	Iran	Store B
B19	Carambola	Fresh	Malaysia	Store F
B20	Dragon fruit	Fresh	Vietnam	Store F
B21	Grape	Fresh	Brazil	Store F
B22	Kiwi	Fresh	New Zealand	Store F
B23	Grapefruit	Fresh	Israel	Store F
B24	Raspberry	Frozen	Germany	Store C
B25	Strawberry	Frozen	Germany	Store C
B26	Grape	Fresh	Spain	Store G
B27	Grape	Fresh	Brazil	Store G
B28	Pineapple	Fresh	Costa Rica	Store C
B29	Grape	Fresh	Brazil	Store H
B30	Grape	Fresh	Brazil	Store H
B31	Grape	Fresh	Brazil	Store H
B32	Grape	Fresh	Peru	Store H
B33	Grape	Fresh	Peru	Store H
B34	Grape	Fresh	Peru	Store H
B35	Grape	Fresh	Spain	Store H
B36	Grape	Fresh	Brazil	Store H
B37	Grape	Fresh	Brazil	Store H
B38	Grape	Fresh	Brazil	Store H
B39	Raspberry	Fresh	Portugal	Store H
B40	Raspberry	Fresh	Portugal	Store H
B41	Raspberry	Fresh	Portugal	Store H
B42	Raspberry	Fresh	Portugal	Store H
B43	Highbush blueberry (organic)	Fresh	Chile	Store H
B44	Highbush blueberry (organic)	Fresh	Chile	Store H
B45	Highbush blueberry (organic)	Fresh	South Africa	Store H
B46	Highbush blueberry (organic)	Fresh	South Africa	Store H
B47	Highbush blueberry (organic)	Fresh	South Africa	Store H
B48	Highbush blueberry (organic)	Fresh	South Africa	Store H
B49	Blackberry	Fresh	Mexico	Store H
B50	Blackberry	Fresh	Mexico	Store H

C = meat

Sample ID	Product	Fresh/frozen	Country of origin	Store of purchase
C1	Dry-cured ham (Jamón serrano)	Dry-cured	Spain	Store A
C2	Turkey cold cuts	Precooked	Brazil	Store B
C3	Chicken cold cuts	Precooked	Brazil	Store B
C4	Pork sirloin steak	Raw	Germany	Store B
C5	Chorizo cuts	Dry-cured	Spain	Store B
C6	Bacon	Raw	Poland	Store B
C7	Game meat	Raw, frozen	New Zealand	Store F

C8	Chicken , whole, skinned	Raw, frozen	France	Store F
C9	Lamb	Raw, frozen	New Zealand	Store F
C10	Turkey	Smoked	Brazil	Store F
C11	Black Forest ham (Schwarzwälder Schinken)	Dry-cured	European Union	Store F
C12	Dry-cured ham (Jamón serrano, Jamón ibérico)	Dry-cured	Spain	Store F
C13	Dry-cured ham	Dry-cured	Unknown	Store F
C14	Duck, half	Precooked, frozen	Hungary	Store C
C15	Beef, pieces	Raw	Germany	Store C
C16	British bacon	Raw	The United Kingdom	Store C
C17	Black Forest ham (Schwarzwälder Schinken)	Dry-cured	Germany	Store G
C18	Dry-cured ham (Jamón serrano)	Dry-cured	Spain	Store G
C19	Dry-cured ham	Dry-cured	Italy	Store G
C20	Turkey cold cuts	Precooked	Brazil	Store G
C21	Chicken cold cuts	Precooked	Brazil	Store G
C22	Turkey cold cuts	Precooked	Brazil	Store G
C23	Chicken cold cuts	Precooked	Brazil	Store G
C24	Pork tenderloin	Raw	Denmark	Store G
C25	Bacon	Raw	Belgium	Store G
C26	Quails	Raw, frozen	Italy	Store E
C27	Duck legs	Raw, frozen	Hungary	Store E
C28	Pork tenderloin	Raw	Denmark	Store H
C29	Pork tenderloin	Raw	Denmark	Store H
C30	Pork tenderloin	Raw	Denmark	Store H
C31	Turkey ligament	Raw, frozen	Poland	Store E
C32	Turkey ligament	Raw, frozen	Poland	Store E
C33	Turkey ligament	Raw, frozen	Poland	Store E
C34	Duck legs	Raw, frozen	The Netherlands	Store E
C35	Duck legs	Raw, frozen	The Netherlands	Store E
C36	Turkey meat, precut	Raw	Germany	Store B
C37	Turkey meat, precut	Raw	Germany	Store B
C38	Turkey meat, precut	Raw	Germany	Store B
C39	Turkey meat, precut	Raw	Germany	Store B
C40	Turkey meat, precut	Raw	Germany	Store B
C41	Turkey meat, precut	Raw	Germany	Store B
C42	Turkey meat, precut	Raw	Germany	Store B
C43	Turkey meat, precut	Raw	Germany	Store B
C44	Turkey meat, precut	Raw	Germany	Store B
C45	Turkey meat, precut	Raw	Germany	Store B
C46	Turkey meat, precut	Raw	Germany	Store B
C47	Turkey meat, precut	Raw	Germany	Store B
C48	Turkey meat, precut	Raw	Germany	Store B
C49	Pork sirloin steak	Raw	Germany	Store B
C50	Pork sirloin steak	Raw	Germany	Store B
C51	Broiler meat (chicken wings)	Raw	Lithuania	Store I

C52 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C53 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C54 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C55 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C56	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C57 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C58 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C59 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C60 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C61	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C62 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C63 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C64 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C65 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C66	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C67 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C68 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C69 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C70 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C71	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C72 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C73 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C74 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C75 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C76	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C77 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C78 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C79 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C80 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C81	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C82 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C83 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C84 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C85 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C86	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C87 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C88 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C89 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I

C90 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C91	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C92 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C93 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C94 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C95 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C96	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C97 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I
C98 subsample	Broiler meat (chicken wings)	Raw	Lithuania	Store I

D = seafood

Sample ID	Product	Fresh/frozen	Country of origin	Store of purchase
D1	Shrimp (*Pandalus borealis*)	Frozen	Norway	Store A
D2	Squid rings	Frozen	Spain	Store B
D3	Blue mussel	Cooked, frozen	Unknown	Store B
D4	Scampi	Cooked, frozen	Vietnam	Store B
D5	Pangasius	Frozen	Vietnam	Store B
D6	Coalfish	Frozen	Pacific Ocean	Store B
D7	Tuna steak	Frozen	Pacific Ocean	Store F
D8	Grooved carpet shell (*Ruditapes decussatus*)	Frozen	Italy	Store F
D9	King prawn (*Melicertus latisulcatus)*	Frozen	Indian Ocean	Store F
D10	Rainbow trout caviar	Fresh	France	Store F
D11	Whiteleg shrimp (*Litopenaeus vannamei*)	Cooked	Vietnam	Store F
D12	Shrimp (*Pandalus borealis / Pandalus jordani)*	Cooked	Pacific Ocean	Store F
D13	Surimi sticks	Cooked	Unknown	Store F
D14	Surimi pieces	Cooked	Unknown	Store F
D15	King prawn rings	Cooked, frozen	Vietnam	Store C
D16	Codfish fillet	Fresh	Norway	Store C
D17	Cold smoked salmon (*Salmo salar*) cuts	Cold smoked	Norway	Store G
D18	Pink salmon (*Oncorhynchus gorbuscha*) fillet	Frozen	Pacific Ocean	Store G
D19	Pink salmon (*Oncorhynchus gorbuscha*) fillet	Frozen	Pacific Ocean	Store G
D20	Pike perch (*Stizostedion lucioperca*) fillet	Frozen	Estonia	Store G
D21	Surimi sticks	Frozen	Unknown	Store G
D22	Small cuttlefish	Frozen	Pacific Ocean	Store E
D23	Red tailed tinfoil barb	Frozen	Vietnam	Store E
D24	Wahoo steak	Frozen	Pacific Ocean	Store E
D25	Japanese scad	Frozen	Pacific Ocean	Store E
D26	Bullet tuna (*Auxis rochei*)	Frozen	Pacific Ocean	Store E
D27	Surimi sticks	Frozen	Unknown	Store B
D28	Pangasius	Frozen	Vietnam	Store B
D29	Pangasius	Frozen	Vietnam	Store B
D30	Scallops	Frozen	Canada	Store B

APPENDIX II

Country/region of origin for samples per food category in Study III.

Country of origin	Number of samples (total)	Sample category			
		Vegetables	Fruit and berries	Meat	Seafood
Belgium	4	3		1	
Brazil	19		12	7	
Canada	1				1
Chile	2		2		
China	3	3			
Colombia	1		1		
Costa Rica	3		3		
Denmark	4			4	
Egypt	1	1			
Estonia	1				1
European Union	1			1	
France	4	2		1	1
Germany	22	2	2	18	
Hungary	2			2	
India	1		1		
Indian Ocean	1				1
Iran	1		1		
Israel	1		1		
Italy	6	3		2	1
Kenya	2	2			
Laos	6	6			
Lithuania	11	1		10	
Malaysia	4	3	1		
Morocco	1		1		
Mexico	3		3		
New Zealand	3		1	2	
Norway	3				3
Pacific Ocean	9				9
Peru	5	2	3		
Poland	4			4	
Portugal	4		4		
South Africa	5		5		
Spain	12	4	3	4	1
Thailand	6	6			

The Netherlands	3	1		2	
Turkey	1	1			
UK	1			1	
Vietnam	8		1		7
Unknown	31	20	5	1	5
Total:	200	60	50	60	30

APPENDIX III

Previously sequenced ESBL/AmpC-producing *Escherichia coli* isolates collected in Finland in this thesis or deposited to public databases included for genomic comparison in Study IV.

Isolate	**Source**	**MLST**	***bla* gene(s)**	**Isolation year**	**Reference**
CH20	Wastewater	ST1674**	bla_{CMY-2}	2012	Unpublished
192A	Cattle	ST1727	$bla_{CTX-M-1}$	2014	Päivärinta et al., 2016
EL24E	Veterinarian	ST131	$bla_{CTX-M-27}$	2016	Verkola et al., 2019
EL120E	Veterinarian	ST450	bla_{TEM-1}, $bla_{CTX-M-14}$	2016	
EL158E	Veterinarian	ST10	$bla_{CTX-M-1}$	2016	
EL216E	Veterinarian	ST131	$bla_{CTX-M-15}$	2016	
EL233E	Veterinarian	ST80	$bla_{CTX-M-14}$	2016	
EL245E	Veterinarian	ST963	$bla_{CTX-M-1}$	2016	
EL256E	Veterinarian	ST131	bla_{TEM-1}, $bla_{CTX-M-15}$	2016	
EL259E	Veterinarian	ST871	bla_{SHV-12}	2016	
EL298E	Veterinarian	ST648	$bla_{CTX-M-15}$	2016	
5	Broiler meat	ST373	bla_{CMY-2}	2015	Päivärinta et al., 2020
33	Broiler meat	ST373	bla_{CMY-2}	2015	
34	Broiler meat	ST117	bla_{CMY-2}	2015	
47	Broiler meat	ST101	bla_{CMY-2}	2015	
53	Broiler meat	ST351	$bla_{CTX-M-1}$	2015	
A12	Broiler caecum	ST1594	$bla_{CTX-M-1}$	2015	
C15	Broiler caecum	ST1594	bla_{CMY-2}	2015	
M11	Broiler caecum	ST101	bla_{CMY-2}	2015	
Q11	Broiler caecum	ST1594	$bla_{CTX-M-1}$	2015	
W20	Broiler caecum	ST212	bla_{TEM-1}, bla_{CMY-2}	2015	
PS57-R	Broiler production (Broiler parent caecum)	ST429	bla_{CMY-2}	2017	Stuy I
PS174-R	Broiler production (Broiler parent caecum)	ST2040	bla_{CMY-2}	2017	

PS310-R	Broiler production (Broiler parent caecum)	ST429	*bla*$_{CMY-2}$	2017	
PS371-R	Broiler production (Broiler parent caecum)	ST2040	*bla*$_{CMY-2}$	2017	
PS61-R	Broiler production (Broiler parent caecum)	ST2040	*bla*$_{CMY-2}$	2017	
PS148-R	Broiler production (Broiler parent caecum)	ST429	*bla*$_{CMY-2}$	2017	
PS184-R	Broiler production (Broiler parent caecum)	ST429	*bla*$_{CMY-2}$	2017	
PS378-R	Broiler production (Broiler parent caecum)	ST429	*bla*$_{CMY-2}$	2017	
2E177-R	Broiler production (Egg surface)	ST2040	*bla*$_{CMY-2}$	2017	
1E61-R	Broiler production (Egg surface)	ST2040	*bla*$_{CMY-2}$	2017	
1E74-R	Broiler production (Egg surface)	ST2040	*bla*$_{CMY-2}$	2017	
2E151-R	Broiler production (Egg surface)	ST429	*bla*$_{CMY-2}$	2017	
Y13-R	Broiler production (Hall environment)	ST429	*bla*$_{CMY-2}$	2017	
H5	Barnacle goose	ST359	*bla*$_{TEM-1}$, *bla*$_{CTX-M-1}$	2017	Study II
H11	Barnacle goose	ST58	*bla*$_{TEM-1}$, *bla*$_{CTX-M-1}$	2017	
H21	Barnacle goose	ST115	*bla*$_{TEM-32}$, *bla*$_{CMY-2}$	2017	
H29	Barnacle goose	ST453	*bla*$_{CTX-M-15}$	2017	
H58	Barnacle goose	ST1594	*bla*$_{CMY-2}$	2017	
H68B	Barnacle goose	ST3580	AmpC promoter mutation (-42 C>T)	2017	
H98	Barnacle goose	ST453	*bla*$_{TEM-1}$, *bla*$_{CTX-M-15}$	2017	
H163	Barnacle goose	ST2178	*bla*$_{CMY-2}$	2018	
H193	Barnacle goose	ST2178	*bla*$_{CMY-2}$	2018	
A40.2-1	Imported food (Coriander from Malaysia)	ST155	*bla*$_{TEM-1}$, *bla*$_{CTX-M-55}$	2018	Study III
A41.2-1	Imported food (Coriander from Malaysia)	ST12713	*bla*$_{CTX-M-65}$	2018	
C51-1	Imported food (Raw broiler from Lithuania)	ST189	*bla*$_{SHV-12}$	2018	
C56.1-1	Imported food (Raw broiler from Lithuania)	ST4994	*bla*$_{TEM-52}$	2018	
C61-1	Imported food (Raw broiler from Lithuania)	ST1011	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
C66-1	Imported food (Raw broiler from Lithuania)	ST423	*bla*$_{CARB-2}$, *bla*$_{SHV-12}$	2018	
C71.1-1	Imported food (Raw broiler from Lithuania)	ST1485	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
C76.1-1	Imported food (Raw broiler from Lithuania)	ST201	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
C76.1-2	Imported food (Raw broiler from Lithuania)	ST12712	*bla*$_{SHV-12}$	2018	
C81.1-1	Imported food (Raw broiler from Lithuania)	ST38	*bla*$_{CTX-M-1}$	2018	
C81.3-1	Imported food (Raw broiler from Lithuania)	ST1638	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
C81.1-2	Imported food (Raw broiler from Lithuania)	ST641	*bla*$_{CARB-2}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$	2018	
C86.1-1	Imported food (Raw broiler from Lithuania)	ST641	*bla*$_{CARB-2}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$	2018	
C86.1-2	Imported food (Raw broiler from Lithuania)	ST38	*bla*$_{CTX-M-1}$	2018	

C91-2	Imported food (Raw broiler from Lithuania)	ST117	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
C96-1	Imported food (Raw broiler from Lithuania)	ST88	*bla*$_{TEM-1}$, *bla*$_{CMY-2}$	2018	
SRR11638565*	Healthy adult	ST38	*bla*$_{CMY-2}$, *bla*$_{OXA-1}$,	2016	Gröndahl-Yli-Hannuksela et al., 2020
SRR11638568	Healthy adult	ST58	*bla*$_{CTX-M-55}$	2016	
SRR11638569	Healthy adult	ST131	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$	2016	
SRR11638570	Healthy adult	ST11038	*bla*$_{CTX-M-15}$	2016	
SRR11638571	Healthy adult	ST1312	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$	2016	
SRR11638572	Healthy adult	ST131	*bla*$_{CTX-M-15}$	2016	
SRR11638573	Healthy adult	ST10	*bla*$_{SHV-12}$	2016	
SRR11638574	Healthy adult	ST131	*bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$	2016	

bla genes determined in Ridom SeqSphere+ software with NCBI AMRFinder.
* Sequence Read Archive (SRA) Run accession
** = imperfect hit

APPENDIX IV

Plasmids from GenBank database included for comparison with plasmids obtained in Study II.

Plasmid	Inc group / pMLST	*bla* gene	Plasmid name (GenBank)	Query coverage (%)	Identity (%)	*bla* gene	Inc group / pMLST	Bacterial species / ST	Accession number	Country	Source	Year of isolation
pZPK-H5	IncI1/ ST38 CC-3	*bla*$_{CTX-M-1}$	pCOV15	96	99.79	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli*	MG648932.1	France	Healthy broiler caecal sample	2010 - 2012
			pH2291-112	96	99.90	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli* / ST1638	KJ484629.1	Switzerland	Healthy human	2013
			p22638	95	99.83	*bla*$_{CTX-M-1}$	IncI1-Iγ/ST3	*E. coli* / ST1638	MN419437.1	Norway	Poultry feces	2016
			pEC7	95	99.87	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli* / ST196	CP053679.1	Guadeloupe / France	Rat	2013
			pEC38	92	99.90	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli* / ST349	CP053677.1	Guadeloupe / France	Human blood sample (pyelonephritis)	2013
			p08-1118	94	99.90	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli*	MH847511.1	France	Pig digestive tract	2008
			pCOV12	94	99.90	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli*	MG648914.1	France	Healthy broiler caecal sample	2010 - 2012
			p2305	94	99.86	*bla*$_{CTX-M-1}$	IncI1 /ST3	*E. coli*	MG948334.1	Switzerland	Dog	2012 - 2016

			p14011252	92	99.91	*bla*$_{CTX-M-1}$	IncI1/ST3	*E. coli* / ST602	MK181561.1	Denmark	Chicken meat	2014
			pESBL26	92	99.91	*bla*$_{CTX-M-1}$	IncI1/ST3	*E. coli*	MT230257.1	France	River	2014
pZPK-H163, pZPK-H193	IncI1/ST23 CC-2	*bla*$_{CMY-2}$	pCMY-136	100	99.95	*bla*$_{CMY-136}$	IncI1	*E. coli*	MG844436.1	France	Human urinary tract sample	2002-2005
			pCE1628_I1	100	99.96	*bla*$_{CMY-2}$	IncI1/ST23	*E. coli* / ST457	MT468651.1	Australia	Australian silver gull	2012
			pJIE512b	100	98.75	*bla*$_{CMY-2}$	IncI1/ST2	*E. coli*	HG970648.1	Australia	Human clinical sample	N/A
			p87	97	99.99	*bla*$_{CMY-2}$	IncI1/ST23	*E. coli*	CP023385.1	Scotland	Dog urinary tract infection	2002
			p17437	93	98.44	*bla*$_{CTX-M-1}$	IncI1-Iγ/ST3	*E. coli* / ST57	MN419430.1	Norway	Poultry feces	2016
			pIFM3804	92	98.95	*bla*$_{CTX-M-1}$	IncI1/ST108	*E. coli*	KF787110.1	UK	Pig	2009
			p07-024	92	99.09	*bla*$_{CTX-M-1}$	IncI1/ST3	*E. coli*	MH847502.1	France	Pig digestive tract	2006
			pCOV30	92	99.10	*bla*$_{CTX-M-1}$	IncI1/ST3	*E. coli*	MG649043.1	France	Broiler (colibasillosis)	2010-2012
			pDE105	90	99.06	*bla*$_{TEM-1}$	IncI1	*Shigella sonnei*	MG569891.1	Vietnam	Human fecal sample	2000
			p15076331	92	98.51	*bla*$_{CTX-M-1}$	IncI1/ST3	*E. coli* / ST156	MK181558.1	Denmark	Cattle/pork meat	2015
pZPK-H21	IncK	*bla*$_{CMY-2}$, *bla*$_{TEM-32}$	p4809.66	94	99.85	*bla*$_{CMY-2}$	IncK2	*E. coli* / ST1431	KR905389.1	Switzerland	Human urinary tract infection	2011
			pDV45	94	99.81	*bla*$_{CMY-2}$	IncK2	*E. coli* / ST1564	KR905384.1	Switzerland	Poultry meat	2013
			p23C57-3	94	99.84	*bla*$_{CMY-2}$	IncB/O/K/Z	*E. coli* / ST648	LC501565.1	Japan	Broiler fecal sample	2011
			p5312.29	92	99.88	*bla*$_{CMY-2}$	IncK2	*E. coli* / ST131	KR905385.1	Switzerland	Human urinary tract infection	2014
			pMbl488	90	94.81	*bla*$_{TEM-1B}$ (also *mcr-1*)	IncK2	*E. coli* / ST38	KY565558.1	Switzerland	Broiler meat (imported from Germany)	2014
			pCOV9	88	99.87	*bla*$_{CMY-2}$	IncB/O/K/Z	*Escherichia coli*	MG648907.1	France	Healthy broiler caecal sample	2010-2012
			pNVI1292	88	99.86	*bla*$_{CMY-2}$	IncK2	*Escherichia coli*/ST38	KU312044.1	Norway	Broiler meat	2012
			pDV10	88	99.99	*bla*$_{CMY-4}$	IncK2	*Escherichia coli*/ST38	KR905390.1	Switzerland	Poultry meat	2013
			pMbl536	88	97.82	*bla*$_{TEM-1B}$ (also *mcr-1*)	IncK2	*Escherichia coli*/ST226	KY689635.1	Switzerland	Broiler meat (imported from Germany)	2014
			pTMSA970	86	99.86	*bla*$_{CMY-2}$	IncK2	*Escherichia coli*/ST420	KR905388.1	Switzerland	Poultry cloacae	2012
pZPK-H58	IncK	*bla*$_{CMY-2}$	p23C16-2	99	99.98	*bla*$_{CMY-2}$	IncB/O/K/Z	*Escherichia coli*/ST23	LC501559.1	Japan	Broiler fecal sample	2011
			p17C9-3	99	99.95	*bla*$_{CMY-2}$	IncB/O/K/Z	*Escherichia coli*/ST373	LC501529.1	Japan	Broiler fecal sample	2005

			p22C48-3	98	99.78	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST362	LC501547.1	Japan	Broiler fecal sample	2010
			p24C117-3	98	99.94	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST155	LC501577.1	Japan	Broiler fecal sample	2012
			p24C25-2	97	99.97	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST57	LC501570.1	Japan	Broiler fecal sample	2012
			p16C96-3	97	99.95	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST155	LC501526.1	Japan	Broiler fecal sample	2004
			p22C25-2	97	99.95	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST10	LC501544.1	Japan	Broiler fecal sample	2010
			p18C3-2	97	99.95	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST90	LC501531.1	Japan	Broiler fecal sample	2006
			p19C79-2	97	99.95	bla_{CMY-2}	IncB/O/K/Z	*Escherichia coli*/ST354	LC501535.1	Japan	Broiler fecal sample	2007
			pTMSA992	97	99.96	bla_{CMY-2}	IncK2	*Escherichia coli*/ST420	KR905387.1	Switzerland	Poultry cloacae	2012
pZPK-H29, pZPK-H98	IncY	$bla_{CTX-M-15}$, bla_{TEM-1B}	p2018K-0756	87	99.92	$bla_{CTX-M-15}$, bla_{TEM-1B}	IncY	*Salmonella* Typhi	CP044008.1	Pakistan	Human clinical sample	2018
			p60006	87	99.98	$bla_{CTX-M-15}$	IncY	*Salmonella* Typhi	LT906492.1	Pakistan	Human blood culture	2016-2017
			pR19.2839_83k	86	99.92	$bla_{CTX-M-15}$, bla_{TEM-1B}	IncY	*Salmonella* Typhi	CP046430.1	Taiwan	Human blood culture	2019
			pPGR46	71	99.98	$bla_{CTX-M-15}$, bla_{TEM-1B}	IncY	*Escherichia coli*	KM023153.1	Nigeria	Human fecal sample	2011
			pEco-CTX-M-15	70	99.90	$bla_{CTX-M-15}$, bla_{TEM-1B}	IncY	*Escherichia coli*	MF510423.1	France	Human bile sample (cholangitis)	2015
			pRC960-1	61	99.88	bla_{TEM-1B}	IncY	*Shigella flexneri*	KY848295.1	China	Pig fecal sample	2009
pZPK-H11	IncN+IncR/ST1(IncN)	$bla_{CTX-M-1}$	p100_NDM5_IncN	60	99.97	bla_{NDM-5}	IncF-IncN	*Escherichia coli*/ST167	MT199177.1	Italy	Human urine	2018
			pH1038-142	59	99.97	$bla_{CTX-M-1}$, bla_{TEM-1}	IncF-IncN/ST1	*Escherichia coli*	KJ484634.1	Switzerland	Healthy human	2013
			pC5_41608	54	99.96	$bla_{CTX-M-1}$	IncN	*Klebsiella pneumoniae*/ST2748	MF953243.1	USA	Cow	2016
			pHHA45	54	99.93	$bla_{CTX-M-1}$	IncN/ST1	*Escherichia coli*	JX065630.1	Denmark	Pig	2006
			pL2-43	54	99.96	$bla_{CTX-M-1}$	IncN/ST1	*Escherichia coli*/ST295	KJ484641.1	Switzerland	Lamb	2012
			pYUHAP5-2	54	99.95	bla_{TEM-1B}	IncN	*Salmonella enterica* serovar London	CP060136.1	China	Slaughtered pig	2016
			pVQS1	53	99.93	bla_{TEM-1} (also *qnrS1*)	IncN	*Salmonella enterica* serovar Wirchow	JQ609357.1	Switzerland	Human clinical sample	2005-2009
			pRSB203	47	99.89	bla_{TEM-1}	IncN	Uncultured bacteria	JN102342.1	Germany	Wastewater treatment plant effluent	N/A
			pABWA45_3	51	99.99	bla_{TEM-1B}	IncN	*Escherichia coli*/ST635	CP022157.1	Switzerland	Wastewater	2016

	pKC396	55	99.53	*bla*$_{CTX-M-65}$, *bla*$_{TEM-1}$	IncN	*Escherichia coli*/ST131	HM138653.1	Germany	Human clinical sample	2006
	pBK31551	61	99.70	*bla*$_{KPC-4}$, *bla*$_{TEM-1}$	IncN	*Klebsiella pneumoniae*/ST834	JX193301.1	USA	Human clinical sample (blood culture, bacteremia)	2005
	pKC394	56	99.45	*bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$	IncN	*Escherichia coli*/ST131	HM138652.1	Germany	Human clinical sample	2006
	pKT58A	54	99.77	(*qnrS1*)	IncN/ST3	*Escherichia coli*	JX065631.1	Slovakia	Wild water bird	2010
	pMUR050	53	99.60	(*armA*)	IncN	*Escherichia coli*	AY522431.4	Spain	Pig clinical isolate	2002
	p15ODMR	47	99.99	*bla*$_{TEM-1B}$	IncN	*Escherichia coli*/ST10	MG904997.1	Switzerland	Pig (diarrhea)	2014-2015
	pRSB206	48	99.99	*bla*$_{TEM-1}$	IncN	Uncultured bacteria	JN102344.1	Germany	Wastewater treatment plant effluent	N/A
	pRSB205	47	99.99	*bla*$_{TEM-1}$	IncN	Uncultured bacteria	JN102343.1	Germany	Wastewater treatment plant effluent	N/A
	plasmid IncN	48	99.78	*bla*$_{TEM-1A}$	IncN	*Klebsiella pneumoniae*/ST258	CP027050.1	Greece	Human clinical sample (stool)	2012-2014
	pRSB201	50	99.70	*bla*$_{TEM-1}$	IncN	Uncultured bacteria	JN102341.1	Germany	Wastewater treatment plant effluent	N/A
	pQNR2078	53	99.61	(*qnrB19*)	IncN	*Escherichia coli*	HE613857.1	Germany	Horse clinical sample (genital tract infection)	2005

APPENDIX V

Accession numbers for sequence data deposited to public database repositories in Studies II–IV.

STUDY II

European Nucleotide Archive (ENA) accession numbers
Study accession number: PRJEB42655 (Secondary accession: ERP126547)

Isolate	**Sample primary accession**	**Sample secondary accession**	**Illumina run accession**	**Oxford Nanopore run accession**	**Plasmid sequence accession**
H5	ERS5602973	SAMEA7856498	ERR5188293	ERR5190298	ERZ1738234
H11	ERS5602974	SAMEA7856499	ERR5188294	ERR5190299	ERZ1738235
H21	ERS5602975	SAMEA7856500	ERR5188295	ERR5190300	ERZ1738236
H29	ERS5602976	SAMEA7856501	ERR5188296	ERR5190301	ERZ1738237
H58	ERS5602977	SAMEA7856502	ERR5188297	ERR5190302	ERZ1738238
H68B	ERS5602978	SAMEA7856503	ERR5188298	ERR5208198	No ESBL-plasmid
H98	ERS5602979	SAMEA7856504	ERR5188299	ERR5190303	ERZ1738239
H163	ERS5602980	SAMEA7856505	ERR5188300	ERR5190304	ERZ1738240
H193	ERS5602981	SAMEA7856506	ERR5188301	ERR5190305	ERZ1738241

STUDY III

European Nucleotide Archive (ENA) accession numbers
Study accession number: PRJEB37779 (secondary accession: ERP121109)

Isolate	Sample primary accession	Sample secondary accession	Illumina run accession	MinION run accession
A35-1	ERS4425123	(SAMEA6675587)	ERR4027016	N/A
A35.2-2	ERS4425124	(SAMEA6675588)	ERR4027017	N/A
A40.2-1	ERS4425125	(SAMEA6675589)	ERR4027018	ERR4027800
A41.2-1	ERS4425126	(SAMEA6675590)	ERR4027019	ERR4027801
C32.1-2	ERS4425127	(SAMEA6675591)	ERR4027020	N/A
C51-1	ERS4425128	(SAMEA6675592)	ERR4027021	ERR4027802
C51.2-2	ERS4425129	(SAMEA6675593)	ERR4027022	N/A
C56.1-1	ERS4425130	(SAMEA6675594)	ERR4027023	ERR4027803
C61-1	ERS4425131	(SAMEA6675595)	ERR4027024	ERR4027804
C66-1	ERS4425132	(SAMEA6675596)	ERR4027025	N/A
C71.1-1	ERS4425133	(SAMEA6675597)	ERR4027026	N/A
C76.1-1	ERS4425134	(SAMEA6675598)	ERR4027027	N/A
C76.1-2	ERS4425135	(SAMEA6675599)	ERR4027028	N/A
C81.1-1	ERS4425136	(SAMEA6675600)	ERR4027029	ERR4027805
C81.3-1	ERS4425137	(SAMEA6675601)	ERR4027030	N/A
C81.1-2	ERS4425138	(SAMEA6675602)	ERR4027031	N/A
C86.1-1	ERS4425139	(SAMEA6675603)	ERR4027032	ERR4027806
C86.1-2	ERS4425140	(SAMEA6675604)	ERR4027033	N/A
C86.3-1	ERS4425141	(SAMEA6675605)	ERR4027034	N/A
C91-2	ERS4425142	(SAMEA6675606)	ERR4027035	N/A
C96-1	ERS4425143	(SAMEA6675607)	ERR4027036	N/A

N/A = not applicable

STUDY IV

European Nucleotide Archive (ENA) accession numbers

Study accession number: PRJEB47797

Sample	Accession
D1	ERS7651650 (SAMEA9973367)
D2	ERS7651651 (SAMEA9973368)
D3	ERS7651652 (SAMEA9973369)
D4	ERS7651653 (SAMEA9973370)
D5	ERS7651654 (SAMEA9973371)
D6	ERS7651655 (SAMEA9973372)

D7	ERS7651656 (SAMEA9973373)
D8	ERS7651657 (SAMEA9973374)
D9	ERS7651658 (SAMEA9973375)
D10	ERS7651659 (SAMEA9973376)
D11	ERS7651660 (SAMEA9973377)
D12	ERS7651661 (SAMEA9973378)
D13	ERS7651662 (SAMEA9973379)
D14	ERS7651663 (SAMEA9973380)
D15	ERS7651664 (SAMEA9973381)
D16	ERS7651665 (SAMEA9973382)
D17	ERS7651666 (SAMEA9973383)
D18	ERS7651667 (SAMEA9973384)
D19	ERS7651668 (SAMEA9973385)
D20	ERS7651669 (SAMEA9973386)
D21	ERS7651670 (SAMEA9973387)
D22	ERS7651671 (SAMEA9973388)
D23	ERS7651672 (SAMEA9973389)
D24	ERS7651673 (SAMEA9973390)
D25	ERS7651674 (SAMEA9973391)
D26	ERS7651675 (SAMEA9973392)
D27	ERS7651676 (SAMEA9973393)
D28	ERS7651677 (SAMEA9973394)
D29	ERS7651678 (SAMEA9973395)
D30	ERS7651679 (SAMEA9973396)

APPENDIX VI

Virulence and antimicrobial resistance genes other than *bla* identified in 30 ESBL-producing *Escherichia coli* isolates obtained from Finnish patients collected in the Eastern Finland healthcare district during 2018–2020 in Study IV.

Sample	Acquired resistance genes other than *bla*	Fosfomycin resistance mutations	Quinolone resistance mutations	Virulence genes
D1	*aadA5, aph(3'')-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*

D2	*aph(3'')-Ib*, *aph(6)-Id*, *mph*(A), *sul2*, *dfrA14*	Not found	Not found	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entD, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, ibeA, kpsD, kpsM, ompA, pic, set1A, set1B, vat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D3	*aadA5*, *aph(3'')-Ib*, *aph(6)-Id*, *mph*(A), *sul1*, *sul2*, *tet*(A), *dfrA17*	Not found	*gyrA* (D87N), *gyrA* (S83L), *parC* (S80I)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entD, entE, entS, espL1, espL4, espR1, espX1, espX4, espX5, espY1, espY2, espY3, espY4, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D4	*tet*(M)	Not found	*marR* (S3N)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, cnf1, entB, entC, entD, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, focC, focD, focF, focI, hlyA, hlyB, hlyC, hlyD, ibeA, iroB, iroC, iroD, iroE, iroN, kpsD, kpsM, kpsT, ompA, papB, papC, papD, papF, papI, papJ, papK, sfaA, sfaB, sfaC, sfaD, sfaE, sfaF, sfaG, sfaH, sfaS, sfaY, vat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D5	Not found	*uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *marR* (S3N), *parC* (S80I), *parE* (L416F)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, kpsT, ompA, papB, papI, papX, sat, sfaX, vat, yagV/ecpE, yagW/ecpD, yagX/ec, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D6	*aadA5*, *aph(3'')-Ib*, *aph(6)-Id*, *mph*(A), *sul1*, *sul2*, *tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D7	*aac(6')-Ib-cr*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, cnf1, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyA, hlyB, hlyC, hlyD, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papC, papD, papF, papG, papJ, papK, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D8	*aadA5*, *aph(3'')-Ib*, *aph(6)-Id*, *mph*(A), *sul1*, *sul2*, *tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entS, fdec, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, vat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*

D9	Not found	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (S83L), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D10	*aadA2, mph*(A), *sul1, tet*(A), *dfrA12*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyA, hlyB, hlyC, hlyD, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papC, papD, papF, papG, papJ, papK, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D11	*aadA5, aph(3'')-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D12	*aadA5, aph(3'')-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A)	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, sat, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D13	Not found	Not found	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D14	*aadA2, aph(3'')-Ib, aph(6)-Id, mph*(B), *cmlA1, sul1, sul2, tet*(A), *dfrA1*	Not found	*gyrA* (D87N), *gyrA* (S83L), *parC* (S80I)	*astA, east1, entB, entC, entD, entE, entS, espX1, espX4, espX5, fdeC, fepA, febB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, ompA, papC, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D15	Not found	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D16	*aadA5, aph(3'')-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*

D17	Not found	Not found	*marR* (S3N)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, ibeA, kpsD, kpsM, ompA, pic, set1A, set1B, vat, yagV/ecpE, yagW/ecpD, yagX/eC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D18	Not found	*uhpT* (E350Q)	Not found	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entS, espL1, espR1, espX1, espX4, espY2, espY4, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iucD, iutA, kpsD, kpsM, kpsT, ompA, papX, sat, sfax*
D19	Not found	Not found	Not found	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entD, entE, entS, espL1, espL4, espX1, espX4, espX5, espY2, espY3, espY4, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyA, hlyB, hlyC, hlyD, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papC, papD, papG, papI, papJ, papK, sat, yagV/ecpE, yagW/ecpD, yagX/eC, yagZ/ecpA, ykgK/ecpR*
D20	*aph(3'')-Ib, aph(6)-Id, sul2, tet*(A)	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfax, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D21	Not found	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, papB, papI, papX, sat, sfax, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D22	*aadA5, aph(3'')-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D23	*aadA2, mph(A), sul1, tet*(A), *dfrA12*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyD, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papC, papD, papF, papG, papJ, papK sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D24	*aac(6')-Ib-cr*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*

D25	*aadA5, aph(3")-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfax, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D26	Not found	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D27	*aadA2, mph*(A), *sul1, tet*(A), *dfrA12*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, cnf1, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyA, hlyB, hlyC, hlyD, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papC, papD, papF, papG, papJ, papK, sat, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D28	*aadA5, aph(3")-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (S83L), *parE* (I529L)	*afaA, afaB-I, afaC-I, afaC-III, afaD, aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, daaA, daaC, daaD, daaF, draA, draB, draC, draD, draP, entB, entC, entE, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimB, fimC, fimD, fimE, fimF, fimG, fimH, fimI, hlyA, hlyB, hlyC, hlyD, kpsD, kpsM, ompA, papB, papI, papX, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D29	*aadA5, aph(3")-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*
D30	*aadA5, aph(3")-Ib, aph(6)-Id, mph*(A), *sul1, sul2, tet*(A), *dfrA17*	*ptsI* (V25I), *uhpT* (E350Q)	*gyrA* (D87N), *gyrA* (S83L), *parC* (E84V), *parC* (S80I), *parE* (I529L)	*aslA, chuA, chuS, chuT, chuU, chuV, chuW, chuX, chuY, entB, entC, entE, entF, entS, fdeC, fepA, fepB, fepC, fepD, fepG, fes, fimA, fimC, fimD, fimE, fimF, fimG, fimH, fimI, iucA, iucB, iucC, iutA, kpsD, kpsM, ompA, papB, papI, papX, sat, sfaX, yagV/ecpE, yagW/ecpD, yagX/ecpC, yagY/ecpB, yagZ/ecpA, ykgK/ecpR*