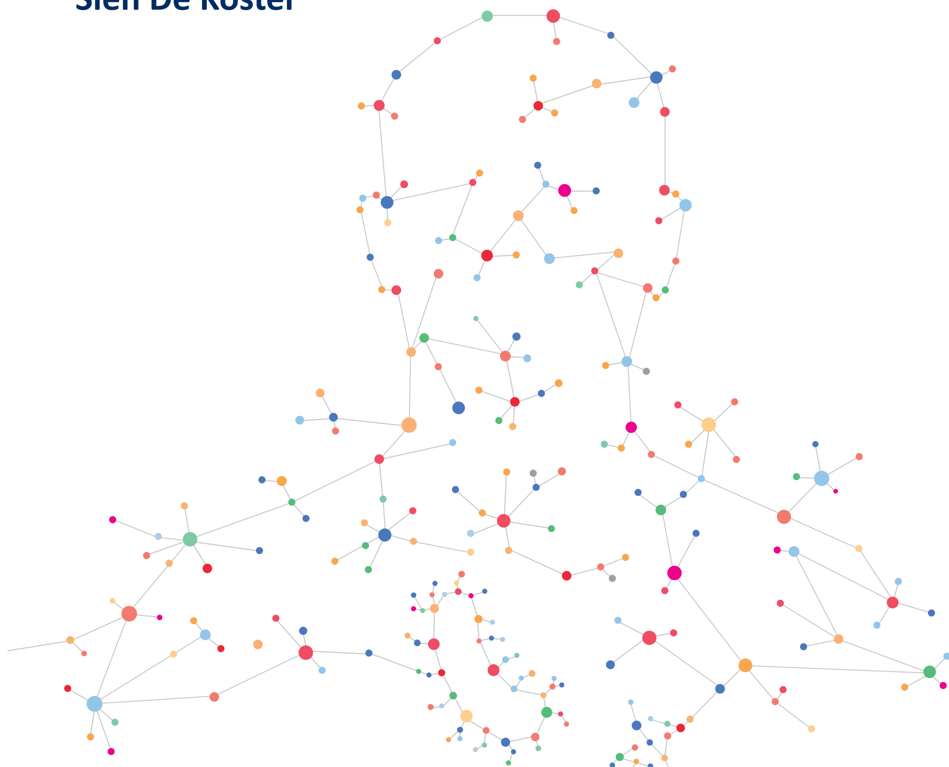
Use of phenotypic and genomic tools to study the prevalence and transmission of antibiotic resistance in a One Health concept

Dissertation submitted for the degree of Doctor of Medical Sciences
at the University of Antwerp to be defended by

Sien De Koster



Supervisor: Prof. Dr. Herman Goossens

Laboratory of Medical Microbiology
Faculty of Medicine and Health Sciences
Antwerp, 2023

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Het gebruik van fenotypische en genotypische methoden om de prevalentie en transmissie van antibiotica resistentie te bestuderen in een ‘One Health’ concept

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Abstract

Multidrug resistance is increasingly observed in human and veterinary medicine worldwide. This major public health challenge is a One Health issue connecting humans, animals and the environment. Insights into the levels of antibiotic resistance in One Health sectors and the pathways that lead to the spread of antibiotic resistance will guide control strategies leading to improved patient care as well as public and animal health.

We employed both phenotypic methods and genomics to investigate the occurrence of antibiotic resistance, the antibiotic use and the dynamics of transmissible resistance genes or isolates within the human and veterinary sectors in Belgium and the Netherlands using a One Health approach.

In this work, we identified and characterized extended-spectrum beta-lactamase (ESBL)-producing, ciprofloxacin-resistant and colistin-resistant Enterobacterales from animals and humans. The carriage of colistin-resistant bacteria by hospitalized patients, healthy individuals from the community and livestock showed that resistance is present in all sectors examined. However, remarkable differences in antibiotic use and resistance were observed between countries and farms. Livestock is a reservoir for a large variety of antimicrobial resistance genes, virulence genes and plasmids. Resistance was spread within a multifaceted landscape of transmission pathways involving both dissemination or a common source of resistant clones and horizontal transfer of plasmids. The complex epidemiology of antibiotic resistance in farms makes it difficult to translate these findings to the impact on human health. However, the pandemic multidrug-resistant clone *E. coli* ST131 and *bla*$_{\text{CTX M-15}}$ commonly associated with human infections was rarely found in livestock. Additionally, animal-to-human transmission or vice versa was not detected. Genomic analysis of a global collection of *K. pneumoniae* ST101 identified ICE*Kp* harboring the yersiniabactin siderophore as a key virulence factor present in hospital-associated isolates. The absence of this siderophore in livestock-associated, community-

associated, and food-associated isolates indicates a lower virulence capacity compared to hospital-associated isolates. Taken together, the presence of resistant bacteria in the examined One Health sectors seems to reflect the antibiotic pressure in each sector rather than transmission of resistant isolates between sectors.

In conclusion, this thesis provides insights into the carriage of antibiotic-resistant Gram-negative bacteria by humans and animals and contributes to an improved understanding of the underlying resistance mechanisms and spread of resistance in all One Health sectors involved.

Samenvatting

Multidrug resistente bacteriën worden wereldwijd in toenemende mate waargenomen in de humane en veterinaire geneeskunde. Deze belangrijke uitdaging voor de volksgezondheid is een 'One Health' kwestie die mensen, dieren en het milieu met elkaar verbindt. Inzicht in de niveaus van antibioticaresistentie en de verspreiding van antibioticaresistentie in One Health sectoren zal leiden tot controlestrategieën voor een verbeterde patiëntenzorg alsook verbeterde dier-en volksgezondheid.

We gebruikten fenotypische en genotypische methoden om het voorkomen van antibioticaresistentie, het antibioticagebruik en de dynamiek van overdraagbare resistentiegenen of isolaten binnen de humane en dierlijke sectoren in België en Nederland te onderzoeken met behulp van een 'One Health' benadering.

In deze thesis identificeerden en karakteriseerden we 'extended-spectrum beta-lactamase' (ESBL)-producerende, ciprofloxacine resistente en colistine resistente Enterobacterales geïsoleerd uit dieren en mensen. Het dragerschap van colistine resistente Enterobacterales door gehospitaliseerde patiënten, gezonde personen en vee toonde aan dat resistentie aanwezig is in alle onderzochte sectoren binnen het One Health kader. Er werden echter opmerkelijke verschillen in antibioticagebruik-en resistentie waargenomen tussen landen en veehouderijen. Voedselproducerende dieren zijn een reservoir voor een grote verscheidenheid aan antimicrobiële resistentiegenen, virulentiegenen en plasmiden. De resistentie werd verspreid via transmissie van resistente klonen of via blootstelling aan een gemeenschappelijke bron van resistente klonen en via horizontale overdracht van plasmiden. De complexe epidemiologie van antibioticaresistentie in veehouderijen bemoeilijkt de interpretatie om de gevolgen voor de menselijke gezondheid in te schatten. De wijdverspreide, multiresistente kloon *E. coli* ST131 and $bla_{\text{CTX-M-15}}$ die vaak in verband wordt gebracht met infecties bij de mens, werd echter zelden bij vee aangetroffen. Bovendien werd geen overdracht van dier op mens of omgekeerd vastgesteld.

Genoomanalyse van een globale collectie van *K. pneumoniae* ST101 identificeerde ICE*Kp* met het yersiniabactin siderofoor als een belangrijke virulentiefactor in isolaten van ziekenhuispatiënten. Isolaten uit veedieren, gezonde personen en voedsel bevatten dit siderofoor niet, wat wijst op een lagere virulentiecapaciteit in vergelijking met ziekenhuisgeassocieerde isolaten. De aanwezigheid van resistente bacteriën in sectoren binnen het One Health kader lijkt eerder de antibioticadruk in elke sector te weerspiegelen en niet de overdracht van resistente isolaten tussen sectoren.

Kortom, dit proefschrift biedt inzicht in het dragerschap van antibioticaresistente Gram-negatieve bacteriën door mens en dier. Het draagt bovendien bij tot een beter begrip van de onderliggende resistentiemechanismen en de verspreiding van resistentie in alle sectoren binnen het One Health kader.

List of Abbreviations

AFLP	Amplified fragment length polymorphism
AIEC	Adherent-invasive *E. coli*
AMCRA	Antimicrobial Consumption and Resistance in Animals
AMR	Antimicrobial resistance
APEC	Avian pathogenic *E. coli*
AST	Antibiotic susceptibility testing
BD100	Number of treatment days per 100 days
BE	Belgium
BeH-SAC	Belgian Hospitals- Surveillance of Antimicrobial Consumption
BelVet-SAC	Belgian Veterinary Surveillance of Antimicrobial Consumption
BFP	Bundle-forming pili
bla	β-lactamase
cg	Core genome
CI	Confidence interval
CiproR	Ciprofloxacin-resistant
CLSI	Clinical and Laboratory Standard Institute
ColR	Colistin-resistant
DAEC	Diffusely adherent *E. coli*
DDD	Defined daily doses
DEC	Diarrheagenic *E. coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *E. coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre of Disease Prevention and Control
EEA	European Economic Area
EFSA	European Food Safety Authority

EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMA	European Medicines Agency
EPEC	Enteropathogenic *E. coli*
ESAC-NET	European Surveillance of Antimicrobial Consumption Network
ESBL	Extended-spectrum beta-lactamase
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
ETEC	Enterotoxigenic *E. coli*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogenic *E. coli*
FTIR	Fourier-transform infrared spectroscopy
HAI	Healthcare-associated infection
hvKP	Hypervirulent *K. pneumoniae*
ICE	Integrative and conjugative elements
IPEC	Intestinal pathogenic *E. coli*
IRIS	Infection Risk Scan
IS	Insertion sequence
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	Lipopolysaccharides
LTCF	Long-term care facility
MALDI-TOF	Matrix-assisted laser desorption ionization-time-of-flight
mcr	Mobile colistin resistance
MDR	Multidrug-resistant
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
(N)ICU	(Neonatal) intensive care unit
NL	The Netherlands

NMEC	Neonatal meningitis-associated *E. coli*
OIE	World Organization for Animal Health
OR	Odds ratio
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCU	Population correction unit
pEtN	Phosphoethanolamine
PFGE	Pulsed-field gel electrophoresis
PMQR	Plasmid-mediated quinolone resistance
PPS	Point prevalence study
QRDR	Quinolone resistance determining region
SEPEC	Sepsis-associated *E. coli*
SNP	Single nucleotide polymorphism
ST	Sequence type
STEC	Shiga toxin-producing *E. coli*
T4SS	Type IV secretion system
TI	Treatment incidence
TSB	Tryptic Soy Broth
UPEC	Uropathogenic *E. coli*
UTI	Urinary tract infection
VNTR	Variable-number tandem repeat
VTEC	Verotoxigenic *E. coli*
wg	Whole genome
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

Definitions

Accessory genome	Part of the genome containing non-essential genes present in a subset of the strains and strain-specific genes.
Artificial intelligence	A field which combines computer science and robust datasets to enable problem-solving by using computer systems to perform tasks normally requiring human intelligence, such as visual perception, speech recognition, decision-making, and translation between languages.
Antimicrobial resistance	The capacity of bacteria to survive antibiotic concentrations designed to inhibit or kill these bacteria.
Clonal group	Isolates of bacterial species that are indistinguishable in genotype and that descended from the same recent ancestor.
Core genome	The set of homologous genes present in all genomes of a set of strains.
Clinical breakpoint	The concentration of an antibiotic used to define whether the infection by a particular bacterial isolate is likely to be treatable in a patient.
Epidemic	An unexpected increase in the number of disease cases in a specific geographical area.
Epidemiology	The method or study used to find the causes of health outcome and diseases in populations. It is the study of the distribution and determinants of diseases within populations, as well as the development of knowledge and strategies on how to prevent and control diseases.
Genotype	The genetic makeup of an organisms or group of organisms at a given location (i.e. locus) in the genome. The genotype determines or contributes to its phenotype.

Genomic plasticity	The property by which an organism can exchange DNA to adapt their genomes to environmental changes and occupy novel niches.
Metagenomic sequencing	Determining the sequence of all the genetic material of microorganisms presents in a sample, consisting of the genomes of the microbial community in the sample.
Microbiota	The community of microorganisms (such as bacteria, fungi and viruses) present in a defined environment (refers to the taxonomy of microorganisms present).
Microbiome	The community of microorganisms (such as bacteria, fungi and viruses) and their genes present in a defined environment (refers to the bacteria and their genes).
Mobile genetic element	Segments of DNA that encode enzymes and other proteins that mediate the movement of DNA within genomes or between bacterial cells.
Multi-drug resistant	The lack of susceptibility to at least one agent in three or more antibiotic classes.
Pandemic	The exponential growth in disease cases with a wide geographic reach.
Pathogenicity	The ability of an organism to cause disease and harm the host.
Phenotype	A set of observable physical traits or characteristics of an organism resulting from the expression of a genotype.
Plasmidome	The complete set of plasmids present in bacterial isolates or samples.
Prevalence	In general, the prevalence is the proportion of a population who have a specific characteristic in a given time frame. In epidemiology, prevalence is the proportion of a population that are affected by a medical condition in a given time period.
Resistome	The collection of all the antibiotic resistance genes (acquired and intrinsic resistance genes) and their precursors in pathogenic and nonpathogenic bacteria in a given microbial ecosystem.

Transmission	The spreading or transfer of micro-organisms.
Virulence	The degree to which a pathogenic organism can cause disease in a host.
Whole genome sequencing	Also known as full genome sequencing. The process of determining the DNA sequence of an organism’s genome. Short-read sequencing: Also referred to as second generation sequencing. Sequencing of short fragments of DNA (50-500 bp) by synthesis or ligation using a DNA polymerase or ligase enzyme, respectively. Long read sequencing: Also referred to as third generation sequencing. Sequencing of a single molecule and generating longer lengths (5000 bp->5 kb).

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CHAPTER 1
Scope and objectives of the thesis

1.1 Rationale

Enterobacteriaceae are important causes of urinary tract infections (UTI), bloodstream infections, healthcare-associated pneumonia, and intra-abdominal infections. Multidrug resistance in Enterobacteriaceae is increasingly observed in human and veterinary medicine and is considered one of the major public health challenges worldwide (1). Multidrug-resistant (MDR) or extensively drug-resistant (XDR) Enterobacteriaceae residing in the human and animal gut can be disseminated via direct contact, agricultural and human waste as well as via unhygienically slaughter practices or contaminated food. This can potentially result in complex transmission paths between humans, the environment and animals and from one country to another (2). This interlinkage between human and animal health requires an integrated, multisectoral approach, labeled as the One Health approach, to battle antibiotic resistance in the hospital, community and livestock sectors. Belgium and the Netherlands have high population densities combined with one of the highest densities of livestock (cattle, pigs, poultry) in Europe. In addition, both countries are interconnected via patient exchange, movement of persons and goods and have comparable intensive food production practices. These factors make active monitoring of antibiotic use and resistance in a One Health perspective in this region important. However, national monitoring systems differ in data collection, analyses and reporting, making comparisons difficult. The lack of fully integrated and standardized measurements of antibiotic use and resistance in humans and livestock do not allow for accurate comparison between humans, livestock species and countries. Harmonized and comparable data on antibiotic use and resistance is needed to improve guidelines, guide infection control policies and intervention strategies and steer research agendas. The combination of phenotypic and genotypic methods provides accurate information on

antibiotic resistance and underlying genetic mechanisms for resistance. However, most of the surveillance systems rely on phenotypic antibiotic resistance profiles and the current assays lack, unlike whole genome sequencing (WGS), the ability to track MDR and clones with a high-risk for transmission and infection. WGS, a technique to unravel the DNA sequence of an organism, enabled the investigation of antibiotic resistance mechanisms on a DNA level (3) and provides effective discrimination of epidemiologically unrelated strains. A better understanding of the occurrence and spread of resistant bacteria in humans and animals will allow for better implementation of control strategies.

1.2 Objectives

Antibiotic resistance is a global health problem that needs studying of emerging microorganisms and resistance mechanisms. Within the scope of understanding and mapping antibiotic resistance, this PhD project explores the presence and spread of antibiotic-resistant Enterobacteriaceae in the border region of Belgium and the Netherlands. The project is part of the i-4-1-Health study, funded by the European Union, which aims to reduce antibiotic resistance in human and animal sectors in the Belgian-Dutch border region using active monitoring of resistance and antibiotic stewardship. The **general objective of this thesis** was to investigate the occurrence of antibiotic resistance, the antibiotic use and the dynamics of transmissible resistance genes or isolates within the human and veterinary sectors in Belgium and the Netherlands using a One Health approach. The human sectors included in this thesis were hospitals, long-term care facilities (LTCFs) and day care centres. While weaned pigs, broilers and veal calves are the animal categories with the highest antibiotic use (4), and therefore, of utmost importance to investigate, our research focused on broilers and pigs. Cattle farming was excluded in the studies reported in this thesis. Following resistance mechanisms in Enterobacterales were included in the study: ESBL- and carbapenemase production, ciprofloxacin resistance, and colistin resistance.

The **specific objectives** of this work were:

(i) to investigate the occurrence of ESBL-producing *E. coli*, carbapenem-resistant and ciprofloxacin-resistant *E. coli* in Belgian and Dutch pig and poultry farms with a history of high antibiotic use (**Chapter 3**)

(ii) to genotypically characterize and trace ESBL-producing and ciprofloxacin-resistant *E. coli* of Belgian food animals (**Chapter 4**)

(iii) to investigate colistin resistance in Enterobacterales from humans and animals in the border region of Belgium and the Netherlands using a One Health approach (**Chapter 5**).

(iv) to assess the genetic relationship of *K. pneumoniae* ST101 isolated from hospitalized patients, the environment, the community and livestock (**Chapter 6**).

1.3 Outline of the thesis

This thesis is divided into seven chapters. **Chapter 2** provides an introduction to antibiotic resistance with a special focus on critically important antimicrobials for human and animal medicine. We discuss the Enterobacteriaceae family as a reservoir for resistance in the gastrointestinal tract of humans and animals with a particular focus on their molecular makeup. We zoom in on the molecular mechanisms of resistance to critically important antibiotics and discuss pandemic and epidemic lineages of two of the most prevalent Enterobacteriaceae members causing serious infections, *E. coli* and *K. pneumoniae.* The chapter continues with an overview of the phenotypic and genotypic methods for the detection of resistance and a summary of the available information on antibiotic use and resistance in Europe, Belgium and the Netherlands. Finally, we introduce the concept of One Health as an approach to tackle antibiotic resistance across borders and across sectors.

Chapter 3 shows unified information on the quantity of antibiotic use and the presence of antibiotic resistance at the level of the farm in two neighboring countries with different antibiotic policies, Belgium and the Netherlands. By using comparable methods for sampling, sample processing, data collection and data analysis in this cross-sectional and cross-border study, we determined the occurrence of ESBL-producing, ciprofloxacin-resistant and carbapenem-resistant *E. coli* in farm animals and explored the association between on-farm antibiotic use and antibiotic resistance.

Chapter 4 characterizes the molecular makeup and epidemiology of ESBL-producing and ciprofloxacin-resistant *E. coli* from Belgian broiler and pigs. In this study, resistant *E. coli* isolates were subjected to short-read sequencing in order to assess the genetic diversity, resistance genes (resistome) and plasmids (plasmidome) and elucidate the inter- and intra-farm transmission of bacteria and plasmids. In addition, associations of genetic markers with a resistance phenotype for fluoroquinolones and cephalosporins were investigated in detail.

Chapter 5 investigates the presence and spread of colistin-resistant Enterobacteriaceae in Belgium and the Netherlands using a One Health approach. Samples were collected cross-sectionally from patients in hospitals, residents in LTCFs, children attending day care centres, and broilers and pigs in farms. A single survey was conducted in LTCFs and daycare centers. Two rounds of repeated measurements with a one-year interval were performed in hospitals and farms to longitudinally assess the presence of colistin resistance in hospitals and farms as well as the colistin use in farms. Both phenotypic antibiotic resistance testing and short-and long-read sequencing were used to fully characterize the colistin-resistant isolates and investigate the resistome. This integrated, multisectoral study provides a better understanding on the occurrence and molecular basis of colistin resistance in these One Health settings.

In **Chapter 6**, special focus lies on the *K. pneumoniae* ST101 clone, an emerging high-risk pathogen which is highly adapted to the hospital environment and is causing outbreaks in several countries. While high-risk *K. pneumoniae* clones in animals remains scarce, we detected *K. pneumoniae* ST101 in a broiler and pig in two Belgian farms. The two livestock isolates and eight hospital-associated isolates from the laboratory collection were subjected to short-read and long-read sequencing to gain insights into the genetic diversity between hospital-associated and non-clinical *K. pneumoniae* ST101. The study also includes all available *K. pneumoniae* sequences (n=586, September 2021) originating from healthy individuals in the community, hospital patients, the environment and animals to provide insights into the variability in the genome and the virulence and resistance traits of this high-risk clone in all One Health sectors.

Finally, **Chapter 7** concludes this thesis by discussing the findings in the context of available literature, discussing the potential implications of our findings as well as reviewing potential future research perspectives.

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CHAPTER 2
Introduction

2.1 Public health burden of antimicrobial resistance

Antibiotics are small molecules that can inhibit or kill bacteria and are used to prevent and treat bacterial infections. The use of antibiotics in intensive food production systems, hospitals and the community creates a selective pressure leading to the increasing prevalence of microorganisms resisting these antibiotics (1). Antibiotic resistance is defined as the capacity of bacteria to survive antibiotic concentrations applied to inhibit or kill these bacteria (2). With the overlap of antibiotics used in human and animal medicine, antibiotic resistance is a threat to human as well as animal health and is one of the greatest challenges of the 21st century on a global level (1,3). In addition, the lack of the development of new antimicrobial medicines is enhancing the health threat. The inability of antibiotics to treat infections puts pressure on modern medicine making even simple procedures more dangerous. It is also leading to longer duration of illness, longer hospital stays, increasing health care costs and increased mortality. Globally, antimicrobial resistance (AMR) is a leading cause of death. In 2019, an estimated 4.95 million deaths were associated with bacterial antibiotic resistance (4). In Europe, annually 33,000 people die from an infection due to resistant bacteria, which is a burden comparable to that of influenza, tuberculosis and HIV/AIDS combined (5). Healthcare costs and lost productivity due to AMR measure at least 1.5 billion euros per year (6). According to a population-based modelling analysis based on data from the European Antimicrobial Resistance Surveillance Network (EARS-Net), the number of deaths attributed to AMR was estimated at 530 per year in Belgium and 206 deaths per year for the Netherlands (5). The costs of AMR to the Belgian health system are approximately 24 million euros each year according to the Organization for Economic Co-operation and Development (7).

Six pathogens were identified as the dominant causes of AMR-associated deaths: *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (4). These microorganisms have been labeled as priority pathogens by the World Health Organization (WHO) (8) that can cause severe and deadly infections such as bloodstream infections and healthcare-associated pneumoniae (9). The most critical pathogen-drug combinations that require action are MDR tuberculosis, third-generation cephalosporin-resistant *E. coli*, carbapenem-resistant *A. baumannii*, fluoroquinolone-resistant *E. coli*, carbapenem-resistant *K. pneumoniae*, and third-generation cephalosporin-resistant *K. pneumoniae* (4). The WHO published a list of the critically important antimicrobials for human medicine. Examples are carbapenems (e.g. meropenem), 3rd, 4th and 5th generation cephalosporins (e.g. ceftriaxone and cefepime), penicillins (e.g. piperacillin, ampicillin, amoxicillin-clavulanic acid), quinolones (e.g. ciprofloxacin) (**Figure 2.1**) and polymyxins (e.g. colistin) (10).

Continued use of these antibiotics in human medicine, horticulture, livestock and aquaculture drives the selection of resistant bacterial populations.

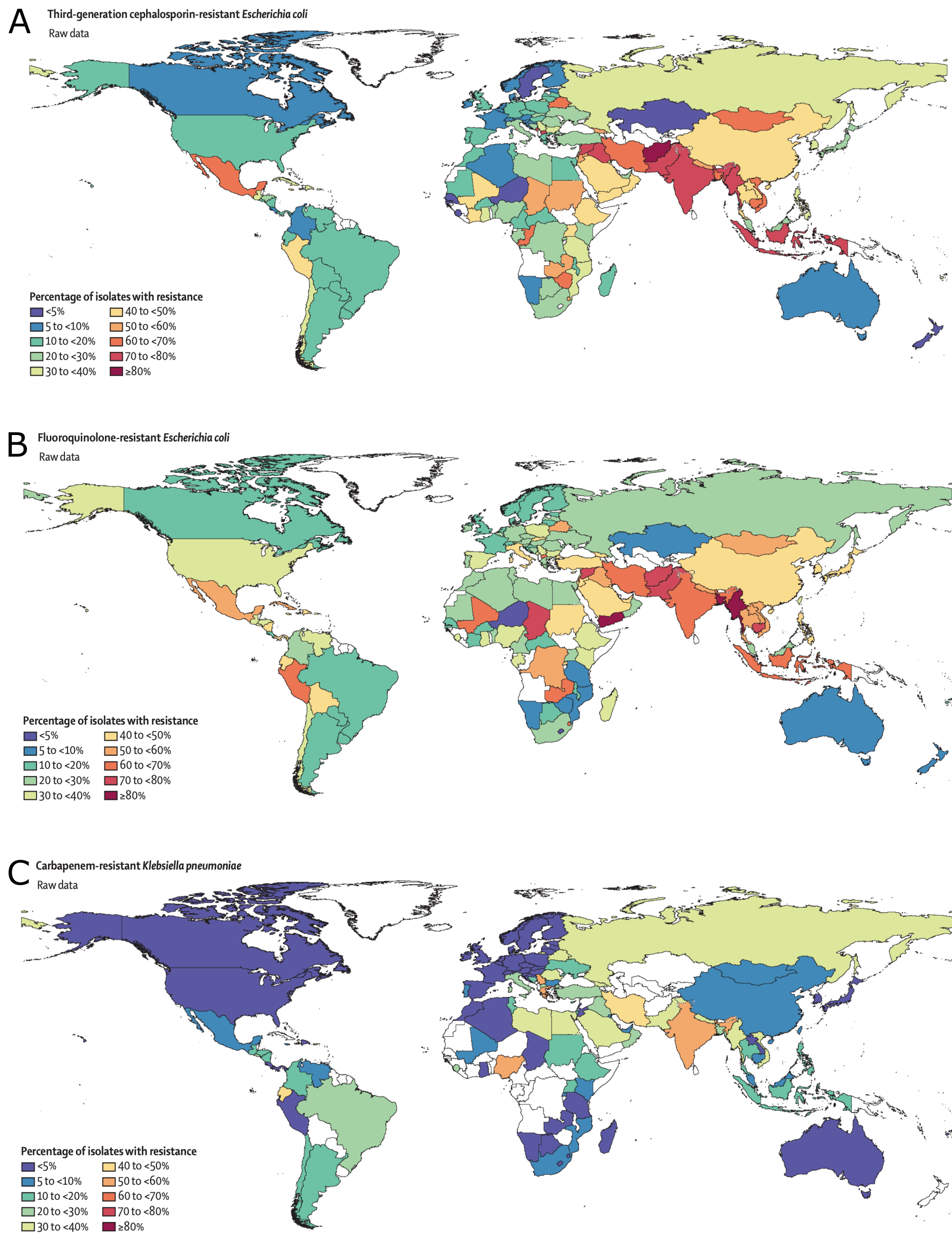

Figure 2.1: Estimated percentage of pathogen isolates that are resistant to third-generation cephalosporins (A), fluoroquinolones (B) and carbapenems (C) in 2019. Locations with no data are presented in white. Figure adapted from (4).

2.2 Gastrointestinal microbiota as a reservoir for antimicrobial resistance

The human microbiota is the vast number of microorganisms in various sites of the body and is known as “the hidden organ” with 150 times more genetic information than that of the entire human genome (11). The gastrointestinal tract is colonized by an abundant microbial community with approximately 100 trillion microorganisms and over 35,000 bacterial species (12) serving several functions such as food fermentation, vitamin production, protection against pathogens and modulating immune responses (11). Hence, the gut microbiota plays a major role in health and disease. The gut microbiota is generally composed by six phyla including *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria* and *Verrucomicrobia* among which the first two are dominant (11). The bacterial population in the gut may be altered by the intake of antibiotics leading to reduced species diversity, altered metabolic activity, the selection of antibiotic-resistant bacteria and potential horizontal gene transfer of AMR genes among bacteria in the gut. The gastrointestinal tract is, therefore, an important reservoir for the emergence of antibiotic resistance (2,13). The presence of antibiotic-resistant organisms in the gut has been associated with a risk of invasive infections and transmission (14). The incidence of resistant bacteria found in the gut is clearly increasing (15). Broad spectrum antibiotics or antibiotics that are largely eliminated via the bile and gut (e.g. ceftriaxone) cause worse collateral damage to the gut microbiota (13). The gut may contain high concentrations of resistant Gram-negative and -positive bacteria such as Enterobacteriaceae, *Pseudomonas*, *Acinetobacter* and vancomycin-resistant enterococci during and after antibiotic treatment (13). In particular, Enterobacteriaceae, widely present in the human and animal gut, have a remarkable genome plasticity and have the capacity to accumulate resistance genes, often via horizontal gene transfer. MDR or XDR Enterobacteriaceae residing in the human and animal gut can be disseminated via direct contact, agricultural and human waste as well as via unhygienic conditions during slaughtering or contaminated food (16). Multidrug resistance in Enterobacteriaceae is increasingly observed in human and veterinary medicine and is considered one of the major public health challenges worldwide (17).

2.3 The Enterobacteriaceae family

2.3.1 Phenotypic and genotypic features

Enterobacteriaceae are a heterogeneous family of enteric, Gram-negative, facultative aerobic or anaerobic, non-spore-forming, rod-shaped bacteria. The bacteria are either motile with a flagella or nonmotile (18). The Enterobacteriaceae are classified within the order of the Enterobacterales, the class of the Gammaproteobacteria and the phylum of the Proteobacteria (19). Members of the Enterobacteriaceae family share common features, such as phenotypes (Gram-negative, oxidase negative, enterobacterial common antigen positive, nitrate reductase positive, catalase positive), habitats (gastrointestinal tract of vertebrates, soil, water, food) and disease patterns (diarrhea, blood stream infections, UTIs, etc.) (20). To date, over 150 species of Enterobacteriaceae are identified, but only a small portion are clinically significant (such as *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia* species) (18,20,21). Historically, the members of the family were differentiated based on structural and biochemical features (19,20). Enterobacteriaceae also possess complex antigenic structures on their outer membrane creating a basis for serologic classification or serotyping. Three major types of antigens are present on the outer membrane: the O-antigen (lipopolysaccharides, LPS), the K-antigen (polysaccharide capsular) and H-antigen (flagellar protein). The H-antigen plays a role in motility; the K-and O-antigen give structural support to the cell and interact with the environment. The O-antigen is a chain of carbohydrates which is highly variable and thus serotype specific (22).

Afterwards, bacteria have been classified using molecular methods based on the small subunit of ribosomal RNA, 16s rRNA gene sequencing (23). This was followed by other molecular techniques such as matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry and WGS (18). The introduction of WGS has had an enormous impact as a tool for bacterial genotyping with an unprecedented resolution. Analysis of the entire genome via WGS facilitated discrimination of highly related bacterial lineages and changed outbreak analysis.

The Enterobacteriaceae genome size depends on the species. The size of the *E. coli* genome varies from ~ 4.5 to 5.5 Mb (24). *K. pneumoniae* genome size is around 5.5 Mb. Both *E. coli* and *K. pneumoniae* have a considerable genomic plasticity and a large accessory genome often harboring many resistance and virulence genes (25,26). The *E. coli* genome contains 4,000 to 5,000 genes of which 2,000 are part of the core genome while around 3,000 genes are part of the accessory genome allowing *E. coli* to adapt to different ecological niches (17,23,27). The ability to acquire and disseminate a wide variety of antibiotic resistance and virulence genes makes some members of the Enterobacterales family among the most significant public health problem worldwide (28).

2.3.2 Pathogenesis of enteric Gram-negative bacteria

Bacterial pathogenicity depends on both human and bacterial factors such as the host immune status and the bacterial virulence. Virulence mechanisms include adhesins, invasins, capsules, type three secretion systems, outer membrane proteins, toxins, capsules, iron acquisition systems, and biofilm formation. These mechanisms are used by bacteria to cause infection (29,30).

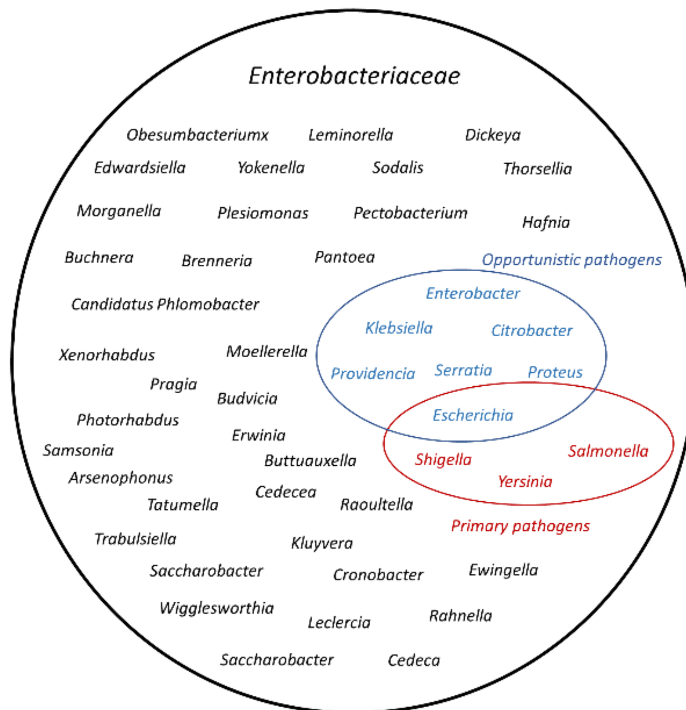

Figure 2.2: Different genera belonging to the Enterobacteriaceae family. Primary pathogens are organisms capable of causing disease in all carriers through the production of virulence factors. Opportunistic pathogens are organisms capable of causing disease under certain conditions or in certain hosts (e.g. immunocompromised hosts or persons with underlying conditions). Figure adapted from (31).

Many members of the Enterobacteriaceae family are commensal organisms colonizing the gut in healthy conditions, however, can also be pathogenic in humans and animals causing life-threatening intestinal and extra-intestinal infections (**Figure 2.2**) (18). Among the Enterobacteriaceae family, known pathogens in the gut are *Salmonella*, *Shigella, Escherichia, Yersinia*, *Klebsiella*, *Shigella*, *Proteus, Serratia* and *Citrobacter* species (**Table 2.1**) (21,30). These are important causes of UTIs, bloodstream infections, hospital-and healthcare-associated pneumonia and intra-abdominal infections (17). Enterobacteriaceae can also be pathogenic for animals, for example, *E. coli* can cause colibacillosis in broiler chickens (avian pathogenic *E. coli* or APEC), diarrhea in pigs

(mostly during the first 3-5 days of life and 3-10 days after weaning), colibacillosis and colisepticemia in newborn calves and mastitis in cows. Diarrhea is considered one of the major diseases in livestock as it can propagate among animals at herd/flock level and sometimes cause high mortality (17,20,32). Clinically important infections are usually caused by *E. coli* (18) (**Figure 2.3**). *E. coli* causing clinical syndromes outside of the gut are termed extraintestinal pathogenic *E. coli* (ExPEC). ExPEC strains are important causes of UTI (uropathogenic *E. coli* or UPEC), neonatal meningitis (neonatal meningitis-associated *E. coli* or NMEC) or sepsis (sepsis-associated *E. coli* or SEPEC) (27). *E. coli* causing diarrhea are called intestinal pathogenic *E. coli* (IPEC) or diarrheagenic *E. coli* (DEC) and are classified by their virulence properties. Their cell adherence properties and toxin associated genes are often plasmid or phage mediated (18). These pathogenic *E. coli* are grouped into several pathotypes based on the presence of specific virulence traits: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (18,27,33) (**Table 2.1**). STEC and to a lesser extent, EPEC, ETEC and EAEC are zoonotic pathogens widely recognized as a very important cause of foodborne illness (34). The last decade, hybrid pathogenic *E. coli* have been identified carrying combinations of virulence factors of both DEC- and ExPEC-defining virulence factors (such as ExPEC/STEC and ExPEC/EPEC). Also hetero-pathogenic *E. coli* harboring virulence genes of two or more DEC pathotypes (such as EAEC/STEC and EPEC/ETEC) are known. Some of these combinations of virulence factors may lead to more severe diseases (33).

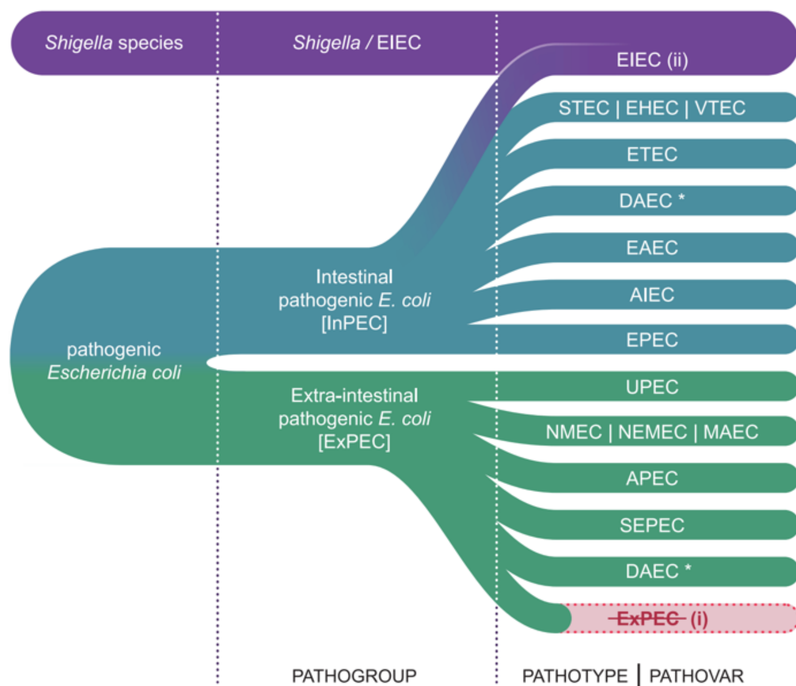

Figure 2.3: Classification of pathogenic *E. coli* causing acute disease. * pathotype is associated with both intestinal and extraintestinal disease. AIEC: adherent-invasive *E. coli*, APEC: avian pathogenic *E. coli*, DAEC: diffusely adherent *E. coli*, EAEC: enteroaggregative *E. coli*, EHEC: enterohemorrhagic *E. coli*, EIEC: enteroinvasive *E. coli*, EPEC: enteropathogenic *E. coli*, ETEC: enterotoxigenic *E. coli*, NMEC/NEMEC/MAEC: neonatal meningitis-associated *E. coli*, SEPEC: sepsis-associated *E. coli*, STEC: Shiga toxin-producing *E. coli*, UPEC: uropathogenic *E. coli*, VTEC: verotoxigenic *E. coli*. Figure from (35).

Other Enterobacteriaceae such as *K. pneumoniae* also have pathogenic capacities at different body sites. *K. pneumoniae* causes hospital-associated infections such as bacterial pneumonia, UTI and bacteremia and community-associated infections such as liver abscess, endophthalmitis, and meningitis (36). In animals, *K. pneumoniae* is a common cause of septicemia, pneumoniae and mastitis in pigs (37), respiratory infections in broilers (38), urinary tract infection in domestical animals (39), pneumonia in horses (40) and bovine mastitis in dairy cattle (41).

Highly invasive clones of *K. pneumoniae* are termed hypervirulent *K. pneumoniae* (hvKP). They belong to a small collection of clonal groups; the most dominant lineage

among hvKP is clonal group 23 which includes ST23, ST26, ST57 and ST1633. Hypervirulence factors include capsule, siderophores, LPS and fimbriae. The capsule type (K) is one of the identifiers of kvKP. Capsule types K1, K2, K5 and K57 are most commonly associated with hvKP. Another defining feature of hvKP is the presence of a plasmid containing virulence genes (e.g. pK2044 and pLVPK) encoding siderophore systems and a mucoid phenotype (regulated by *rmpA* and *rmpA2*). Integrative and conjugative elements (ICEs) in the chromosome are also important for the acquisition of virulence factors in hvKP; these elements can encode siderophore yersiniabactin, siderophore salmochelin and genotoxin colibactin (damages DNA and disrupts the host cell cycle) (42). Siderophore systems enhance the ability to survive and replicate within the host by scavenging iron from the host transport proteins. Siderophores detected in *K. pneumoniae* are enterobactin (*ent*) and yersiniabactin (*ybt*). HvKP also harbor salmochelin (*iro*) and aerobactin (*iuc*) siderophores which improve the growth efficiency of the bacteria (36).

These hvKP show a hyper mucoid phenotype and are mainly associated with community-acquired infections (36). However, both in the community and the hospital setting, convergence of virulence and resistance is found resulting in MDR-hypervirulent *K. pneumoniae* (MDR-hvKP) (mainly belonging to ST11). The combination of high virulence and resistance in high-risk clones poses a serious health threat (36).

Although, virulence and invasiveness have been studied in *E. coli* and *K. pneumoniae*, the exact mechanisms are less well understood in other Enterobacterial species such as *Enterobacter* and *Citrobacter* (30). *Enterobacter* species (mainly *Enterobacter cloacae* complex) cause a broad range of hospital-acquired infections such as pneumonia, UTI as well as wound and device infections. *Serratia marcescens* and *Citrobacter* species are also common opportunistic pathogen in hospitalized patients. UTIs can also be caused by *Proteus* and *Providencia* species (18).

Table 2.1: Clinically relevant Enterobacteriaceae, their most important virulence determinants and disease manifestation in humans and livestock.

Species	Pathotype	Important virulence determinants	Disease in humans	Disease in animals
Escherichia coli	APEC	ColV plasmids, *hlyE, cvaC, iss, fimC, tsh, iucC, sitA* (43)	potential foodborne zoonotic pathogen (UTI, meningitis) (43)	colibacillosis in avian species (43)
	UPEC	PAI (simultaneous presence of *yfcV, vat, chuA, fyuA*) (33)	UTI (44,45)	coliform mastitis in sows (46)
	DAEC	Ada/Dr adhesins (*afa, dra, daa*) (27,33)	diarrhea (44)	colibacillosis (*E. coli* diarrhea) (32,47)
	EAEC	pAA (*aggR* (typical), *aatA, aaiG* (atypical)) (27,33)	diarrhea (44)	colibacillosis (*E. coli* diarrhea) (32,48)
	EHEC/STEC	Shiga toxins (*stx1, stx2*) (27,33)	hemolytic-uremic syndrome (44)	colibacillosis (*E. coli* diarrhea) and colisepticemia (32,48)
	EIEC	pINV (*ipaH*) (27,33)	dysentery (44)	colibacillosis (*E. coli* diarrhea) (32,48)
	EPEC	LEE PAI (*eae* and *bfp* (typical), *eae* alone (atypical)) (27)	diarrhea (44)	colibacillosis (*E. coli* diarrhea) and colisepticemia (32,47)
	ETEC	ST or LT toxins (*elt, est*) (27,33)	diarrhea (44)	colibacillosis (*E. coli* diarrhea) and colisepticemia (32,47,49)
	SEPEC	*ibe*A, B, C, *traT*, *iss*, *colV*, *cvaC*, *gimB*, *sfa/foc* genes encoded on plasmids (45)	sepsis (45)	hemorrhagic septicemia (50)
	NMEC	*ibeABC, traT, iss, colV, cvaC, gimB, sfa/foc, mat* genes encoded on plasmids (45)	neonatal meningitis (45)	

Enterobacter, Serratia, Citrobacter species			UTI, pneumonia, sepsis (44)	Mastitis in cows (51)
Klebsiella pneumoniae	Classical *K. pneumoniae*	capsular polysaccharide (44,52) , fimbriae, siderophores (enterobactin, yersiniabactin) (36)	pneumonia, UTI, bacteremia, liver abscess, endophtalmitis, meningitis (44)	septicemia, pneumoniae and mastitis in pigs (37); respiratory infections in broilers (38); mastitis in cows (51)
	Hypervirulent *K. pneumoniae*	Hypermucoviscosity (RmpA), capsules (mainly K1 and K2), siderophores (enterobactin, yersiniabactin, aerobactin, salmochelin), genotoxin colibactin (36)	Pyogenic liver abscess, meningitis, endophtalmitis, necrotizing fasciitis (36)	septicemia, pneumoniae and mastitis in pigs and cows (37); respiratory infections in broilers (38)
Proteus species		urease, proteus toxic agglutinin, fimbriae (44,53)	UTI (44)	UTI (46)
Salmonella species		*Salmonella* PAI (SPI) (54)	gastroenteritis, sepsis, typhoid fever (44)	systemic septicemia in cattle, enteritis in calves, lambs, pigs (46), pullorum disease and fowl typhoid in poultry (51)
Shigella species		SHI-1,-2,-3,-O PAI, Shiga toxin (55)	dysentery, hemolytic-uremic syndrome (44)	dysentery (46)
Yersinia species		Yersinia virulence plasmid (pYV) (56)	plague, mesenteric adenitis, enteric fever (44)	diarrhea (rarely) (46)

APEC: avian pathogenic *E. coli*, DAEC: diffusely adherent *E. coli*, EAEC: enteroaggregative *E. coli*, EHEC: enterohemorrhagic *E. coli*, EIEC: enteroinvasive *E. coli*, EPEC: enteropathogenic *E. coli*, ETEC: enterotoxigenic *E. coli*, LEE: enterocyte effacement, LT: heat-labile toxin, NMEC: neonatal meningitis-associated *E. coli*, PAI: pathogenicity island, SEPEC: sepsis-associated *E. coli*, SPI: *Salmonella* pathogenicity island, ST: heat-stable toxin, STEC: shiga toxin-producing *E. coli*, UPEC: uropathogenic *E. coli*, UTI: urinary tract infection.

A variety of antibiotics are used to treat these Gram-negative infections such as cephalosporins (ceftriaxone, cefotaxime, ceftazidime), fluoroquinolones (ciprofloxacin, levofloxacin), tetracyclines, aminoglycosides (gentamicin, amikacin), carbapenems, broad-spectrum penicillins with or without β-lactamase inhibitors (amoxicillin-clavulanic acid, piperacillin-tazobactam), fosfomycin, and trimethoprim-sulfamethoxazole (**Figure 2.4**) (57). However, despite the availability of several classes of antibiotics, Gram-negative infections are associated with high mortality and morbidity. This is due to MDR in Gram-negatives which limits the therapeutic options (57).

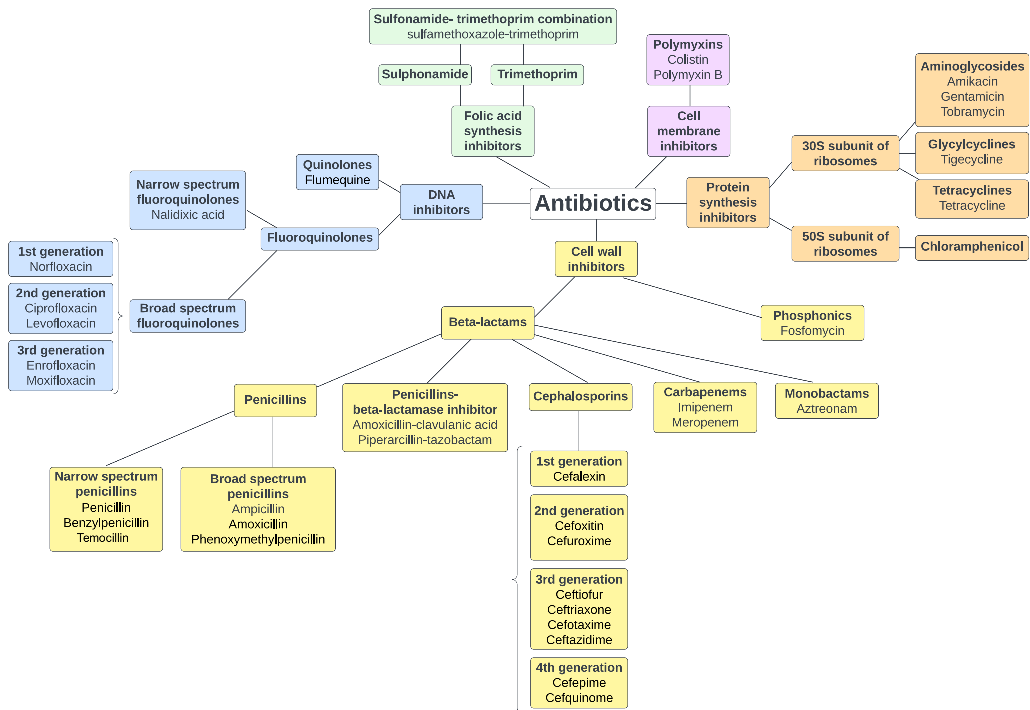

Figure 2.4: Schematic overview of the antibiotic classes and antibiotic compounds described in this thesis. Classes are color-coded based on the mechanism of action. Antibiotic classes are shown in bold, active compounds are listed under each class.

2.3.3 Molecular mechanisms of antibiotic resistance in Enterobacteriaceae

The remarkable genetic plasticity of bacteria permits them to respond to a wide variety of threats, including antibiotics. Antibiotic resistance can either be innate by inherent resistance genes present in the bacteria or acquired via horizontal gene transfer or via gene mutation(s) (17,58,59). Horizontal gene transfer is the most important mechanism for the acquisition and spread of antimicrobial resistance. Three ways of horizontal gene transfer are known: conjugation (*i.e.* transfer of mobile genetic elements (MGEs) such as plasmids, transposons and integrons), transduction (*i.e.* transfer of DNA by bacteriophages), and transformation (*i.e.* the uptake of environmental DNA) (59,60).
The most important resistance mechanisms are: (i) antibiotic inactivation by enzymatic modification or elimination, (ii) antibiotic target alteration to reduce binding capacity, (iii) reducing cell permeability (e.g. porin loss) and increasing efflux to reduce intracellular accumulation of the antibiotic and (iv) modification of metabolic pathways and regulatory network to circumvent the effect of the antibiotic (58,61).

2.3.3.1 β-lactam mode of action and resistance

β-lactam antibiotics are the most widely prescribed bactericidal antibiotics. These antibiotics have a β-lactam ring in their structure and are derivatives of penicillin (*i.e.* cephalosporins, monobactams, carbapenems and β-lactamase inhibitors) (62). They interrupt the synthesis of the cell wall by binding to transpeptidases, also known as penicillin-binding proteins (PBPs), followed by acylation of an active-site serine in the transpeptidase domain of the PBP (63,64). In Gram-negative bacteria, β-lactam resistance mechanisms include changing permeability (OmpC and OmpF porins in *E. coli* and OmpK35-K36 in *K. pneumoniae)*, activating efflux pumps (e.g. AcrAB-TolC) and to a lesser extent alteration of PBPs in the periplasmatic space of Gram-negative bacteria (59,62). The most prevalent resistance mechanism is the production of β-lactamases, which inactivate β-lactam antibiotics by binding and hydrolysing the β-lactam ring of penicillins, and first-and second-generation cephalosporins (59,62,64). Major β-

lactamase families include ESBLs, AmpC cephalosporinases and carbapenemases (**Figure 2.5A**) (64).

ESBLs arose from point mutations in class A β-lactamases TEM-1, TEM-2 and SHV-1 in the 1980s. These enzymes are active against penicillins, first-, second- and third-generation cephalosporins and aztreonam, but not cephamycins (e.g. cefoxitin). ESBLs are inhibited by β-lactamase inhibitors (59). TEM (Temoneira β-lactamases)- and SHV (sulfhydryl reagent variable β-lactamases)-ESBLs were dominant until 2000 after which CTX-M-(cefotaxime-hydrolyzing β-lactamases) ESBLs emerged which are predominantly identified in human and animal isolates worldwide (17). The different ESBL family enzymes have different activities. For example, CTX-M ESBLs typically hydrolyze cefotaxime better then ceftazidime and are more susceptible to inhibition by tazobactam than clavulanic acid unlike the TEM and SHV ESBLs. The cephalosporin-hydrolyzing OXA type ESBLs are rarely found in Enterobacteriaceae (59,63). CTX-M-type enzymes are now the most prevalent ESBLs in Enterobacteriaceae worldwide (63).

Another important type of β-lactamases that confer MDR patterns are carbapenemases (63). Carbapenemases can be divided into three different classes based on the molecular classification (65). Class A carbapenemases include KPC (*Klebsiella pneumoniae* carbapenemase), SME (*Serratia marcescens* enzyme), Nmc-A (non-metallo-carbapenemase-A), IMI (Imipenemase), and GES-type enzymes. Class B, the zinc-dependent metallo-beta-lactamases include enzymes such as NDM (New Delhi MBL), VIM (Verona integron-encoded metallo- β-lactamase) and IMP (imipenem-resistant *Pseudomonas*). Class D β-lactamases are termed 'oxacillinases'. Of the OXA-type carbapenemases, OXA-48 enzymes are widely found on plasmids in Enterobacteriaceae. The other carbapenemases can be either plasmid-encoded (NDM, IMP, GES and KPC), chromosomally encoded (SME and Nmc-A) or both (IMI and VIM) (63).

β-lactamase (*bla*) genes carried on plasmids pose an important risk for transmission of resistance (64). The spread of ESBLs, especially CTX-M enzymes, and carbapenemases, in Enterobacteriaceae has become a serious public health problem worldwide (59).

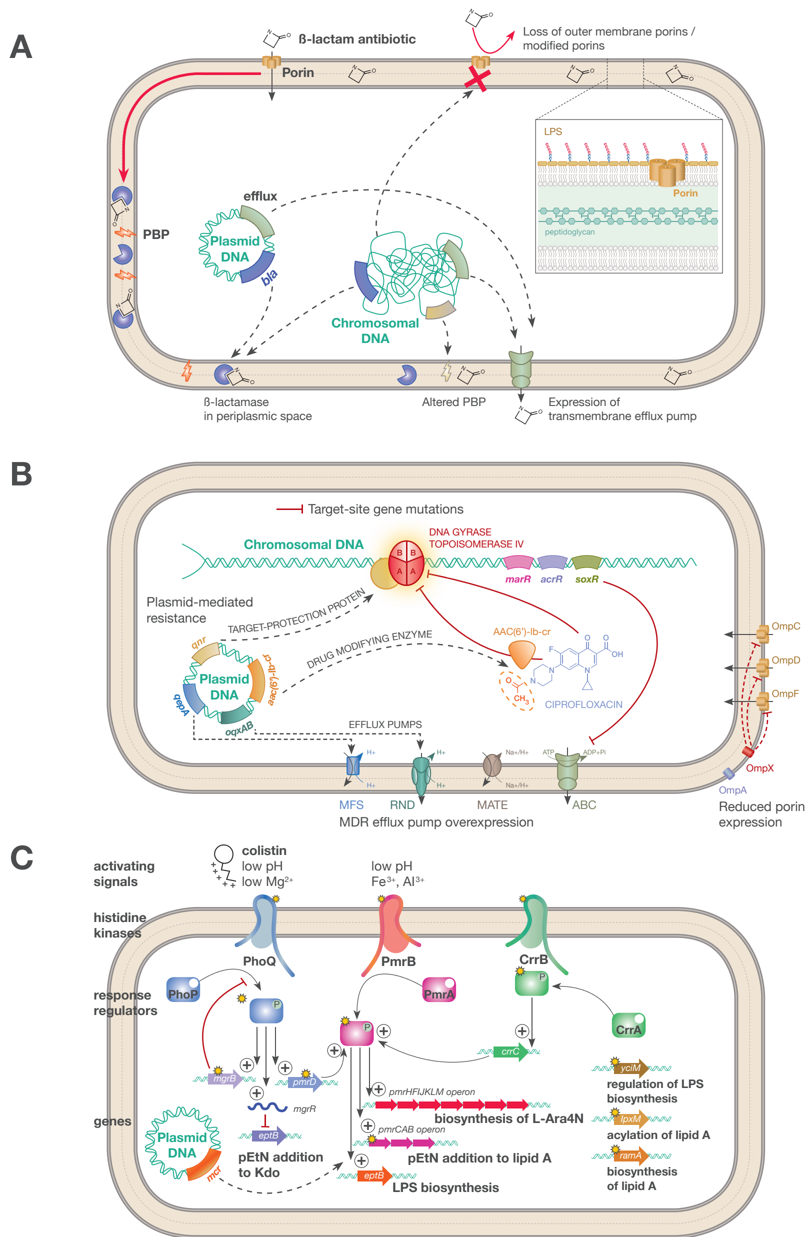

Figure 2.5: The major mechanisms of β-lactam (A), fluoroquinolone (B) and colistin resistance (C) in bacteria. *bla*: β-lactamase, Kdo: 3-deoxy-D-manno-octulosonic acid, L-Ara4N: 4-amino-4-deoxy-L-arabinose, LPS: lipopolysaccharides, *mcr*: mobile colistin resistance, MDR: multidrug resistance, PBP: penicillin-binding protein, pEtN: phosphoethanolamine. Figure adapted from (28,59,64,66).

2.3.3.2 Fluoroquinolone mode of action and resistance

Quinolones are synthetic, broad-spectrum antibiotics that prevent the bacterial cell growth by interfering with the DNA replication, recombination and repair. These antibiotics have a bactericidal effect against virtually all bacteria (17). Ciprofloxacin is a second-generation quinolone with activity against Gram-negatives and a notable activity against Gram-positive bacteria. Levofloxacin and moxifloxacin are second- and third-generation quinolones with improved Gram-positive antibacterial activity. The newest quinolone molecules, for example clinafloxacin, have a significant anaerobic activity (67). (Fluoro)quinolones sold in veterinary medicine include for example norfloxacin, enrofloxacin, marbofloxacin and flumequine (68). Quinolones are widely used for multiple clinical indications because of their potency, spectrum of activity, oral bioavailability and excellent tissue penetration. However, (fluoro)quinolone use should be restricted as it can cause serious and potentially permanent side effects involving muscles, tendons or joints and the nervous system (69). Quinolones target two essential heterotetramer enzymes, DNA gyrase (encoded by *gyrA* and *gyrB*) and DNA topoisomerase IV (encoded by *parC* and *parE*). Amino acid changes in gyrase and/or topoisomerase IV can cause quinolone resistance which are commonly localized between amino terminal domains of GyrA (residues Ala67-Gln106) or ParC (residues Ala64-Gln103) and are near the active site tyrosines of the enzymes (Tyr122 for GyrA and Tyr120 for ParC) (**Figure 2.5B**). The region has been termed the quinolone resistance determining region (QRDR) of GyrA and ParC (70,71). In this region, amino acid substitutions at residues S83 and D87 in GyrA and residues S80 and E84 in ParC are strongly associated with fluoroquinolone resistance in clinical *E. coli* isolates. Ciprofloxacin MIC steeply increases when mutations accumulate in *gyrA* and *parC* (71). The triple mutations (S83L, D87N in *gyrA* and S80I in *parC*) confer high-level ciprofloxacin resistance and are overrepresented in clinical isolates which suggest a selective advantage and low fitness costs (72).

Fluoroquinolones can also be exported from *E. coli* by efflux pumps including AcrAB-TolC (73), AcrEF (73), MdfA and YdhE/NorE (74). Chromosome-mediated mutations in

the operons of endogenous transmembrane efflux pump AcrAB-TolC can lead to overexpression of this efflux pump and contribute to resistance. Mutations that inactivate *marR* (a repressor of *marA*, a transcriptional activator of *acrAB* and *tolC*), *acrR* (a repressor of *acrAB*) or *soxR* (repressor of soxS, an activator of superoxide stress genes) can increase the efflux activity of the AcrAB-TolC multidrug efflux pump (**Figure 2.5B**) (75,76). The efflux pump regulator mutations cause, in contrast to the relatively cost-free drug-target mutations, a fitness cost (72,77,78).

Besides chromosomal mutations also plasmid-mediated quinolone resistance (PMQR) can cause increases in fluoroquinolone minimum inhibitory concentrations (MICs) (**Figure 2.5B**). To date, three mechanisms of PMQR are known. The first mechanisms involves the protection of DNA gyrase and topoisomerase IV from quinolone inhibition and is mediated by *qnrA*, *qnrB*, *qnrC, qnrD, qnrS* and *qnrVC* genes (79). More recently, CrpP, a novel ciprofloxacin-phosphorylating enzyme was detected on a plasmid harbored by *P. aeruginosa* (80). Secondly, acetylation of quinolones by a variant of aminoglycoside acetyltransferase Aac(6')-Ib-cr which reduces quinolone susceptibility. The third mechanisms involves the decreased quinolone accumulation due to quinolone efflux pumps QepAB and OqxAB. These mechanisms provide low-level resistance, however, are usually present on MDR plasmids and facilitate selection of higher-level resistance making infections with PMQR-carrying pathogens harder to treat (79).

2.3.3.3 Colistin mode of action and resistance

Colistin or polymyxin E is a penta-cationic polypeptide antibiotic discovered in 1949. The originally named "colimycin" was isolated from *Paenibacillus* (formerly named *Bacillus) polymyxa var. colistinus* by Koyama and collegues (81). The bactericidal effect of colistin is based on the interaction between the positively charged diaminobutyric acid residues of colistin with the negatively charged phosphate groups of lipid A and compete in the replacement of Ca^{2+} and Mg^{2+} ions, thereby destabilizing the LPS. Next, the N-terminal fatty acid chain and the hydrophobic domain of colistin (Leu^6-L-Leu^7) insert into

the outer membrane leading to an increase membrane permeability, leakage of cell contents and eventually cell death (28).

Colistin use in human medicine was first restricted to topical use due to its systemic toxicity. However, the last decade, colistin is increasingly used as a last-resort drug for the treatment of infections with carbapenemase-producing Enterobacteriaceae (*E. coli* and *Klebsiella* species) and MDR *Pseudomonas* and *Acinetobacter* species which are increasingly causing hospital outbreaks (82).

Colistin is also administered in animals for gastrointestinal infections caused by Enterobacteriaceae in intensive husbandry systems, mainly in prevention and group treatments of diarrhea caused by *E. coli* and *Salmonella* species in pig and poultry production and in cattle (82–84). Polymyxins have no activity towards Gram-negative cocci, Gram-positive cocci, Gram-positive bacilli, anaerobic bacteria and intrinsically resistant species including *Neisseria*, *Serratia*, *Stenotrophomonas* spp., *Providencia* spp., *Proteus* spp., *Bulkholderia pseudomallei*, *Morganella morganii* and *Edwardsiella tarda* (28).

Colistin resistance is a result of modification of LPS by addition of cationic groups phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) leading to a reduction of the net negative charge of LPS by shielding the phosphate and carboxyl groups which reduces the electrostatic interaction with the antibiotic (28). Several genera of the Enterobacteriaceae, such as *Klebsiella*, *Escherichia*, *Enterobacter* and *Salmonella,* can acquire resistance to polymyxins. Proteins responsible for the synthesis and (regulation of the) addition of these cationic groups to LPS are chromosomally encoded in the *pmrC* gene (lipid A pEtN phosphotransferase for addition of pEtN group to LPS), the *pmrE* gene and the *pmrHFIJKLM* operon (both required for the synthesis and transfer of Ara4N to LPS), *pmrA/B* and *phoP/Q* two-component systems consisting of a sensor kinase protein (PmrB and PhoQ) and a response regulator (PmrA and PhoP), *pmrD* gene (encoding an adaptor protein between two-component systems pmrAB and PhoPQ), *mgrB* (feedback inhibitor of PhoPQ system) and *crrAB* (regulation of PmrAB system) (28,85).

Mutations leading to the constitutive activation of PmrAB and PhoPQ lead to the upregulation of the *pmrCAB* operon, *pmrHFIJKLM* operon and *prmE* gene, and therefore, the synthesis and transfer of pEtN and L-Ara4N to lipid A of LPS (28,85). Other alterations to proteins YciM, LpxM, RamA and OmpW in *K. pneumoniae* are associated with the colistin-resistant phenotype (**Figure 2.5C**) (28). Stepwise increases in the number of polymyxin resistance genes with mutations and the polymyxin MIC were shown (86).

In *K. pneumoniae*, the overexpression and shedding of anionic capsule polysaccharide and the overexpression of efflux pumps, such as AcrAB and KpnEF play a role in colistin resistance (28).

In 2015, the first mobile colistin resistance (*mcr*) gene was described in a plasmid carried by an *E. coli* in China (87). The MCR-proteins are members of the pEtN transferase enzyme family. Resistance is a result of the addition of pEtN to lipid A similar to the chromosomal mutations (**Figure 2.5C**).

2.3.4 Pandemic and epidemic *E. coli* and *K. pneumoniae* lineages

The prevalence of ESBL- or carbapenemase producing *E. coli* and *K. pneumoniae* is increasing worldwide of which certain clonal lineages have an epidemic potential with their so-called 'high-risk' characteristics. Epidemic and pandemic clones are easily transmitted, persistent and able to adapt to the host environment, providing a greater opportunity for the acquisition of antibiotic resistance genes. A series of stepwise evolutionary events leading to enhanced colonization and competitiveness contribute to the success of these MDR clones (88,89).

Firstly, the rapid expansion of carbapenem resistance in *K. pneumoniae* has made carbapenem-resistant *K. pneumoniae* the fastest growing antibiotic resistance threat in Europe. Carbapenem-resistant *K. pneumoniae* has a very high impact on public health accounting for > 90 000 infections and > 7000 deaths annually in Europe alone. Carbapenem-resistant *K. pneumoniae* are able to cause major nosocomial outbreaks through dissemination of high-risk clones but also impose the risk of horizontal transfer

of MGEs carrying carbapenemase genes (9). Recent outbreaks of carbapenemase-producing and colistin-resistant *K. pneumoniae* in European Union (EU)/European Economic Area (EEA) countries show the increase in virulence, transmissibility and antimicrobial resistance among certain *K. pneumoniae* strains (90). Carbapenemase-producing isolates usually belong to four lineages of sequence type (ST) ST11, ST15, ST101 and ST258/512 which are widely distributed across Europe (89). The global spread of bla_{KPC} has been linked to the dissemination of *K. pneumoniae* ST258 (91). Two ST258 clades with distinct capsule polysaccharide gene regions have been associated with carriage of specific bla_{KPC} genes, namely, clade I with bla_{KPC-2} and clade II with bla_{KPC-3} (63). The genome of this successful pathogenic clone is a hybrid of ST11 (80% homology) and ST442 (20% homology) strains (91). Carbapenem-resistant *K. pneumoniae* of ST11 is a lineage associated with the spread of bla_{KPC-2} (92). *K. pneumoniae* ST101 is another lineage of carbapenemase-producing isolates associated with bla_{OXA-48}, bla_{KPC} and bla_{NDM}. Healthcare-associated infections with carbapenem-resistant ST101 isolates occur worldwide (93). Colistin-resistant isolates within the ST101 lineage have also been identified limiting the treatment options for this pathogen and posing a risk for the dissemination of colistin resistance (93,94). On the other hand, the detection of high-risk *K. pneumoniae* clones in animals remains scarce (95–97).

Secondly, globally disseminated MDR pathogenic clones of *E. coli* are ST131, ST1193, ST167, ST410 and ST648 (**Figure 2.6**) (63,88). *E. coli* strains are divided into phylogenetic groups A, B1, B2, C, D, E or F. Pathogenic strains generally belong to group B2 and carry virulence genes more frequently compared to group A and B1 strains, the latter are generally associated with commensal *E. coli* (98). Nowadays, *E. coli* ST131 is highly virulent in humans. It is the predominant *E. coli* lineage causing extraintestinal infections and possibly the most widely distributed resistance clone. The dissemination of *E. coli* ST131 led to a global increase in fluoroquinolone-resistant and CTX-M-type beta-lactamase-producing *E. coli* (62,64). Sequential acquisition of virulence and resistance genes was essential to the global dissemination of ST131. First, acquisition of virulence factors such as *fim*H genes, encoding the type 1 fimbrial adhesin, and

siderophore-related genes causes the bacterium to have enhanced colonization capabilities through better adhesion and cellular invasion. Next, the acquisition of fluoroquinolone resistance through chromosomal mutations confers a greater fitness advantage when the duration of carriage is extensive. The final step in the evolution of pandemic ST131 is the acquisition of MDR-inducing MGEs such as CTX-M class ESBL genes typically carried on IncF-type plasmids (88). *E. coli* ST131 is also found in animals, primarily in dogs and cats, rarely in horses, in poultry and occasionally in pigs (99). Certain human- and animal-associated ST131 isolates show a high degree of similarity with respect to resistance characteristics, virulence traits and genomic background. However, evidence for direct inter-species transfer of ST131 is currently limited (99). *E. coli* ST1193 is an emerging global MDR high-risk clone following the footsteps of ST131 and is the second most frequent clone among fluoroquinolone/cephalosporin-resistant *E. coli* (100). Another example of a pandemic *E. coli* clone in humans is ST410. ST410-B4/H24RxC gained carbapenem-resistance by acquisition of $bla_{\text{OXA-181}}$ and $bla_{\text{NDM-5}}$ in addition to the $bla_{\text{CTX-M-15}}$ harbored by ST410-B3/H24Rx.

Monitoring the prevalence and understanding the resistance mechanisms and spread of antibiotic resistance in clinical samples as well as in healthy humans, animals and the environment is essential to gain insight in the baseline levels of AMR in these niches and to implement control measures. To gain these insights, it is important to perform both genotypic and phenotypic screening and association studies (101).

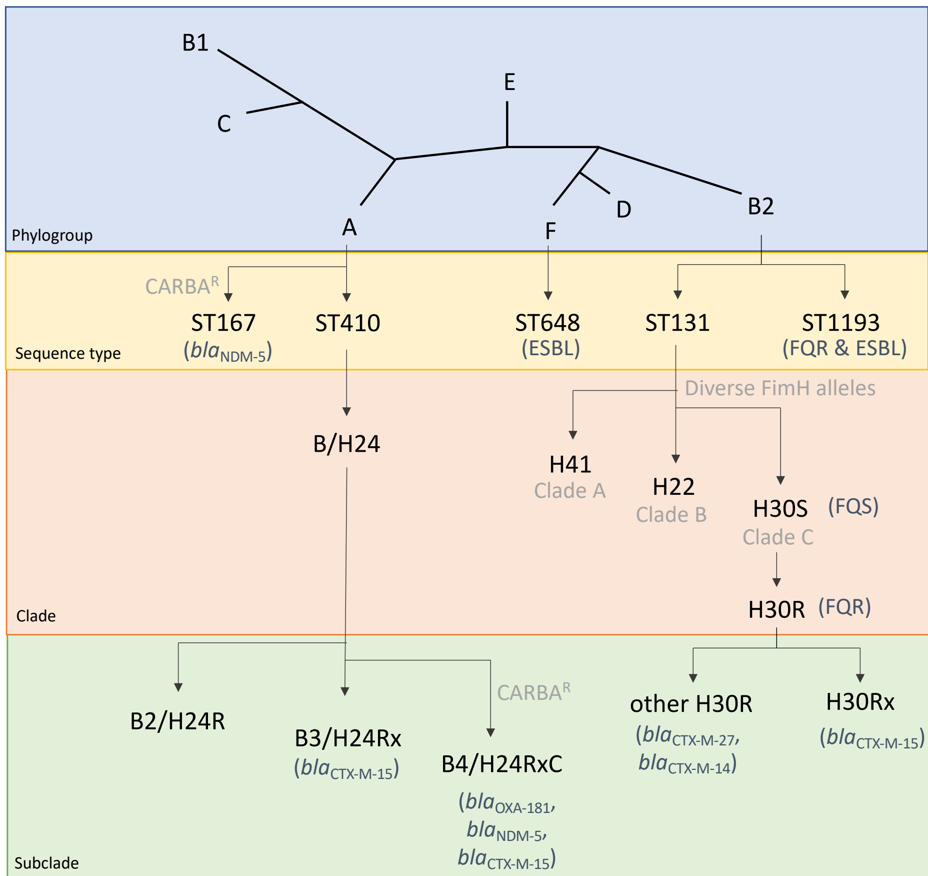

Figure 2.6: Pandemic *E. coli* and their subdivision into clades and subclades. Genetically and phenotypically diverse *E. coli* species can be categorized in phylogroups (A, B1, B2, C, D, E or F) and into sequence types (ST). *E. coli* from a monophyletic group can be split into clades and bacteria that share common phenotypic or genotypic traits can be defined as clones. CARBA^R: carbapenem-resistant, FQS: fluoroquinolone-sensitive, FQR: fluoroquinolone-resistant. Figure adapted from (88).

2.4 Detection and characterization of antibiotic-resistant bacteria

Accurate and fast detection of antibiotic-resistant bacteria are crucial for appropriate antibiotic treatment selection and to reduce the misuse of antibiotics (102). In addition, data on the susceptibility or resistance of bacterial isolates are collected and analyzed for surveillance purposes and antimicrobial stewardship programs. A range of methods are available to detect antibiotic resistance both phenotypically and genotypically. Routinely used methods in diagnostic laboratories involve plating of samples (e.g. feces or sputum) and antibiotic susceptibility testing (AST). More recently, innovative tools such as surface-enhanced Raman scattering spectroscopy, Fourier-transform infrared spectroscopy (FTIR), and MALDI-TOF mass spectrometry were introduced for the fast identification and characterization of micro-organisms. Pathogen identification by Raman spectroscopy can be used to detect single bacterial cells directly in samples by using magnetic separation, centrifugation and filtration to isolate bacteria and amplifying signals using nanoparticles. FTIR can generate spectra based on the absorption of infrared radiation by proteins, lipids and sugars in the bacterial cell. By discriminating isolates based on the differences in the surface cell polysaccharides, fast bacterial typing and outbreak analysis has become possible. MALDI-TOF mass spectrometry allows fast and accurate bacterial identification as well as the detection of specific resistance biomarkers by detecting a resistance peak pattern, by detecting the mass shifts during hydrolysis of β-lactam antibiotics or by detecting bacterial growth in the presence and absence of antibiotics (103–105). Furthermore, basic molecular methods such as nucleic acid amplification-based techniques like polymerase chain reaction (PCR) for resistance gene detection are used in diagnostic laboratories. Other, state-of-the-art molecular methods are mainly used as research tools. Examples are WGS and metagenomics, techniques to determine the complete genetic content of a cell or sample (101). In the next sections, we focus on conventional culture-based methods and molecular methods to detect and characterize antibiotic-resistant bacteria.

2.4.1 Phenotypic methods for the detection of antibiotic-resistant bacteria

Conventional culture-based methods employ phenotypic resistance detection by assessing bacterial growth in the presence of antibiotics and inform clinical treatment decisions. Typically, pure culture isolates from the sample (blood, urine, mucosal, fecal) are used for AST (**Figure 2.7A**). These growth-based screening methods require several cultivation steps such as plating samples on non-selective or antibiotic-selective agar plates to obtain pure cultures which is sometimes preceded by enrichment to increase the number of bacteria before AST can be applied. Antibiotics are tested using agar dilution, gradient test, disk diffusion and broth microdilution methods (102,106) (**Figure 2.7B**). This can be done manually or in an automated manner. Clinical breakpoint or epidemiological cut-off values are determined by European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI) to determine if a bacterial isolate is sensitive or resistant to the antibiotics (101,106). Advantages of these methods are the low cost and the ability to detect the expressed resistance levels, measured by the MIC (gold standard) or the zone diameter in disk diffusion testing. However, disadvantages are phenotypic testing may be generally lengthy (usually days), is only viable for cultivable bacteria and, unlike the molecular methods, phenotypic methods lack the ability to provide insights into the underlying molecular mechanisms of resistance (107).

2.4.2 Molecular methods for the detection and typing of antibiotic-resistant bacteria

Molecular methods can offer a more precise characterization of resistant bacteria and gives insights into the resistance mechanisms by detecting antibiotic resistance-coding genes, gene products or mutations associated with resistance. Currently, molecular tests for AMR include (i) WGS of resistant bacteria, (ii) (shotgun or targeted) metagenomics for direct sample testing, (iii) hybridization-based test to detect hybridized nucleic acid probes of target gene sequences (such as DNA microarray), (iv) amplification-based tests to allow detection of a number of copies of target gene sequence (such as PCR) and (v)

immunoassays to detect AMR gene products by binding to specific antibodies (such as lateral flow immunoassays) (107,108).

Of these molecular tests, sequencing has the highest discriminatory power (102,109). Sequencing can be culture-dependent and applied to DNA from bacterial isolates (referred to as WGS) or culture-independent and applied to the whole community of microorganism DNA from a sample without isolating or culturing a specific organism (referred to as metagenomics) (**Figure 2.7C**). Sequencing technologies and computational methods are facilitating rapid pathogen identification, epidemiological typing and detection of antibiotic resistance genes in bacterial genome and metagenome sequencing data (110). This technique improves the understanding of bacterial evolution, outbreaks and transmission events as well as molecular mechanisms of resistance, virulence and pathogenicity (**Figure 2.7D**) (102,109).

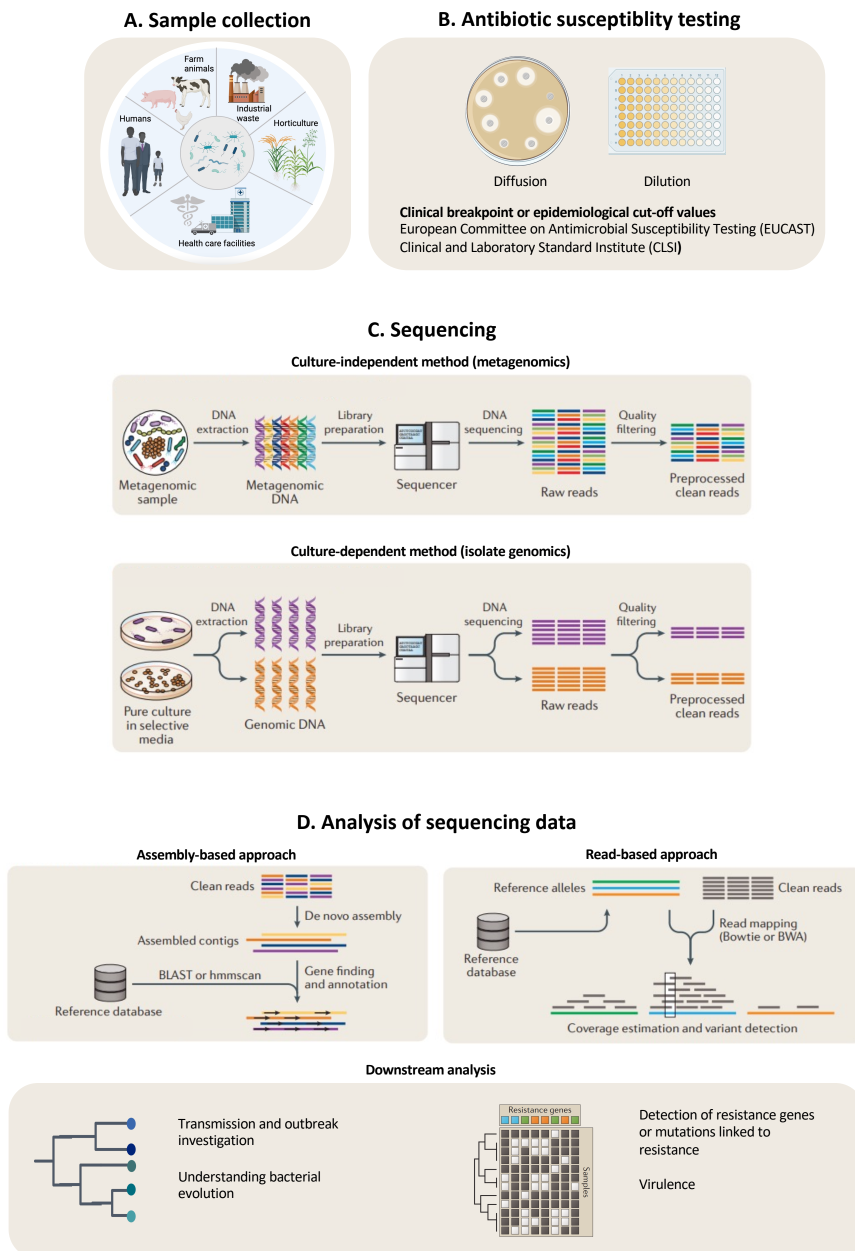

Figure 2.7: Methods to detect and characterize antibiotic-resistant bacteria. (A) Sample collection, (B) phenotypic detection of antibiotic resistance, (C) sequencing approaches to detect antibiotic resistance genes and antibiotic-resistant bacteria, (D) downstream analysis methods of *in silico* characterization and profiling of resistant bacteria. Figure adapted from (110). Created with BioRender.com.

2.4.2.1 Whole genome sequencing and metagenomic sequencing to investigate and control antimicrobial resistance

(This section is part of a publication by De Koster, S.; Rodriguez Ruiz, J.P.; Glupczynski, Y.; Goossens, H.; Xavier, B.B.. Methodological guidance for clinical metagenomics and antimicrobial resistance research. Microb Health Dis 2022, 4, 3. doi:10.26355/MHD_20229_773)

WGS and metagenomic sequencing data are generated by short-read sequencing technologies (Illumina/Ion Torrent) or long-read sequencing technologies (nanopore sequencing (MinION) and single-molecule real-time sequencing (SMRT-seq, PacBio)). For antimicrobial resistance gene analysis, two approaches can be used. The first, assembly-based approach involves sequencing reads to be assembled into contiguous fragments (contigs) followed by annotation and comparison to reference databases. When using the second, read-based approach, sequencing reads can be directly mapped to a reference database and is mostly used for metagenomics data (110) (**Figure 2.7D**).

Shotgun metagenomic sequencing allows for the identification of all the genes present in the sample without the selection of a specific gene (2,111). Thus, metagenomic sequencing allows for an in-depth characterization of the microbiota and all AMR genes (resistome) directly from samples, for example, fecal, food, environmental samples and samples that are recalcitrant to culture (2,112). Determining all AMR genes in the sample will help understand the complex interactions between organisms, genes and their environment. However, for effective AMR surveillance, the focus should be on clinically relevant yet low abundant AMR genes (such as ESBLs and carbapenemases) and horizontal gene transfer events, which are generally rare (113). The abundance of critical resistant pathogens, such as ESBL or carbapenemase-producing Enterobacteriaceae, is often below the detection threshold of direct sequencing. This is a major limitation when characterizing the gut resistome (14,114). In this case, direct metagenomic shotgun sequencing might suffer from low sensitivity in detecting minority populations harboring resistance genes and/or low specificity in identifying allelic variants (61,114). All resistance sequences account for less than 1% of the total sequenced DNA, indicating that

the proportion of these genes is relatively low, and even deep sequencing may not be able to capture these elements in the total gene pool present in the samples (113,115,116). Selective culture-enrichment of stool samples can be used to identify low abundance pathogens within the microbiome but hampers the quantification of the resistome because of differential growth rates of bacteria (14). Merging culture-dependent and- independent techniques could provide more profound resolution and help better understand microbial communities, including low abundant species (111). However, there is currently a lack of validated enrichment methods, and, additionally, the combined use of such techniques would significantly increase the complexity and costs. Another method to enrich resistance genes and genes involved in DNA mobilization is the use of a capture library in targeted metagenomics. Target capture enriches resistance genes directly from standard metagenomic DNA extractions and increases the proportion of sequenced reads mapping to resistance genes. This can increase sensitivity and improve the identification of resistance genes within a complex metagenome background (114,115). However, resistance genes that are not present in the reference database when the probe libraries are designed might not be captured and might be missed. This emphasizes the challenge of continuously updating the probe libraries to include all known resistance genes and shows that genes might be missed.

In summary, these techniques are limited because only known resistance genes or mutations are detected and presence of the gene does not necessarily mean that the gene is always expressed to cause antibiotic resistance (107). Comprehensive databases that link specific antimicrobial resistance genes to specific AST results are needed. To date, accurately predicting phenotypic antibiotic resistance from genotypic data is still inconclusive, because there is no consensus in phenotypic methods and expression of the gene is not always tested. Therefore, molecular tests are nowadays complementing traditional culture-based methods for clinical and surveillance applications and provide insight into the global distribution of antimicrobial resistance genes (110).

2.4.2.2 Whole genome sequencing to type bacterial isolates

In addition to detecting antibiotic resistance genes in bacteria or samples, WGS of bacterial isolates can be used to type and discriminate different bacterial isolates. Traditional typing methods based on phenotypes such as serotype and phage-type and other typing methods such as 'pulsed-field' gel electrophoresis (PFGE), amplified fragment length polymorphisms (AFLP) and variable-number tandem repeat (VNTR) typing are now replaced by WGS to investigate bacterial transmission and outbreaks. WGS can discover genome-wide variations. In outbreak investigations, WGS provides the discriminatory power to distinguish all epidemiologically unrelated isolates to reveal person-to-person transmission (117). Determining the ST is widely used in when typing bacteria because of the internationally standardized nomenclature and high reproducibility. In multilocus sequence typing (MLST), internal sequences of mostly seven housekeeping genes are amplified and sequenced. Each locus is assigned arbitrary numbers and based on the combination of identified alleles (called the allelic profile), the ST is determined. Using WGS, this conventional MLST can be expanded to core genome (cg) or whole genome (wg) MLST composed of thousands of different alleles from the core or whole genome to obtain higher discriminatory power making it possible to distinguish very closely related isolates. Alternatively, this can also be done by comparing different genomes and identifying single nucleotide polymorphisms (SNPs). Thus, WGS allows an accurate characterization of transmission events and outbreaks (117). Altogether, WGS became an essential tool to monitor and trace antibiotic resistance. Applications range from the diagnostic tests to antibiotic stewardship via surveillance and elucidation of the emergence and persistence of resistance. WGS has already provided insights into the history of the emergence and spread of antibiotic resistance. This technique is also able to shed light on transmission between humans and animals and can greatly enhance surveillance programs for antibiotic resistance (118,119).

2.5 National and international surveillance of antibiotic use and resistance in hospitals, the community and livestock

Surveillance of antibiotic use and resistance is one of the cornerstones in the control of infectious diseases. The link between antibiotic consumption and the development of antibiotic resistance has been demonstrated by several studies (120,121). In addition, resistance to one specific antibiotic agent can lead to resistance to a whole related class and resistance can spread rapidly from one organism or location to another through exchange of genetic material (6). Therefore, monitoring of antibiotic consumption and resistance is essential for evidence-based risk assessment and guidance of interventions to reduce AMR. Several national, European and global surveillance reports on antibiotic use and antibiotic resistance exist. In Belgium, the Belgian Veterinary Surveillance of Antimicrobial Consumption (BelVet-SAC) report addresses antibiotic sales data and usage data in farms, while BeH-SAC monitors the antimicrobial consumption in Belgian hospitals. In addition, the BELMAP report summarizes the antimicrobial use and resistance across sectors (9,122,123). In the Netherlands, surveillance of antibiotic resistance and use in the livestock industry and humans is summarized in the Nethmap-MARAN report (124). The European Centre of Disease Prevention and Control (ECDC) provides data on antimicrobial consumption in the European Union (EU)/European Economic Area (EEA) via the European Surveillance of Antimicrobial Consumption Network (ESAC-NET) (125) and on the invasive, resistant bacteria in humans (EARS-NET) (126). Point prevalence studies (PPS) of healthcare-associated infections (HAIs) and antimicrobial use in hospitalized inpatients are organized in Europe (ECDC-PPS) and on a worldwide level (Global-PPS) (127). The ECDC organizes HALT studies which are European point prevalence surveys to monitor antimicrobial use and healthcare-associated infections in LTCFs (128). The European Medicines Agency (EMA) monitors the sales of veterinary antimicrobials in European countries in the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) (129). The European Food Safety Authority (EFSA) joint with ECDC and EMA monitors the antimicrobial use in humans

and food-producing animals in the EU/EEA. On a global level, the WHO and World Organization for Animal Health (OIE) monitor antibiotic use and resistance.

2.5.1 Antibiotic use and resistance in farms globally, in Europe and in Belgium and the Netherlands

In veterinary medicine, data on antibiotic use is based on sales or delivery, prescription, and administration data. The use of antibiotics as growth promotors was banned in the EU since 2006 (129). In contrast, antibiotic growth promotors in animals are still used by 40/157 countries participating in the OIE annual report (mainly in the Americas, Far East Asia and Oceania). Globally, tetracycline and penicillin antibiotics are used most often in animals (130). On the other hand, antimicrobial use in food-producing animals is reducing over time. While the livestock production expressed in biomass is increasing (+1.88% between 2020 and 2021 and +3.6% compared to 2011, mainly in dairy cattle and broiler production), the antibiotic use is drastically decreasing in Belgium (-5.6% in the total sales of antibacterial products between 2020 and 2021 and -42.6% compared to 2011). However, increased efforts are needed as the reduction goal for 2024 is up to -65% (122) and compared to neighboring countries (France, Luxemburg, Germany, UK, the Netherlands) with relatively comparable livestock farming structures, the use in Belgian livestock remains high (103.4 mg/population correction unit (PCU) in Belgium and 50.2 mg/PCU in the Netherlands) (122,129). In the Netherlands, a spectacular decrease in sales of 70.8% was obtained between 2009 and 2021. Both in the Dutch broiler and pig farming sectors, the lowest sales since the start of the monitoring was recorded in 2021 (124).

In Belgium, veal calves, weaned piglets and broilers are the animal categories with the highest use of antibacterials (122,131). The median farm-level number of treatment days per 100 days (BD100) is 14.18 in weaners, 3.49 in broilers and 16.26 in veal calves in 2021 (122,131). In these species, macrolides, sulphonamides, polymyxins, tetracyclines and penicillins are predominantly used (9).

Both in Belgium and the Netherlands, the number of farms with persistently high usage antibiotic levels (i.e. higher than the BD100 action value based on the national

benchmark) remains high with 9% of the Belgian pig farms (9) and 13.8% of the Dutch farms (132) being repeatedly high antibiotic users. In contrast to the piglets and fatteners, the persistently high use in the conventional broiler farms has decreased in both countries (122,131,132).

In Belgium, the use of the critically important molecules (such as 3rd and 4th generation cephalosporins and fluoroquinolones, products of highest importance for human medicine that should be avoided in veterinary medicine as much as possible) dropped by -82.9% which is well under the reduction goal of -75% by 2024 (122,131). Also in the Netherlands, the use of drugs of last resort for human medicine remained low in 2021 (124).

In Belgium and the Netherlands, a decreasing or stabilizing trend in the prevalence of antibiotic resistance is observed. An increase in the prevalence of sensitive *E. coli* isolates is seen in pigs (+8.2%) and broilers (+3.8%) from 2014 to 2021 (124,131). In Belgium, the prevalence of ciprofloxacin, colistin and cephalosporin resistance in commensal *E. coli* from food-producing animals remained stable and below 10% over the years (2011-2020). The levels of MDR *E. coli* are highest in poultry in both countries (9,124).

2.5.2 Antibiotic use and resistance in the community and hospital sector in Europe, Belgium and the Netherlands

For both the community and the hospital sector, consumption data per country in the EU was based on the sales of antibiotics or a combination of sales and reimbursement data and is often quantified using ‘defined daily doses (DDD) per 1000 inhabitants per day’.

Both acute care hospitals and LTCFs have high numbers of HAIs up to a total of 8.9 million HAI episodes annually in the EU/EEA (133).

The median prevalence of residents in LTCFs treated with antibiotics was 5.9% in Belgium and 4.4% in the Netherlands in 2016 which was higher than the EU/EEA median prevalence of 3.6%. In contrast to the Netherlands, Belgium does not have national guidelines on antibiotic prescription specific for the elderly patient population. In LTCFs,

antimicrobials are commonly prescribed for the treatment of infections (around 65%), predominantly for respiratory tract infections, urinary tract infections and skin/wound infections and for uroprophylaxis (around 28%). The most frequently prescribed antibiotics are beta-lactams, penicillins, quinolones, sulfonamides and trimethoprim (134,135). In the Netherlands, large variations in the total antibiotic use across different LTCF organizations was observed and the increasingly high use of fluoroquinolones is LTCFs is worrisome (124).

Overall, in Europe, total antibiotic use in the community and hospitals decreased by 17% between 2019 and 2020 based on DDD per 1000 inhabitants per day, most likely a result of the COVID-19 pandemic. These decreases are generally larger in the community (18.3%) than in the hospitals (4.5%). However, an overall shift towards higher consumption of broad-spectrum antibiotics is observed for both the community and hospital sector (125).

Although decreasing trends in antibiotic use in the community over the last 10 years are observed in Belgium and the Netherlands, the consumption of antibiotics for systemic use in the community in Belgium (15.3 DDD per 1000 inhabitants per day) is almost double compared to the Netherlands (7.8 DDD per 1000 inhabitants per day) and is slightly higher than the mean consumption in EU/EEA countries (15.0 DDD per 1000 inhabitants per day). Besides Austria, the Netherlands have the lowest consumption of antibiotics in the community in Europe. Similarly, the Netherlands have the lowest consumption of antibiotics in the hospitals in the EU/EEA (0.76 DDD per 1000 inhabitants per day) which is half of the amount that is used in Belgium (1.4 DDD per 1000 inhabitants per day) and the EU (1.57 DDD per 1000 inhabitants per day). Amoxicillin, amoxicillin/clavulanic acid, nitrofurantoin, azithromycin and cefuroxime were the most used in the Belgian ambulant setting. The overall ratio of broad-to-narrow spectrum antibiotics declined significantly, however was still far from the BAPCOC target. In the Belgian hospitals, the total consumption of antibiotics is higher compared to the Netherlands, but comparable with France, Sweden and Denmark. In the Belgian acute care hospitals, a decrease in overall consumption of antibiotics for systemic use (-17%) was observed over the last

decade based on the indicator DDDs per 1000 patient days. Amoxicillin/clavulanic acid, cefazolin, piperacillin/tazobactam, flucloxacillin and ciprofloxacin are the five most used products in these hospitals (136). In Belgium, BeH-SAC allows for the comparison on antimicrobial consumption between hospitals (benchmarking). High variations in antibiotic consumption between acute care hospitals was observed along with the high use of broad-spectrum antibiotics (especially fluoroquinolones such as ciprofloxacin, moxifloxacin and levofloxacin). Wards using the most antibiotics are intensive care units (ICUs), burn units and pediatrics. An increase in the use of piperacillin with a beta-lactamase inhibitors, carbapenems, beta-lactamase resistant penicillins and penicillins was observed over the last decade (9,123).

2.5.3 Cephalosporin and carbapenem use and resistance in the veterinary and human sectors in Europe, Belgium and the Netherlands

Third and fourth generation cephalosporins are products of the highest importance for human medicine and should be used under very strict conditions in livestock (122). Overall in the veterinary sector, third and fourth generation cephalosporins account for 0.2% of the total sales of antibiotics in the EU in 2020. A decreasing trend of 32.8% in these cephalosporin sales is seen in the EU between 2010 and 2020 (129). In the Belgian farms, a decrease in the sales of third and fourth generation cephalosporins of -13.3% was observed in 2021 compared to 2020. In Belgian pigs, the use of these cephalosporins completely ceased in 2021 (122,131). Also in the Netherlands, sales of third and fourth generation cephalosporins dropped to very low amounts (<0.01 mg/PCU) (129).

In Belgium, non-selective monitoring of ESBL-producing *E. coli* (ESBL-*E. coli*) from pigs and broilers showed relatively low prevalences (maximally 10%), while selective monitoring showed prevalences of 78.5% in broilers and 40.3% in pigs in 2021 (131). In the Netherlands, randomly selected indicator *E. coli* were not resistant to extended-spectrum cephalosporins in cecal samples of broilers or in slaughter pigs. Active screening by selective isolation of resistant *E. coli* showed 11.3% ESBL-producing *E.*

coli in broilers and 9.3% in pigs. The prevalence of ESBL-*E. coli* was 1.2% in pork and 19.3% in chicken meat in 2021. *bla*$_{CTX-M}$ was the most prevalent ESBL gene (124). Resistance to meropenem was not observed in broiler chickens and slaughter pigs and no carbapenemase-producing Enterobacteriaceae were detected in livestock (124).

Carbapenems are not used in veterinary medicine (129), however, the EU/EEA population-weighted mean consumption in human medicine showed a statistically significant increase. The use of carbapenems in the hospitals was 0.05 DDD per 1000 inhabitants per day in 2020. In contrast, the use of carbapenems significantly decreased in Belgium between 2011 and 2020 (125).

As in livestock, parentally administered drugs (intramuscular or intravenous) such as cephalosporins and carbapenems are only being prescribed very infrequently in LTCF residents (10% of prescribed antimicrobials in European LTCFs) (135,137). The prevalence of ESBL-producing Enterobacteriaceae among nursing home residents in Belgium was 6.2% in 2011 (138) and increased to 11.3% in 2015 (137). CTX-M-1 group ESBLs and especially CTX-M-15 were predominant among these ESBL producers. The prevalence of CPE was low (< 0.1%) in Belgian LTCF in 2015 (137). This was in line with another study which did not detect any carbapenem-resistant Enterobacteriaceae in LTCF in Belgium and the Netherlands in 2016 and 2017 (133).

The use of 2nd and 3rd generation cephalosporins increased in the Dutch hospitals (124). The percentage of MDR in Enterobacteriaceae in Dutch primary care, hospital departments and LTCF was generally low (<10%). In the Netherlands, the percentages of ESBLs slightly decreased for *E. coli* and *K. pneumoniae* from 2019 to 2021 except for the ICU where a sharp increase in ESBL-producing *K. pneumoniae* was observed (12% in 2019 to 15% in 2021). The prevalence of carbapenem-resistant Enterobacteriaceae in acute care hospitals was 1.2% in Belgium and 2.7% in the Netherlands (133). The most frequently identified carbapenemase-encoding gene is *bla*$_{OXA-48}$ (124).

Among human pathogens in Belgium, the prevalence of invasive *E. coli* resistant to 3rd generation cephalosporins (10% in 2019) and carbapenem-resistant *K. pneumoniae* (1.2% in 2019) increased over time. This is due to the spread of CTX-M-family ESBLs causing

resistance to 3rd generation cephalosporins. Another major event is the global spread of the very successful clone *E. coli* ST131 over the last two decades. Detailed molecular surveillance on carbapenem-resistant *K. pneumoniae* is not performed and therefore the proportion of carbapenemase producers among carbapenem-resistant Enterobacteriaceae and the distribution of the carbapenemase family types is currently unknown (9).

2.5.4 Fluoroquinolone use and resistance in the veterinary and human sectors in Europe, Belgium and the Netherlands

The median value of the sales of fluoroquinolones in veterinary medicine overall accounted for 2.6% of the total antibiotics sales in the EU in 2020 (129). Fluoroquinolones are used predominantly in broilers (9). A decreasing trend in the fluoroquinolone sales is seen in Europe, Belgian and Dutch livestock from 2010 to 2020. An overall reduction of 92.1% in the sales of fluoroquinolones was realized in the Netherlands since 2011 resulting in very low sales (<0.01 mg/PCU) in veterinary medicine in the Netherlands (124,129). In Belgium, a spectacular decrease in the use of quinolones of -45.9% was observed in veterinary medicine in 2021 which is largely due to the reduced use of flumequine (mainly applied in poultry) and a moderate decrease in enrofloxacin and marbofloxacin. The use of fluoroquinolones in pigs remained stable and very low (122). Although fluoroquinolones are still used predominantly in poultry, the use of fluoroquinolones in veal calves increased strongly (27.0 kg in 2021 to 62.3 kg used in 2022). In both the poultry and veal calve sectors, increased efforts to reduce fluoroquinolone use are needed (139).

In Dutch livestock, the highest levels of resistance to fluoroquinolones were found in *E. coli* from broilers. Resistance to ciprofloxacin was detected in 27.3% of *E. coli* from broilers and in 2% of *E. coli* from pigs. Fluoroquinolone resistance was also detected in bovine meat (8.1%), but was absent in pig meat (124).

In the hospital sector, the use of quinolones significantly decreased in the 10-year trends of the EU/EEA mean (125). In the Netherlands, there was a significant and clinically

relevant decrease in resistance to ciprofloxacin from 11% to 6% in *K. pneumoniae* from primary care patients, was lower than 10% in *K. pneumoniae* and *E. cloacae* complex from hospital patients and decreased from 28% in 2017 to 23% in 2021 in *E. coli* from urology patients (124).

2.5.5 Polymyxin use and resistance in the veterinary and human sectors in Europe, Belgium and the Netherlands

Although the sales of polymyxins in animals decreased by nearly 70% between 2011 and 2018 and are restricted for use to treat clinical conditions when no other antibiotics are available in veterinary medicine, the use of colistin in food-producing animals by far outweighed the consumption in humans in 2017 (140). In addition, although colistin is considered a highest priority critically important antimicrobial for use in humans, it is still reported to be used as growth promotor in animals in six countries in of which one in Africa, four countries in the Americas and one country in Asia, Far East and Oceania (130).

The median consumption of polymyxins in animals was 1.8 mg/kg estimated biomass compared to 0.04 mg/kg estimated biomass in humans in Europe. This use can be explained by the limited availability of alternative antibiotics for the treatment of colibacillosis (e.g. weaning diarrhea in pigs) caused by resistant bacteria. The use of colistin was significantly associated with resistance to polymyxins in *E. coli* from food-animals, especially from poultry and pigs (140).

However, a decreasing trend in the polymyxin sales is seen in livestock from 2010 to 2020. During this period, a decrease of 76.5% was observed (from 10.98 mg/PCU to 2.58 mg/PCU) in the EU (129). Both in Belgium and the Netherlands, colistin sales decreased more than 75% since 2011 (122,124,131). Overall, the sales of polymyxins accounted for 2.8% of the total sales of antibiotics in 2020 in the EU (129). Pigs remain the species with the largest use of colistin, especially weaner pigs, which are treated with colistin against enteropathogenic *E. coli* infections. The majority of colistin is used in oral group

treatment (122,124,131). Almost no resistance to colistin was observed in the Belgian farms according to the Antimicrobial Consumption and Resistance in Animals (AMCRA) report (122,131). No colistin resistance was detected in indicator *E. coli* from broiler chickens and slaughter pigs in 2021. Using PCR, *mcr-1* positive *E. coli* were identified in 2% of the broiler samples and 0.3% of the samples from pigs (124).
In contrast to veterinary medicine, colistin use in intensive care in EU/EEA hospitals is increasing due to the limited choice of treatment for serious invasive infections caused by MDR Gram-negative bacteria. Currently, the EARS-NET report does not include testing on colistin resistance in the human sector (140). According to BeH-SAC, the consumption of polymyxins in Belgian acute care hospitals was stable between 2013 and 2017 (2.5 DDD/1000 patient days in 2013 and 2.6 DDD/1000 patient days in 2017) (123). Polymyxin use was lower in Dutch hospitals (0.001 DDD/1000 patient days in 2017 and 2020). In Dutch LTCF, polymyxins for systemic use was 0-0.1 DDD/1000 residents/day (141). In Belgium, colistin resistance in pathogenic *E. coli* from humans and animals remains below 1% (136).

These findings show that major efforts have been taken to reduce the antibiotic use and resistance in Europe. However, it also shows that there are several knowledge gaps and that there is still room for improvement. The WHO prepared a global action plan on AMR involving five objectives: (i) improving awareness and understanding of AMR, (ii) strengthen knowledge and evidence through surveillance and research, (iii) reducing the incidence of infections, (iv) optimizing the use of antimicrobials in human and animal health, and (v) develop the economic case for interventions (new antimicrobial medicines, diagnostic tools and vaccines). These objectives can only be achieved by a 'One Health' approach involving coordination among different sectors and actors such as human and veterinary medicine, agriculture, finance, environment and well-informed consumers (6).

2.6 The One Health approach: tackling antibiotic resistance across borders and across sectors

Bacteria can be found in water, soil, air, humans, animals and plants either in a commensal (non-disease causing) or pathogenic (infection causing) relationship. The main drivers for the exchange of AMR bacteria across these niches are the inappropriate use of antibiotics, inadequate infection prevention and control measures, poor farming husbandry, food processing and distribution practices and inadequate sewage and waste management (142). MDR or XDR Enterobacteriaceae residing in the human and animal gut can be disseminated via direct contact, agricultural and human waste as well as via unhygienically slaughter practices or contaminated food (16). Thus, human and animal health are interconnected as bacteria are transmitted from humans to animals and vice versa (**Figure 2.8)**. Circulation of resistant bacteria is influenced by trade, travel and both human and animal migration. Also, groups of people living in close proximity such as in daycare centers or in LTCFs are affected by antibiotic use that select for colonizing or infecting resistant pathogens that can freely move between the hospital and the community (64). This can result in complicated transmission paths between humans, the environment and animals and from one country to another, making antibiotic resistance a complex epidemiological issue (6,16). Antibiotic resistance is a global risk beyond the capacity of any organization or nation to manage or mitigate alone. Besides action across borders, it also requires action across sectors including human and animal health sectors, agriculture, food security and economic development (6). Thus, the interlinkage between human and animal health requires a transdisciplinary approach. An integrated, multisectoral One Health approach is needed to battle antibiotic resistance in the hospital, community and livestock sectors (**Box 2.1**).

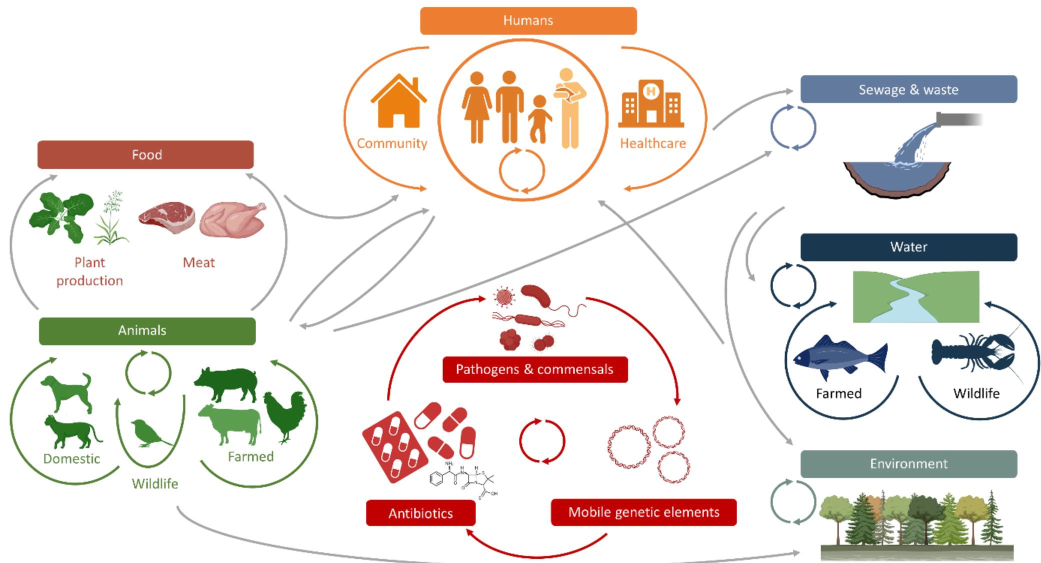

Figure 2.8: Possible transmission routes of antibiotic resistance. The integrated ecosystems of humans, animals and the environment illustrate the importance of the One Health approach to battle antibiotic resistance. Created with BioRender.com. Figure adapted from (16,143).

Cross-sectorial and coordinated actions are needed to better understand the epidemiology, the emergence and prevalence of antibiotic resistance in healthcare, animal husbandry and food production sectors and the investigation of how resistance develops and spreads (144). All countries should have a national action plan on AMR (6). Strategies to reduce AMR, slow down the development of AMR and preserve the effectiveness of antimicrobials is referred to as antimicrobial stewardship (144). Accurate detection and quantification of the gut resistome can guide such antimicrobial stewardship strategies (14). Both the EU One Health Action plan against AMR and the WHO global action plan recommend an enhanced detection and epidemiological surveillance of resistant microorganisms and disclosure of this information to guide control strategies (6,144).

Box 2.1: One Health definitions

“***One Health** is a collaborative, multisectoral, and transdisciplinary approach- working at the local, regional, national and global levels- with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants and their shared environment.”*- Centers for Disease Control and Prevention (145)

“***One Health** is an integrated, unifying approach to balance and optimize the health of people, animals and the environment. The approach mobilizes multiple sectors, disciplines and communities to work together. The One Health approach is particularly relevant for food and water safety, nutrition, the control of zoonoses, pollution management and combatting antimicrobial resistance.”*- WHO (146)

“*The **One Health** approach is a collaborative, whole of society, whole of government approach to understanding, anticipating and addressing the risk to global health*”- World Organization for Animal Health (147)

“***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.*” – One Health High Level Expert Panel of the joint Food and Agriculture Organization of the United Nations (FAO), OIE, the United Nations Environment Programme (UNEP) and the WHO (148).

Using harmonized monitoring and research, insights in the transmission of AMR between animals and humans and the risk to human and animal health should be developed. However, currently, harmonized data on AMR in a One Health context is very limited or not existing. Harmonization will improve the understanding of the transmission within and between sectors of human, animal and environmental health (9).

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CHAPTER 3

ESBL-producing, carbapenem- and ciprofloxacin-resistant *Escherichia coli* in Belgian and Dutch broiler and pig farms: a cross-sectional and cross-border study

Published as **De Koster, S**.; Ringenier, M.; Lammens, C.; Stegeman, A.; Tobias, T.; Velkers, F.; Vernooij, H.; Kluytmans-van den Bergh, M.; Kluytmans, J.; Dewulf, J.; Goossens H., on behalf of the i-4-1-Health Study Group*. ESBL-Producing, Carbapenem- and Ciprofloxacin-Resistant Escherichia coli in Belgian and Dutch Broiler and Pig Farms: A Cross-Sectional and Cross-Border Study. Antibiotics 2021,10, 945. doi: 10.3390/antibiotics10080945

*see p 241-242

3.1 Abstract

Background: The use of antibiotics in food production selects for resistant bacteria and may cause a threat to human and animal health. Belgium and the Netherlands have one of the highest densities of broilers and pigs in Europe, making active monitoring of antibiotic use and resistance in this region vital. This study aimed to quantify ESBL-producing (ESBL-*E. coli*), carbapenem- and ciprofloxacin-resistant (CiproR) *Escherichia coli* in animal feces on broiler and pig farms with a history of high antibiotic use in Belgium and the Netherlands.

Methods: A total of 779 broiler and 817 pig fecal samples, collected from 29 conventional broiler and 31 multiplier pig farms in the cross-border region of Belgium and the Netherlands, were screened for the presence of antibiotic-resistant *E. coli* using selective culturing.

Results: Carbapenem-resistant *E. coli* were not detected. ESBL-*E. coli* were remarkably more prevalent in samples from Belgian than Dutch farms. However, CiproR-*E. coli* were highly prevalent in broilers of both countries. The percentage of samples with ESBL- and CiproR-*E. coli* was lower in pig compared to poultry farms and varied between farms. No clear association with the on-farm antibiotic use in the year preceding sampling was observed. Multidrug resistance was frequently observed in samples from both countries, but ESBL-production in combination with ciprofloxacin resistance was higher in samples from Belgium.

Conclusions: This study demonstrated marked differences in antibiotic resistance between countries, farms and within farms. The observed variation cannot be explained straightforward by prior quantity of antibiotic use suggesting that it results from more complex interactions that warrant further investigation.

3.2 Introduction

Pig and poultry meat is often produced in specialized and intensive livestock systems with high animal densities, large production units with application of strict biosecurity measures, use of preventive vaccinations and antibiotic treatments (1). The use of antibiotics in farm animals may select for bacteria resistant to antibiotics, possibly including those used in human medicine. A considerable amount of applied antibiotic substances ends up in the intestines (2). Consequently, the gastrointestinal tract of livestock is an important reservoir for the selection of antibiotic resistance.

Currently, the increasing resistance in Gram-negative enteric bacilli receives special attention because of the potential horizontal spread to pathogens (3–5). In *Escherichia coli,* ESBL-production and carbapenem and fluoroquinolone resistance result in a decreased efficiency of critically important antibiotics, such as third- and fourth-generation cephalosporins, meropenem and ciprofloxacin (6,7). Resistance to these substances in intestinal bacteria of animals has become a threat to human health because of the potential risk of spread to humans (8). Dissemination can occur via direct contact, exposure to feces via agricultural and human waste, fecal contamination of carcasses during slaughter and contaminated food or drinking water (4,9). Although livestock and food-associated reservoirs are not major contributors to the ESBL occurrence in humans (10), transmission between reservoirs is likely to occur (11–14).

The south and central parts of the Netherlands and Flanders (Belgium) have one of the highest livestock densities in Europe (15). Both countries have comparable farming practices (15), yet total antimicrobial use in food-producing animals in Belgium is still relatively high (113.1 mg/PCU compared to the Netherlands (57.5 mg/PCU) in 2018 (16). Overall, in line with the reduction in use (17-18), a reduction in the prevalence of antibiotic resistance in commensal *E. coli* bacteria in animals in the Netherlands (17) and in Belgium is observed (19). Still, considerable variations in antibiotic use between farms and between countries have been observed for pigs and broilers (20–22). To better understand factors affecting antibiotic resistance and to implement stewardship actions more effectively, understanding antibiotic use and resistance on animal species and farm

level in each country is essential. National (farm-level) monitoring systems from distinct countries differ in data collection, analyses and reporting, making comparison of outcomes difficult. In this study, harmonized and comparable data on antibiotic use and resistance in food-producing animals at farm level in Belgium and the Netherlands is used, providing opportunities to compare antibiotic use and resistance and to study the origin and relevance of these differences. The aim of this study was to investigate the percentage of samples with ESBL-producing *E. coli* (ESBL-*E. coli*), carbapenem-resistant and ciprofloxacin-resistant *E. coli* (CiproR-*E. coli*) in Belgian and Dutch pig and poultry farms with a history of high antibiotic use.

3.3 Results

3.3.1 Antibiotic use in Belgian and Dutch broiler and pig farms

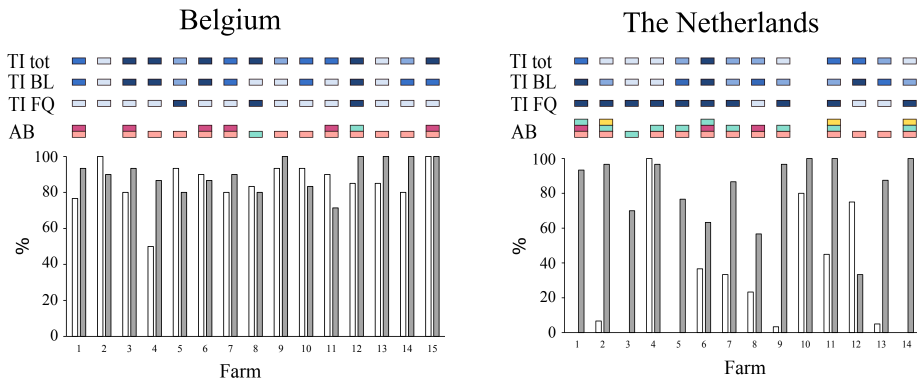
The total treatment incidence (TI), TI of beta-lactams and fluoroquinolones and active substances of these antibiotics used one year before sampling per farm are shown in **Figure 3.1** and **Supplementary Figure 3.1, Table 3.1 and 3.2**. In the year before sampling, no carbapenems, third- or fourth-generation cephalosporins were used in the Belgian and Dutch broiler farms. In ten out of fourteen Dutch broiler farms the active compound flumequine was used, and enrofloxacin was additionally used in three of these farms. In Belgium, two out of fifteen broiler farms used flumequine. Carbapenems, third- and fourth-generation cephalosporins or (fluoro)quinolones were not used in Dutch pig farms in the study period. In the Belgian pig farms third-generation cephalosporines (ceftiofur and cefquinome) were used in one farm, no (fluoro)quinolones or carbapenems were used. Beta-lactam antibiotics were prescribed in 92% of the studied farms. In general, the total TI and TI of beta-lactams was higher in Belgium compared to the Netherlands, both in weaned pigs and broilers. The type of beta-lactams prescribed in broilers were the penicillinase-sensitive beta-lactam phenoxymethylpenicillin and the broad-spectrum beta-lactam amoxicillin. In pigs, amoxicillin was frequently used in Belgium, while in the Netherlands procaine benzylpenicillin, ampicillin and amoxicillin were prescribed.

3.3.2 ESBL-producing, carbapenem-resistant and ciprofloxacin-resistant *E. coli* in Belgian and Dutch broiler and pig farms

A total of 779 broiler and 817 pig fecal samples were tested, covering 89% of the total aimed number of samples. Due to invalid sampling (n=2) and limitation of laboratory materials for selective culturing, the envisaged total number of 1800 samples could not be achieved. Of all resistant bacterial isolates (1855 isolates from 1596 samples), 91.4% were identified as *E. coli*. Other Enterobacteriaceae were present in low numbers, namely *Citrobacter freundii* (0.05%), *Escherichia fergusonii* (0.6%), *Klebsiella pneumoniae* (1.78%), *Morganella morganii* (0.16%), *Proteus spp.* (5.90%), and *Providencia rettgeri* (0.05%) and were excluded from further analysis.

In none of the samples, carbapenem-resistant Enterobacteriaceae were detected. In general, the percentage of samples positive for resistant bacteria in pig farms was notably lower compared to broiler farms after selective culturing. In pigs, ESBL-*E. coli* and CiproR-*E. coli* were more prevalent in Belgium than in the Netherlands. In Belgian broilers, the percentage of ESBL-*E. coli* was high compared to Dutch broilers (**Table 3.1**). The within-farm percentage of ESBL-*E. coli* was above 70% in 14/15 Belgian broiler farms compared to 3/14 of the broiler farms in the Netherlands (**Figure 3.1**). In contrast, the percentage of CiproR-*E. coli* in broilers was high in both countries. All participating broiler farms tested positive for the presence of CiproR-*E. coli* and 26 out of 29 farms showed a percentage of positive samples of 70% or higher after selective culturing of resistant bacteria. The percentage of resistant bacteria varied greatly between farms. Moreover, variations in resistance between different units of the same farm were observed (**Supplementary Figure 3.2**).

Broiler



Pig

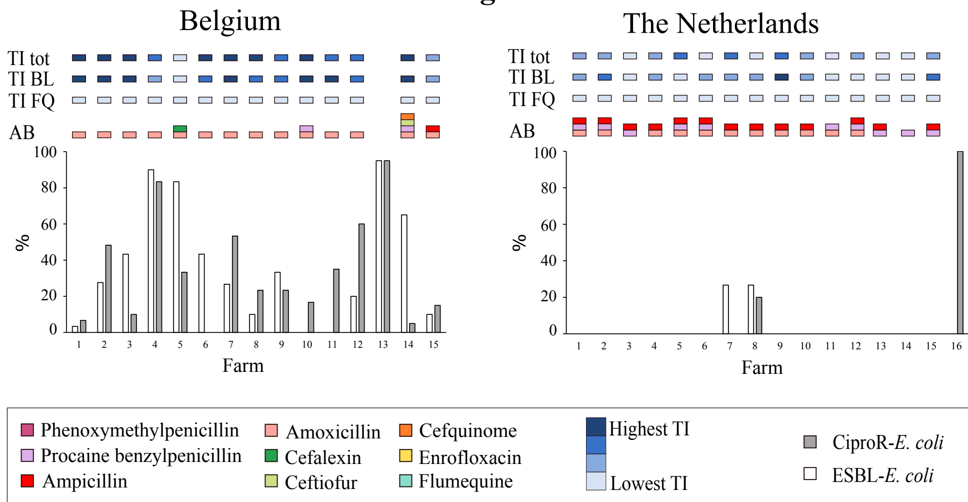


Figure 3.1. Percentages of ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* in Belgian and Dutch broiler and pig farms and the use of anti-biotics on farm-level. Antibiotic use in the year preceding sampling is presented as treatment incidence (TI) of total antibiotic use (TI tot), beta-lactam (TI BL) and fluoroquinolone (TI FQ) antibiotics. Colors indicate the active substance of the antibiotic (AB) used. Lowest to highest TI was indicated with a blue gradient. The total TI and beta-lactam TI was categorized based on quartiles. The TI of fluoroquinolones was categorized based on use or no use. For Dutch pig farm ID ten, eleven and twelve prevalence of ciprofloxacin-resistant E. coli was not determined. For Belgian pig farm ID 13 and Dutch pig farm ID 16, data on antibiotic use was not available for publication. CiproR-*E. coli*: ciprofloxacin-resistant *E. coli,* ESBL-*E. coli*: ESBL-producing *E. coli*.

Table 3.1. Distribution of farm level percentage of positive samples for ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* in Belgian and Dutch broiler and pig farms with estimated odds ratio for a positive sample.

Broiler								
		Number of samples	Percentage positive samples (%)	Number of positive farms	Min-max within farm percentage (percentage positive samples per farm)	Median percentage (%)	Interquartile range (%)	OR NL vs BE (95% CI)
ESBL-*E. coli*	**BE**	399	85	15/15	50-100	85	80-93	1 (reference)
	NL	380	27	10/14	0-100	15	0.83-43	0.007 (0.001-0.048)
CiproR-*E. coli*	**BE**	283	88	15/15	71-100	90	85-100	1 (reference)
	NL	303	82	14/14	33-100	90	72-97	0.60 (0.24-1.47)

(Continued)

		Pig						
		Number of samples	Percentage positive samples (%)	Number of positive farms	Min-max within farm percentage (percentage positive samples per farm)	Median percentage (%)	Interquartile range (%)	OR NL vs BE (95% CI)
ESBL-*E. coli*	**BE**	399	37	13/15	0-95	28	10-54	1 (reference)
	NL	418	4.0	2/16	0-27	0	0-0	0.004 (0-0.042)
CiproR-*E. coli*	**BE**	399	33	14/15	0-95	23	13-51	1 (reference)
	NL	328	11	2/13	0-100	0	0-0	0.006 (0-0.098)

BE: Belgium, NL: the Netherlands, OR: odds ratio, CI: confidence interval, CiproR-*E. coli:* ciprofloxacin-resistant *E. coli,* ESBL-*E. coli:* ESBL-producing *E. coli*

3.3.3 Associations between antimicrobial use and resistance

No association between the level of antibiotic use and the percentage of resistant samples on farm level in broiler and pig farms was found (**Table 3.2**). When studying the association between the total antibiotic use and the percentage of ESBL-*E. coli* and CiproR-*E. coli* positive samples, a lower odds for a positive sample was observed in farms with a higher use compared to farms with the lowest use in this study. One exception was the positive, yet not significant, association between total antibiotic use and the percentage of *E. coli* positive samples in the 3rd quartile category of antibiotic use (odds ratio (OR) 1.2). The presence of ESBL-*E. coli* was generally not associated with higher beta-lactam use in farms. In contrast, although not significant, a higher odds for the presence of CiproR-*E. coli* was found in broiler farms that used fluoroquinolones in the year preceding sampling.

Table 3.2. Associations between antibiotic use and prevalence of resistant samples in broiler and pig farms using a mixed effects logistic regression model. The model showed no association of any level of antibiotic use with prevalence. The quantity of antibiotic use in the year preceding sampling was categorized in quartiles of treatment incidence (TI) of total antibiotic use and beta-lactam use and use or no use of fluoroquinolone antibiotics.

	ESBL-*E. coli*			**CiproR-*E. coli***		
	Category total TI	**OR**	**95% CI**	**Category total TI**	**OR**	**95% CI**
Broiler	Belgium, total TI <2.9	1 (reference)		Belgium, total TI <2.9	1 (reference)	
	The Netherlands	0.02	0-0.09	The Netherlands	0.46	0.19-1.07
	Total TI 2nd quartile [2.9- <6.2]	0.80	0.07-8.03	Total TI 2nd quartile [2.9- <6.2]	0.33	0.10-0.95
	Total TI 3rd quartile [6.2- <12.2]	1.20	0.1-12.22	Total TI 3rd quartile [6.2- <12.2]	0.40	0.11-1.24
	Total TI 4th quartile [12.2- <28]	0.95	0.08-11.54	Total TI 4th quartile [12.2- <28]	0.31	0.09-0.98
	Category TI beta-lactam	**OR**	**95% CI**	**Category TI fluoroquinolone**	**OR**	**95% CI**
	Belgium, TI_BL <1.2	1 (reference)		Belgium, no fluroquinolone use	1 (reference)	
	The Netherlands	0.02	0-0.11	The Netherlands	0.45	0.16-1.22
	TI beta-lactam 2nd quartile [1.2- <3.4]	0.28	0.02-3.30	Fluoroquinolone use	1.69	0.63-4.77
	TI beta-lactam 3rd quartile [3.4- <7.4]	0.27	0.03-2.28			
	TI beta-lactam 4th quartile [7.4- <16]	0.33	0.03-2.81			
	ESBL-*E. coli*			**CiproR-*E. coli***		
	Category total TI	**OR**	**95% CI**	**Category total TI**	**OR**	**95% CI**
Pig	Belgium, total TI <12.9	1 (reference)		Belgium, total TI <12.9	1 (reference)	
	The Netherlands	0.01	0.00-0.11	The Netherlands	0.01	0-0.05
	Total TI 2nd quartile [12.9- <23.2]	0.04	0.00-1.77	Total TI 2nd quartile [12.9- <23.2]	0.07	0-1.61
	Total TI 3rd quartile [23.2- <44]	0.63	0.03-15.90	Total TI 3rd quartile [23.2- <44]	0.48	0.03-5.04
	Total TI 4th quartile [44- <82]	0.20	0.01-7.40	Total TI 4th quartile [44- <82]	0.10	0.01-1.14
	Category TI beta-lactam	**OR**	**95% CI**	**Category TI fluoroquinolone**	**OR**	**95% CI**
	Belgium, TI beta-lactam <3.2	1 (reference)		no fluoroquinolone use		
	The Netherlands	0	0-0.03			
	TI beta-lactam 2nd quartile [3.2- <12.1]	6.68	0.34-350.81			
	TI beta-lactam 3rd quartile [12.1- <22.7]	0.47	0.01-27.10			
	TI beta-lactam 4th quartile [22.7- <54]	0.22	0.00-9.93			

CiproR-*E. coli:* ciprofloxacin-resistant *E. coli,* ESBL-*E. coli*: ESBL-producing *E. coli*, CI: confidence interval, TI: Treatment Incidence, OR: odds ratio

3.3.4 Antibiotic resistance in ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* from broiler chickens and pigs

No meropenem resistance was found in *E. coli* from the feces of broilers and pigs (**Figure 3.2**). ESBL-*E. coli* were resistant to ampicillin, cefuroxime and ceftriaxone (BE) or cefotaxime (NL). Resistance to piperacillin-tazobactam, cefoxitin, fosfomycin and amikacin/gentamycin was generally low. In broilers, 33.4% of the Belgian ESBL-*E. coli* were co-resistant to ciprofloxacin, whereas in the Netherlands 12.6% of the isolates showed ESBL-production in combination with ciprofloxacin resistance. No resistance to ciprofloxacin was found in ESBL-*E. coli* isolates from Dutch pigs. In Belgian pigs, 17.4% of the ESBL-*E. coli* were co-resistant for ciprofloxacin.

Resistance to ampicillin was high (>80%) in CiproR-*E. coli* in both animal species and both countries. Resistance exclusive to ciprofloxacin was found in 4.0% of the Belgian broilers whereas 14.9% of the Dutch CiproR-*E. coli* from broilers were resistant exclusively to ciprofloxacin. In pigs, this is the case for 6.7% of the Belgian and none of the Dutch CiproR-*E. coli*. The most common combination of AMR phenotype in Belgian CiproR-*E. coli* was ampicillin- ciprofloxacin-trimethoprim/sulfamethoxazole (38.9% and 28.7% of the isolates from broilers and pigs respectively) and ampicillin-amoxicillin/clavulanic acid- ciprofloxacin- trimethoprim/sulfamethoxazole in Dutch CiproR-*E. coli* isolates from broilers (42.5% of the isolates) and pigs (84.6% of the isolates).

The percentage of MDR *E. coli* was high in pigs and broilers in both countries (**Table 3.3**). Resistance levels of the strains varied. In some farms, resistance to eight antibiotic classes was observed, while in other farms, bacteria resistant to only one class were isolated (**Supplementary Figure 3.3**).

Table 3.3. Multidrug resistance in *E. coli* from broilers and pigs. Number of isolates tested (N) and the percentage (%) of MDR isolates. A total of 12 antibiotic agents were included per country, namely ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, cefuroxime, ceftriaxone (Belgium)/cefotaxime (the Netherlands), ceftazidime, meropenem, amikacin (Belgium)/gentamycin (the Netherlands), ciprofloxacin, fosfomycin and trimethoprim-sulfamethoxazole.

		ESBL-*E. coli*		**CiproR-*E. coli***	
		N	% MDR[A]	N	% MDR
Broiler	Belgium	523	89.7	303	77.2
	The Netherlands	143	68.5	301	75.9
Pig	Belgium	201	99.5	164	73.8
	The Netherlands	16	100	39	100

CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*, MDR, multidrug-resistant; [A] MDR: resistant to at least one agent in at least three antimicrobial categories.

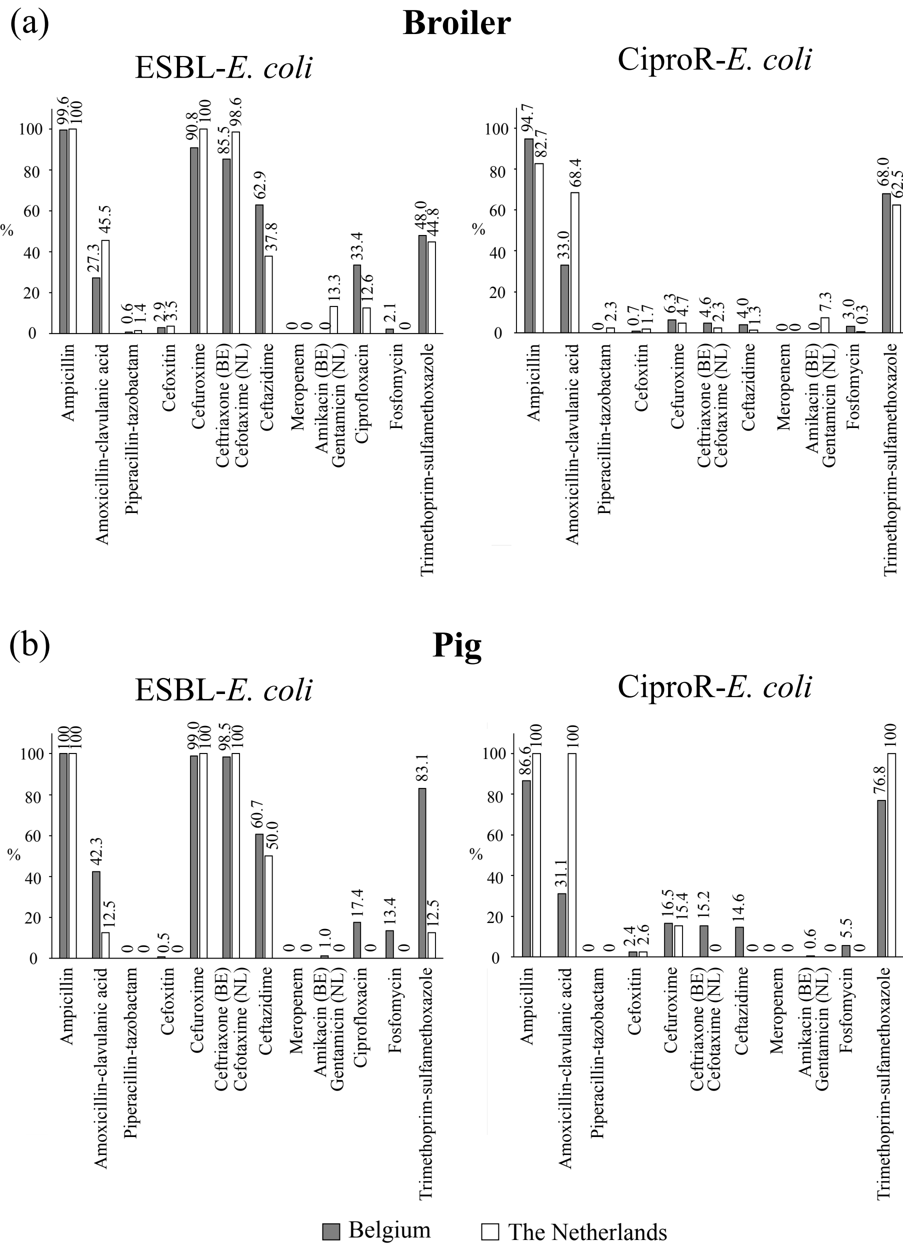


Figure 3.2. Percentage of antibiotic resistance per type of antibiotic in all ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* isolates from broiler chickens (A) and weaned pigs (B) in Belgium and the Netherlands. Number of ESBL-*E. coli* from broilers: N BE= 523, N NL= 143, number of CiproR-*E. coli* from broilers: N BE= 303, N NL= 301. Number of ESBL-*E. coli* from pigs: N BE= 201, N NL= 16, number of CiproR-*E. coli* from pigs: N BE= 164, N NL= 39. CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*, BE: Belgium, NL: the Netherlands.

3.4 Discussion

This study compared antibiotic use and resistance in broiler and pig farms in two bordering regions with comparable farming practices using similar data collection and analytical methods (15). Carbapenems are not authorized for use in animals in the EU (8) and these drugs were not used in the year before sampling in the studied farms.

Carbapenem-resistant *E. coli* were not detected in samples from broilers and pigs in Belgium and the Netherlands. However, among samples from Belgian broilers, 85% and 88% were positive for ESBL-*E. coli* and CiproR-*E. coli*, respectively; whereas among samples from Belgian pigs, 37% and 33% were positive for ESBL-*E. coli* and CiproR-*E. coli*, respectively. High rates of ESBL-*E. coli* have been previously reported in Belgian broilers (45%) (23) and in pigs (>70%) (24). Similarly, high rates of CiproR-*E. coli* from Belgian broilers have been previously reported in 2015 (>60%) (19), 2017 (25) and 2018 (>50%) (8). The rates of ESBL-*E. coli* and CiproR-*E. coli* were lower in samples from Dutch broilers (27% and 82% respectively) and pigs (4.0% and 11%, respectively). Similar rates of ESBL/AmpC-producing *E. coli* in feces of Dutch broilers (i.e. 33%) and slaughter pigs (i.e. 11%) were reported in 2017 by the Dutch monitoring system, MARAN (26). However, this MARAN survey of 2017 reported only 34% of CiproR-*E. coli* from fecal samples of broilers and 2% of the *E. coli* from pig fecal samples (26). The higher rates of CiproR-*E. coli* in our study might be explained by differences in farm selection. Indeed, in the MARAN survey, a stratified random sampling strategy was used, whereas in our study, farms with a history of high antibiotic use were selected. Finally, we also showed that the rates of ESBL-*E. coli* co-resistant to ciprofloxacin was higher in Belgium (33% in broilers and 17% in pigs) compared to the Netherlands (13% in broilers and 0% in pigs).

The veterinary sales of critically important antibiotics to human health care (3rd and 4th generation cephalosporins and fluoroquinolones) fell sharply in both Belgium and the Netherlands (16,18,26). However, the restriction of these antibiotics for veterinary use was implemented earlier in the Netherlands (in 2013) (27) than in Belgium (in 2016) (28).

These differences in antibiotic policy between Belgium and the Netherlands could explain the observed differences of ESBL-*E. coli* and CiproR-*E. coli.* The high rates of CiproR-*E. coli* in samples from Dutch broilers could be explained by the higher use of flumequine and fluoroquinolones (29) in most Dutch farms compared with Belgian farms.

Several studies have shown an association between antibiotic use and resistance at national level (30) and animal level (2). However, we could not demonstrate a clear link between the level of antibiotic use on farms during the year preceding sampling and the rates of antibiotic-resistant *E. coli* from fecal samples per farm. Our study was not powered to establish relationships between these variables. Moreover, we selected farms with a higher than average antibiotic use which introduced a bias. Several other factors account for emergence of antibiotic resistance, not necessarily related to antibiotic use on farms during the year preceding sampling, such as antibiotic use in earlier stages of the production chain and the farm environment. Indeed, high rates of antibiotic-resistant *E. coli* in the studied farms could also be due to the use of antibiotics in the primary breeding companies at the top of the pyramid in the broiler production systems. The Netherlands Veterinary Medicines Authority (SDa) reported high fluoroquinolone use in poultry farming subsectors, mainly due to the use in broiler parent and grandparent stock (31). Dierikx *et al*. (2013) showed the presence of ESBL/AmpC- producing *E. coli* isolates in the grandparent stock, one-day-old parent stock chicks and broiler chickens (32). The same study also reported the use of enrofloxacin in the grandparent stock to prevent mortality from *E. coli* infection. Contamination of consecutive flocks could be caused by recirculation of resistant strains present in the farm environment (32). High antibiotic resistance rates in fecal samples may also be explained by exposure to cumulated, resistance genes in litter or dust, or by additional introduction from non-poultry sources, such as water or other animals present on the farms (33,34).

Our study has several methodological specificities and limitations. We estimated the percentage of resistant samples based on selective culturing of bacteria followed by phenotypic antibiotic resistance determination. Hence, a sample is considered positive when resistant Enterobacteriaceae are present in the sample. This method is different from

studies where estimation is based on randomly isolated resistant bacteria as a percentage of a population of bacteria. In addition, the number of samples investigated for presence of CiproR-*E. coli* was reduced to six samples per farm in six Belgian broiler farms (ID 9-15) and five Dutch broiler farms (ID 10-14), which might lead to a less accurate estimation of the presence of CiproR-*E. coli* in these farms. Antibiotic susceptibility testing was performed separately for Belgian and Dutch isolates with two distinct methods (disc diffusion and broth dilution). However, both methods provide a qualitative assessment of the susceptibility or resistance of the isolates and should not impact the resistance rates in each country. Finally, because of low prevalence of enterobacterial species other than *E. coli* (8.6%), these were excluded from the analysis.

In conclusion, we provide unified information on the quantity of antibiotic use and presence of antibiotic resistance at the level of the farm in two neighboring countries, with different antibiotic policies. Based on comparable and harmonized data on antibiotic use and resistance, we demonstrated clear differences in antibiotic resistance in farms with a history of high antibiotic use between the border regions of Belgium and the Netherlands. Harmonized data on antibiotic use and resistance leads to improved comparability of results and could lead to better implementation of stewardship actions. The study provides opportunities to create awareness among farmers, veterinarians and stakeholders of alarming rates of antibiotic resistance.

3.5 Materials and methods

3.5.1 Study design, farm selection and farm characteristics

In this cross-sectional study, 60 farms were included in Belgium and the Netherlands, comprising 29 conventional broiler farms (Belgium: N= 15, the Netherlands: N= 14) and 31 multiplier pig farms (Belgium: N= 15, the Netherlands: N= 16). Farms were recruited between March 2017 and July 2017. The farms were required to be located in either Flanders (Belgium) and the three southern provinces of the Netherlands and participation

was voluntary. The farms were included based on the relative level of antibiotic use; meaning that antibiotic use was higher than average compared to the national benchmark value in the respective countries as described previously (22). The farm characteristics are summarized in **Supplementary Tables 3.1 and 3.2** and are described by Caekebeke *et al.* (2020) (22).

3.5.2 Antibiotic use

Antibiotic use was calculated from registration documents provided by national quality assurance organizations, the farmers or farm veterinarians. Antibiotic use was quantified as the TI per 100 days for pigs and per production round for broilers (35) as described by Caekebeke *et al.* (2020) (22). Total TI (referred to as TI tot) was defined as the average TI per round (broilers) or per 100 days (pigs) in the year preceding sampling. Likewise, TI of beta-lactams (phenoxymethylpenicillin, procaine benzylpenicillin, ampicillin, amoxicillin, cefalexin, ceftiofur, cefquinome) and TI of fluoroquinolones (enrofloxacin, flumequine) is hereafter referred to as TI BL and TI FQ (**Supplementary Table 3.1 and 3.2**).

3.5.3 Collection of fecal samples

The sampling period lasted six months, from the end of September 2017 to the beginning of April 2018 with the specific dates of sampling shown in **Supplementary Tables 3.1 and 3.2**. Samples were collected in a stratified-random sampling design based on the number of available units (broiler houses or rooms with weaned pigs). Within a farm, samples were collected from different units when more than one unit was present to take into account intra-farm variability. A maximum of three units were sampled per farm. The collection of 30 fecal samples per farm was aimed, evenly distributed over the selected units resulting in a total of 1800 samples. Fresh fecal droppings were collected from the stable floors using a nylon-flocked swab with 2 ml Cary-Blair transport medium (FecalSwab^{TM}, Copan Italy, Brescia, Italy).

Broilers were sampled at approximately 35 days of age and weaned pigs between 8 and 10 weeks of age. After testing the first broiler farms, the observed high percentage of samples with CiproR-*E. coli* allowed for the reduction to six samples per farm in six remaining Belgian broiler farms (ID 9-15) and five remaining Dutch broiler farms (ID 10-14) for reasons of costs and workload in the laboratory (**Supplementary Table 3.1**).

3.5.4 Microbiological methods

Fecal samples were submitted for microbiological analysis as described by Kluytmans-van den Bergh *et al.* (2019) (36). A non-selective enrichment in tryptic soy broth (TSB) (Copan Italy, Brescia, Italy) was followed by subculturing 10 µL of TSB on selective agars, namely CHROMID® ESBL, CHROMID® CARBA, CHROMID® OXA-48 (bioMérieux, Marcy l'Etoile, France) and MacConkey agar (Oxoid, Thermo Fisher Scientific, Basingstoke, UK) supplemented with 2 mg/L ciprofloxacin (Sigma-Aldrich, Saint Louis, USA). TSB and plates were incubated for 18-24h at 35-37 °C under aerobic conditions. Distinctive colonies on the agar plates were selected for species identification with MALDI Biotyper IVD (Bruker, Massachusetts, USA) for Belgian isolates and VITEK® MS (bioMérieux, Marcy l'Etoile, France) for Dutch isolates.

Subsequently, antibiotic susceptibility testing was performed on all isolates identified as *E. coli* (between one and five distinct *E. coli* per sample). Antibiotic susceptibility testing was performed in two laboratories with a separate panel for antibiotic susceptibility testing. For isolates originating from Dutch farms, minimum inhibitory concentrations for the following antibiotics were determined by broth microdilution VITEK® 2 (N344) (bioMérieux, Marcy l'Etoile, France): ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, cefoxitin, cefuroxime, ceftazidime, cefotaxime, ciprofloxacin, gentamicin, meropenem, trimethoprim-sulfamethoxazole (1:19) and fosfomycin. Antimicrobial susceptibility of Belgian isolates was tested for ampicillin (10 µg), amoxicillin-clavulanic acid (20/10 µg), piperacillin-tazobactam (30/6 µg), cefoxitin (30 µg) and cefuroxime (30 µg), ceftriaxone (30 µg) and ceftazidime (10 µg), ciprofloxacin (5 µg), meropenem (10 µg), amikacin (30 µg), trimethoprim-sulfamethoxazole

(1.25/23.75 µg), fosfomycin (200 µg) using disk diffusion (Rosco, Taastrup, Denmark). Individual isolates were classified as susceptible, intermediate or resistant according to the EUCAST (v8.1) clinical breakpoints (37). The combination disk diffusion method was used to confirm the presence of ESBL-*E. coli*. For this, the antibacterial activity of cefepime (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg, Rosco, Taastrup, Denmark) was assessed. The reduction of bacterial growth (reduction of inhibition zone ≥ 5 mm) when the cephalosporin is combined with clavulanic acid was considered indicative for ESBL production (38).

3.5.5 Data analysis

Statistics were performed for broilers and pigs separately in statistical program R version 4.0.2. (39). The odds of a positive sample was analyzed using a mixed effects logistic regression model (40) with country and categorized antibiotic use as explanatory variables and with the number of positive samples from the total samples as outcome variable. Quantity of antibiotic use in the year preceding sampling was categorized in quartiles of treatment incidence (TI) of total antibiotic use and beta-lactams and use or no use of fluoroquinolone antibiotics (**Supplementary Table 3.3**). Farm was added to the model to account for the correlation between the sample results within a farm. The odds ratio (OR) was calculated with 95% confidence interval.

The percentage of samples with resistant bacteria was calculated as the number of positive samples divided by the total number of samples. MDR was determined based on the antimicrobial categories as described by Magiorakos *et al.* (2012) (41). MDR was defined as resistance to at least one agent in at least three antimicrobial categories.

3.6 Addendum

3.6.1 Acknowledgements

We are grateful to the farmers, the veterinarians and all collaborators in the participating farms for their contribution to the collection of the epidemiological data. We are grateful to the microbiology technicians in the participating laboratories for their contribution to the collection of the microbiological data.

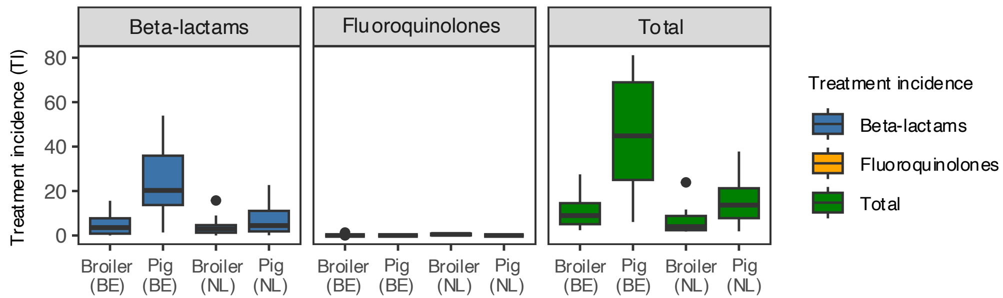
3.6.2 Contribution to authorship

Conceptualization: C.L., A.S., T.T., F.V., M.K.-v.d.B., J.K., J.D., H.G.; Data curation: M.K.-v.d.B.; Formal analysis: S.D.K., M.R., T.T., H.V.; Funding acquisition: J.K., J.D., H.G.; Investigation:S.D.K., M.R.; Methodology: C.L., A.S., T.T., F.V., H.V., M.K.-v.d.B., J.K., J.D., H.G.; Project administration: M.K.-v.d.B., J.K., H.G.; Supervision: C.L., J.D., H.G.; Visualization: S.D.K., H.V.; Writing – original draft: S.D.K; Writing – review & editing: M.R., C.L., A.S., T.T., F.V., H.V., M.K.-v.d.B., J.K., J.D., H.G.

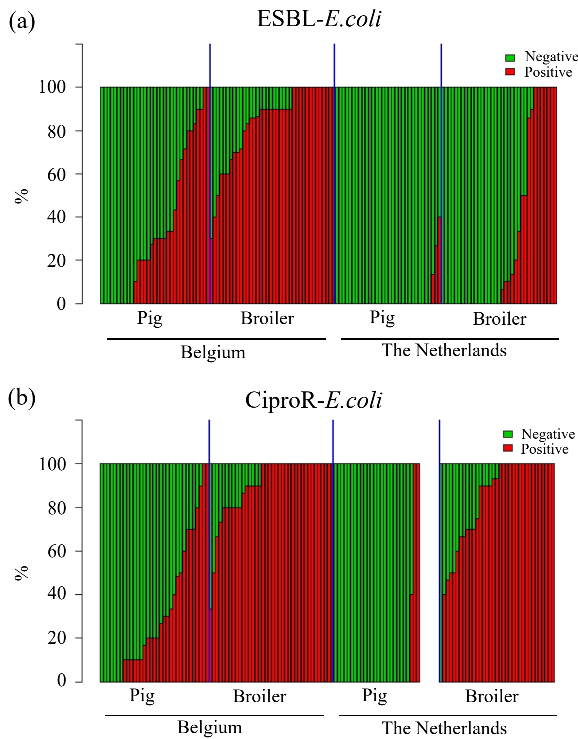
3.6.3 Funding

The i-4-1-Health project was financed by the Interreg V Flanders-The Netherlands program, the cross-border cooperation program with financial support from the European Regional Development Fund (ERDF) (0215). Additional financial support was received from the Dutch Ministry of Health, Welfare and Sport (325911), the Dutch Ministry of Economic Affairs (DGNR-RRE/14191181), the Province of Noord-Brabant (PROJ-00715/PROJ-01018/PROJ-00758), the Belgian Department of Agriculture and Fisheries (no reference), the Province of Antwerp (1564470690117/1564470610014) and the Province of East-Flanders (E01/subsidie/VLNL/i-4-1-Health).The authors are free to publish the results from the project without interference from the funding bodies. Selective and non-selective agar plates, Etests and Vitek2 AST cards were provided by bioMérieux; FecalSwabs and tryptic soy broths were provided by Copan. The authors were free to publish the results from the project without interference by bioMérieux or Copan.

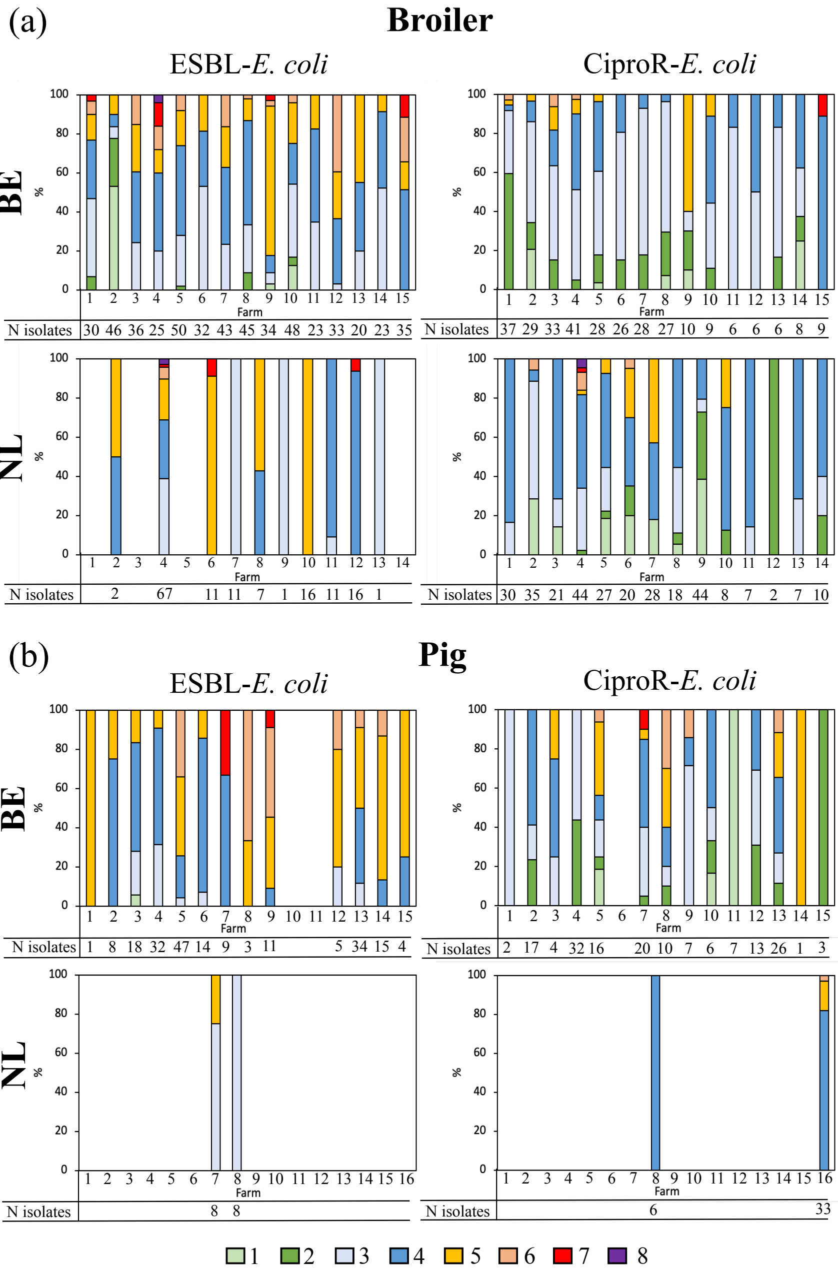
3.6.4 Supplementary information



Supplementary Figure 3.1: Boxplots of within-farm treatment incidence of beta-lactams, fluoroquinolones and total antibiotic used in the year prior to sampling. BE: Belgium, NL: the Netherlands.



Supplementary Figure 3.2: Percentage of samples positive for ESBL-*E. coli* (A) and CiproR-*E. coli* (B) per unit for Belgian and Dutch pig and broiler farms. BE: Belgium, NL: the Netherlands. CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*.



Supplementary Figure 3.3: Percentage of isolates that show antibiotic resistance to a number (1-8) of antibiotic classes (colours) per farm (x-axis) in ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* isolates from broiler chickens (A) and pigs (B) in Belgium (BE) and the Netherlands (NL). N is the number of isolates evaluated. CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*.

Supplementary Table 3.1: Farm characteristics and antibiotic use in terms of treatment incidence (TI) in the broiler farms.

Belgium								The Netherlands							
Farm ID	**Date of sampling**	**Total number of broilers**	**Number of units**	**Number of rounds per year**	**TI tot**	**TI BL**	**TI FQ**	**Farm ID**	**Date of sampling**	**Total number of broilers**	**Number of units**	**Number of rounds per year**	**TI tot**	**TI BL**	**TI FQ**
1	25/09/'17	180,000	4	6	7.44	5.96	0	**1**	27/09/'17	41,500	1	7	11.69	9	1
2	26/09/'17	50,000	1	5	2.37	0	0	**2**	09/10/'17	150,000	6	7.5	2.55	1.26	0.36
3	03/10/'17	125,000	4	6.5	18.13	13.75	0	**3**	16/10/'17	107,000	4	7.5	2.26	0	0.87
4	09/10/'17	79,500	3	6-7	17.49	14.17	0	**4**	06/11/'17	91,000	3	7.5	1.75	0.98	0.74
5	11/10/'17	85,000	3	7.4	4.32	2.99	0.03	**5**	24/10/'17	51,000	3	6.5	6.08	4.5	0.96
6	18/10/'17	90,000	3	6.5	12.57	8.26	0	**6**	19/10/'17	490,000	10	7.5	23.9	15.75	1.5
7	30/10/'17	130,000	4	6.5	12.13	7.23	0	**7**	10/11/'17	140,000	4	7	2.94	1.22	0.63
8	30/10/'17	87,000	2	7.5	14.42	0	1.33	**8**	13/11/'17	70,000	2	7	4	3.45	0
9	09/11/'17	82,000	3	7.2	5.33	0.36	0	**9**	20/11/'17	77,700	2	7.5	1.88	1.59	0.06
10	21/11/'17	84,000	2	7.5	8.95	5.45	0	**10**	14/02/'18		5				
11	02/02/'18	75,000	3	7	6.27	1.17	0	**11**	15/02/'18	63,000	2	7	8.79	2.95	0.2
12	05/02/'18	60,000	2	7.5	27.51	15.62	0.14	**12**	14/02/'18	23,400	2	6.5	9.63	7.93	0
13	19/02/'18	85,000	3	7.8	2.84	0.46	0	**13**	14/03/'18	50,000	2	7.4	5.53	4.67	0
14	05/03/'18	53,000	2	7	4.85	3.54	0	**14**	26/03/'18	165,000	4	7.4	2.38	1.87	0.49
15	06/04/'18	85,000	2	7	14.68	3.41	0	**15**	NA						

TI tot: average TI per round in the year preceding sampling; TI BL: TI of beta-lactams in the year preceding sampling; TI FQ: TI of fluoroquinolones in the year preceding sampling

Supplementary Table 3.2: Farm characteristics and antibiotic use in terms of treatment incidence (TI) in pig farms.

Belgium						The Netherlands					
Farm ID	**Date of sampling**	**Number of weaned pigs**	**TI tot**	**TI BL**	**TI FQ**	**Farm ID**	**Date of sampling**	**Number of weaned pigs**	**TI tot**	**TI BL**	**TI FQ**
1	16/11/'17	1,404	75.33	38.77	0	**1**	31/01/'18	1,700	13.66	9.36	0
2	24/10/'17	725	60.32	41.03	0	**2**	07/02/'18	1,300	21.56	14.71	0
3	27/10/'17	6,000	52.81	27.34	0	**3**	23/02/'18	1,400	7.19	0.67	0
4	08/11/'17	2,220	25.9	12.09	0	**4**	17/10/'17	936	12.94	7.26	0
5	08/11/'17	688	6.07	1.36	0	**5**	27/11/'17	3,600	34.33	3	0
6	14/11/'17	1,275	45.57	15.3	0	**6**	16/11/'17	2,800	12.02	12.02	0
7	16/11/'17	1,104	81.14	50.05	0	**7**	14/11/'17	2,400	24.51	10.12	0
8	22/11/'17	705	44.1	14.57	0	**8**	01/11/'17	1,824	8.4	3.23	0
9	23/11/'17	1,200	33.35	17.46	0	**9**	10/10/'17	8,000	37.79	22.71	0
10	23/01/'18	2,100	71.81	23.1	0	**10**	17/01/'18	3,500	20.97	4.45	0
11	06/02/'18	200	23.25	23.25	0	**11**	22/01/'18	1,400	4.52	0.08	0
12	07/02/'18	1,855	24.71	13.41	0	**12**	15/01/'18	3,000	14.52	2.24	0
13	16/02/'18	1,400				**13**	20/02/'18	800	1.87	1.42	0
14	26/02/'18	750	74.56	53.95	0	**14**	06/02/'18	2,500	5.86	0.38	0
15	08/03/'18	827	21.32	7.42	0	**15**	08/02/'18	1,300	17.64	17.43	0
						16	11/10/'17				

TI tot: average TI per 100 days in the year preceding sampling; TI BL: TI of beta-lactams in the year preceding sampling; TI FQ: TI fluoroquinolones in the year preceding sampling.

Supplementary Table 3.3: Categories of the quantity of antibiotic use in the year preceding sampling, presented as quartiles of treatment incidence (TI) of total antibiotic use and beta-lactam use and use or no use of fluoroquinolone antibiotics.

	Categories			
	Quartile	**Total TI**	**TI beta-lactam**	**TI fluoroquinolone**
Broiler	1st	[0- <2.9]	[0- <1.2]	no use
	2nd	[2.9- <6.2]	[1.2- <3.4]	use
	3rd	[6.2- <12.2]	[3.4- <7.4]	
	4th	[12.2- <28]	[7.4- <16]	
	Quartile	**Total TI**	**TI beta-lactam**	**TI fluoroquinolone**
Pig	1st	[0- <12.9]	[0- <3.2]	no use
	2nd	[12.9- <23.2]	[3.2- <12.1]	
	3rd	[23.2- <44]	[12.1- <22.7]	
	4th	[44- <82]	[22.7- <54]	

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CHAPTER 4

Genetic characterization of ESBL-producing and ciprofloxacin-resistant *Escherichia coli* from Belgian broilers and pigs

Published as **De Koster, S**.; Xavier, B.B.; Ringenier, M.; Lammens, C.; Kluytmans-Van den Bergh, M.; Kluytmans, J.; Dewulf, J.; Goossens, H.; on behalf of the i-4-1-Health Study Group*. Genetic characterization of ESBL-producing and ciprofloxacin-resistant *Escherichia coli* from Belgian broilers and pigs. Frontiers in Microbiology 2023, doi: 10.3389/fmicb.2023.1150470.

*see p 241-242

4.1 Abstract

Background: The increasing number of infections caused by Escherichia coli resistant to clinically important antibiotics is a global concern for human and animal health. High overall levels of ESBL-producing and ciprofloxacin-resistant (ciproR) *E. coli* in livestock are reported in Belgium. This cross-sectional study aimed to genotypically characterize and trace ESBL-and ciproR-*E. coli* of Belgian food-producing animals.

Materials and methods: A total of 798 fecal samples were collected in a stratified-random sampling design from Belgian broilers and sows. Consequently, 77 ESBL-*E. coli* and 84 ciproR-*E. coli* were sequenced using Illumina MiSeq. MIC for fluoroquinolones and cephalosporins were determined. Molecular in silico typing, resistance and virulence gene determination, and plasmid identification was performed. Scaffolds harboring ESBL or PMQR genes were analyzed to detect MGEs and plasmid origins. Core genome allelic distances were used to determine genetic relationships among isolates.

Results: A variety of *E. coli* STs (n=63), resistance genes and virulence profiles was detected. ST10 was the most frequently encountered ST (8.1%, n=13). The pandemic multidrug-resistant clone ST131 was not detected. Most farms harbored more than one ESBL type, with *bla*$_{\text{CTX-M-1}}$ (41.6% of ESBL-*E. coli*) being the most prevalent and least prevalent *bla*$_{\text{CTX M-15}}$ (n=3) being the least prevalent. ST10 was the most frequently encountered ST (8.1%, n=13). The pandemic multidrug-resistant clone ST131 was not detected and *bla*$_{\text{CTX M-15}}$ (n=3) was rarely found. IncI1-I(alpha) replicon type plasmids carried different ESBL genes (*bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{TEM-52C}}$). PMQR genes (15.5%, n=13) played a limited role in the occurrence of ciproR-*E. coli*. More importantly, sequential acquisition of mutations in QRDR of *gyrA* and *parC* led to increasing MICs for fluoroquinolones. GyrA S83L, D87N and ParC S80I mutations were strongly associated with high-level fluoroquinolone resistance. Genetically related isolates identified within the farms or among different farms highlight transmission of resistant *E. coli* or the presence of a common reservoir. IncI1-I(alpha) replicon type plasmids carried

different ESBL genes (*bla*$_{CTX-M-1}$, *bla*$_{CTX-M-32}$ and *bla*$_{TEM-52C}$). In addition, the detection of plasmid replicons with associated insertion sequence (IS) elements and ESBL/PMQR genes in different farms and among several STs (e.g. IncI1-I(alpha)/IncX3) underline that plasmid transmission could be another important contributor to transmission of resistance in these farms.

Conclusions: Our findings reveal a multifaceted narrative of transmission pathways. These findings could be relevant in understanding and battling the problem of antibiotic resistance in farms.

4.2 Introduction

Escherichia coli remains one of the most important pathogens for humans (1), as evidenced by its contribution to mortalities due to drug resistance. Fluoroquinolones and beta-lactam antibiotics are life savers in both human (2) and animal healthcare (3): these medications are essential for treating severe illnesses. Resistance to extended-spectrum cephalosporins and fluoroquinolones constitutes a major public health problem because this limits the treatment options for serious bacterial infections (2) and drives the use of the last resort of antibiotic therapy, i.e. carbapenems. The gastrointestinal tract of animals serves as a reservoir of AMR, which can spread via MGEs (4). The presence of resistance genes on MGEs enables their dispersion, posing a great hazard to food safety (5). Clinically significant ESBL genes, belonging to the *bla*$_{CTX-M}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ gene families, can successfully disseminate because they are commonly located on plasmids (IncA/C, IncF, IncHI1, IncHI2 IncI, IncK, IncN, IncX plasmids) (6). In addition, three mechanisms of PMQR are known: protection of DNA gyrase and topoisomerase IV from quinolone inhibition by *qnr* genes (ColE plasmids) (7), acetylation of quinolones by aminoglycoside acetyltransferase Aac(6')-Ib-cr (8) and quinolone accumulation due to quinolone efflux pumps QepAB (9) and OqxAB (ColE plasmids, IncX plasmids) (6,10,11). These mechanisms provide low-level resistance (ciprofloxacin MIC range:

0.06-0.25 mg/L); however, they are usually present on MDR plasmids and facilitate the selection of higher-level resistance making infections with PMQR-carrying pathogens harder to treat (11). Quinolone resistance in Gram-negative bacteria can also be caused by single amino acid changes in QRDRs in DNA gyrase (*gyrA*) and DNA topoisomerase IV (*parC*) (12,13). Another mechanism contributing to (fluoro)quinolone resistance is the increased expression of the AcrAB-TolC efflux pump which is regulated by repressor AcrR and other regulators of drug efflux MarAR and SoxRS as well as RNA polymerase RpoB (14–18) and the AcrB component of the efflux pump itself (17,19).

A previous study indicated a high occurrence of ESBL-producing and ciprofloxacin-resistant *E. coli* in fecal samples of broilers and pigs in Belgian farms (De Koster et al., 2021). Possible explanations for these observations include the dissemination of resistant *E. coli* vertically along the production chain from one generation to another (21,22) and resistant *E. coli* residing in the farm environment (23) along with the dissemination of resistant *E. coli* or their resistance genes between farm animals (24). However, the research into the genetic diversity and antibiotic resistance of *E. coli* that colonize livestock in Belgian farms has been limited. Most studies of commensal *E. coli* in livestock, such as the AMCRA reports (25), the EFSA and ECDC reports (26) rely on phenotypic AMR profiles. The lack of WGS to track MDR and high-risk clones was acknowledged in the latest BELMAP report, which aims to summarize monitoring programs in Belgium and recommends improving monitoring (27). An interdisciplinary One Health strategy is essential for tracking AMR's spread between humans, animals and their shared environment. Data on *E. coli* found in food-producing animals should be utilized to identify potential pathways of transmission through which the risk may reach human populations through consumption. To investigate the molecular epidemiology of ESBL-*E. coli* and ciproR-*E. coli,* we used WGS to identify resistance genes, mutations and potential transmission pathways between and among farms.

4.3 Results

4.3.1 ESBL and plasmid-mediated quinolone resistance genes in ESBL-producing and ciprofloxacin-resistant *E. coli*

The most abundant ESBL genes detected in *E. coli* isolated from broilers were $bla_{CTX\text{-}M\text{-}1}$ (40.5%, n=17) followed by $bla_{SHV\text{-}12}$ (31.0%, n=13). Other ESBL genes detected in broiler isolates were $bla_{CTX\text{-}M\text{-}32}$ (2.4%), $bla_{CTX\text{-}M\text{-}55}$ (2.4%), $bla_{SHV\text{-}2}$ (2.4%), $bla_{TEM\text{-}15}$ (2.4%), $bla_{TEM\text{-}52B}$ (4.8%) and $bla_{TEM\text{-}52C}$ (7.1%). Three isolates (7.1%) from different broiler farms harbored $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$. $Bla_{CTX\text{-}M\text{-}1}$ was also the most common in *E. coli* from pigs (34.3%, n=12), followed by $bla_{CTX\text{-}M\text{-}32}$ (22.9%), $bla_{TEM\text{-}52C}$ (11.4%), $bla_{CTX\text{-}M\text{-}3}$ (8.6%), $bla_{CTX\text{-}M\text{-}14}$ (8.6%), $bla_{CTX\text{-}M\text{-}15}$ (5.7%), $bla_{SHV\text{-}2}$ (5.7%), $bla_{TEM\text{-}52B}$ (2.9%) in pig isolates (**Figure 4.1A**). Eight of the ciproR-*E. coli* also harbored $bla_{CTX\text{-}M\text{-}1}$ (n=2), $bla_{CTX\text{-}M\text{-}32}$ (n=2), $bla_{CTX\text{-}M\text{-}15}$ (n=1), $bla_{SHV\text{-}12}$ (n=2) and one isolate with both $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$). PMQR genes were found in a relatively low number of ciproR-isolates (14.3%, n=12) (**Figure 4.1B**). Of the 84 ciproR-*E. coli*, 12 isolates harbored *qnrS1* (8.9% of the broiler isolates and 15.4% of the pig isolates). Two pig isolates (5.1%) additionally contained the efflux pump OqxAB. A total of 9.5% of the ESBL-*E. coli* from broilers and 8.6% of the ESBL isolates from pigs harbored *qnrS1*. Also, *qnrB19* was detected in 5.7% of porcine ESBL-*E. coli*.

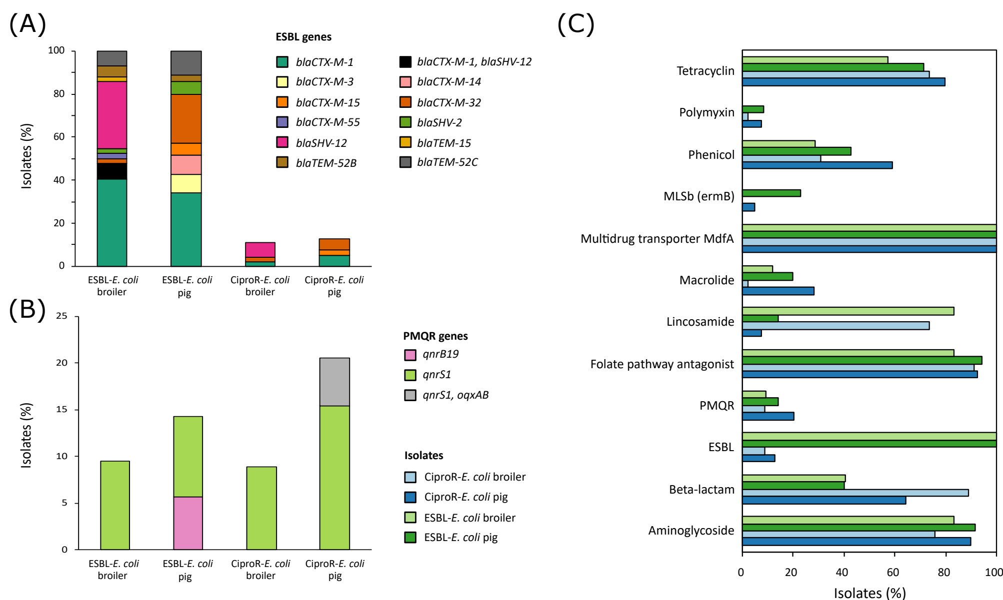


Figure 4.1: The percentage of isolates carrying ESBL genes (A), plasmid-mediated quinolone resistance (PMQR) genes (B) and genes conferring resistance to other antibiotic classes (C).

4.3.2 Other resistance genes, virulence genes and plasmids

In total, 95.8% of the isolates were MDR (i.e., resistant to at least 3 antibiotic classes (28)). Genes conferring resistance to aminoglycosides were abundant (overall in 84.5% of the isolates), folate pathway antagonists were present in 90.1% of the isolates, and all isolates harbored multidrug transporter MdfA. Lincosamide resistance was often detected in broiler isolates (ciproR-*E. coli*: 73.3%, ESBL-*E. coli* 83.3%) and beta-lactam resistance was often detected in ciproR-*E. coli* (pig: 64.1%, broiler: 88.9%) (**Figure 4.1C**). Plasmid-mediated colistin resistance was found in three pig farms (*mcr-1.1* (n=1), *mcr-2.1* (n=2), *mcr-9* (n=1)) and in one broiler farm (*mcr-9* (n=1)). Both *mcr-9*- containing isolates did not have the complete *qseC-qseB* two-component system to induce colistin resistance. Highly diverse resistance gene profiles (131 different profiles among 161 isolates) were detected within the same farm and between farms.

The mean number of resistance genes was significantly higher ($p<0.05$) in ciproR-*E. coli* from pigs (9.44 ± 4.01) compared to ciproR-*E. coli* from broilers (7.51 ± 2.85) **(Figure**

4.2A). Resistance genes that are a current threat to public health, referred to as Rank I resistance genes, were more abundantly present in ciproR-*E. coli* compared to ESBL-*E. coli* and more in pig isolates (4.6 ± 2.4 Rank I resistance genes) compared to broiler isolates (2.8 ± 1.4 Rank I resistance genes) ($p<0.01$) (**Figure 4.2B**). Similar observations can be made for Rank II resistance genes (considered future threats) which were present in higher numbers in porcine ciproR-*E. coli* compared to ESBL-*E. coli* from both broilers and pigs ($p<0.05$) (**Figure 4.2C**). On the other hand, broiler isolates contain a higher number of virulence genes (ciproR-*E. coli*: 4.62 ± 2.23; ESBL-*E. coli*: 5.45 ± 2.60) compared to pig isolates (ciproR-*E. coli*: 3.10 ± 2.25; ESBL-*E. coli*: 3.97 ± 2.81) (**Figure 4.2D**). This divergence of resistance and virulence was observed in the higher number of virulence genes (up to twelve genes) and lower number of Rank I resistance genes in ESBL-*E. coli*, while the opposite was seen for most ciproR-*E. coli,* which can carry a higher number of Rank I resistance genes (up to 10 Rank I resistance genes) (**Supplementary Figure 4.1**). Fourteen isolates showed a convergence of virulence and resistance (at least 3 Rank I resistance genes and more than six virulence genes) which belonged to ST117, ST189 (n=2), ST648, ST88, ST1011, ST75, ST624, ST115 (n=3), ST48 and ST350 (n=2). Overall, a large diversity was seen in the number of virulence and Rank I resistance genes ranging from lower-risk (one resistance gene and one virulence gene) to high-risk isolates (five Rank I resistance genes and nine virulence genes) (**Supplementary Figure 4.1**). On average, four plasmids were detected per isolate and no significant differences in the number of plasmids between the isolates of different origins were detected **(Figure 4.2E)**. The most common replicon markers (>10% in one or more categories) were IncFIB (52.9%), IncI1-I (gamma) (38.2%), Col (MG828) (30.1%), IncFII (27.7%), IncX1 (25.6%), IncFIC(FII) (23.6%) and p0111 (18.9%). Plasmid replicon IncB/O/K/Z was exclusively detected in broiler isolates (in 23.0% of CiproR-*E. coli* and in 28.9% of ESBL-*E. coli*) (**Supplementary Figure 4.2**). Most virulence genes were involved in adherence and invasion (**Supplementary Figure 4.3)**. The most prevalent virulence genes were *iss* (75%), *gad* (57%), *lpfA* (37%) and *iroN* (37%). A total of 120 different virulence profiles were detected within farms.

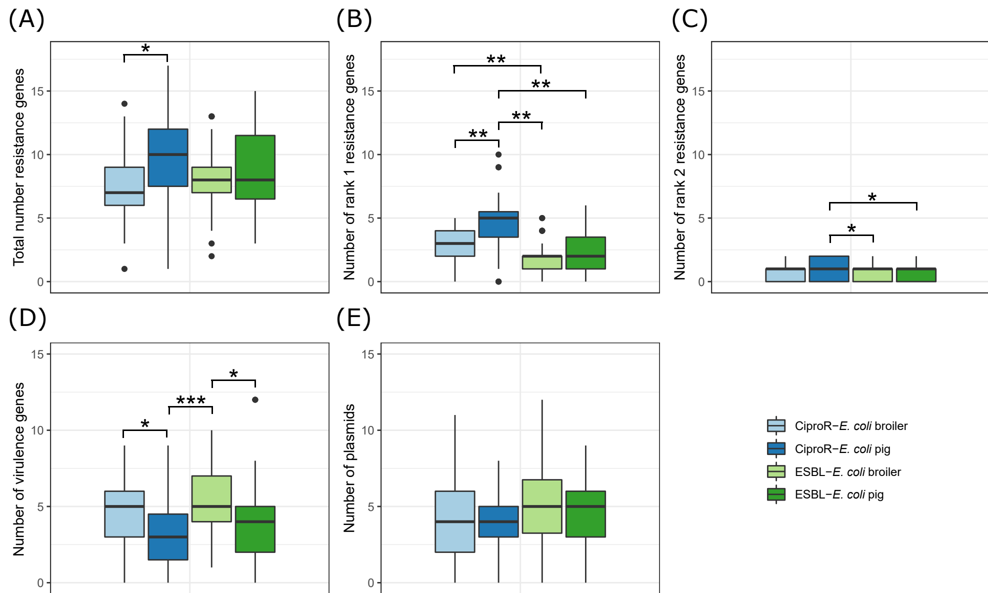


Figure 4.2: Number of resistance genes (A-C), virulence genes (D) and plasmids (E) in ESBL-producing *E. coli* (ESBL-*E. coli*) and ciprofloxacin-resistant *E. coli* (CiproR-*E. coli*) isolated from broilers and pigs. Statistically significant differences are indicated according to the level of significance: * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) (ANOVA with TukeyHSD or Games-Howell post-hoc tests).

4.3.3 Genotype-phenotype correlations for resistance in ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli*

More than one type of ESBL gene was detected in most of the sampled farms (73.3%; 22/30 farms). All ESBL genes were associated with very high ampicillin (MIC ≥ 32 mg/L) and cefotaxime (MIC 8 to ≥64 mg/L) resistance levels ($p<0.001$), except for two *bla*$_{SHV-2}$-harboring porcine isolates which showed cefotaxime MICs below breakpoint (MIC ≤ 1 mg/L). Strong levels of agreement between ESBL genotype and phenotype were detected for cefuroxime (89.44%, phi coefficient: 0.76), and ceftazidime (86.96%, phi coefficient: 0.77) and an almost perfect level of agreement was detected for cefotaxime (98.14%, phi coefficient: 0.96) (**Table 4.1**).

Table 4.1: Concordance between ESBL genotypes and cephalosporin phenotypes in *E. coli* isolates from livestock

Antibiotic	Susceptible phenotype		Non-susceptible phenotype		Agreement (%)	Phi coefficient (95% CI)	P-value
	ESBL gene presence	ESBL gene absence	ESBL gene presence	ESBL gene absence			
Cefuroxime	7 (4.3%)	66 (41.0%)	78 (48.5%)	10 (6.2%)	89.44	0.76 (0.69-0.88)	*** (<0.001)
Cefotaxime	2 (1.2%)	75 (46.6%)	83 (51.6%)	1 (0.6%)	98.14	0.96 (0.91-1)	*** (<0.001)
Ceftazidime	19 (11.8%)	75 (46.6%)	65 (40.4%)	1 (0.6%)	86.96	0.77 (0.67-0.87)	*** (<0.001)

CI: confidence interval

Mutations in QRDR of *gyrA* and *parC* were found in all ciproR-*E. coli*. Sequential acquisition of individual mutations in QRDR of *gyrA* and *parC* led to increasing MICs for all tested fluoroquinolone antibiotics. Predicted amino acid change S83L in GyrA caused low-level resistance to enrofloxacin and moxifloxacin, but not to ciprofloxacin and levofloxacin. Triple or quadruple mutations in QRDR caused high-level fluoroquinolone resistance (MIC>4 mg/L). QnrS1 or QnrB19 alone leads to low-level resistance to enrofloxacin and moxifloxacin and a sensitive/intermediate phenotype for ciprofloxacin and levofloxacin. The presence of both *oqxAB* and *qnrS1* genes lead to a non-susceptible phenotype for all four fluoroquinolones (**Figure 4.3**).
GyrA S83L, D87N and ParC S80I were strongly and significantly associated with resistance to fluoroquinolones. Triple mutations in *gyrA* (S83L and D87N/Y/G) and *parC* (S80I/R or E84K) were detected in 88% of the ciproR-*E. coli* and confer resistance to all tested fluoroquinolones. Two isolates contained a fourth mutation (GyrA S83L and D87N, ParC S80I and E84G) and one isolate additionally contained the *qnrS1* gene that showed MIC>32 mg/L for all fluoroquinolones. Outside of the QRDR in *gyrA* and *parC,* other mutations were detected in *gyrA*, *parC*, *gyrB*, *parE*, *acrB*, *acrR*, *marR*, *rpoB*, *soxR* and *soxS,* yet, were not positively associated with fluoroquinolone resistance (**Figure 4.4**). No mutations were detected in *marA*.

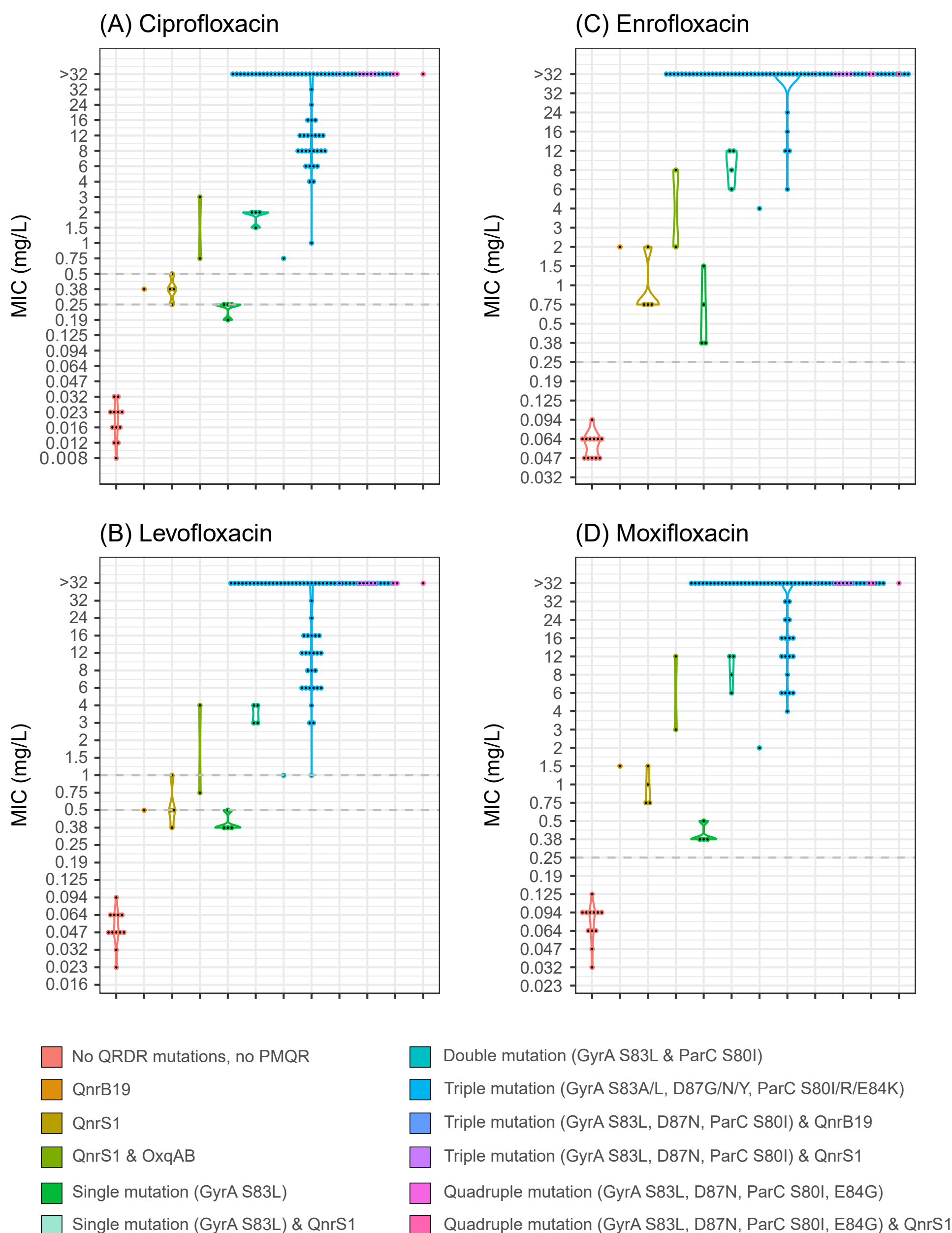


Figure 4.3: Minimum inhibitory concentration values for ciprofloxacin (A), levofloxacin (B), enrofloxacin (C) and moxifloxacin (D) of 106 isolates from Belgian broilers and pigs in association with the mutations in quinolone resistance determining region (QRDR) of GyrA and ParC and the presence of plasmid-mediated quinolone resistance (PMQR) genes. EUCAST breakpoints are indicated with a horizontal, dotted grey line. MIC: minimum inhibitory concentration.

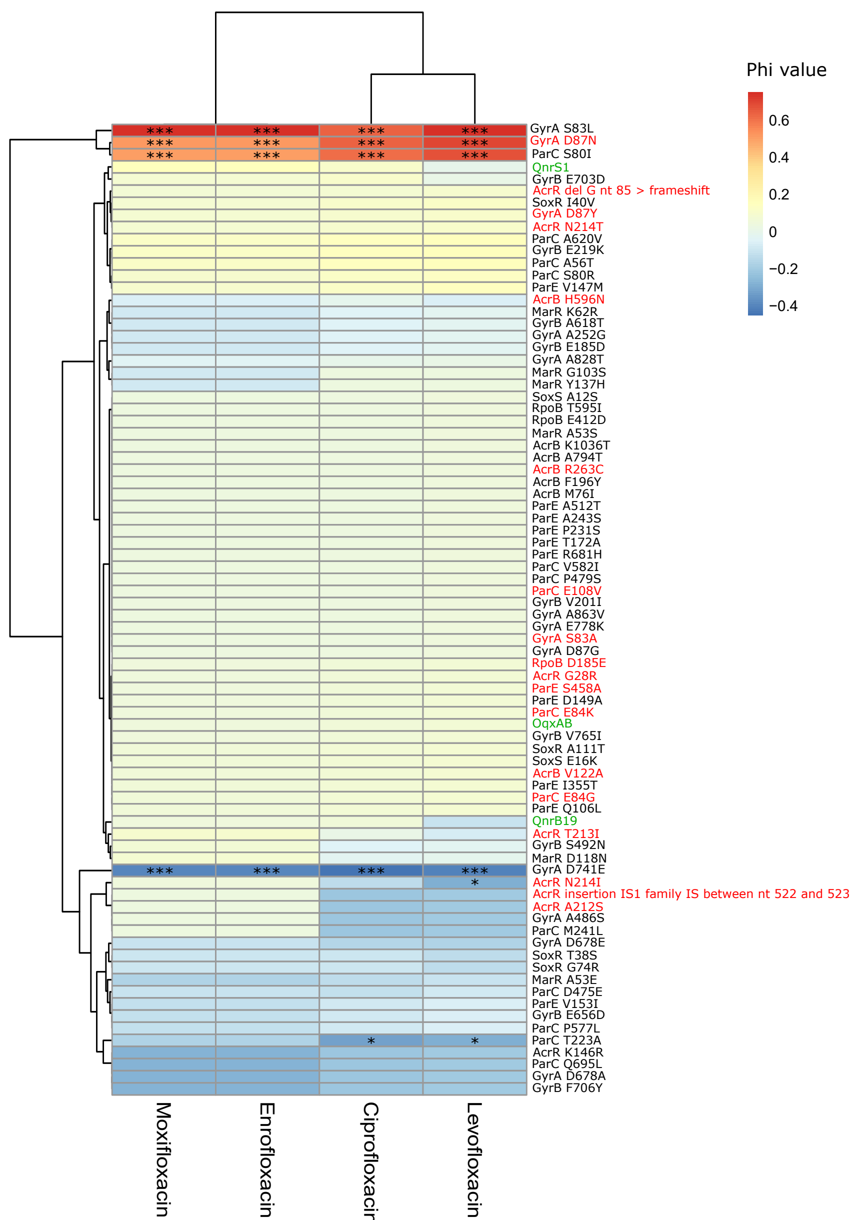


Figure 4.4: Heatmap of the association between the presence of plasmid-mediated quinolone resistance genes and mutations and fluoroquinolone non-susceptibility. Colors represent the phi values. Negative phi values represent negative associations, positive values represent positive associations between the genes/mutations and the non-susceptibility to the fluoroquinolone antibiotics. Plasmid-mediated quinolone resistance genes are indicated in green, predicted amino acid changes that are likely deleterious for the protein function according to SIFT are indicated in red. * (p<0.05), *** (p<0.001) (Chi-squared test). IS: insertion sequence, nt: nucleotide.

4.3.4 Genetic context of ESBL genes and plasmid-mediated quinolone resistance genes in ESBL-producing and ciprofloxacin-resistant *E. coli*

The specific genetic context of ESBL and PMQR genes (closest MGE and, if possible, identification of plasmid origin or replication) could be identified for 66 isolates (**Figure 4.5**). MGEs tended to be present at a fixed distance from the resistance gene. ESBL gene $bla_{\text{CTX-M-1}}$ was commonly found in association with IS*Ecp1* upstream of the gene (n=30) and was always detected on plasmids (**Figure 4.5A**). The plasmid IncI1-I(alpha) could be detected in twelve $bla_{\text{CTX-M-1}}$-producing strains and, using pMLST, six of the IncI1-I(alpha) plasmids showed ST3, clonal complex 3. Evidently, this particular MGE circulates in six pig and thirteen broiler farms amongst various *E. coli* genotypes, showcasing the remarkable distribution reach of this $bla_{\text{CTX-M-1}}$ harboring plasmid (**Figure 4.5B**). Other resistance genes detected on a subset of the $bla_{\text{CTX-M-1}}$-containing sequences are: *aadA5* (n=2), *dfrA17* (n=2), *mdtG* (n=1), *mdtH* (n=1), *mexA* (n=1), *mexB* (n=1), *qnrS1* (n=1), *sul2* (n=6) and *tetA* (n=1), as well as virulence gene *cib* (n=10). One porcine isolate harbored $bla_{\text{CTX-M-1}}$ associated with IS*5* on an IncI1-I(alpha), ST3, CC3 plasmid. The IncI1-I(alpha) plasmid origin of replication could also be detected in association with other ESBL genes, such as $bla_{\text{TEM-52C}}$ (n=3) and $bla_{\text{CTX-M-32}}$ (n=1). The $bla_{\text{SHV-12}}$ gene was detected on an IncN plasmid, without any association of IS elements in four broiler isolates from four different farms or in association with IS26 137 bp upstream of the $bla_{\text{SHV-12}}$ gene on an IncB/O/K/Z plasmid in two isolates from a broiler farm. A composite transposon IS*26* surrounded the $bla_{\text{SHV-2}}$ gene in isolates (n=2) from a pig farm. Most ESBL genes were located on a plasmid. However, seven ESBL genes ($bla_{\text{CTX-M-3}}$ (n=3) associated with IS*Ecp1*, $bla_{\text{CTX-M-14}}$ associated with IS903 (n=1), $bla_{\text{CTX-M-15}}$ (n=1) and $bla_{\text{CTX-M-32}}$ (n=2)) were predicted to be located on the chromosome. Different IS elements/transposons flanked the $bla_{\text{CTX-M-32}}$ gene (upstream ISKpn26 (n=2) on an IncX plasmid (n=1) or downstream IS*Sbo1* on an IncI1-I(alpha) plasmid (n=1) or upstream ISVas3 (n=1)) and the $bla_{\text{TEM-52C}}$ (upstream ISSbo1 (n=2), upstream Tn2 (n=1), downstream IS*Ror2* (n=2)) in different isolates. The $bla_{\text{TEM-52B}}$ gene was flanked by Tn2 in one porcine isolate and was located on an IncX1 plasmid. Co-localization of QnrS1

with *bla*$_{CTX-M-15}$ (n=1) or *bla*$_{CTX-M-55}$ (n=1) on a predicted plasmid contig was detected (**Supplementary Figure 4.4**). In 14 out of 75 isolates (18.7%), co-localization of virulence factor colicin Ib (*cib* gene, polypeptide toxins against *E. coli* and closely related bacteria) with an ESBL gene was detected.

The PMQR gene *qnrS1* was flanked by downstream IS*Knp19* (n= 6) and upstream either by ISEc*36* (n= 7) or by IS*26* (n=1). For one porcine isolate, the plasmid replicon could be identified as IncX1 harbouring *bla*$_{TEM-1B}$. For two broiler isolates from two different farms, QnrS1 could be located on an IncX3 plasmid (**Figure 4.5B**). QnrB19 was found to be located on a Col(pHAD) plasmid (n=2); however, no IS elements flanking the gene could be identified. Also, no flanking MGEs could be identified for *oqxAB* genes.

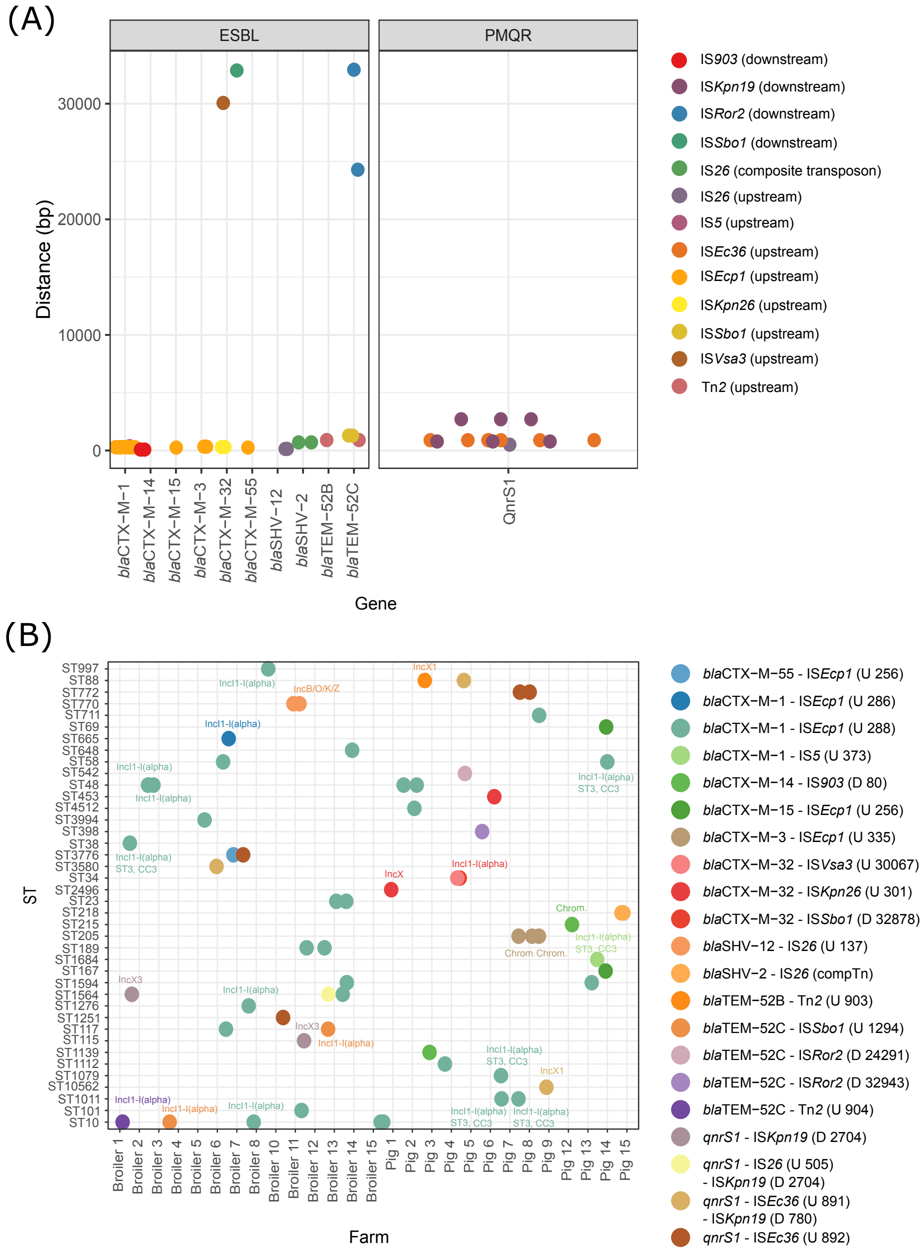


Figure 4.5: Mobile genetic elements and their association to ESBL genes and plasmid-mediated quinolone resistance (PMQR) genes. (A) Distance of mobile genetic elements to the ESBL or PMQR gene. (B) The combination of the ESBL/PMQR gene with the closest mobile genetic element for every farm and ST element. The plasmid origins of replication are indicated in the figure. The distance and upstream (U)/downstream (D) location of the mobile genetic element are indicated in the figure legend.

4.3.5 Typing and possible transmission events of resistant *E. coli* within and between farms

A highly diverse population of *E. coli* was isolated from broiler and pig farms (**Figure 4.6**). Overall, 63 different *E. coli* STs were detected with ST10 being the most abundant (13 out of 161 isolates, 8.1%). Phylogroup A was most common among ESBL-*E. coli* from pigs (57.1%) and broilers (47.6%), and ciproR-*E. coli* from pigs (53.8%), while B1 was most common among ciproR-*E. coli* from broilers (31.1%). The number of virulence genes in phylogroups A and B1 was lower compared to phylogroups D and G (**Supplementary Figure 4.5**). FimH54 was the most common among ESBL-*E. coli* from broilers (16.7%) and pigs (40.0%) and ciproR-*E. coli* from pigs (41.0%), and fimH32 was most common among ciproR-*E. coli* from broilers (22.2%). With 85 different serotypes among 161 isolates, serotypes were widely diverse.

To determine the genetic relatedness of the isolates, a study specific cgMLST scheme with 3012 loci was developed. Genetically linked bacterial clusters, with a maximal difference of ten alleles among them (29,30), were identified on several pig (n=8) and broiler farms (n=3) (ST10, ST34, ST205, ST215, ST345, ST453, ST683, ST744, ST1011, ST1140, ST1158). Moreover, the presence of genetically similar resistant bacteria was detected between different broiler farms (n=5) (ST115, ST48, ST155). These results suggest either transmission or a common reservoir between broiler farms. Transmission of *E. coli* ST1594 has likely occurred between a broiler farm and a pig farm as an allelic difference of 3 loci was shown between the two isolates (**Figure 4.6**).

4.3.6 Pathotypes detected in Belgian farm animals: ESBL-producing and ciprofloxacin-resistant enterotoxigenic *E. coli* and ESBL-producing enteropathogenic *E. coli*

Most of the *E. coli* isolates were non-pathogenic. However, twelve pathogenic *E. coli* (7.45%) were detected in five pig farms and two broiler farms. ESBL-producing ETEC were detected in pig farms six (n= 2; phylogroup B1, CTX-M-32-producing) and fifteen (n= 2 from the same pig; phylogroup A; SHV-2-producing) and ciprofloxacin-resistant ETEC were detected in pig farms eight (n= 2, ST772, phylogroup A, FimH54) and nine (n= 1, ST10, phylogroup A, FimH54). Enterotoxins *sta* and *stb* were present in 4 ETEC strains, *sta* was present in one ETEC strain and *stb* was present in two ETEC strains. The *stb*-containing contigs of the ETEC strains from pig farm fifteen also contained the *astA* gene encoding the heat-stable enterotoxin (EAST1) and IS100, an IS21 family insertion element.

ESBL-producing EPEC were detected in pig farm two (n= 2) and broiler farms four (n= 1) and twelve (n= 2). All EPEC strains were atypical because of the lack of bundle-forming pili (BFP). All EPEC strains were fimH54 belonging to phylogroup A; two were ST48 and CTX-M-1-producing strains, one was ST10 and TEM-52C producing strain and two were ST189 and CTX-M-1-producing strain. The latter two contained the IS256 composite transposon to mobilize the cassette of pathogenic virulence genes (*eae*, *espA*, *espB*, *espF*, *astA*, *tir*).

Figure 4.6: Minimum spanning tree of ciprofloxacin-resistant and ESBL-producing *E. coli* from broilers and pigs. The minimum spanning tree is distance-based and was generated by iTOL using cgMLST profile data (3012 loci). Colored clusters indicate genetically related isolates with ≤ 10 allelic differences from different broilers/pigs. The isolate IDs are shown in the first ring. The farm is indicated in colored strips in the second ring. Achtmann ST and phylogroups are indicated in rings three and four, respectively. The origin of the isolate is indicated with black (pig), grey (broiler) or white (*E. coli* K12 and *E. coli* O157-H7 reference strains) nodes.

4.4 Discussion

The study showed that livestock is a reservoir for a large variety of AMR genes, virulence genes and plasmids. More than one type of ESBL gene was detected in most farms and *E. coli* belonging to a variety of STs was found in Belgian broilers and pigs.

The large collection of STs and serotypes of commensal *E. coli* in animals was described before (31–36). However, the pandemic multidrug-resistant clone ST131 commonly associated with human infections was not detected and $bla_{CTX\ M\text{-}15}$ was rarely found (n=3 from two pig farms (ST4981, ST69, ST167). *E. coli* ST131 was also not detected in pig farms in Switzerland during a longitudinal study (4). The spread of $bla_{CTX\text{-}M\text{-}15}$ in human-associated *E. coli* is globally linked to IncFII plasmids in ST131 (6). IncFII plasmids were commonly detected (27.7% of the isolates) in this study but could not be linked to $bla_{CTX\text{-}M\text{-}15}$ or ST131. Instead, CTX-M-1 predominates in *E. coli* from food-producing animals and food in Europe (32,37). We found that the most common ESBL genes were $bla_{CTX\text{-}M\text{-}1}$ and $bla_{SHV\text{-}12}$ and ST10 was the most abundant sequence type. This is in line with other reports (4,31–34,37–41). ST10 has been found in both humans, animals, retail meat and the environment (40,42–45), is associated with ESBL production (4,45), and has been reported as an emerging extra-intestinal pathogen in humans, pigs and broilers (46–48). The results from our study combined with published data confirm that ST10 is a potential dominant clonal group of commensal *E. coli* in food-producing animals globally. Other high-risk lineages (ST69, ST117, ST23, ST58, ST648, ST744) of *E. coli* were identified among our isolates. A total of twelve (7.45%) pathogenic *E. coli* strains were detected (ETEC and atypical EPEC), one ST10 TEM-52C-producing strain and two ST189 CTX-M-1-producing strains which contained an IS256 composite transposon to mobilize the cassette of pathogenic virulence genes (*eae*, *espA*, *espB*, *espF*, *astA*, *tir*). These composite transposons can move as a single unit to move these pathogenic virulence genes and disseminate them among bacteria.

The spread of ESBL genes is highly linked to epidemic and highly transmissible plasmids (6,49). Most ESBL genes were predicted to be located on plasmids (91%) and were in the proximity of an IS element or transposon that was usually located at a fixed distance from

the ESBL gene. The *bla*$_{CTX-M-1}$ gene was often associated with IS*Ecp1* and IncI1-I(alpha)-ST3 in several broiler and pig farms, as described before (5,6,22,37,50,51). IS*Ecp1* is known to be associated with ESBL genes. Genes downstream of this IS element can be mobilized through transposition (including chromosomal integration) and are able to enhance ESBL gene expression under its own promotor (35,37,52). In our study, the IncI1-I(alpha) plasmid was also found to carry other ESBL genes (*bla*$_{CTX-M-32}$ and *bla*$_{TEM-52C}$). These results indicate that the IncI1-I(alpha) plasmid is a major plasmid type contributing to the spread of ESBLs in Belgian farms. Other ESBL-plasmid origin-of-replication combinations were: bla$_{SHV-12}$ on an IncN plasmid or IncB/O/K/Z plasmid, bla$_{CTX-M-32}$ on an IncX plasmid and bla$_{TEM-52B}$ on an IncX1 plasmid. QnrS1 seems to be flanked by different IS elements and was located on IncX1 in a pig farm or IncX3 plasmids in two broiler farms. IncX plasmids were described as widely distributed and to be associated with fluoroquinolone resistance (53). The presence of QnrS1 on IncX1 or IncX3 plasmids was shown before in Germany's pork and beef production chain (54). QnrB19 could be located on a Col(pHAD) plasmid in two isolates in our study, which was also the case in *Salmonella* spp. from poultry in Nigeria (55).

Co-localization of ESBL genes with virulence factor *cib* was detected in 14/75 isolates (18.7%) and co-localization with other resistance genes (such as *aadA* genes, *dfrA* genes, *aph(3')-Id*, *aph(6)-Id*, *bla*$_{TEM-1B}$, *cmlA1*, *sul* genes, *tetA*, *and qnrS*) was detected. PMQR and ESBL genes localized on the same presumed plasmid contig (*qnrS1* with *bla*$_{CTX-M-15}$ (n=1) or *bla*$_{CTX-M-55}$ (n=1)) is concerning. Plasmids co-harboring multiple resistance determinants to critically important antibiotics for human medicine limit treatment options for severe infections and are a threat to public health.

PMQR genes were found in a remarkably low number of isolates and play a limited role in the occurrence of ciproR-*E. coli* in Belgian farms. Ciprofloxacin resistance was caused by mutations in the QRDR region of *gyrA* and *parC* in all ciproR-*E. coli*, of which most showed triple mutations (GyrA S83L and D87N and ParC S80I) significantly associated with high-level fluoroquinolone resistance. In contrast, QnrS1 or QnrB19 alone leads to low-level resistance to enrofloxacin and moxifloxacin and a sensitive/intermediate

phenotype for ciprofloxacin and levofloxacin. Despite strong negative correlations between the presence of *qnr* genes and *gyrA* mutations shown previously and the hypothesis that Qnr proteins have a protective effect on quinolone targets (36), the presence of QnrS1 combined with GyrA S83L amino acid change was almost always detected in our study. Only two porcine ciproR-*E. coli* isolates did not contain any mutations in the QRDR of *gyrA* and *parC*, instead harbored two PMQR (OqxAB and QnrS1). Although PMQR mechanisms provide low-level resistance (11), the combination of OqxAB and QnrS1 was sufficient to result in fluoroquinolone resistance above breakpoint.

Pig isolates showed a higher mean number of resistance genes, especially for porcine ciproR-*E. coli*, which could reflect the higher use of antibiotics in pigs compared to broilers (27). In contrast, virulence genes were more abundantly present in broiler isolates. Most virulence genes were involved in adherence and invasion (most prevalent virulence genes were *iss, gad, lpfA*), which can contribute to successful colonization and enhanced survival in the gut and the environment (56). Also, the presence of ExPEC-associated virulence factors (such as *astA*, *iss*, *iha*, and *iroN*) is an indication that these commensal *E. coli* in Belgian farms may have pathogenic potential (57). Phylogroups A and B1 were the most common and are associated with commensal phenotypes (58). In line with this, phylogroups A and B1 carried a lower number of virulence genes compared to phylogroups D and G. However, the pathogenic *E. coli* (ETEC and EPEC) detected in this study belonged to phylogroups A and B1 showing that these phylogroups also have the potential to cause extraintestinal infections.

We identified multiple genetically related clones in different animals of the same farm and of distinct farms. The presence of clonally-related bacteria in different poultry farms suggests a common reservoir or transmission of resistant bacteria. The vertical spread of resistant bacteria from the top to the bottom of the broiler production pyramid (21,22) and resistant *E. coli* residing in the farm environment (23) were previously identified as important transmission routes of resistant bacteria. The diverse profiles of resistance genes, virulence genes and plasmid profiles reflect complex epidemiology. In addition,

the detection of plasmid replicons with associated IS elements and ESBL/PMQR genes in different farms and among several STs (such as IncI1-I(alpha) and IncX3) underline that plasmid transmission could be another important contributor to the transmission of resistance.

Our data show the complex epidemiology of ESBL-production and ciprofloxacin resistance in *E. coli* from livestock, suggesting the spread of these resistances involves both dissemination of resistant clones and horizontal transmission of plasmids. This emphasizes how critical it is to curtail the unnecessary use of antibiotics across all levels of the livestock production chain to preserve antibiotic effectiveness. Additionally, further research into plasmid involvement should include sequencing over longer reads to better understand its circulation on farms. The study supports that commensal *E. coli* in livestock should be monitored using WGS. Although not all resistance genes could be associated with MGEs or plasmids and we only sequenced a sub-selection of the resistant strains per farm, we gained valuable information on the genetic characteristics of ESBL-*E. coli* and ciproR-*E. coli* and the transmission of clones and resistance genes in Belgian farms using genomic data.

4.5 Materials and methods

4.5.1 Setting, study period and sample/isolate collection

Within the framework of the i-4-1-Health project, a total of 798 fecal samples were collected in a stratified-random sampling design from conventional broiler (n=15) and multiplier sow farms (n=15) in Flanders, Belgium (September 2017–April 2018). When present, sampling was conducted in different units (broiler houses or rooms with weaned pigs) with a maximum of three units per farm. The farms were included based on the relative level of antibiotic use, meaning that antibiotic use was higher than average compared to the national benchmark value in the respective countries. Farm characteristics and antibiotic use were described previously (59).

4.5.2 ESBL-producing and ciprofloxacin-resistant *E. coli*

Isolation of ESBL- and ciproR-*E. coli* was performed as described by Kluytmans-van den Bergh *et al.* (2019) (60). A total of 724 ESBL-*E. coli* and 467 ciproR-*E. coli* were isolated from the fecal samples. To investigate the molecular epidemiology, three ESBL-*E. coli* and three ciproR-*E. coli* from each farm were chosen for in-depth analysis including phenotypic characterization and whole genome sequencing. In particular, the first ESBL-*E. coli* and ciproR-*E. coli* isolated from each farm unit were selected.In farms with one sampled unit, three ESBL-*E. coli* and ciproR-*E. coli* with a distinct antibiotic susceptibility profile were selected from that unit. Using these selection criteria, 82 ESBL-*E. coli* (broiler (n=45), pig (n= 37)) and 84 ciproR-*E. coli* (broiler (n= 45), pig (n=39)) were selected for MIC determination and whole genome sequencing.

4.5.3 Whole genome sequencing

A single colony was inoculated in 4 mL Mueller Hinton broth and incubated overnight at 35-37°C. The MasterPure Complete DNA & RNA Purification kit (Epicentre, Madison, WI, USA) was used to extract genomic DNA. Libraries were prepared using the Nextera XT sample preparation kit (Illumina, San Diego, CA, USA) and sequenced with 2x 250 bp paired-end sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). The sequencing data were submitted to NCBI under BioProject PRJNA905236. **Supplementary Table 4.1** provides an overview of ESBL-*E. coli* and ciproR-*E. coli* sequences and their genetic characteristics used in this study.

4.5.4 *De novo* assembly, genotyping and phylogenetic analysis

Sequences were trimmed with TrimGalore v.0.4.4 (https://github.com/FelixKrueger/TrimGalore) and assembled *de novo* using SPAdes v.3.13.0 (Bankevich et al., 2012). Assembly quality was assessed with Quast (62). The assembled genome was annotated using Prokka v.1.12 (63). Additional analysis was performed using BacPipe v1.2.6 (64) including the PubMLST database (Achtman scheme) (65), the CARD database (66), ResFinder v4.1 (67), VirulenceFinder v2.0.3 (68)

and PlasmidFinder v2.0 (69). Serotype and pathotype were determined using BioNumerics v7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium). The identification of pathotypes was performed according to the virulence factor database (VFDB) (70). In silico prediction of fimH type and H and O serotypes was performed using FimTyper 1.0 (71) and SeroTypeFinder (72), respectively. Phylogroups were determined using ClermonTyping (73). For core genome multilocus sequence typing (cgMLST), a gene-by-gene approach was employed by generating a study-specific scheme and analyzing allelic loci distances of cgMLST using ChewBBACA (74) and visualizing the tree using iTOL v6 (75).

4.5.5 Phenotypic and genotypic antibiotic resistance determination

ESBL production was phenotypically confirmed using the combination disk diffusion method. Ciprofloxacin resistance was confirmed by ciprofloxacin MIC determination using VITEK® MS system (bioMérieux, Marcy l'Etoile, France). In addition, MICs for amoxicillin-clavulanic acid, ampicillin, cefuroxim, cefotaxime, ceftazidime, cefoxitin, fosfomycin, gentamicin, imipenem, meropenem, nitrofurantoin, piperacillin-tazobactam,tobramycin, trimethoprim were determined using VITEK® MS system (bioMérieux, Marcy l'Etoile, France). Furthermore, ciprofloxacin, enrofloxacin, levofloxacin and moxifloxacin were tested for 106 *E. coli* of which 18 were ciprofloxacin-susceptible *E. coli* and 88 were ciprofloxacin non-susceptible *E. coli* using E-tests (bioMérieux, Marcy l'Etoile, France) to identify genome-wide associations between genetic markers and fluoroquinolone resistance levels. Results were interpreted using the EUCAST breakpoint tables v12.0 (76) and an enrofloxacin breakpoint of MIC≤0.25 mg/L (77). After sequencing, known ESBL genes could not be detected in five phenotypic ESBL-*E. coli* (5/82; 6%) (from broiler farms one, four and eight and pig farms three and fifteen); therefore, these isolates were excluded, resulting in 77 ESBL-*E. coli* for further analysis. QRDRs were investigated for mutations conferring resistance within gyrase *gyrA* and *gyrB* and topoisomerases IV *parC* and *parE.* In addition, mutations in *acrB*, *acrR*, *marA*, *marR*, *rpoB*, *soxR*, *soxS* were considered. Mutations and predicted amino

acid changes were aligned using clustalw, inbuilt within the CLC genomics workbench v.9.5.3 (CLC bio, Denmark). Prediction of whether amino acid changes affect protein function was performed by Sorting Intolerant From Tolerant (SIFT) (78). Scaffolds containing ESBL or PMQR genes were analyzed using MGEFinder v1.0.3 (79), and ISFinder (80) to detect MGEs and replicon types of plasmids. Scaffolds containing ESBL genes or PMQR represent plasmid sequences were analyzed further on NCBI using blastn search with default settings to the blast database v5. Resistance genes were classified as Rank I (human-associated, mobile antibiotic resistance genes, in ESKAPE pathogens, current threats) or Rank II (human-associated, mobile antibiotic resistance genes emerging from non-pathogens, future threats) (81) (**Supplementary Table 4.2**).

4.5.6 Statistical tests and visualization

Statistical tests and visualization of the presence of resistance genes, virulence genes and plasmids were performed using R version 4.2.0 (82). Differences in the presence of genes were tested using a One-way ANOVA and TukeyHSD test in case of equal variances or a Welch ANOVA and the Games-Howell test in case of unequal variances (mean ± standard deviation and p-values are shown). Associations of genetic markers with a phenotype were examined using phi and chi-squared test.

4.6 Addendum

4.6.1 Acknowledgements

We are grateful to the farmers, the veterinarians and all collaborators in the participating farms for their contribution to the collection of the epidemiological data. We are grateful to the microbiology technicians in the participating laboratories for their contribution to the collection of the microbiology data.

4.6.2 Contribution to authorship

Original draft writing: S.D.K; Review and editing: M.R., B.B.X, C.L., D.D.C, K.D.B, K.M., J.D., M.K-v.d.B, J.K., H.G.; Data collection: S.D.K, M.R., Data analysis: S.D.K, B.B.X, D.D.C., K.D.B., K.M.; Funding acquisition: H.G., J.K.; Project administration: M.K-v.d.B., J.K., H.G, Supervision: H.G.,C.L., M.K.-v.d.B., J.K., Data curation: S.D.K., M.K.-v.d.B.

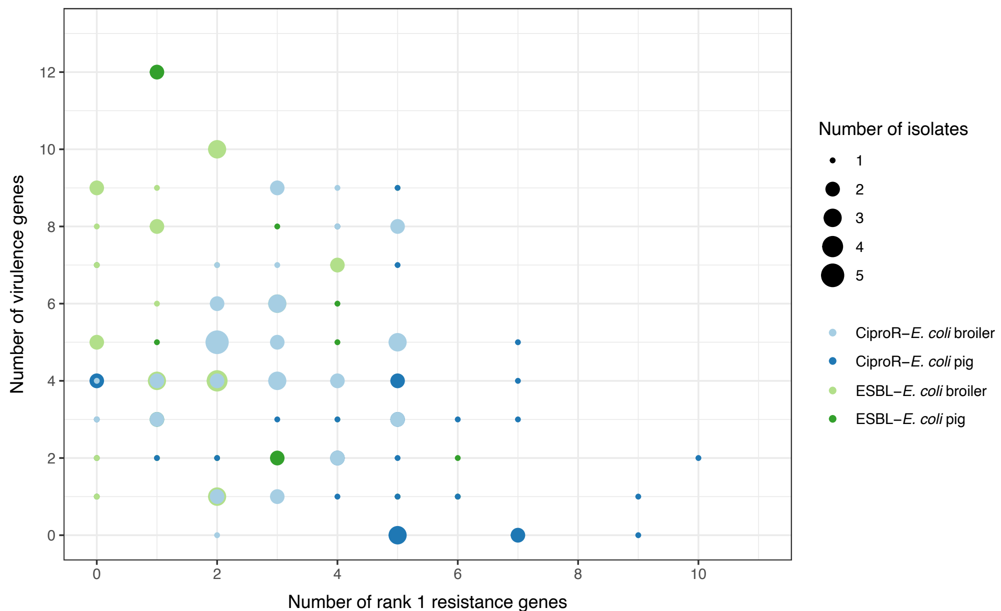
4.6.3 Funding

The i-4-1-Health project was financed by the Interreg V Flanders-The Netherlands program, the cross-border cooperation program with financial support from the European Regional Development Fund (ERDF) (0215). Additional financial support was received from the Dutch Ministry of Health, Welfare and Sport (325911), the Dutch Ministry of Economic Affairs (DGNR-RRE/14191181), the Province of Noord-Brabant (PROJ-00715/PROJ-01018/PROJ-00758), the Belgian Department of Agriculture and Fisheries (no reference), the Province of Antwerp (1564470690117/1564470610014) and the Province of East-Flanders (E01/subsidie/VLNL/i-4-1-Health).The authors are free to publish the results from the project without interference from the funding bodies. Selective and non-selective agar plates, Etests and Vitek2 AST cards were provided by bioMérieux; FecalSwabs and tryptic soy broths were provided by Copan. The authors were free to publish the results from the project without interference by bioMérieux or Copan.

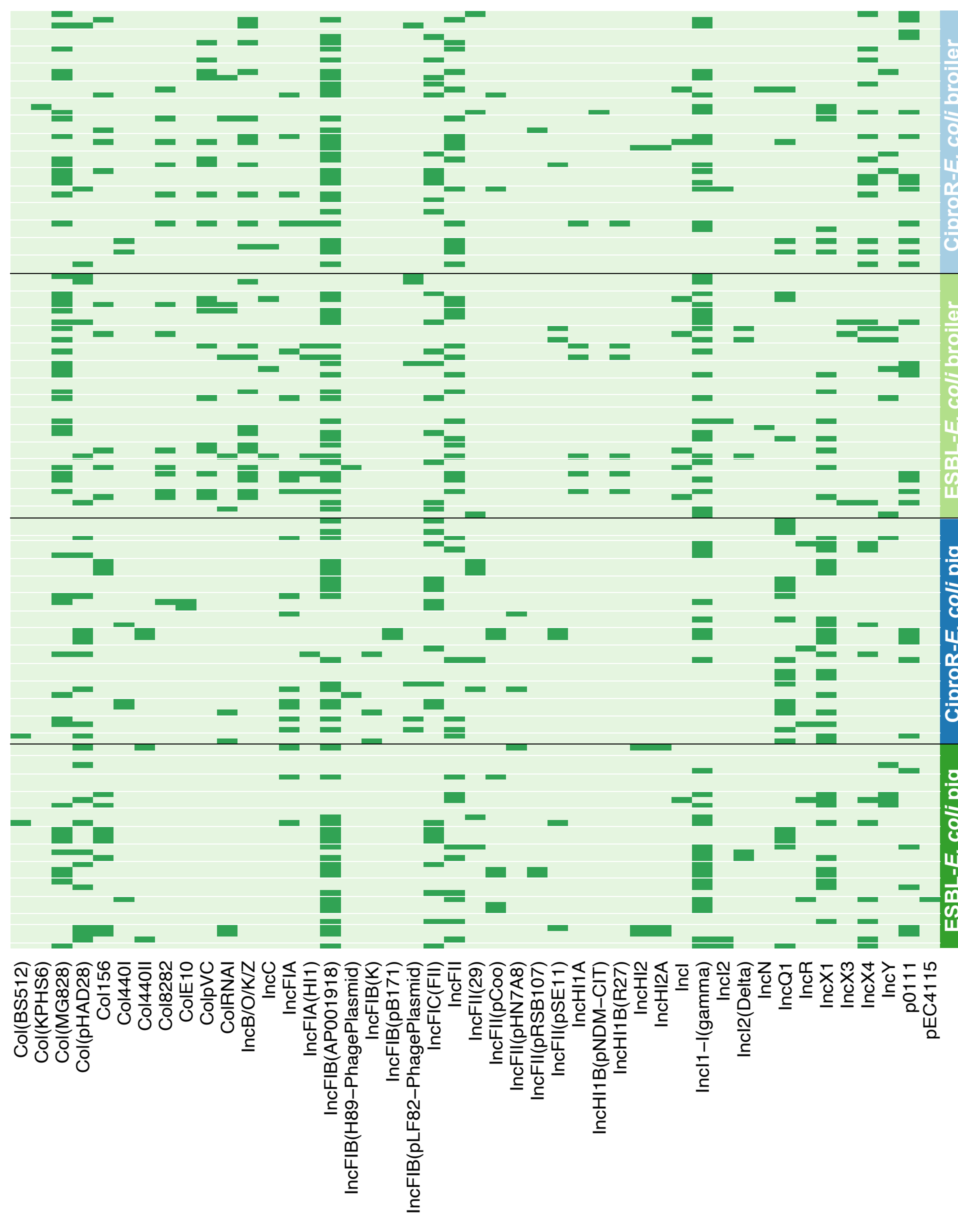
4.6.4 Supplementary information

Supplementary Table 4.1: Overview of ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* sequences and their genetic characteristics used in this study. Available online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1150470/full#supplementary-material.

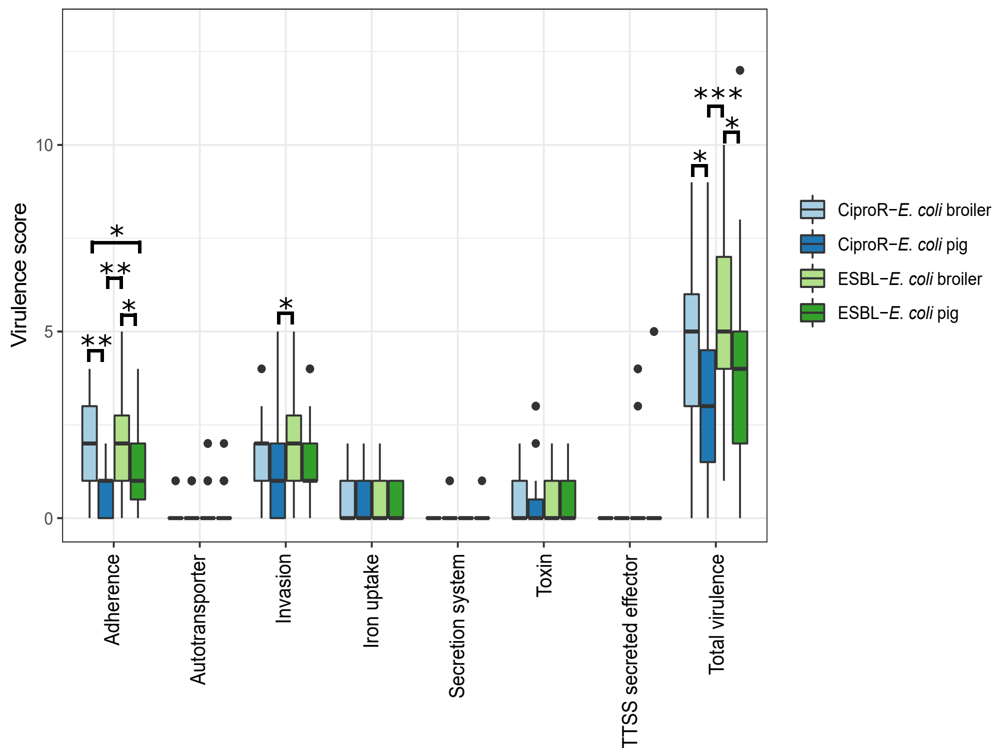
Supplementary Table 4.2: Classification of all detected resistance genes in this study by rank according to Zhang et al. 2021 (81). Available online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1150470/full#supplementary-material.



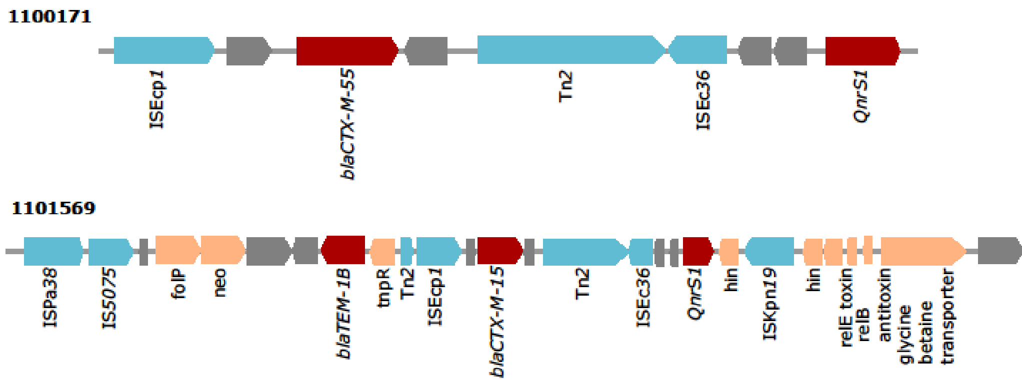
Supplementary Figure 4.1: Number of virulence genes and rank I resistance genes (current threats). The size of the bubbles represents the number of isolates, colors indicate origin of the isolates (pig: dark color, broiler: light color) and resistance mechanism (ESBL-producing *E. coli* (ESBL-*E. coli*): green, ciprofloxacin-resistant *E. coli* (CiproR-*E. coli*): blue).



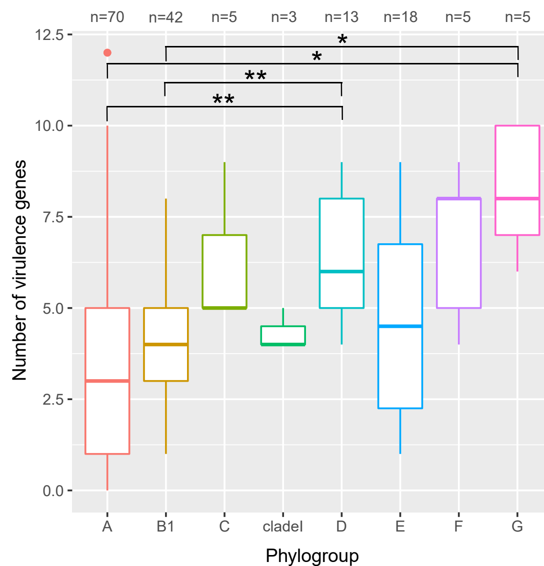
Supplementary Figure 4.2: Heatmap of presence (dark green) and absence (light green) of plasmid origin of replications in ESBL-producing and ciprofloxacin-resistant *E. coli* isolated from broilers and pigs. Each row relates to an isolate and each column represents a plasmid origin of replication. White vertical lines separate isolates from the same farm. CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*.



Supplementary Figure 4.3: Number of virulence genes according to the role of the gene in pathogenesis and life-style in ESBL-producing *E. coli* and ciprofloxacin-resistant *E. coli* isolated from broilers and pigs. Statistically significant differences are indicated according to the level of significance: * (p<0.05), ** (p<0.01), *** (p<0.001) (ANOVA with TukeyHSD or Games-Howell post-hoc tests). CiproR-*E. coli*: ciprofloxacin-resistant *E. coli*, ESBL-*E. coli*: ESBL-producing *E. coli*.



Supplementary Figure 4.4: Genetic context of ESBL and plasmid-mediated quinolone resistance (PMQR) genes co-localized in the same genetic region. Blue bars represent mobile genetic elements, grey bars are hypothetical proteins and red bars are ESBL genes or PMQR.



Supplementary Figure 4.5: Number of virulence genes for each phylogroup. Statistically significant differences are indicated according to the level of significance: * ($p<0.05$), ** ($p<0.01$) (ANOVA with Games-Howell post-hoc test).

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CHAPTER 5

One Health surveillance of colistin-resistant Enterobacterales in Belgium and the Netherlands between 2017 and 2019

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*see p 241-242

5.1 Abstract

Background: Colistin serves as the last line of defense against multidrug resistant Gram-negative bacterial infections in both human and veterinary medicine. This study aimed to investigate the occurrence and spread of colistin-resistant Enterobacterales (ColR-E) using a One Health approach in Belgium and in the Netherlands over the course of a twelve-month period.

Methods: In a transnational research project, a total of 998 hospitalized patients, 1430 long-term care facility (LTCF) residents, 947 children attending day care centres, 1597 pigs and 1691 broilers were sampled for the presence of ColR-E in 2017 and a second round twelve months later except in LTCF residents and children which were sampled once in 2018. The colistin TI in livestock at farm level was used to determine the association between colistin use and resistance. Selective culturing and colistin MIC were employed to identify ColR-E. A combination of short-read (Illumina) and long-read (PacBio) sequencing technologies were utilized to investigate the molecular characteristics and genetic makeup of 562 colistin-resistant isolates. The presence of chromosomal mutations and of *mcr*-genes that mediate colistin resistance as well as the resistome and virulome was determined. Core genome multi-locus sequence typing (cgMLST) was applied to examine potential transmission events within and between the One Health sectors examined.

Results: The presence of ColR-E was observed in all examined One Health sectors. In Dutch hospitalized patients, ColR-E proportions (11.3 and 11.8% in both measurements) were higher than in Belgian patients (4.4 and 7.9% in both measurements), while the occurrence of ColR-E in Belgian LTCF residents (10.2%) and children in day care centres (17.6%) was higher than their Dutch counterparts (5.6% and 12.8%, respectively). Colistin was used in the majority of the pig farms (26 of the 31 farms) and colistin use was associated with the occurrence of colistin resistance in these pig farms. The percentage of pigs carrying ColR-E was 21.8 and 23.3% in Belgium and 14.6 and 8.9%

in the Netherlands during both measurements. The proportion of broilers carrying ColR-E was higher in the Netherlands (5.3; 1.5%) compared to Belgium (1.5; 0.7%). Colistin resistance was mainly detected in *E. coli* (63.2%, n=473), *Klebsiella* spp. (22.5%, n=166), and *Enterobacter* spp. (10.0%, n=75). *E. coli* is the most important species for the spread of colistin resistance genes (*mcr-1.1*, *mcr-2.1*, *mcr-2.2* and *mcr-5.1*) in Belgian pig farms. *mcr*-harboring *E. coli* were detected in 17.4% (31/178) of the screened pigs from 7 Belgian pig farms. Concurrently, four human-related *Enterobacter* spp. isolates harbored *mcr-9.1* and *mcr-10* genes. The majority of colistin-resistant isolates (419/473, 88.6% *E. coli*; 126/166, 75.9% *Klebsiella* spp.; 50/75, 66.7% *Enterobacter* spp.) were susceptible to the critically important antibiotics (extended-spectrum cephalosporins, fluoroquinolones, carbapenems and aminoglycosides).
Chromosomal colistin resistance mutations have been identified in globally prevalent high-risk clonal lineages, including *E. coli* ST131 (n=17) and ST1193 (n=4). Clonally related isolates were detected in different patients, healthy individuals and livestock animals of the same site suggesting local transmission. Clonal clustering of *E. coli* ST10 and *K. pneumoniae* ST45 was identified in different sites from both countries suggesting that these clones have the potential to spread colistin resistance through the human population or were acquired by exposure to a common (food) source. In pig farms, the continuous circulation of related isolates was observed over time. Inter-host transmission between humans and livestock animals was not detected.

Conclusions: In this study, we have identified ColR-E in all examined One Health sectors in both Belgium and the Netherlands. The findings of this study contribute to a broader understanding of ColR-E prevalence and the possible pathways of transmission, offering insights valuable to both academic research and public health policy development.

5.2 Introduction

Colistin (polymyxin E) has been classified as critically important for human medicine with the highest priority by the WHO (1). It is also recognized as an antibiotic of high importance in veterinary medicine by the World Organization for Animal Health (OIE) (2). Colistin is administered orally in animals for the treatment of gastrointestinal infections and septicemia caused by Enterobacterales in intensive husbandry systems, mainly in swine and poultry (3–5). In healthcare settings, colistin is a reserve antibiotic for multidrug-resistant (MDR) Gram-negative infections (1,4,6) and it is also used for the treatment of *P. aeruginosa* infections in cystic fibrosis patients, topical treatment of otitis externa or ophthalmic infections (4) and for selective decontamination in critically ill patients (7,8). With the increasing number of hospital outbreaks with carbapenemase-producing Enterobacteriaceae (mostly *Klebsiella* species) and MDR non-fermentative Gram-negative bacteria (*Pseudomonas* and *Acinetobacter* species), colistin plays a key role for public health (3,9). The escalating incidence of MDR and colistin-resistant Gram-negative Enterobacteriaceae among the human and animal populations has led to a lack of effective therapeutic approaches for these infections, resulting in suboptimal clinical outcomes (4).

The emergence of colistin resistance is primarily due to alterations in LPS, the primary target site for this antibiotic (10,11). Such modification can result from chromosomal mutations that cause overexpression of the *pmrHFIJKLM* operon, *pmrCAB* operon and the *pmrE* gene, as well as the presence of plasmid-mediated mobile colistin resistance (*mcr*) genes. As many as eleven plasmid replicon types, including IncI2, IncX4, IncP, IncX, and IncFII, have been linked to the transmission of colistin-resistance genes (12,13). Furthermore, these plasmids exhibit a high degree of stability (14). Colistin resistance genes have been isolated from poultry, pigs, cattle, animal-derived food products and human isolates (15).

In the context of the global dissemination of colistin resistance, key contributing factors are the international trade of food animals and meat, as well as the worldwide movements

of colonized or infected individuals (16). A meta-analysis has revealed that the primary reservoirs of *mcr*-harboring *E. coli* were found in chickens and pigs with estimated global prevalences of 15.8% and 14.9%, respectively. Lower prevalences of plasmid-mediated colistin resistance were observed in *E. coli* isolates from healthy human populations (7.4%) and clinical samples (4.2%) (13). Evidence of clonal transmission within the livestock sectors and into the meat sectors exists (17,18). *mcr* genes were also detected in wastewater, rivers and seawater (14,19,20) and in dog feces and flies (14). This highlights the importance of an integrated, multisectoral approach that fits within the concept of One Health-*i.e.* across human, animal and environmental health. However, currently, surveillance systems in livestock and humans are heterogeneous in Europe (21). In 2014, European monitoring for colistin resistance in *Salmonella* and indicator *E. coli* from animals became mandatory (Regulation 2013/652/EU) (3). In contrast, surveillance of colistin resistance in Gram-negative clinical isolates from humans is not yet monitored in Europe (22). Consequently, it is crucial to monitor the presence and transmission of antibiotic resistance in key reservoirs, such as humans, chickens and pigs in order to effectively combat the emergence and spread of colistin-resistant bacteria and colistin resistance genes. Current literature on global studies describing the circulation of colistin-resistant bacteria among humans, animals, food and the environment is scarce.

Utilizing a One Health approach with harmonized and comparable methodology, our study examines the prevalence and possible dissemination of colistin-resistant Enterobacterales (ColR-E) in hospital patients, LTFC residents and healthy children in day care centres, as well as broilers and pigs on farms in Belgium and the Netherlands. We also aimed to elucidate the molecular basis of colistin resistance in different human healthcare settings and in livestock farming environments.

5.3 Results

5.3.1 Presence of colistin-resistant Enterobacterales in hospitals, long-term care facilities, day care centres and farms in Belgium and the Netherlands

Of the 1268 Enterobacterales isolates picked from the selective colistin agar plate, 748 (58.9%) were confirmed as colistin resistant (MIC ≥4 mg/L). These colistin-resistant isolates were distributed in 24 bacterial species, the majority being *Escherichia coli* (63.2%), followed by *Klebsiella* spp. (22.5%), three quarter of which were *Klebsiella pneumoniae,* and 10.0% of *Enterobacter* spp. A larger variety in bacterial species was carried by humans compared to livestock animals (**Supplementary Figure 5.1**).

While a single survey was conducted in long-term care facilities and day care centres, two rounds of repeated measurements with a one-year interval were performed in hospitals and farms to longitudinally assess the presence of ColR-E in these sectors. ColR-E isolates were found in all investigated One Health sectors, albeit with different frequency of occurrence by sector (**Figure 5.1A**). Each measurement, the percentage of patients carrying ColR-E at one Belgian hospital (7/160 (4.4%) and 16/202 (7.9%)) was significantly lower compared to the prevalence observed among patients at two Dutch hospitals (43/382 (11.3%) and 30/254 (11.8%)) ($p<0.001$) (**Table 5.1**). Similar occurrences were observed between the two Dutch hospitals and the two measurements (9.09-12.2%)

On the other hand, the prevalence of ColR-E colonization was significantly higher in Belgian LTCF residents (67/656, 10.2%) as opposed to their Dutch counterparts (43/774, 5.6%). A total of 11/13 Belgian LTCF and 14/17 Dutch LTCF were positive for ColR-E with up to 21.6% and 16.7% of the residents colonized within a Belgian and Dutch LTCF, respectively. Similarly, the ColR-E colonization rate was higher in children attending day care centres in Belgium (79/448, 17.6%) than in those attending similar institutions in the Netherlands (64/499, 12.8%). Fifteen out of seventeen Belgian and 22/28 Dutch day care centres were ColR-E positive with up to 35.7% and 31.6% of the children colonized in a Belgian and Dutch day care centre, respectively.

Table 5.1: Comparison of colistin resistance between Belgium and the Netherlands by measurement and sector

Sector (measurement)	Number of samples		Colistin resistance (%) (range of within site percentage positive samples)		Number of positive sites		Risk difference (%)	95% CI	P-value
	BE	NL	BE	NL	BE	NL			
Hospital (1)	160	382	4.4	11.3 (9.1-11.3)	1/1	2/2	6.9	3.9-9.9	*** (<0.001)
Hospital (2)	202	254	7.9	11.8 (11.7-12.2)	1/1	2/2	3.9	2.2-5.5	*** (<0.001)
LTCF	656	774	10.2 (1.9-21.6)	5.6 (0-16.7)	11/13	14/17	-4.7	-7.9 - -1.4	** (<0.01)
Day care	448	499	17.6 (0-35.7)	12.8 (0-31.6)	15/17	22/28	-4.8	-9.6 - -0.1	* (<0.05)
Broiler (1)	399	380	1.5 (0-10)	5.3 (0-16.7)	3/15	9/14	3.8	0.9-6.6	** (<0.01)
Broiler (2)	450	390	0.7 (0-3.3)	1.5 (0-10)	3/15	4/13	0.9	-0.6 - 2.4	ns
Pig (1)	399	328	21.8 (0-86.7)	14.6 (0-46.7)	11/15	11/13	-7.2	-18.0- 3.7	ns
Pig (2)	420	450	23.3 (0-93.3)	8.9 (0-46.7)	12/14	12/15	-14.4	-26.8 - -2.1	* (<0.05)

CI: confidence interval, BE: Belgium, NL: the Netherlands, ns: not significant..

The lowest occurrences were detected in the broiler farms in Belgium and the Netherlands. Each measurement, a larger proportion of the broilers were colonized in the Netherlands (20/380 (5.3%) and 6/390 (1.5%)) compared to Belgium (6/399 (1.5%) and 3/450 (0.7%)). ColR-E isolates were detected in 3/15 Belgian broiler farms. Within-farm occurrences ranged from 0 to 10% in the first and 0 to 3.3% in the second measurement. The number of Dutch broiler farms positive for ColR-E declined from 9/14 in the first measurement to 4/13 farms in the second measurement. Within- farm occurrences in the Dutch broiler farms ranged from 0 to 16.7% in the first and from 0 to 10% in the second measurement.

The proportion of positive samples (i.e. showing the presence of colistin-resistant bacteria) was higher in the Belgian pig farms than in the Dutch pig farms at each measurement: 87/399 (21.8%) and 98/420 (23.3%) vs 48/328 (14.6%) and 40/450 (8.9%), respectively. However, the percentage of positive samples varied greatly between different pig farms (0%-93.3% in Belgium and 0-46.7% in the Netherlands) (**Figure 5.1B**). Two Belgian pig farms showed consistently high occurrence of colistin resistance

(≥70%) over a period of one year. On the other hand, ten Belgian broiler farms, one Dutch pig farm and 4 Dutch broiler farms consistently showed no colistin resistance over the two measurements.

When investigating carriage of indicator bacteria (*E. coli*, *Klebsiella* spp. and *Enterobacter* spp.) individually, few ColR-*E. coli* were detected in Belgian hospitalized patients (1.3%) compared to Dutch patients (7.1%) in the first measurement. The percentage of hospitalized patients carrying MDR isolates was similar in Belgium and the Netherlands (3.8-5.9%), while slightly higher percentages of elderly (3.2%) and children (6.3%) carried MDR isolates in Belgium compared to those in the Netherlands (1.9% of the elderly and 3.4% of the children). Similarly, MDR isolates were more prevalent in Belgian pigs (18.1-19.1%) compared to Dutch pigs (8.8-7.1%) in both measurements (**Supplementary Figure 5.2**).

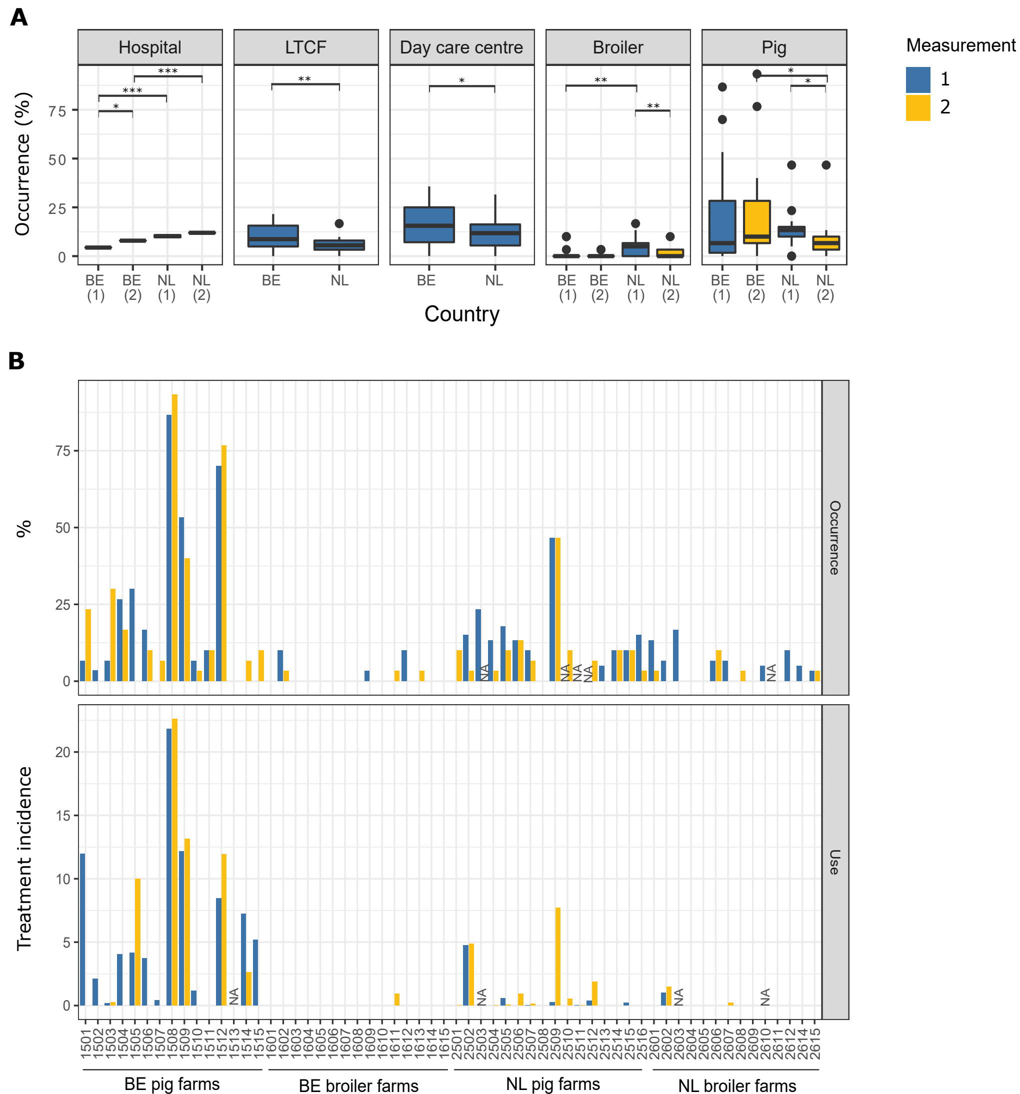


Figure 5.1: Occurrence of colistin resistance in examined One Health sectors (A) and colistin treatment incidence in farms (B). (A) Boxplots of the occurrence of colistin-resistant Enterobacterales in hospitalized patients, healthy individuals in day care centres and long-term care facilities, broilers and pigs. Differences in the occurrences of colistin resistance were tested using generalized linear models with negative binomial distribution. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$). (B) Occurrence of colistin-resistant Enterobacterales and colistin treatment incidence per farm. Colistin treatment incidence includes prescriptions one year before the first measurement (1) and between the first and second measurement (2). BE: Belgium, NL: the Netherlands, LTCF: long-term care facility, NA: data not available.

5.3.2 Colistin use in broiler and pig farms

In the study period, colistin TI was higher in the pig populations in comparison to broiler chickens. Among the surveyed farms, nearly all Belgian (14/15) and the majority of Dutch (11/15) pig farms employed colistin as a treatment six months before or during the study period (**Figure 5.1B**). In contrast, its use was limited to only one Belgian and two Dutch broiler farms. Notably, the colistin TI within the farms displayed variability on a per-farm basis. In particular, three Belgian pig farms (farm IDs 1508, 1509 and 1512) showed a high TI of colistin during and between the measurement periods which was linked to a high occurrence of colistin resistance (>50% of the pigs positive for carriage of colistin-resistant Enterobacterales) (**Figure 5.1B**). Colistin resistance was positively associated with the prior use of colistin within pig farms (**Table 5.2**).

Table 5.2: Association of colistin resistance with prior colistin use in pig farms in Belgium (n=14) and the Netherlands (n=15). A total of 379 and 420 Belgian as well as 298 and 450 Dutch pigs were screened for the carriage of colistin-resistant Enterobacterales in the first and second measurement, respectively. Associations were assessed using a generalized linear model.

Measurement	Use	Country	Estimated change in odds of colistin resistance for each unit increase in colistin use[A]	95% CI	p-value
Measurement 1	1 year before measurement	Belgium	1.13	1.04-1.23	* (<0.05)
		Netherlands	1.08	0.71-1.66	ns
Measurement 2	2-3 years before measurement	Belgium	1.12	1.02-1.22	* (<0.05)
		Netherlands	0.87	0.47-1.61	ns
	6 to 15 months before measurement	Belgium	1.22	1.09-1.36	** (<0.01)
		Netherlands	1.85	1.20-2.86	* (<0.05)
	6 months before measurement	Belgium	1.18	1.03-1.35	* (<0.05)
		Netherlands	1.39	1.09-1.76	* (<0.05)

CI: confidence interval, ns: not significant. [A] The estimated change in odds represents the odds of colistin resistance after colistin use compared to the odds of colistin resistance without colistin use. Odds ratio above one indicated that colistin exposure is associated with higher odds of colistin resistance.

5.3.3 Chromosomal mutations and plasmid-mediated colistin resistance detected in colistin-resistant *E. coli, Klebsiella* spp. and *Enterobacter* spp.

A total of 343 *Escherichia coli,* 112 *Klebsiella pneumoniae,* 28 *Enterobacter (quasi)roggenkampii*, 24 *Klebsiella variicola*, 13 *Enterobacter cloacae,* 10 *Enterobacter asburiae*, 8 *Enterobacter kobei, 6 Klebsiella michiganensis*, 5 *Enterobacter hormaechei,* 5 *Enterobacter ludwigii*, 4 *Klebsiella quasipneumoniae*, 2 *Klebsiella aerogenes,* 2 *Klebsiella oxytoca* were sequenced to study the molecular make-up of colistin-resistant isolates.

Overall, mutations were most prevalent in *pmrB* (440/562, 78.3%), followed by *pmrA* (222/562, 39.5%) and *phoQ* (186/562, 33.1%). Mutations in *phoP* were less prevalent (27/562, 4.8%) (data not shown). Alterations in *mgrB* or its promotor region were detected in *E. coli* (76/343, 22.2%), *Enterobacter* spp. (32/69, 46.4%) and *Klebsiella* spp. (93/150, 62.0%) (**Supplementary Table 5.1**). Concurrent mutations in two component system PmrAB and PhoPQ or its regulators were present in most isolates (508/571), however, single mutations led to colistin resistance in 49 isolates (8.7%) (**Supplementary Table 5.2**).

Plasmid-mediated *mcr*-genes were detected in 36 of the 562 sequenced colistin-resistant isolates (6.4%). The *mcr* genes were detected in 31/178 (17.4%) of the screened pigs, none of the broilers, 1/96 (1.0%) of the hospitalized patients, 2/112 (1.8%) of the residents in LTCF and 1/146 (0.7%) of the children. Bacterial species were 31 *E. coli* (83.8%), 1 *E. asburiae* (2.7%), 1 *E. roggenkampii* (2.7%), 2 *E. kobei* (5.4%) and 1 *E. hormaechei* (2.7%). Plasmid-mediated colistin resistance genes were not detected in any *Klebsiella* species isolates. Genes *mcr-1.1*, *mcr-2.1, mcr-2.2* and *mcr-5.1* were all detected in *E. coli* isolated from Belgian pig farms, while *mcr-9* and *mcr-10* were detected in *Enterobacter* isolates from hospitalized patients and healthy individuals (from a Belgian hospital, day care center and LTCF, and a Dutch hospital) (**Supplementary Figure 5.3**).

Different MGEs were flanking these *mcr*-genes: IS26 flanked *mcr-1.1*, *mcr-5.1*, *mcr-9.1* and *mcr-10,* ISApI1 flanked *mcr-1.1*, while *mcr-2* was flanked by IS*Ec*69. The presence of *mcr-1.1* and *mcr-2.1* genes was observed on IncX4 and IncHI2 plasmids, while the

mcr-5.1 genes could be identified on an IncFII (29) plasmid (**Figure 5.2**). Aligning the reads to the most similar reference plasmid sequence according to blastn, showed that several *mcr-1.1*-harboring sequences from Belgian pig farm 12 were highly similar (query coverage 100%, >99.70% identity) to pMFDS2258.1 (accession number MK869757.1), a plasmid isolated from chicken meat from Brazil in 2017 (**Figure 5.2A**). Similarly, an *mcr-1*-haboring plasmid from pig farm 7 was aligned to a plasmid from an Italian stream (accession number MF449287.1) (**Figure 5.2D**). Other *mcr-1.1* plasmid sequences from Belgian pigs could be aligned to various IncX4 and IncHI2 plasmids with lower query coverages (1%-77%) (**Figure 5.2B-E**). In addition, high query coverage (>99%) was found between *mcr-2*-harboring sequences from Belgian pig farms 4 and 9 to pKP37 (accession number LT598652.1), an *mcr-2.1*- carrying plasmid isolated from Belgian pigs in 2016 (22) (**Figure 5.2F**). Both the *mcr-1*- and the *mcr-2*-harboring plasmids were detected over time in the same pig farms, suggesting the persistence of these plasmids in the farms over a period of one year. *Mcr-5.1* sequences from Belgian pigs were aligned to a plasmid from human stool in Mexico (pYU07-18_89; CP035549.1, query coverage 95%) and from pork meat in Vietnam (pVE155; AP018354.1, query coverage 57%) (**Figure 5.2G-H**). *Mcr-9* and *mcr-10*- containing plasmids showed similarities with plasmids previously isolated in Egypt, Spain and China (query coverage 0.7-89%) (**Figure 5.2I-L**).

Figure 5.2: Global view of Basic Local Alignment Search Tool (BLAST) comparisons between the *mcr*-harboring sequences and the most similar reference plasmid sequence (in grey) according to blastn. Figure was generated using the BLAST Ring Image Generator (BRIG). Percentages indicate the query coverage of the *mcr*-containing sequence with the reference plasmid. Isolate ID, origin, measurement round (M1/M2) are indicated for each ring. Insertion sequences, plasmid Inc type, resistance and virulence genes are indicated in different colors.

5.3.4 Phenotypic and genotypic resistance identified in colistin-resistant isolates in examined One Health sectors

Phenotypic MDR was detected in 61.5% (291/473) of *E. coli*, 33.1% (55/166) *Klebsiella* spp. and 78.7% (55/75) of *Enterobacter* spp. isolates. Colistin-resistant and MDR human-associated isolates were most commonly resistant to ampicillin (46.8% of the human MDR isolates), amoxicillin- clavulanic acid (71.2%) and cefoxitin (43.2%). MDR livestock-associated isolates were regularly resistant to ampicillin (80.0% of the broiler MDR isolates and 83.0% of the porcine MDR isolates) and trimethoprim-sulfamethoxazole (76.0% of the broiler MDR isolates, 83.0% of the porcine MDR isolates). The percentage of livestock-associated *E. coli* (82.4%) and *Klebsiella* spp. (58.5%) isolates with an MDR phenotype was higher compared to human-associated isolates (30.2% of *E. coli* and 16.8% of *Klebsiella* spp.). For *Enterobacter* isolates, this difference in MDR proportions was not observed (83.3% of the animal-associated and 78.3% of the human-associated isolates) (**Figure 5.3A**). Nonetheless, the majority of the colistin-resistant *E. coli* (419/473, 88.6%), *Klebsiella* spp., (126/166, 75.9%) and *Enterobacter* spp. (50/75, 66.7%) were phenotypically susceptible to the critically important antibiotics (fluoroquinolones, extended-spectrum cephalosporins, carbapenems and aminoglycosides). Carbapenem resistance and carbapenemase genes were not found in any isolate of the different settings. Phenotypic resistance rates to extended-spectrum cephalosporins, fluoroquinolones or aminoglycosides were relatively low (7.2%, 6.2% and 2.9% of the isolates, respectively) (**Figure 5.3B**). Acquired ESBL genes were detected in 3.6% of the isolates, *qnr* genes were detected in 6.9% isolates and mutations in the QRDR were detected in 10.3% of the isolates (**Figure 5.4**).

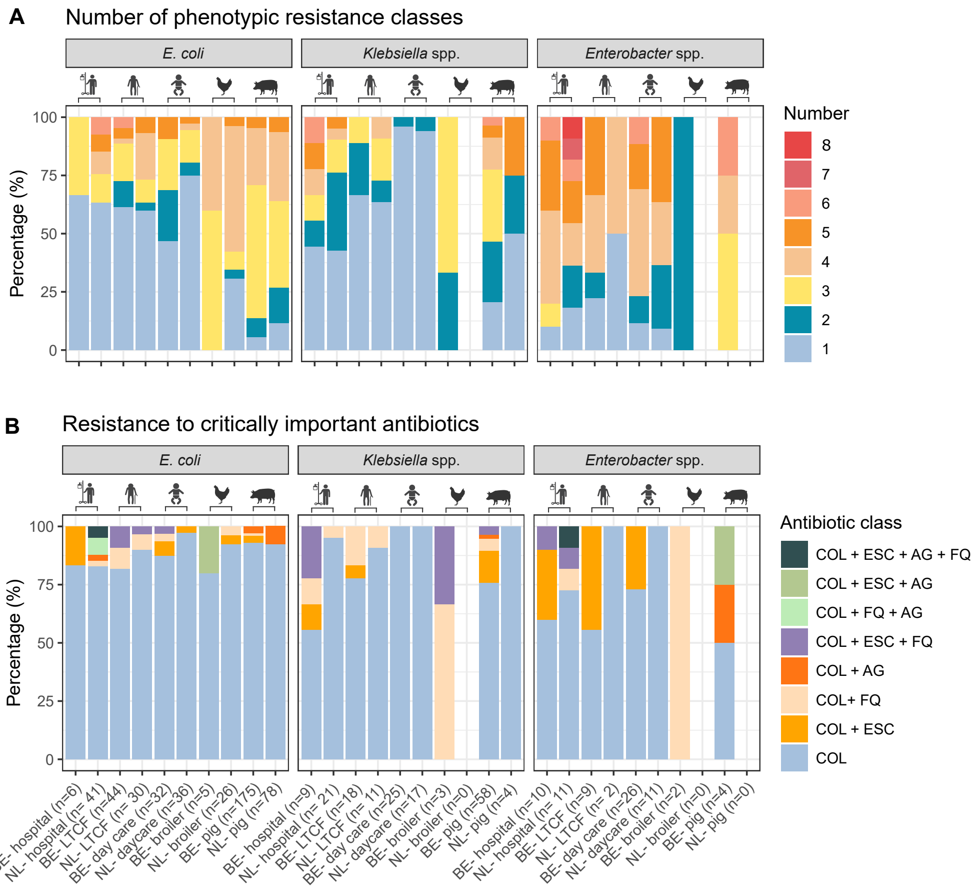


Figure 5.3: Phenotypic antibiotic resistance of colistin-resistant Enterobacterales. (A) Stacked barplots of the proportion of isolates phenotypically resistant to a number of antibiotic classes. (B) Stacked barplots of the proportion of isolates phenotypically resistant to critically important antibiotics. AG: aminoglycosides, COL: colistin, ESC: extended-spectrum cephalosporins, FQ: fluoroquinolones, LTCF: long-term care facility, BE: Belgium, NL: the Netherlands.

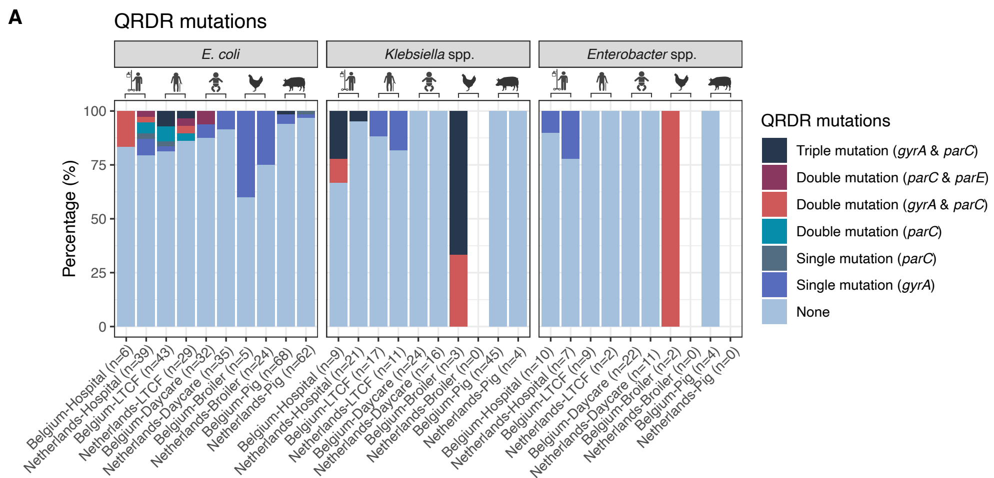
5.3.4.1 Associated resistance in colistin-resistant *E. coli*

Resistance to extended-spectrum cephalosporins was detected in 4.0% (19/473) of the *E. coli* isolates from all sectors, except from Dutch pig farms. ESBL genes (*bla*$_{CTX-M}$ and *bla*$_{OXA}$ type) were acquired by 2.9% (10/343) of the sequenced *E. coli*. Phenotypic resistance to ciprofloxacin was detected in 6.8% (32/473) of the isolated *E. coli*. Plasmid-mediated *qnr* genes were detected in two hospitalized patients from the Netherlands and

two children, one LTCF resident and twelve pigs from Belgium. A total of 12.2% (42/343) harbored one or more mutations in *gyrA*, *parC* and/or *parE* (**Figure 5.4**). Phenotypic aminoglycoside (gentamicin, tobramycin or amikacin) resistance was present in *E. coli* from Dutch hospital patients, Belgian broilers and Belgian and Dutch pigs (3.6%, 17/343). The *aac(3)* family resistance genes was present in 4.7%. Combined resistance to colistin, extended-spectrum cephalosporins, fluoroquinolones and aminoglycosides was detected in 2 *E. coli* isolates (0.4%) from Dutch hospitals.

<u>5.3.4.2 Associated resistance in colistin-resistant *Klebsiella* spp.</u>

Acquired ESBL genes were detected in 5.3% (8/150) of the *Klebsiella* isolates. Ciprofloxacin resistance was present in 16/166 isolates (9.6%). This resistance was linked to mutations in QRDR regions of *gyrA* and *parC* in 11/150 isolates (7.3%) and *qnr* genes in 18/150 isolates (12.0%). A total of 48/150 isolates (32.0%) harbored aminoglycoside resistance genes (**Figure 5.4**).



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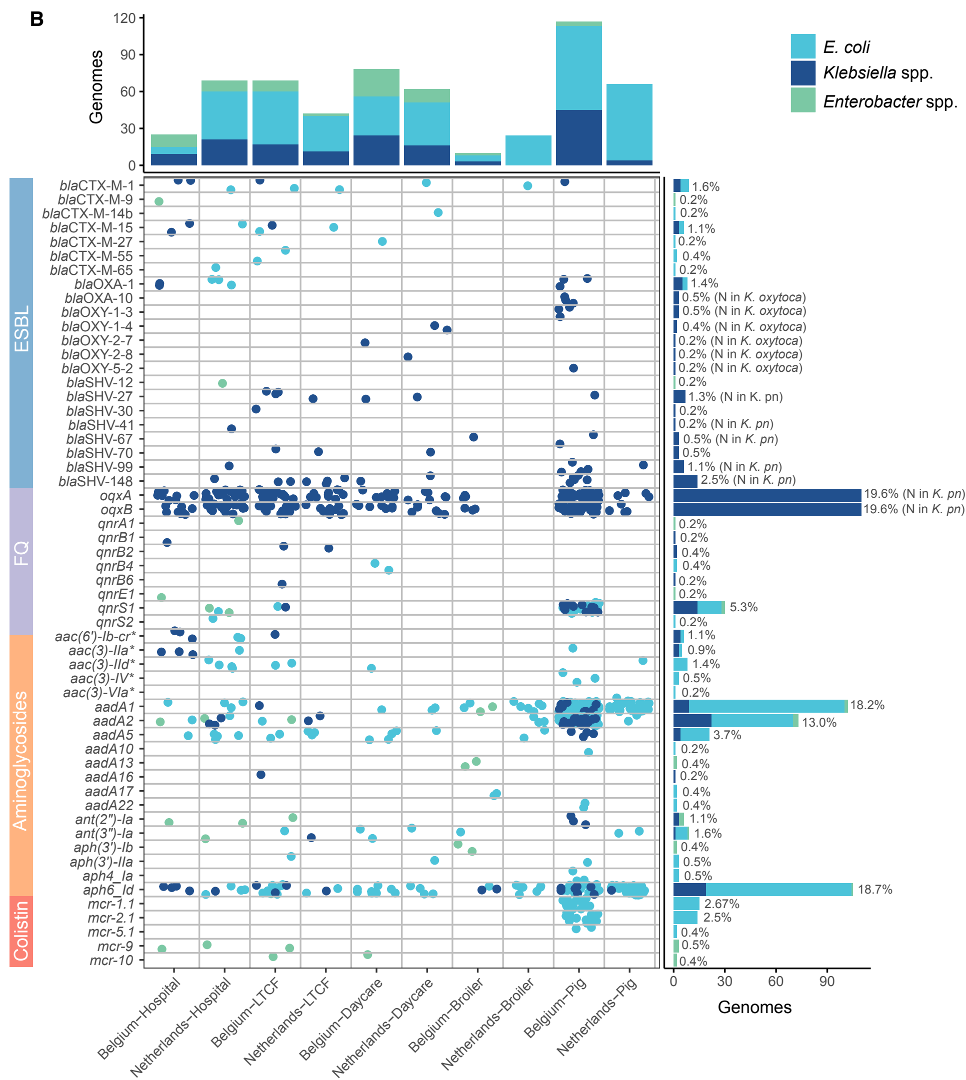


Figure 5.4: Genotypic fluoroquinolone resistance mutations (A) and genes (B) for critically important antibiotics detected in colistin-resistant Enterobacterales. (A) Stacked barplots of the proportion of colistin-resistant *E. coli* and *Klebsiella* spp. with mutations in the quinolone-resistance determining regions (QRDR) linked to fluoroquinolone resistance. (B) Resistance genes for critically important antibiotics detected in colistin-resistant Enterobacterales. Each circle represents a genome (isolate) colored by species. Barplots show the number of genomes from the different sectors (top) and containing the resistance gene (right) colored by species. N: naturally occurring genes, *Kpn*: *K. pneumoniae*, LTCF: long-term care facility, QRDR: quinolone-resistance determining region, FQ: fluoroquinolone, ESBL: extended-spectrum beta-lactamase, * aminoglycoside genes linked to resistance to gentamicin, tobramycin.

5.3.4.3 Associated resistance in colistin-resistant *Enterobacter* spp.

An intermediate phenotype for meropenem was observed in *E. cloacae* from one Belgian broiler and to imipenem from one Dutch child (0.2%). Resistance to extended-spectrum cephalosporins was detected in 24.0% (18/75) *Enterobacter* isolates, while the proportion of isolates resistant to ciprofloxacin (8.0%, 6/75) and aminoglycosides (4.0%, 3/75) was low (**Figure 5.3**). A single mutation in QRDR region of *gyrA* (S83I or S83Y) was detected in 3 isolates (7.2%) from hospitalized patients and both Belgian broiler isolates harbored a mutation in *gyrA* (S83I) and *parC* (S80I). ESBL genes among *Enterobacter* spp. were uncommon: $bla_{CTX\text{-}M\text{-}9}$ was harbored by *E. kobei* from a Belgian patient and $bla_{SHV\text{-}12}$ was harbored by *E. hormaechei* from a Dutch patient (**Figure 5.4B**).

5.3.5 Virulence potential of colistin-resistant *E. coli*, *Klebsiella* and *Enterobacter* isolates from examined One Health sectors

Virulence factors present in all isolates were linked to fimbrial adhesins, inflammatory signaling, invasion and the enterobactin siderophore. Various iron uptake systems such as aerobactin, salmochelin and yersiniabactin were associated mainly with human-associated *Escherichia* isolates and were less prevalent among livestock-associated isolates ($p<0.001$) (**Supplementary Figure 5.4).**

Colistin resistance was also detected in *K. pneumoniae* harboring hypervirulence genes and various *E. coli* pathotypes, suggesting that these commensal bacteria may have pathogenic potential. Investigation of virulence-associated genes have uncovered the presence of virulence plasmid-associated loci, specifically *iuc*, *iro*, and *rmpA/rmpA2*, in three colistin-resistant *K. pneumoniae* strains. These strains have the potential to exhibit hypervirulent characteristics and belong to two distinct sequence types: ST5 (K39, O1 type), originating from two separate swine farms in Belgium, and ST592 (K57, O3b type) obtained from a medical facility in the Netherlands. Colistin resistance was detected in different pathotypes including intestinal and extraintestinal pathogenic *E. coli*. Colistin-resistant *E. coli* pathotypes detected were STEC (porcine *E. coli* n=2), DAEC (human-associated *E. coli*, n=14), atypical EPEC (n=18 from all examined One Health sectors)

and UPEC harboring *papC, papG* and *iucC* (human-associated *E. coli*, n=24). Of these pathogenic *E. coli*, 22 isolates (35.5%) were MDR. Half of these pathogenic, MDR *E. coli* (n=11) belonged to known invasive extraintestinal *E. coli* STs (ST10, ST38, ST69, ST73 and ST131) (23).

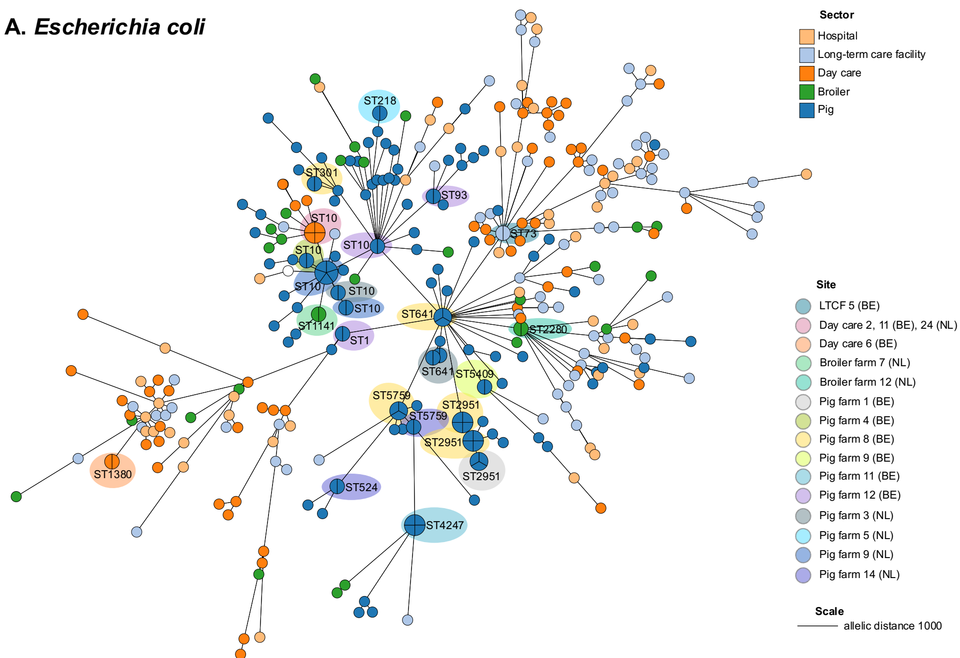
5.3.6 Detection of colistin resistance within pandemic lineages

A diversity of STs was detected among the ColR-E isolates. Several pandemic clones, such as *E. coli* ST10 (n=35) of which three harbored *mcr-1.1* on an IncX4 plasmid, ST38 (n=7), ST131 (n=17), ST405 (n=2), ST648 (n=2), ST1193 (n=4), *Klebsiella pneumoniae* ST15 (n=2), ST45 (n=7), ST101 (n=1), ST147 (n=1) and ST307 (n=1), and *Enterobacter cloacae* ST171 (n=1) were detected. Most of these isolates (n=57/80, 71.3%) were not resistant to fluoroquinolones, extended-spectrum cephalosporins, carbapenems and aminoglycosides. However, human-associated *E. coli* ST131 and ST1193 showed high virulence potential combined with resistance to critically important antibiotics (**Figure 5.5**). Within LTCFs and hospitals, the presence of *E. coli* ST131 strains displaying colistin resistance and possessing *bla*$_{\text{CTX-M-15}}$ and fluoroquinolone resistance mutations (H30Rx) were discovered (n=3), along with the detection of ST131-H30R (fluoroquinolone-resistant) strains (n=3).

Figure 5.5: Insights into the resistance to critically important antibiotics and the number of virulence classes present in colistin-resistant isolates from One Health sectors involved. Dotplot of the resistance and number of virulence classes per isolate. Each circle represents a single isolate. Color indicates species and sequence types of resistant isolates are indicates with labels. LTCF: long term care facility. COL: colistin resistance, ESC: extended-spectrum cephalosporin resistance, CARBA: carbapenem resistance, FQ: fluoroquinolone resistance, AG: aminoglycoside resistance.

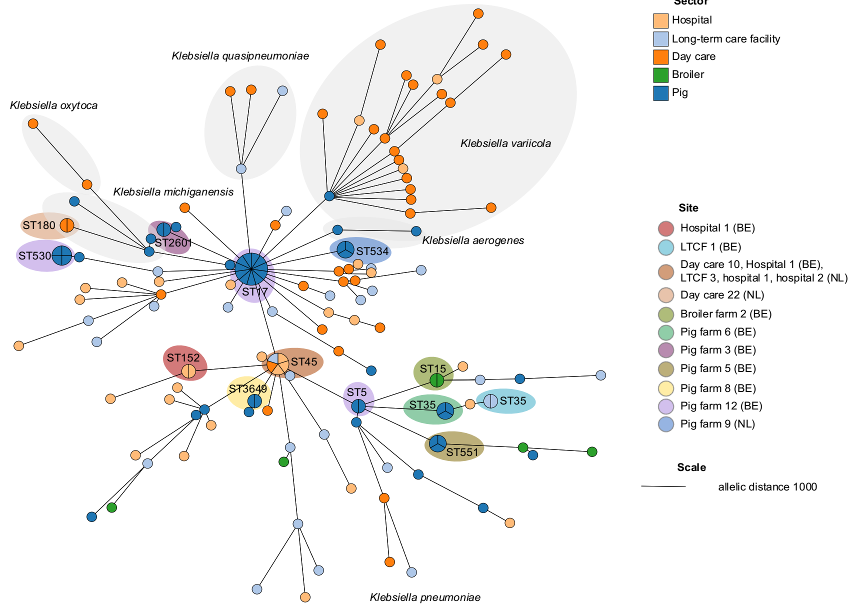
5.3.7 Potential transmission pathways of colistin-resistant Enterobacterales across and within the One Health framework

Inter-host transmission between humans and livestock animals was not detected. However, clusters of related isolates were detected in all sectors indicating that transmission of ColR-E occurred in broiler and in pig farms, between children within the day care centres, and between patients residing in the LTCFs and the hospitals (**Figure 5.6 and Table 5.3**). Related isolates were also detected between different sampling sites. Closely related isolates of *K. pneumoniae* ST45 (n=5) isolates were detected between the Dutch and Belgian hospitals, a Belgian day care centre and a Dutch LTCF. Similarly, a clonal clustering of *E. coli* ST10 (n=4) was identified at two Belgian day care centres and at a Dutch day care centre. The transmission of *mcr-1.1-* and *mcr-2.1-* harboring *E. coli* was also detected amongst Belgian pig farms. A recurrent presence of clonally related strains was noted during both measurements, strongly suggesting the persistent circulation of these particular isolates within the pig farm ecosystem (**Table 5.3**).



(Continued)

B. *Klebsiella* spp.



C. *Enterobacter* spp.

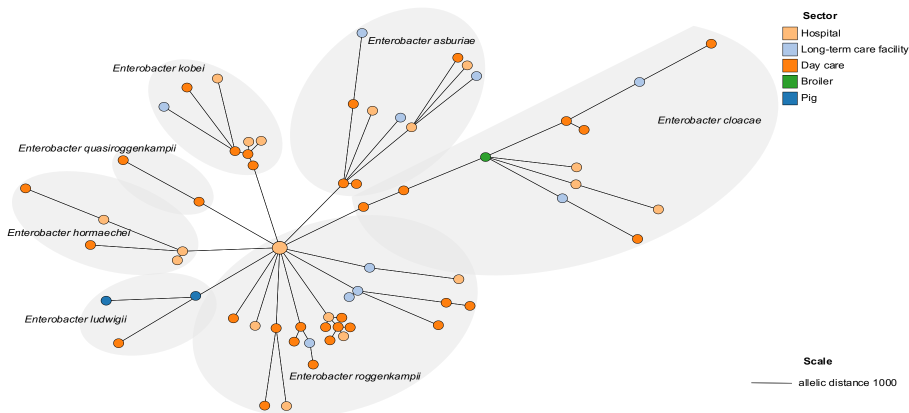


Figure 5.6: Minimum spanning trees of *Escherichia coli* (n=343) (A), *Klebsiella* spp. (n=156) (B) and *Enterobacter* spp. (n=69) (C) isolated from humans in hospitals, long-term care facilities (LTCF), day care centres, broilers and pigs in farms. Minimum spanning trees based on allelic distances of cgMLST profile data (2976 loci for *E. coli*, 3362 loci for *Klebsiella* spp., and 2952 loci for *Enterobacter* spp.). Branch lengths indicate the allelic distance as indicated by the tree scale. Collapsed nodes indicate genetically related isolates with ≤10 and 12 allelic differences for *E. coli* and *Klebsiella* spp. respectively. The sequence type is indicated for each cluster of related isolates. The origin of isolation is shown as colored nodes for each isolate. BE: Belgium, NL: Netherlands, ST: sequence type.

Table 5.3: Origin and characteristics of related isolates. Clonal relatedness was defined as ≤10 and ≤ 12 allelic differences between isolates of *E. coli* and *Klebsiella* spp., respectively.

ST	Species	Nr of isolates	Domain	Site(s)	Measure-ment round	Allelic distance [min-max]	Colistin MIC (mg/L)	*mcr*	Resistome
ST1	*E. coli*	2	Pig	BE pig farm 12	2	3	4/8	-	*blaTEM-1B*
ST10	*E. coli*	5	Pig	NL pig farm 9	1 & 2	[3-9]	4/8	-	*aadA1, blaTEM-1A, dfrA1, tet(B)*
ST10	*E. coli*	2	Pig	BE pig farm 4	1	10	8	-	*aadA22, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, lnu(G), qacE, sul1, sul2, tet(B)*
ST10	*E. coli*	2	Pig	BE pig farm 12	2	1	8	*1.1*	*aadA1, aadA2, blaTEM-1B, cmlA1, dfrA12, mcr-1.1, sul3*
ST10	*E. coli*	4	Day care	BE day care 2 & 11, NL day care 24	1	[2-9]	8/16	-	-
ST10	*E. coli*	2	Pig	NL pig farm 9	2	2	8/16	-	*aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1C, dfrA1, mph(B), qacE, sul1, sul2, tet(B)*
ST10	*E. coli*	2	Pig	NL pig farm 3	1	7	4	-	*blaTEM-1B, sul2, tet(B)*
ST1141	*E. coli*	2	Broiler	NL broiler farm 7	1	8	4/8	-	*blaTEM-1B, dfrA1, sul2*
ST1380	*E. coli*	2	Day care	BE day care 6	1	9	8	-	*blaDHA-1, blaTEM-1B, mph(A), qacE, qnrB4, sul1*
ST218	*E. coli*	2	Pig	NL pig farm 5	1 & 2	7	4/8	-	*aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, dfrA1, qacE, sul1, sul2, tet(A)*
ST2280	*E. coli*	2	Broiler	NL broiler farm 12	1	2	8	-	*sitABCD*

ST2951	*E. coli*	3	Pig	BE pig farm 1	2	[2-7]	16	*1.1*	*aac(3)-IV, aadA1, aadA2, aph(3'')-Ib, aph(3')-Ia, aph(4)-Ia, aph(6)-Id, blaTEM-1B, cmlA1, dfrA12, mcr-1.1, sul2, tet(A), (cmlA1, dfrA12 (n=1))*
ST2951	*E. coli*	4	Pig	BE pig farm 8	2	[0-7]	8	-	*aadA1, aadA2, cmlA1, dfrA12, floR, qnrS1, sul2, sul3, tet(A)*
ST2951	*E. coli*	4	Pig	BE pig farm 8	1 & 2	[3-7]	4/8	-	*aadA1, aadA2, blaTEM-1B, cmlA1, dfrA12, floR, sul2, sul3, tet(A)*
ST4247	*E. coli*	4	Pig	BE pig farm 11	1 & 2	[3-7]	8/16	-	-
ST524	*E. coli*	2	Pig	NL pig farm 14	1 & 2	10	4/8	-	*aadA1, dfrA1, qacE, sul1*
ST5409	*E. coli*	2	Pig	BE pig farm 9	2	6	4	*2.1*	*aadA1, aadA2, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, cmlA1, dfrA12, dfrA8, floR, mcr-2.1, sul2, sul3, tet(A)*
ST5759	*E. coli*	3	Pig	BE pig farm 8	2	[3-6]	8	*1.1*	*aadA2, blaTEM-1B, lnu(F), mcr-1.1, qnrS1*
ST5759	*E. coli*	2	Pig	NL pig farm 14	2	8	8	-	*aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, qacE, sul1, sul2*
ST641	*E. coli*	3	Pig	BE pig farm 8	1	[2-5]	8/16	-	*aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA14, sul2, tet(A)*
ST641	*E. coli*	2	Pig	NL pig farm 3	1	7	4/8	-	*aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, qacE, sul1, sul2, tet(B)*
ST73	*E. coli*	2	LTCF	BE LTCF 5	1	1	8/16	-	-
ST93	*E. coli*	2	Pig	BE pig farm 12	2	5	8/16	-	*aadA1, aadA2, blaTEM-1B, cmlA1, dfrA12, sul3*
ST301	*E. coli*	2	Pig	BE pig farm 8	1	0	8	-	-

ST3649	*K. pneumoniae*	2	Pig	BE pig farm 8	1	2	16/64	-	*blaLAP-2, blaSHV-148, fosA, OqxA, OqxB, qnrS1, sul2, tet(A)*
ST45	*K. pneumoniae*	5	Hospital, LTCF, day care	NL hospital 1 & 2, BE hospital, BE day care 13, NL LTCF 3	2	[1-12]	16/32/64	-	*blaSHV-78/49/148, fosA, OqxA, OqxB, tet(D)*
ST551	*K. pneumoniae*	3	Pig	BE pig farm 5	1	[6-11]	16/32	-	*blaSHV-172,fosA, mph(A), OqxA, OqxB, (aph(3'')-Ib, aph(6)-Id, blaCTX-M-1, blaTEM-1B, dfrA8, sul2 (n=1))*
ST35	*K. pneumoniae*	2	LTCF	BE LTCF 1	1	0	8/64	-	*blaSHV-33, fosA6, OqxA, OqxB*
ST35	*K. pneumoniae*	3	Pig	BE pig farms 6	1	[9-10]	32/64	-	*aadA2, aph(3')-Ia, blaSHV-33, blaTEM-1B, dfrA12, fosA6, lnu(G), mph(A), OqxA, OqxB, qacE, sul1, sul2, tet(D)*
ST15	*K. pneumoniae*	2	Broiler	BE broiler farm 2	1	4	8	-	*aph(3'')-Ib, aph(6)-Id, blaSHV-100, blaTEM-1B, catA1, fosA6, lnu(G), OqxA, OqxB*
ST5	*K. pneumoniae*	2	Pig	BE pig farm 12	1	2	1/8	-	*aph(3'')-Ib, aph(6)-Id, blaSHV-62, OqxA, OqxB, sul2*
ST2601	*K. pneumoniae*	2	Pig	BE pig farm 3	2	6	32/64	-	*aadA2, blaSHV-89, blaTEM-1B, catA1, dfrA12, fosA, OqxA, OqxB, qacE, sul1, tet(B)*
ST180	*K. michiganensis*	2	Day care	NL day care 22	2	2	4/16	-	*aph(3')-Ia, blaOXY-1-4*

ST530	*K. pneumoniae*	3	Pig	BE pig farm 12	1	[6-8]	64/>64	-	*aadA1, aadA5, ant(2")-Ia, blaOXA-10, blaSHV-99, blaTEM-1B, dfrA17, OqxA, OqxB, qacE, qacE, sul1, sul1, tet(A), tet(B)*
ST534	*K. pneumoniae*	3	Pig	NL pig farm 9	1 & 2	[5-10]	64/>64	-	*blaSHV-80, fosA, OqxA, OqxB, tet(D), (aph(3")-Ib, aph(6)-Id, blaTEM-1B, fosA, sul2 (n=1))*
ST17	*K. pneumoniae*	11	Pig	BE pig farm 12	1 & 2	[1-12]	64/>64	-	*blaSHV, fosA6, OqxA, OqxB, tet(D), (aadA2, blaTEM-1B, dfrA16,, qacE, qnrS1, sul1 (n=7))*
ST152	*K. pneumoniae*	2	Hospital	BE hospital	2	5	8/16	-	*aac(3)-IIa, aac(6')-Ib-cr, aac(6')-Ib-cr, aph(3")-Ib, aph(3')-Ia, aph(6)-Id, ARR-3, blaCTX-M-15, blaOXA-1, blaSHV-1b-b, blaTEM-1B, catA1, catB3, dfrA27, fosA, mph(A), OqxA, OqxB, qacE, sul1, sul2, tet(D)*

BE: Belgium, NL: Netherlands, MIC: minimum inhibitory concentration, ST: sequence type.

5.4 Discussion

Using an integrative approach, this study showed the presence of ColR-E among all studied One Health sectors and provides a detailed overview of the phenotypic and molecular makeup of these colistin-resistant isolates from different niches.

This is the first study to have investigated colistin resistance in humans and animals in Belgium and the Netherlands using a One Health approach with a uniform methodology. In the Netherlands, the parallel monitoring of antimicrobial resistance and antibiotic use in animals and humans is reported within Nethmap-MARAN. However, colistin screening in humans in not included (17). In Belgium, the BELMAP report summarizes the antibiotic use and resistance data in the human and veterinary sectors to provide a One Health overview of the Belgian situation (24). These national reports lack whole genome sequencing of colistin-resistant isolates. The available studies on colistin resistance using a One Health concept essentially consist of systematic review and meta-analysis of available literature involving a limited number or specific settings (not using a One Health approach) and using different methodologies (13,25,26).

In this study, we estimated the prevalence of colistin resistance in Belgian and Dutch One Health sectors using selective culturing and whole genome sequencing. Depending on the farm, the percentage of pigs within a farm colonized with ColR-E varied from 0% to 93.3%. The level of colistin resistance was positively associated with prior colistin usage in these pig farms, as was also shown by other studies in food animals (27,28). Although the sales of polymyxins in veterinary medicine is decreasing since 2011 (17,24,27), colistin was used in most of the pig farms in this study. Pigs remain the species with the largest use of colistin, especially weaner pigs for the treatment of enteropathogenic *E. coli* infections (24,29,30). In contrast, colistin was used less frequently in the studied broiler farms which is reflected by the low percentage of broilers carrying ColR-E (2.2%). According to the national and European monitoring systems, investigating resistance in indicator bacteria from healthy food-producing animals, prevalence of colistin resistance remained stable and very low (below 10%) over the years (24,29,31). Colistin resistance

in *E. coli* was not detected in the gastro-intestinal tract of food-producing animals, meat and vegetables in the Netherlands in 2021 when using passive screening (non-selective isolation) (17). The discrepancies with the prevalences found in pig farms in this study can be probably explained by the enrichment step and selective culturing methods we used here and which may have resulted in higher prevalence comparatively to studies using less sensitive methods (32,33). Secondly, the selected farms had higher than average antibiotic use and are not representative for all farms in Belgium and in the Netherlands. Notwithstanding that the use of colistin in food-producing animals outweighs the use of colistin in humans in Europe (27), the prevalence of fecal carriage of ColR-E was detected relatively frequent in three different human sectors assessed in this study. The proportion of hospitalized patients carrying ColR-E was higher in the Netherlands (11.3-11.8%) than in Belgium (4.4-7.9%) though this cannot be considered as representative for the whole country as only three hospitals were involved in the present study. In our study, combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides was not detected in colistin-resistant *K. pneumoniae* from Belgian and Dutch patients nor in *E. coli* from Belgian patients, and therefore lower compared to invasive *K. pneumoniae* isolates from the EARS-NET surveillance (10.3% of the *K. pneumoniae* isolates and 2.9% in *E. coli* from Belgium and 4.3% of the *K. pneumoniae* in the Netherlands). Combined resistance to these critically important antibiotics was detected in 4.9% of the Dutch colistin-resistant *E. coli* isolates compared to 1.9% of invasive *E. coli* isolates from Dutch hospitals in EARS-NET (34). In contrast, proportions of humans colonized with ColR-E in the other human health sectors were higher in Belgium (10.2% in LTCF and 17.6% in day care centres) compared to the Netherlands (5.6% in LTCF and 12.8% in day care centres). The occurrence of colistin resistance in the human population is sparsely studied in Europe. The prevalence of colistin resistance among human clinical Enterobacterales showed a regional variation of 2.4% to 3.4% in Europe (11). In Switzerland, 1.5% of healthy individuals and 3.8% of primary care patients were carriers of ColR-E (35). A recent study showed that 0.3% of the tested *E. coli* and 0.6% of the tested *K. pneumoniae* from clinical samples in the

Netherlands were colistin-resistant (8) and colistin resistance in invasive clinical *E. coli* isolates from hospitalized patients in Belgium remains below 1% (24). A surprisingly high percentage of children in day care centres (15.1%) showed rectal carriage of ColR-E in this study. These high occurrences could possibly partly be explained by factors investigated within our project, such as frequent contact of the studied children with animals (>70% of the children had contact with domestic animals, petting zoo animals and livestock animals), antibiotic use (21% of the Dutch children and 53% of the Belgian children received antibiotics in the last six months before sampling) or hospital stays (7% of the Dutch children and 18% of the Belgian children were admitted in a hospital in the last six months before sampling) (36). A total of 4% and 7% of the Dutch and Belgian residents in LTCF received antibiotic treatment in the last six months before measurement (37). In addition, infection prevention measures (hand hygiene and a clean environment in LTCF as well as hand hygiene, cleaning of toys and avoiding fecal contamination such as cleaning the changing table, use of paper towels in day care centres) could be improved in most day care centres and LTCFs to prevent the spread of resistant bacteria (36,37). Adenosine triphosphate measurements also showed higher levels of environmental contamination in Dutch hospitals compared to Belgian hospitals, likely due to differences in cleaning protocols (38).

Plasmid-mediated colistin resistance was detected in 6.4% of the isolates, which is in line with a previous study (9.7%) (8). *mcr* genes were detected in 1.0% of the hospitalized patients, 1.8% of the LTCF residents, 0.7% of the children attending childcare centres, 17.4% of the screened pigs and none of the broilers which is lower than the estimated prevalences in these sectors worldwide (7.4% in healthy humans, 4.2% in patients, 15.8% in chickens). The estimated prevalence in pigs was higher in our study (17.4%) compared to the meta-analysis (14.9%) (13). Colistin resistance genes *mcr-1.1*, *mcr-2.1* and *mcr-5.1* were reported before in *E. coli* from Belgian pigs between 2012 and 2016 (39). The persistence of the highly related IncX4 plasmids harboring *mcr-1.1* or *mcr-2.1* over a one-year period in these pig farms emphasizes the need for increased efforts to control the spread of *mcr* genes. For example, the ban on free use of colistin in animals has reduced

the incidence of *mcr-1*-harboring IncX4-type plasmids, whose presence is associated with an effective dispersal potential in enterobacteria and among different One Health niches (human, dogs, chickens and flies) (13–15,40). Reports on *mcr-9* in *K. pneumoniae* and *E. cloacae* from clinical samples in the Netherlands were published before (2015-2020) (8,41). In this study, *mcr-9* and *mcr-10* were observed among several *Enterobacter* spp. human isolates from hospitals, day care centres and LTCF in Belgium and the Netherlands suggesting that surveillance of these *mcr* genes is needed. The *mcr*-harboring plasmids showed high levels of similarity to plasmids previously isolated in different countries worldwide showing the global spread of these *mcr*-harboring plasmids. In addition, *mcr* genes were flanked by IS elements, strongly suggesting the potential for mobility of these *mcr* genes.

In contrast to *mcr*-plasmids, chromosomal mutations in the core genome are found to be highly stable and irreversible, even after usage of colistin was stopped (4,11). For the majority of the studied isolates, colistin resistance was caused by chromosomal mutations in genes/operons involved in the biosynthesis of the cell-wall LPS. The presence of these stable chromosomal mutations is worrying when present in key human pathogenic lineages. Indeed, various international high-risk clones, such as *E. coli* ST1193 and ST131 harbored chromosomal mutations, meaning that spread of colistin resistance is possible if these mutations are stable and transmitted to the descendants within that clone. In addition, genetically related clones of *K. pneumoniae* ST45 and *E. coli* ST10 were found at different sites, suggesting that these clones might have the potential to spread colistin resistance through the human population or were acquired by exposure to a common (food) source. Clusters of *E. coli* ST10 were also prevalent in several pig farms some of which harbored the *mcr-1.1* (n=3). *E. coli* ST10 was described as a reservoir for *mcr-1* genes before (42) and has the potential to disseminate this gene among food-producing animals.

Fortunately, inter-host transmission between humans and livestock animals was not observed in this study nor in other studies (40,43,44) and resistance to fluoroquinolones, extended-spectrum cephalosporins, aminoglycosides and carbapenems remained low

(<10%), providing several alternative treatment options for these colistin-resistant isolates.

Our study has several limitations. Firstly, this study lacks extensive epidemiological data leaving gaps in our understanding of pathogen transmission. As a result strict thresholds for clonal relatedness were applied. Secondly, the chromosomal mutations were found by *in silico* analysis and were not experimentally confirmed. Thirdly, very few hospitals were included in the study and farms were not representative for the country as we selected farms with higher than average total antibiotic use making the occurrences of colistin resistance in these sectors not representative for the country. Finally, inter-and intra-laboratory differences in the selection of colonies to isolate, bacterial identification and antibiotic testing might have influenced our results, yet, we attempted to minimize these differences by using similar protocols for microbiological methods and by performing the colistin MIC testing and whole genome sequencing centrally in a single laboratory. Nonetheless, to the best of our knowledge, this is the first One Health study to combine harmonized data on colistin use as well as phenotypic and molecular methods and provide detailed insights into the epidemiology of colistin resistance in the clinical setting, the community and livestock animals in Europe. The present research offers valuable insights into the multifaceted impact of colistin resistance across various One Health sectors involved, ultimately informing strategies related to food production, prudent antibiotic use, and safeguarding public health.

5.5 Materials and methods

5.5.1 Setting, study period and sample/strain collection

As part of the i-4-1-Health Interreg project, an analysis of 6591 fecal, perianal or gastrointestinal stoma samples was conducted. These samples were obtained from hospitalized patients (n=998), LTCF residents (n=1430), children attending day care centres (n=947), pigs (n=1597) and broilers (n=1619) across Belgium (Flanders) and the

Netherlands. The collection period spanned from October 2017 to February 2019. The samples originated from different sites: three hospitals (one from Belgium and two from the Netherlands), 30 LTCFs (thirteen from Belgium and seventeen from the Netherlands), 45 day care centres (seventeen from Belgium and 28 from the Netherlands), 31 multiplier pig farms (fifteen from Belgium and sixteen from the Netherlands) and 29 broiler farms (fifteen from Belgium and fourteen from the Netherlands) (**Supplementary Table 5.3**). Screened patients were hospitalized in different wards including at least one surgical unit and an internal medicine ward in each hospital. Screening for rectal carriage was performed on a single day every two weeks in a two month's time period.
Samples were collected cross-sectionally using a nylon-flocked swab with 2 mL Cary-Blair transport medium (FecalSwab™, Copan Italy, Brescia, Italy). Two rounds of repeated surveys, with a one-year interval between each sampling round, were performed in hospitals and in farms. A single survey was performed in long-term care facilities and in day care centres.
The farms were included based on the relative level of antibiotic use which exceeded the average use compared to the national benchmark value in the respective countries. Farm characteristics and antibiotic use were described previously (34).

5.5.2 Colistin use in farms

Colistin use in the farms was calculated from registration documents provided by national quality assurance organizations, the farmers or farm veterinarians. Antibiotic use was quantified as the TI per 100 days for pigs and per production round for broilers described by Caekebeke and colleagues (2020) (45).

5.5.3 Isolation of colistin-resistant Enterobacterales and antibiotic susceptibility testing

Protocols followed for collection and culturing of specimens were similar in the two countries. Selective isolation of ColR-E was performed as previously described by Kluytmans-van den Bergh and colleagues (46). All non-intrinsically resistant

Enterobacterales species were subjected to broth microdilution (Micronaut MIC-Strip Colistin, Merlin Diagnostika GmbH, Bornheim, Germany) for colistin minimum inhibitory concentration (MIC) determination. Reference strains *E. coli* ATCC25922 (colistin MIC: 0.25 mg/L), *P. aeruginosa* ATCC27853 (colistin MIC: 1 mg/L), *E. coli* NCTC 13846 (*mcr-1* positive, colistin MIC: 4 mg/L) and in-house *K. pneumoniae* 08400 (colistin MIC: 64 mg/L) were used as quality controls. Besides colistin, antibiotic susceptibility testing was performed with a distinct local panel for antibiotic susceptibility testing: by Amphia Hospital (Breda, Netherlands) for the Dutch isolates and by University of Antwerp and Antwerp University Hospital for the Belgian isolates as described before (46). The EUCAST breakpoints v12.0 (January 2022) were used for the interpretation of antibiotic susceptibility and resistance. Multidrug resistance is defined as resistance to at least one antimicrobial drug in three or more antibiotic classes (47).

5.5.4 Short-and long-read sequencing of colistin-resistant Enterobacterales

Whole genome sequencing was performed on isolates identified as *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. Selection for sequencing was based on unique isolates exhibiting variations in susceptibility or resistance for at least one antibiotic class as well as two-fold (or larger) differences in colistin MIC, when multiple isolates were obtained from each individual or farms. This selection led to the whole genome sequencing of 562 colistin-resistant isolates. Additionally, 3 colistin-susceptible *E. coli* and 6 colistin-susceptible *K. pneumoniae* were sequenced and were used for comparison with resistant isolates within the study setting. Two colistin-resistant *K. pneumoniae* (1103990 and 1101433) and one colistin-susceptible *K. pneumoniae* (1101124) were selected for long-read sequencing on PacBio Sequel 1 (Pacific Biosciences, CA, USA). All other isolates were sequenced using the short-read Illumina MiSeq (Illumina, San Diego, CA, USA).

For short-read sequencing, a single colony was inoculated in 4 mL Mueller Hinton broth and incubated overnight at 35-37°C. The MasterPure Complete DNA & RNA Purification kit (Epicentre, Madison, WI, USA) was used to extract genomic DNA. Libraries were

prepared using the Nextera XT sample preparation kit (Illumina, San Diego, CA, USA) and sequenced with 2x 250 bp paired end sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

For long-read sequencing, high-molecular-weight DNA was isolated from fresh overnight cultures. Briefly, a single bacterial colony was inoculated in 10 mL Mueller-Hinton broth and incubated overnight at 35-37°C. DNA was extracted using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations were measured using Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were prepared using the SMRTbell Express Template Prep kit 2.0 (Pacific BioSciences, CA, USA) and whole-genome sequencing was performed on the PacBio Sequel I using the Sequel Sequencing kit 3.0 (Pacific BioSciences, CA, USA). The sequences were submitted to NCBI under BioProject PRJNA927131.

5.5.5 De novo assembly and genotyping

Short-read data was trimmed with TrimGalore v.0.4.4 (https://github.com/FelixKrueger/TrimGalore) and assembled *de novo* using SPAdes v.3.13.0 (48) built within BacPipe v1.2.6 (49). Assembly of long-read sequencing data was done using HGAP with default parameters, included in SMRT Link v10.1 (Pacific BioSciences, CA, USA). Assembly quality was assessed with Quast (50). The assembled genome was annotated using Prokka v.1.12 (51). Additional analysis was performed using BacPipe v1.2.6 including the PubMLST database (52), ResFinder (database 2022-05-24) (53), virulence factor database (VFDB) (54) and PlasmidFinder (database 2021-11-29) (55) and PointFinder (database 2021-02-01) (56). Species identification was confirmed based on WGS data using PubMLST (52). Kleborate 2.2.0 was used to genotypically characterize *Klebsiella* spp.(57).

Colistin-susceptible strains used as a reference for detection of colistin resistant mutations are listed in **Supplementary Table 5.4**. For all isolates, mutations in the *pmrAB* and

phoPQ two-component systems and *mgrB* and its promotor were determined. Virulence genes were functionally classified according to the VFDB (54).

For cgMLST, a gene-by-gene approach was utilized by developing a tailor-made scheme for the specific study, and subsequently assessing allelic loci distances using ChewBBACA (58). Clonal relatedness was defined as ≤10, ≤11 or ≤12 allelic differences between isolates of *E. coli* (59,60)*, Enterobacter* spp. (61) and *Klebsiella* spp. (61), respectively. Trees were visualized using Grapetree (62).

5.5.6 Statistical tests and visualization

Statistical tests and visualization were performed using R version 4.2.0 (63). Differences in proportions of colistin resistance between the first and second measurement per examined One Health sector and country were tested using generalized linear models with a negative binomial distribution. Clustering within wards or units was taken into account. Associations between colistin use and resistance in farms were assessed using a generalized linear model. The association between the presence of an iron uptake system and animal-or human-associated isolates was tested with the Fisher's exact test. P values of <0.05 were considered statistically significant.

5.6 Addendum

5.6.1 Acknowledgements

We are grateful to the farmers, the veterinarians and all collaborators in the participating farms for their contribution to the collection of the epidemiological data. We are grateful to the microbiology technicians in the participating laboratories for their contribution to the collection of the microbiological data.

5.6.2 Contribution to authorship

Writing-original draft preparation: SDK; Writing-review and editing: BBX, CL, NPS, SVK, SC, YG, ILR, WD, CH, JD, AS, MK-vdB, JK, HG; Data acquisition: SDK, CL, NPS, SVK, ILR, WD, CH, JD, AS, MK-vdB, JK, HG; Visualization: SDK; Formal analysis: SDK, SC, BBX; Methodology: CL, WD, CH, JD, AS, MK-vdB, JK, HG; Supervision: BBX, CL, HG; Project administration: CL, MK-vdB, HG, JK; Funding acquisition: HG, JK.

5.6.3 Funding

The i-4-1-Health project was financed by the Interreg V Flanders-The Netherlands program, the cross-border cooperation program with financial support from the European Regional Development Fund (ERDF) (0215). Additional financial support was received from the Dutch Ministry of Health, Welfare and Sport (325911), the Dutch Ministry of Economic Affairs (DGNR-RRE/14191181), the Province of Noord-Brabant (PROJ-00715/PROJ-01018/PROJ-00758), the Belgian Department of Agriculture and Fisheries (no reference), the Province of Antwerp (1564470690117/1564470610014) and the Province of East-Flanders (E01/subsidie/VLNL/i-4-1-Health). The authors are free to publish the results from the project without interference from the funding bodies. FecalSwabs and tryptic soy broths were provided by Copan. The authors were free to publish the results from the project without interference by Copan.

5.6.4 Supplementary information

Supplementary Table 5.1: Alterations in *mgrB* or its promoter region in *Escherichia coli*, *Enterobacter* spp. and *Klebsiella* spp.

Species	Proportion of isolates	Colistin MIC (mg/L)	Genetic region	Alteration
Escherichia coli	1/343 (0.3%)	4	*mgrB*	V7G, V12A, S36N, A40T
	65/343 (19.0%)	4 to 32	*mgrB*	V8A
	1/343 (0.3%)	32	*mgrB*	V8A, D31N
	5/343 (1.5%)	4 to 16	*mgrB*	V8A, I41L
	3/343 (0.9%)	4 to 16	*mgrB*	Q33R
	1/343 (0.3%)	8	*mgrB*	Insertion of 4 nt (TGCT) between nt +53 and +58 leading to a frameshift
Enterobacter asburiae	4/10 (40.0%)	16 to >64	*mgrB* promoter	A > T at nt position -38
	2/10 (20.0%)	64	*mgrB* promoter	T > A at nt position -16 & T > C at nt position -17 & insertion of A between nt -79 and -80
	2/10 (20.0%)	8 to >64	*mgrB* promoter	insertion of A between nt -79 and -80*
	1/10 (10.0%)	>64	*mgrB* promoter	deletion of C at nt position -31
Enterobacter cloacae	11/13 (84.6%)	16 to >64	*mgrB* promoter	A > G at nt position -93
	2/13 (15.4%)	32	*mgrB* promoter	A > G at nt position -93 & G > A at nt position -20
Enterobacter hormaechei	1/5 (20.0%)	16	*mgrB*	deletion of complete gene
	1/5 (20.0%)	16	*mgrB* promoter	insertion IS1 family IS element (IS1S) between nt -55 and -56
Enterobacter kobei	1/8 (12.5%)	>64	*mgrB*	I4K
	1/8 (12.5%)	>64	*mgrB*	T21P
	1/8 (12.5%)	>64	*mgrB* promoter	insertion IS5 family IS element (MITEKpn1) between nt -52 and -53
Enterobacter ludwigii	1/5 (20.0%)	16	*mgrB*	P46T*
Enterobacter roggenkampii	1/26 (3.8%)	16	*mgrB*	K2Q
	1/26 (3.8%)	64	*mgrB* promoter	A > T nt position -71

Enterobacter quasiroggenkampii	1/2 (50.0%)	>64	*mgrB* promoter	insertion of A between nt -79 and -80
	1/2 (50.0%)	>64	*mgrB* promoter	G > A at nt -79- & insertion of A between nt -79 and -80
Klebsiella aerogenes	1/2 (50.0%)	4	*mgrB* promoter	A > T at nt position -6 & G > A at nt position -12 & C > T at nt position -43
	1/2 (50.0%)	32	*mgrB* promoter	G > A nt position -12 & A > G nt position -41 & C > T nt position -43
Klebsiella michiganensis	1/6 (16.7%)	32	*mgrB*	insertion of IS5 family IS element (IS5D) between nt +73 and +74
	2/6 (33.3%)	32 to 64	*mgrB*	insertion IS5 family IS element (ISKpn74) between nt +94 and +95*
	1/6 (16.7%)	16	*mgrB* promoter	insertion IS1 family IS element (IS1X2) between nt -29 and -30*
Klebsiella oxytoca	1/2 (50.0%)	32	*mgrB*	deletion of complete gene
Klebsiella pneumoniae	6/112 (5.4%)	32 to >64	*mgrB*	K3Stop
	4/112 (3.6%)	32 to >64	*mgrB*	L4Stop
	1/112 (0.9%)	32	*mgrB*	Q22P
	1/112 (0.9%)	32	*mgrB*	Q22Stop
	1/112 (0.9%)	16	*mgrB*	C28S
	7/112 (6.3%)	16 to 64	*mgrB*	Q30Stop
	1/112 (0.9%)	8	*mgrB*	D31N
	3/112 (2.7%)	32	*mgrB*	deletion of complete gene
	1/112 (0.9%)	16	*mgrB*	I45N
	1/112 (0.9%)	64	*mgrB*	W47C
	2/112 (1.8%)	8	*mgrB*	W47L
	1/112 (0.9%)	4	*mgrB*	W47Stop*
	1/112 (0.9%)	32	*mgrB*	insertion of IS1 family IS element (IS1X2) between nt positions +37 and +38
	1/112 (0.9%)	>64	*mgrB*	insertion of IS1 family IS element (IS1X3) between nt positions +40 & +41
	2/112 (1.8%)	64	*mgrB*	insertion of IS5 family IS element (IS903 group, IS903B) between nt positions +44 and +45

	1/112 (0.9%)	64	*mgrB*	insertion of IS1 family IS element (IS1S) between nt positions +72 and +73
	1/112 (0.9%)	64	*mgrB*	insertion if IS5 family IS element (ISKpn26) between nt positions +74 and +75
	1/112 (0.9%)	32	*mgrB*	insertion of 22 nt between nt positions +77 and +78 leading to a frameshift
	1/112 (0.9%)	16	*mgrB*	insertion of IS1 family IS element (IS1X2) between nt positions +78 and +79
	1/112 (0.9%)	64	*mgrB*	insertion of IS5 family IS element (ISKpn74) between nt positions +85 and +86
	1/112 (0.9%)	64	*mgrB*	insertion of IS5 family IS element (IS903 group, IS903B) between nt positions +86 and +87
	2/112 (1.8%)	16	*mgrB*	insertion of IS5 family IS element (ISKpn74) between nt positions +91 and +92
	1/112 (0.9%)	32	*mgrB*	insertion of T between nt positions +104 and +105 leading to a frameshift
	1/112 (0.9%)	32	*mgrB*	insertion of IS1 family IS element (IS1X2) between nt positions +105 and +106
	1/112 (0.9%)	>64	*mgrB*	deletion of G at nt position +116 leading to frameshift
	5/112 (4.5%)	64 to >64	*mgrB*	insertion of IS5 family (IS903 group, IS903B) IS element between nt positions +116 and +117
	1/112 (0.9%)	32	*mgrB*	insertion of IS1 family IS element (IS1S) between nt positions +119 and +120
	1/112 (0.9%)	64	*mgrB*	insertion of T between nt positions +123 and +124 leading to a frameshift
	1/112 (0.9%)	8	*mgrB*	insertion of IS1 family IS element (IS1S) between nt position +127 and +128
	11/112 (9.8%)	64 to >64	*mgrB* promoter	insertion of IS1 family IS element (IS1X2) between nt positions -6 and -7
	1/112 (0.9%)	64	*mgrB* promoter	G>T at nt position -9
	1/112 (0.9%)	64	*mgrB* promoter	insertion of IS1 family IS element (IS1X2) between nt -11 and -12
	1/112 (0.9%)	16	*mgrB* promoter	insertion of IS5 family IS element (IS903) IS element between nt position -12 and -13

	2/112 (1.8%)	64	*mgrB* promoter	insertion of IS5 family IS element (IS903 group, IS102) IS element between nt positions -14 and -15
	1/112 (0.9%)	64	*mgrB* promoter	A > T at nt position -36 & T > C at nt position -37
	3/112 (2.7%)	32 to >64	*mgrB* promoter	insertion of IS1 family IS element (IS1X2) between nt positions -55 and -56
	1/112 (0.9%)	32	*mgrB* promoter	insertion of IS1 family IS element (IS1S) between nt positions -60 and -61*
	1/112 (0.9%)	64	*mgrB* promoter	insertion of IS1 family IS element (IS1X2) between nt positions -61 and -62*
	1/112 (0.9%)	64	*mgrB* promoter	insertion of IS1 family IS element (IS1R) between nt positions -61 and -62
Klebsiella quasipneumoniae	1/4 (25.0%)	64	*mgrB*	V1S & K2E
	1/4 (25.0%)	32	*mgrB*	K3Stop
	1/4 (25.0%)	>64	*mgrB* promoter	insertion IS5 family IS element (ISKpn74) between nt -35 and -36
Klebsiella variicola	1/24 (4.2%)	32	*mgrB*	insertion of A between nt +9 and +10 leading to a frameshift
	1/24 (4.2%)	64	*mgrB*	deletion of 11 nt between +26 and +36 leading to a frameshift
	1/24 (4.2%)	64	*mgrB*	L4Stop
	1/24 (4.2%)	32	*mgrB*	Q30Stop
	1/24 (4.2%)	32	*mgrB*	K43Stop
	2/24 (8.4%)	32 to 64	*mgrB* promoter	insertion IS5 family IS element (ISKpn26) between nt -17 and -18
	1/24 (4.2%)	16	*mgrB* promoter	G > A at nt position -95

*indicates that no other alterations in PmrAB or PhoPQ were detected. nt: nucleotide, MIC: minimum inhibitory concentration

Supplementary Table 5.2: Single alteration leading to colistin resistance (no other mutations in PmrAB and PhoPQ detected).

Species	Colistin MIC (mg/L)	Genetic region	Alteration
Enterobacter asburiae	8 to >64	*mgrB* promoter	insertion of A between nt -79 and -80
Enterobacter hormaechei	8	*pmrB*	A47T
Enterobacter kobei	>64	*pmrB*	V331G
Enterobacter ludwigii	16	*mgrB*	P46T
Escherichia coli	4	*pmrA*	G15E
	4 to 8	*pmrA*	G53R
	8 to 16	*pmrA*	G53E
	32	*pmrA*	R81H
	8	*pmrA*	L105P
	8	*pmrB*	L14Q
	32	*pmrB*	T17P
	4	*pmrB*	G19R
	16	*pmrB*	C84R
	4 to 8	*pmrB*	T92P
	8	*pmrB*	L98R
	4	*pmrB*	E121K
	4 to 8	*pmrB*	V133L
	4	*pmrB*	T156M
	8 to 32	*pmrB*	A159V
	16	*pmrB*	A159P
	8 to 16	*pmrB*	V161G
	8	*pmrB*	L194R
	8	*pmrB*	Y315F
	4	*pmrB*	Y358N
	8	*eptA*	W141R
	4	*eptA*	E547K
Klebsiella michiganensis	32 to 64	*mgrB*	insertion IS5 family IS element (ISKpn74) between nt +94 and +95
	16	*mgrB* promoter	insertion IS1 family IS element (IS1X2) between nt -29 and -30
Klebsiella pneumoniae	4	*mgrB*	W47Stop
	32	*mgrB* promoter	insertion of IS1 family IS element (IS1S) between nt positions -60 and -61
	64	*mgrB* promoter	insertion of IS1 family IS element (IS1X2) between nt positions -61 and -62

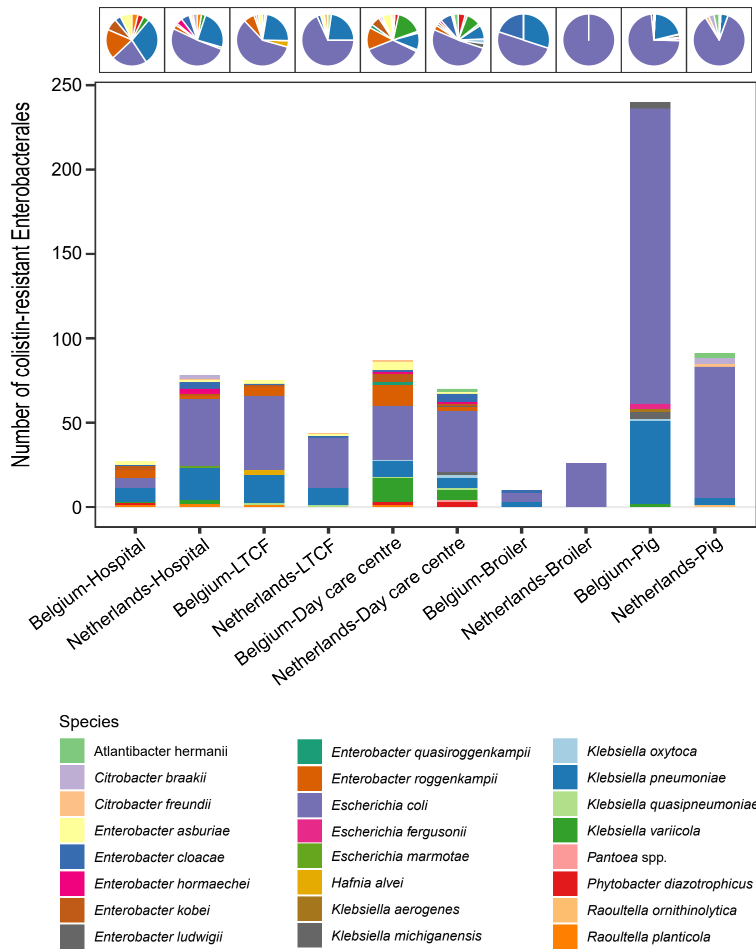
nt: nucleotide, Stop: stopcodon, MIC: minimum inhibitory concentration

Supplementary Table 5.3: Overview of measurement periods, number of sites, number of samples collected per period (measurement 1 and 2) and total number of samples.

Sector	Country	Period 1	Period 2	Nr of sites (1)	Nr of sites (2)	Nr of samples (1)	Nr of samples (2)	Total number of samples	
Hospital	BE	25/10/'17 – 9 /12/'17	3/10/'18- 21/12/'18	1	1	160	202	362	998
	NL	3/10/'17- 12/12/'17	25/9/'18- 4/12/'18	2	2	382	254	636	
LTCF	BE	26/4/ '18- 5/10/'18		13	0	656	0	656	1430
	NL	9/4/ '18- 29/11/'18		17	0	774	0	774	
Daycare	BE	14/11/'18- 8/2/'19		17	0	448	0	448	947
	NL	1/10/'18- 30/1/'19		28	0	499	0	499	
Pig	BE	16/10/'17 - 8/3/'18	6/12/'18- 4 /3/'19	15	14	399	420	819	1597
	NL	10/10/'17- 22/2/'18	3/10/'18 – 11/2/'19	13	15	328	450	778	
Broiler	BE	25/9/'17- 6/4/'18	29/11/'18- 22/2/'19	15	15	399	450	849	1619
	NL	27/9/'17- 9/4/'18	3/10/'18- 20 /2/'19	14	13	380	390	770	
				136	**60**	**4425**	**2166**	**6591**	

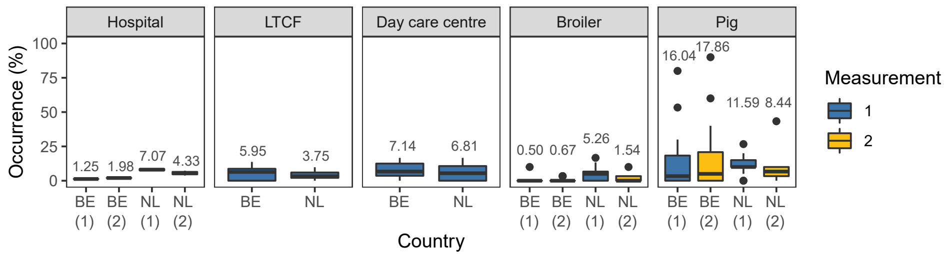
Supplementary Table 5.4: Genomes used as reference for the detection of mutations linked to colistin resistance.

Species	Strain ID	NCBI accession number
Enterobacter asburiae	ATCC35953	CP011863 (64)
	A2563	GCA_011396735.1 (65)
Enterobacter cloacae	ATCC13047	GCA_000025565.1 (66)
Enterobacter hormaechei	K006	SAMN06106854 (67)
	K130	SAMN06106888 (67)
	EC009	SAMN06106832 (67)
	EC001	SAMN06106831 (67)
Enterobacter kobei	73	GCA_021611265.1 (68)
Enterobacter ludwigii	AOUC-8/14	GCA_001263115.1 (69)
	Res13-Abat-PEB19-P1-02-A	GCA_015676575 (70)
Enterobacter roggenkampii	090065	CP045064.2 (71)
Enterobacter quasiroggenkampii	Q2148	GCA_025536215.1 (72)
Escherichia coli	K-12 substr. MG1655	GCA_000005845.2 (73)
	1100008	(This study)
	1100843	(This study)
	2200214	(This study)
Klebsiella aerogenes	HNHF1	CP047669 (74)
Klebsiella michiganensis	KCTC1686	CP003218 (75)
Klebsiella oxytoca	CRKO/UNM	GCF_002508265.1 (76)
Klebsiella pneumoniae	MH78578	CP000647 (77)
	1101124	(This study)
	1101126	(This study)
	1101442	(This study)
	1101454	(This study)
	1101625	(This study)
	1103999	(This study)
Klebsiella quasipneumoniae subsp. *similipneumoniae*	NGKPC-421	ERR3040227 (PacBio) & ERR3039731 (Illumina HiSeq) (78)
Klebsiella variicola	K001	SAMN06106853 (67)

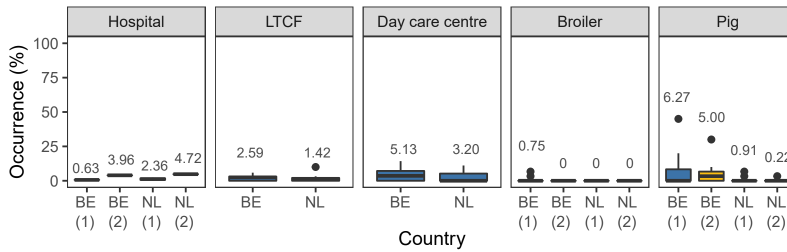


Supplementary Figure 5.1: Distribution of the number of colistin-resistant Enterobacterales species by One Health sector and country.

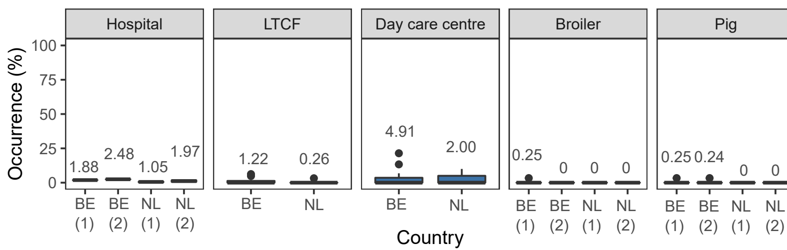
A *Escherichia coli*



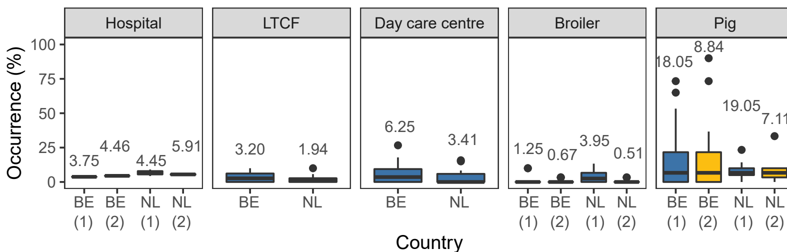
B *Klebsiella* spp.



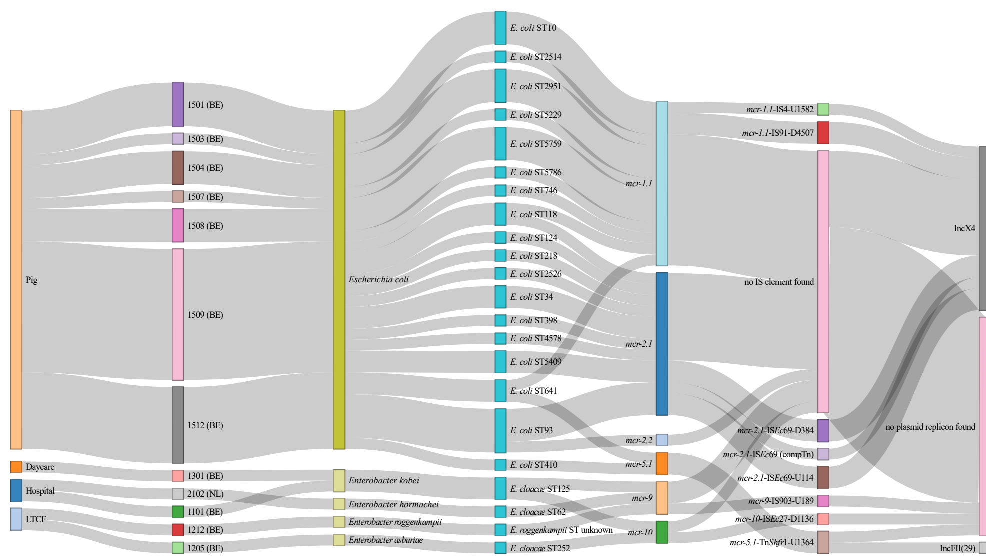
C *Enterobacter* spp.



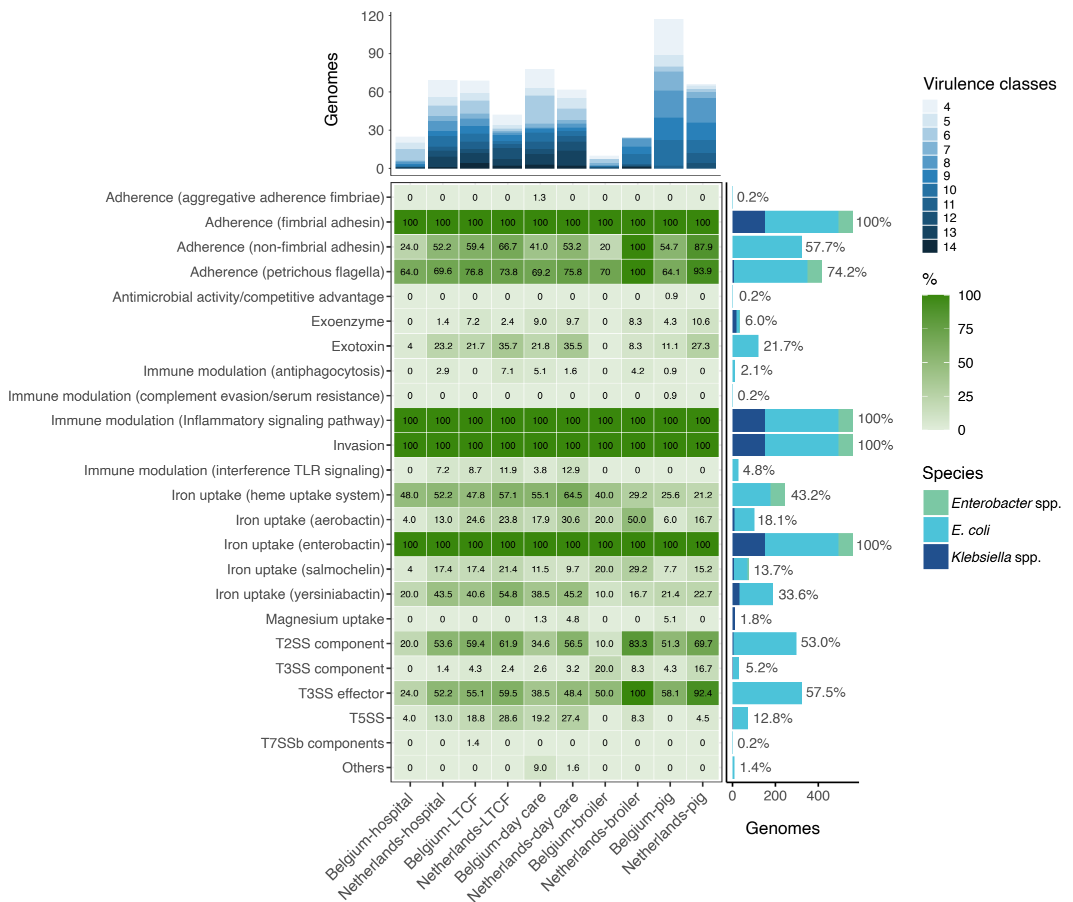
D Multidrug-resistant



Supplementary Figure 5.2: Carriage of colistin-resistant *Escherichia coli* (A), *Klebsiella* spp. (B), *Enterobacter* spp. (C) and multi-drug resistant isolates (D) by humans and animals. The numbers indicated with the boxplots represent the total percentage of positive samples by country, measurement and sector. BE: Belgium, NL: the Netherlands, LTCF: long-term care facility.



Supplementary Figure 5.3: Sankey diagram of the origin and genetic context of mobile colistin resistance (*mcr)*-genes. The closest IS element to the *mcr* gene is indicated together with the upstream (U) or downstream (D) and distance to the *mcr* gene. The width of the lines in the diagram is proportional to the number of isolates. LTCF: long-term care facility, BE: Belgium, NL: the Netherlands, compTn: composite transposon, IS: insertion sequence.



	Belgium-hospital	Netherlands-hospital	Belgium-LTCF	Netherlands-LTCF	Belgium-day care	Netherlands-day care	Belgium-broiler	Netherlands-broiler	Belgium-pig	Netherlands-pig
Adherence (aggregative adherence fimbriae)	0	0	0	0	1.3	0	0	0	0	0
Adherence (fimbrial adhesin)	100	100	100	100	100	100	100	100	100	100
Adherence (non-fimbrial adhesin)	24.0	52.2	59.4	66.7	41.0	53.2	20	100	54.7	87.9
Adherence (petrichous flagella)	64.0	69.6	76.8	73.8	69.2	75.8	70	100	64.1	93.9
Antimicrobial activity/competitive advantage	0	0	0	0	0	0	0	0	0.9	0
Exoenzyme	0	1.4	7.2	2.4	9.0	9.7	0	8.3	4.3	10.6
Exotoxin	4	23.2	21.7	35.7	21.8	35.5	0	8.3	11.1	27.3
Immune modulation (antiphagocytosis)	0	2.9	0	7.1	5.1	1.6	0	4.2	0.9	0
Immune modulation (complement evasion/serum resistance)	0	0	0	0	0	0	0	0	0.9	0
Immune modulation (Inflammatory signaling pathway)	100	100	100	100	100	100	100	100	100	100
Invasion	100	100	100	100	100	100	100	100	100	100
Immune modulation (interference TLR signaling)	0	7.2	8.7	11.9	3.8	12.9	0	0	0	0
Iron uptake (heme uptake system)	48.0	52.2	47.8	57.1	55.1	64.5	40.0	29.2	25.6	21.2
Iron uptake (aerobactin)	4.0	13.0	24.6	23.8	17.9	30.6	20.0	50.0	6.0	16.7
Iron uptake (enterobactin)	100	100	100	100	100	100	100	100	100	100
Iron uptake (salmochelin)	4	17.4	17.4	21.4	11.5	9.7	20.0	29.2	7.7	15.2
Iron uptake (yersiniabactin)	20.0	43.5	40.6	54.8	38.5	45.2	10.0	16.7	21.4	22.7
Magnesium uptake	0	0	0	0	1.3	4.8	0	0	5.1	0
T2SS component	20.0	53.6	59.4	61.9	34.6	56.5	10.0	83.3	51.3	69.7
T3SS component	0	1.4	4.3	2.4	2.6	3.2	20.0	8.3	4.3	16.7
T3SS effector	24.0	52.2	55.1	59.5	38.5	48.4	50.0	100	58.1	92.4
T5SS	4.0	13.0	18.8	28.6	19.2	27.4	0	8.3	0	4.5
T7SSb components	0	0	1.4	0	0	0	0	0	0	0
Others	0	0	0	0	9.0	1.6	0	0	0	0

Supplementary Figure 5.4: Virulence potential of colistin-resistant Enterobacterales from One Health sectors examined in Belgium and the Netherlands**.** Heatmap of the percentage of colistin-resistant Enterobacterales harboring virulence genes related to virulence classes (y-axis) per examined One Health sector in Belgium and the Netherlands (x-axis). Barplots show the number of genomes colored by species per virulence class (right) and colored by the number of virulence class per One Health sector (top). LTCF: long-term care facility.

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CHAPTER 6

Diversity in the characteristics of *Klebsiella pneumoniae* ST101 of human, environmental and animal origin

Published as **De Koster, S**.; Rodriguez Ruiz, J.P.; Rajakani, S.G.; Lammens, C.; Glupczynski, Y.; Goossens, H.; Xavier, B.B.; on behalf of the i-4-1-Health Study Group*. Diversity in the characteristics of *Klebsiella pneumoniae* ST101 of human, environmental and animal origin. Frontiers in Microbiology 2022,13:838207.
doi: 10.3389/fmicb.2022.838207

*see p 241-242

6.1 Abstract

Background: *Klebsiella pneumoniae* ST101 is an emerging high-risk clone which exhibits extensive drug resistance. Bacterial strains residing in multiple hosts show unique signatures related to host adaptation. In this study, we assess the genetic relationship of *K. pneumoniae* ST101 isolated from hospital samples, the environment, community, and livestock using WGS.

Materials and methods: We selected ten *K. pneumoniae* ST101 strains from hospitalized patients in Italy (n=3)(2014) and Spain (n=5)(2015-2016) as well as Belgian livestock animals (n=2)(2017-2018). WGS was performed with 2×250bp paired-end sequencing (Nextera XT) sample preparation kit and MiSeq (Illumina Inc.). Long-read sequencing (Pacbio Sequel I) was used to sequence the two livestock strains and three Italian hospital-associated strains. Furthermore, a public ST101 sequence collection of 586 strains (566 hospital-associated strains, 12 environmental strains, six strains from healthy individuals, one food-associated strain and one pig strain) was obtained. BacPipe and Kleborate were used to conduct genome analysis. ISFinder was used to find IS elements, and PHASTER was utilized to identify prophages. A phylogenetic tree was constructed to illustrate genetic relatedness.

Results: Hospital-associated *K. pneumoniae* ST101 showed higher resistance scores than non-clinical isolates from healthy individuals, the environment, food and livestock (1.85±0.72 in hospital-associated isolates vs 1.14±1.13 in non-clinical isolates, $p<0.01$). Importantly, the lack of integrative conjugative elements ICE*Kp* bearing iron-scavenging yersiniabactin siderophores (*ybt*) in livestock-associated strains suggests a lower pathogenicity potential than hospital-associated strains. Mobile genetic elements appear to be an important source of diversity in *K. pneumoniae* ST101 strains from different origins, with a highly stable genome and few recombination events outside the prophage-containing regions. Core genome MLST based analysis revealed a distinct genetic clustering between human and livestock-associated isolates.

Conclusions: The study of *K. pneumoniae* ST101 hospital-associated and strains from healthy individuals and animals revealed a genetic diversity between these two groups, allowing us to identify the presence of yersiniabactin siderophores in hospital-associated isolates. Resistance and virulence levels in livestock-associated strains were considerably lower than hospital-associated strains, implying that the public health risk remains low. The introduction of an ICE*Kp* into animal strains, on the other hand, might pose a public threat over time.

6.2 Introduction

Klebsiella pneumoniae is part of the Enterobacteriaceae family and is widely present in the gastrointestinal tract of humans and animals as well as in the environment. However, opportunistic, hypervirulent and multidrug-resistant (MDR) *K. pneumoniae* strains have emerged across the world (1). *K. pneumoniae* causes a range of extraintestinal infections in humans, including pneumonia, urinary tract infections and bloodstream infections, usually in the context of opportunistic health-care-associated infections in vulnerable patient groups (1). In the community, hypervirulent strains of *K. pneumoniae* can cause severe infections including pneumonia, pyogenic liver abscess, endophthalmitis, necrotizing fasciitis and meningitis in otherwise healthy persons (2). In animals, *K. pneumoniae* is a common cause of bovine mastitis in dairy cattle (3), pneumonia in horses (4) and urinary tract infections in domestical animals (5) as well as septicemia, pneumonia and mastitis in pigs (6) and respiratory infections in broilers (7).
The global success of the pathogen lies in its accessory genome, which plays an essential role in the emergence of high-risk isolates that are antibiotic-resistant and/or hypervirulent, and are associated with increased pathogenesis, invasive infections and fast adaptation to a specific niche or host (7,8).
Klebsiella pneumoniae is part of the ESKAPE pathogens *(Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp.) which accumulate AMR genes via horizontal gene

transfer of plasmids and MGEs (9). The increasing occurrence of both virulent and MDR isolates (resulting from mutations in core genes as well as from the accumulation of horizontally acquired AMR genes) has led the World Health Organization to consider *K. pneumoniae* as a major global concern (2). Healthcare-associated infections are usually caused by MDR clones with very limited or no treatment options. Especially, the global spread of carbapenemase-producing *K. pneumoniae* has become a reason for concern. *K. pneumoniae* ST101 is one of the major high-risk clonal lineages of carbapanemase-producing isolates ($bla_{OXA\text{-}48}$, bla_{KPC}, bla_{NDM}) and it has been associated with hospital-acquired infections worldwide (10), causing outbreaks in Algeria (11), Czech Republic (12), Greece (13), Italy (14), Spain (15) and Serbia (16). Colistin is a last-resort antibiotic for these infections (17). However, colistin resistance has also emerged following its usage in difficult to treat infections caused by carbapenem- and multidrug-resistant *K. pneumoniae* including the ST101 high-risk clone, leaving almost no alternative treatment options and also leading to the dissemination of colistin resistance (18).

Important virulence factors that contribute to pathogenicity include a capsule, lipopolysaccharides, siderophores, pili, iron uptake systems, efflux pumps and the type VI secretion system (T4SS) (1). Virulence factors may be encoded by genes in the core genome (enterobactin locus *ent*, *fim* and *mrk* loci encoding type 1 and type 3 fimbriae, K and O loci for capsular polysaccharide and LPS biosynthesis) and in the accessory genome (colibactin locus *clb*, salmochelin locus *iro*, aerobactin locus *iuc*, regulators of mucoid phenotypes *rmpA/A2* and the yersiniabactin locus *ybt*) (2). Some of the latter genes are harboured on mobile genetic elements including plasmids, transposons and ICEs. For example, the yersiniabactin (*ybt*)-encoding ICE*Kp* strongly influences the pathogenicity of *K. pneumoniae* strains. The *ybt* siderophore system is a key virulence factor that allows bacterial survival and replication in the host and is therefore significantly associated with pathogenesis and invasive infections (19). The *ybt* and ICE*Kp* structures are highly diverse and are sustained through dynamic horizontal gene transfer events (19).

In addition to reports of invasive infections, contamination of food animals or food products with MDR *K. pneumoniae* has been reported (20–22). Recently, the presence of NDM-1 carbapenemase-producing ST101 *K. pneumoniae* has been reported in chicken meat in Algeria (23). Likewise, other high-risk MDR clones, such as ST11 and ST258, have been detected in animals in China (24). Since *K. pneumoniae* is a colonizing opportunistic pathogen of both humans and animals and a common contaminant of retail meat, an increase in the future of the prevalence of MDR and/or of strains with enhanced virulence might constitute a potential threat for food safety as well as for animal and human health. On the other hand, a large fecal resistome study from slaughter pigs and broilers failed to identify carbapenemase genes suggesting that that these animals would apparently play a role of minor importance as reservoirs of clinical *K. pneumoniae* infections (25). To elucidate zoonoses, pathogen origin, virulence potential, genetic background and epidemiology of emerging infectious diseases, the investigations of bacteria from different origins is fundamental (26). In order to gain a better insight in the antibiotic resistance, virulence, and genetic relatedness between human (hospitalized patients and healthy individuals), animal (livestock-associated), food and environmental *K. pneumoniae* strains, we conducted antibiotic susceptibility testing and a WGS analysis on *K. pneumoniae* ST101 isolates from diverse origins to learn more about their diversity.

6.3 Results

6.3.1 Antibiotic susceptibility of *K. pneumoniae* ST101 from humans and animals

Higher MIC values for third generation cephalosporins, imipenem, fluoroquinolones and aminoglycoside antibiotics were consistently observed among hospital-associated strains compared to the animal strains (**Table 6.1**). Cephalosporin MIC were 24 to >256 mg/L in hospital-associated strains compared to 0.032-0.094 mg/L in animal strains. Resistance to imipenem (4-6 mg/L) and to fluoroquinolones (ciprofloxacin MIC >32 mg/L and norfloxacin MIC >256 mg/L) in most hospital-associated strains was not observed in livestock strains. Resistance to aminoglycoside antibiotics in all but one hospital-associated strain was in contrast to MIC of 0.32-2 mg/L for these antibiotics in animals. Colistin resistance (MIC of 64 mg/L) was detected in one human and in one pig strain. In summary, the hospital-associated study strains showed an MDR phenotype (*i.e.* resistance to at least one agent in at least three antimicrobial categories)(27) whereas animal strains were susceptible to most antibiotics.

Table 6.1: Minimum inhibitory concentrations (MIC) and interpretation for *K. pneumoniae* ST101 of human and animal origin determined by ETEST® with the exception of colistin MIC which were determined using the broth microdilution method.

	MIC (mg/L)									
	Hospital-associated								**Broiler**	**Pig**
Antibiotic	**IT0132A**	**IT0132R1**	**IT0132R2**	**BCR0495**	**BCR0504**	**BCR0133**	**FE1669**	**PS1684E**	**1101124**	**1101433**
Ampicillin	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)
Cefotaxime	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	0,032 (S)	0,032 (S)
Cetriaxone	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	0,032 (S)	0,032 (S)
Ceftazidime	>256 (R)	>256 (R)	>256 (R)	32 (R)	24 (R)	32 (R)	>256 (R)	96 (R)	0,094 (S)	0,094 (S)
Ceftazidime-avibactam	1 (S)	1 (S)	1 (S)	0,5 (S)	0,5 (S)	0,5 (S)	>256 (R)	0,032 (S)	0,094 (S)	0,094 (S)
Imipenem	6 (R)	4 (I)	6 (R)	>32 (R)	6 (R)	6 (R)	>32 (R)	0,25 (S)	0,19 (S)	0,125 (S)
Ciprofloxacin	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	0,016 (S)	0,023 (S)
Norfloxacin	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	>256 (R)	0,125 (S)	0,125 (S)
Amikacin	24 (R)	24 (R)	24 (R)	12 (R)	6 (S)	6 (S)	8 (S)	1,5 (S)	1,5 (S)	2 (S)
Gentamicin	96 (R)	128 (R)	128 (R)	192 (R)	48 (R)	64 (R)	96 (R)	0,25 (S)	0,38 (S)	0,38 (S)
Tobramycin	32 (R)	32 (R)	32 (R)	24 (R)	8 (R)	12 (R)	32 (R)	0,25 (S)	0,38 (S)	0,38 (S)
Trimethoprim-sulfamethoxazole	2 (S)	2 (S)	2 (S)	>32 (R)	>32 (R)	>32 (R)	0,125 (S)	>32 (R)	0,5 (S)	0,75 (S)
Colistin	≤0,125 (S)	0,5 (S)	64 (R)	0,25 (S)	2 (S)	0,25 (S)	2 (S)	0,25 (S)	1 (S)	64 (R)

R= resistant, S= sensitive, I= intermediate.

6.3.2 Resistome and plasmidome analysis and typing of *K. pneumoniae* ST101

In the hospital-associated study strains, resistance to aminoglycosides (gentamicin, tobramycin, amikacin) was correlated with the presence of aminoglycoside acetyltransferases (*aac(3)-IIa* and *aac(6')-Ib*). Resistance to the third-generation cephalosporins (cefotaxime, ceftriaxone and ceftazidime) could be explained by the presence of the ESBL genes $bla_{CTX\text{-}M\text{-}15}$ and imipenem resistance was linked to the carbapenemase gene $bla_{OXA\text{-}48}$ or $bla_{NDM\text{-}1}$. High-level resistance to fluoroquinolone (ciprofloxacin resistance > 32 mg/L and norfloxacin resistance >256 mg/L) was related to triple mutations in quinolone resistance determining regions of *gyrA* and *parC* as amino acid changes S83Y and D87N/D87G in GyrA and S80I in ParC and these were detected exclusively in clinical isolates. One hospital-associated strain (IT0132R2) contained an IS1 family IS1D inserted at position -100 in the promotor region of *mgrB* gene in contrast to the colistin sensitive strains from the same patient. In the pig isolate (1101433), a deletion of guanine at nucleotide position 116th nt led to a frameshift in *mgrB*. Both strains showed colistin resistance with an MIC value of 64 mg/L.

In addition to the study strains, genotypic data was collected from 586 public sequences. Hospital-associated *K. pneumoniae* ST101 showed higher resistance scores compared to isolates from the environment, animals and healthy carriers in the community (1.85 ± 0.72 in hospital-associated isolates vs 1.14 ± 1.13 in non-clinical isolates, $p<0.01$) (**Figure 6.1**). Of all sequences originating from human clinical sources, 556 out of 574 (97%) showed ESBL- or carbapenemase production and 522 of 574 (91%) harbored triple mutations causing fluoroquinolone resistance (ParC S80I, GyrA S83Y and GyrA D87A/G/N). The most common ESBL gene was $bla_{CTX\text{-}M\text{-}15}$ (420 out of 596 isolates, 70.4%) and the most common carbapenemase gene was $bla_{OXA\text{-}48}$ (263 out of 596 isolates, 44.1%), though, $bla_{CTX\text{-}M\text{-}15}$ or any carbapenemase gene were not detected in animal- nor in community-associated strains. The genetic context of bla_{KPC} carbapanemase genes has been further investigated. In this study, 63 out 574 (11%) of the sequences from hospital-associated isolates harbored a bla_{KPC} gene and were flanked by IS elements (ISKpn7/6, 59/63 (93.6%)), with only 4 (6.3%) flanked by transposon (tn2/tn3). The majority of the

bla$_{KPC}$ genes (n=36, 64.8%) were carried on IncFII(K), with only 2 (3.2%) gene harbouring on IncP6 and 5 strains (8%) containing IncFII(pKP91). The median number of plasmids was 6 in hospital-associated strains and strains from hospital sewage, 5 in the pig strains and food-associated strains and 2 or 3 in isolates from broiler, the environment (river water and surface at neonatal intensive care unit (NICU)) and the healthy individuals in the community (**Figure 6.1**). Plasmid replicon IncFIB(K) was detected in all categories and is known to be associated both with MDR and virulence plasmids (2). The IncFIB(pQil) plasmid replicon is known to be associated with the pKPQil plasmid with resistance traits to *bla*$_{KPC-3}$ and the *mer* operon (resistance to mercuric ions) (28) and was detected exclusively in 72 clinical *K. pneumoniae* ST101 isolates (12.5%). Small plasmids (Col) were commonly detected in hospital-associated strains as well as IncR type plasmids (n= 417; 72.4%). Based on long-read sequencing data, Col-type plasmids did not harbor any resistance genes. The *bla*$_{OXA-48}$ gene was carried on IncL plasmids which did not carry other resistance genes. However, various other Inc-type plasmids (such as IncFIB(K), IncFII(K), IncN and IncR) often carried a variety of resistance genes (up to 14 resistance genes) on one plasmid. Five distinct capsular polysaccharide (K loci) and LPS (O antigen) biosynthesis loci were defined among the *K. pneumoniae* ST101 strains (**Figure 6.2A**). Serotype O1v1 and KL17 was the most common among hospital-associated *K. pneumoniae* isolates while O1v2 and KL106 was predominant in animal-associated and community-associated strains. Four hospital-associated isolates and one food-associated isolate from the publicly available databases carried KL2 which is highly conserved in hypervirulent clones and is associated with community-acquired invasive disease and enhanced pathogenicity (2).

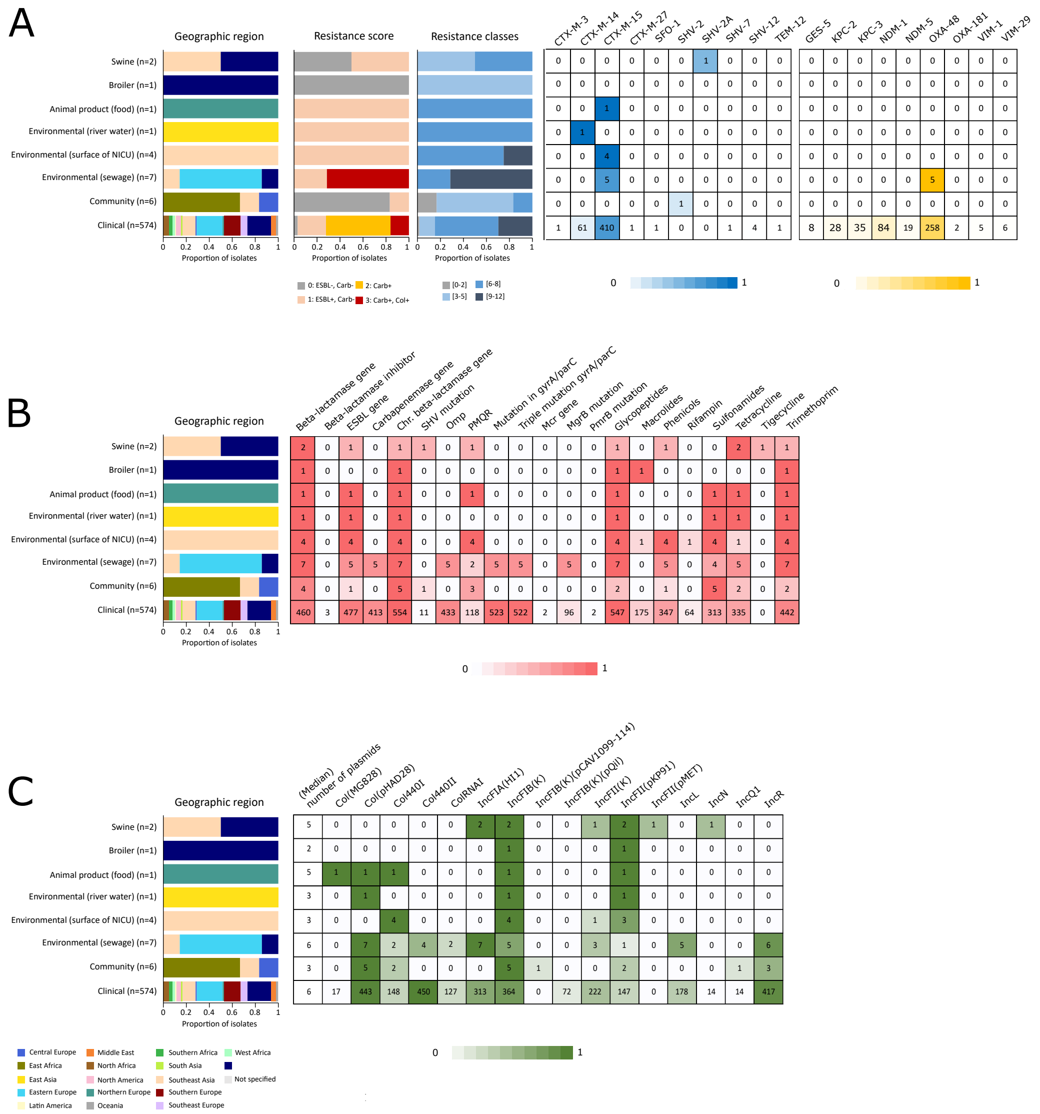
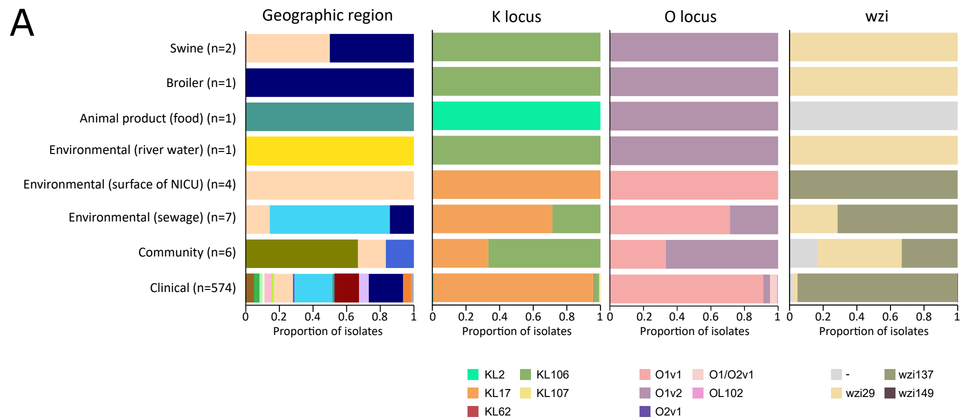


Figure 6.1: Heatmaps of resistance originating from ESBL- and carbapenemase production (A), resistance to different antibiotic classes (B) and most common plasmid origins of replication (C) detected in an international collection of hospital-associated, livestock-associated, healthy community and environmental *K. pneumoniae* ST101. Graphs and heatmaps show the proportion of isolates, numbers in the heatmaps indicate the number of genomes containing the resistance gene. NICU: neonatal intensive care unit.



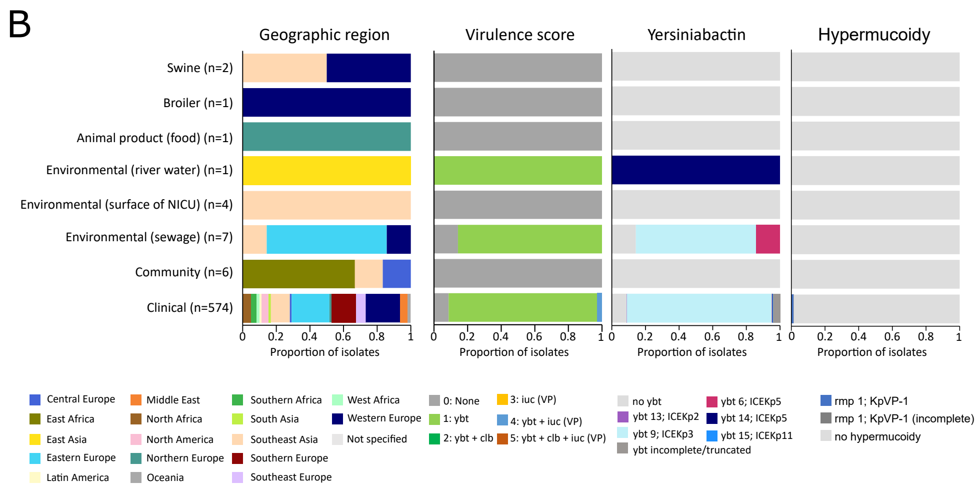


Figure 6.2: Typing (A) and important virulence determinants (B) of *K. pneumoniae* ST101. NICU: neonatal intensive care unit.

6.3.3 Comparative genome analysis of *K. pneumoniae* ST101 of different origin reveals that the mobile genetic elements are an important source of variation

The *K. pneumoniae* ST101 strains display a gene content associated with various horizontal gene transfer mechanisms such as plasmids, phages and MGE (e.g. ICE*Kp*). Intact yersiniabactin, a high-virulence determinant in *K. pneumoniae*, was present in a single genomic island in the majority of hospital-associated isolates (500 of 574; 87%) and 6 out of 7 isolates from hospital sewage (86%), however, yersiniabactin (*ybt* genes and ICE*Kp*) were absent in the six isolates from the healthy community, the two livestock isolates and in the isolate originating from food (**Figure 6.2B**). This virulence factor was mobilized on the integrative conjugative element, ICE*Kp*, containing the *virB* operon of the T4SS and the iron-scavenging siderophore yersiniabactin *ybt* locus. Five distinct *ybt* lineages were detected on four ICE*Kp* variants with *ybt9* on ICE*Kp*3 being the most common (495 (86%) of the hospital-associated isolates) (**Figure 7.2B** & **Supplementary figure 6.1**). The MGE was detected in publicly available sequences collected during an infection as well as in sequences collected for screening. The ICE*Kp* corresponded to a 58-92 kb insertion integrated in an asparagine-tRNA in the chromosome. Based on long-read sequencing data (n=12), the ICE*Kp* was inserted in the third asparagine-tRNA of the 4 or 5 asparagine-tRNA copies present in the chromosome (position 1945074-1945149 in reference sequence IT0132A) (**Supplementary Figure 6.2**). Insertion of ICE*Kp* occurred between a Na+/H+ antiporter and HTH-type transcriptional regulator *argP* (n= 490; 98%), putative FMN/FAD exporter *yeeO* and endoribonuclease *pemK* (n=4; 0,6%), between genes *mtfA* and *yjgH* (n=6; 1,2%) or between *mtfA* and a Na+/H+ antiporter (n=1; 0,2%).

The ICE*Kp* was absent in the chromosomes of livestock-associated strains (n=3). In the livestock-associated strains, the *virB* operon of the T4SS was found on an IncN plasmid containing $bla_{\text{TEM-1B}}$, *dfrA14* and *tetA* resistance genes and an IncFII(pMET) plasmid without resistance genes in the Belgian and in the Thai pig strains, respectively. The *virB* operon of the Belgian broiler strains was found on a IncP-like plasmid containing *aadA1*, $bla_{\text{TEM-1B}}$, *Inu(G)* and *dfrB1* which was previously detected in an *E. coli* strain from pig

caeca (accession number: CP039300.1). Of the 50 sequences from clinical strains in the public databases that lacked the *ybt* locus, five strains harbored the *virB* operon on a plasmid of which two contained the *dfrA14* gene for trimethoprim resistance.

Besides the *ybt* locus, *rmpA/rmpA2* (hypermucoidy) and *iuc* (aerobactin) loci are other notable accessory virulence factors in *K. pneumoniae*. Seven publicly available sequences of hospital-associated strains harbored the *rmpA/rmpA2* genes indicating hypermucoidy. Convergence of resistance and virulence was detected in the sequences originating from 17 hospital-associated isolates from Italy (n=8), Egypt (n=5), Saudi Arabia (n=2), Slovenia (n=1) and Belgium (n=1) (**Figure 6.3**). These sequences harbored the *ybt* and *iuc* loci in addition to ESBL-, carbapenemase genes and/or colistin resistance mutations/genes. Isolates from animals, animal products and the healthy community showed low virulence capacity (no *ybt*, *clb*, *iuc*) and no carbapenemase production.

Based on long-read sequencing data, a total of 4.60-5.89% of the ST101 genomes was composed of genomic islands, and the percentage of prophage sequences was variable from 2.28% to 5.55% in both hospital-associated and in livestock-associated stains (**Supplementary Figure 6.2**). Prophage sequences did not contain any notable virulence factors. Distinct prophage content was detected in animal-associated and hospital-associated strains (**Supplementary Figure 6.2** and **Supplementary Figure 6.3**). A total of 468 polymorphic sites were identified across the *K. pneumoniae* ST101 genome (**Figure 6.4**). Genomic regions containing phage sequences, the ICE*Kp* region and regions harboring T4SS, permease and outer membrane proteins were identified as recombinant. The latter were linked to diversity in K-and O locus types. The *K. pneumoniae* ST101 genome showed to be highly stable with few recombination events outside of these mentioned genomic regions (**Figure 6.4**).

Figure 6.3: Convergence of antibiotic resistance and virulence in the *K. pneumoniae* ST101 population. ESBL: extended-spectrum beta-lactamase production, carba: carbapenemase production, col: colistin resistance, *clb*: colibactin, *iuc*: aerobactin, VP: virulence plasmid, NICU: neonatal intensive care unit, *ybt*: yersiniabactin.

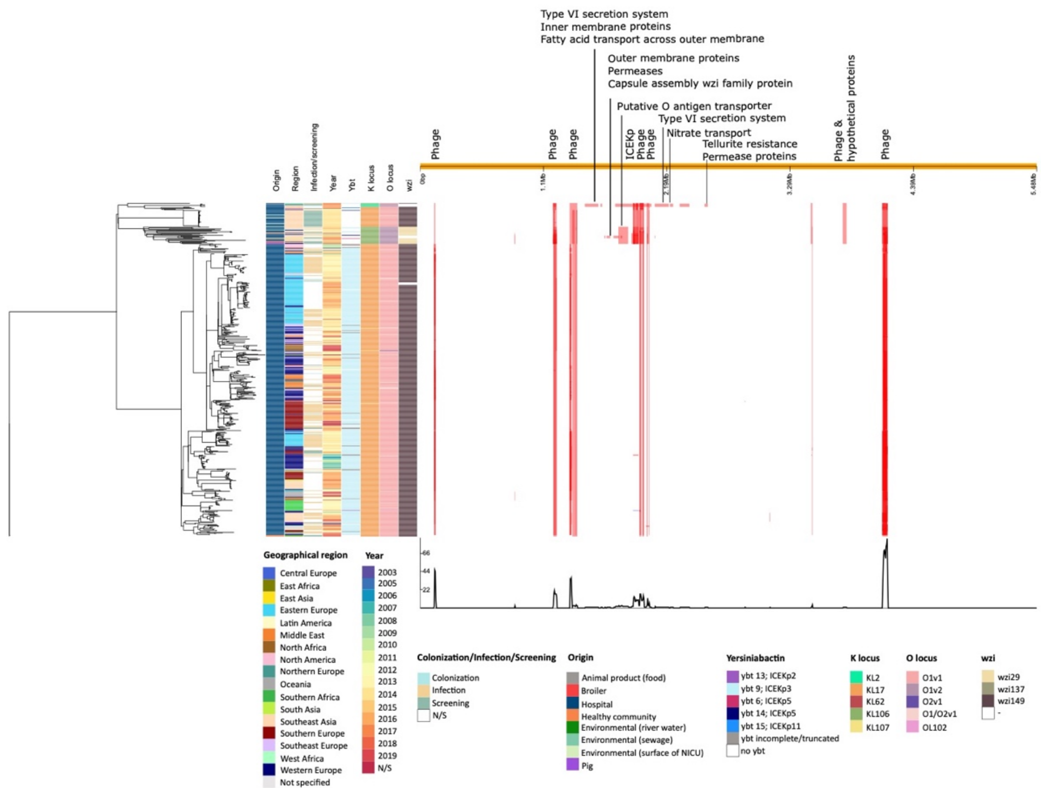
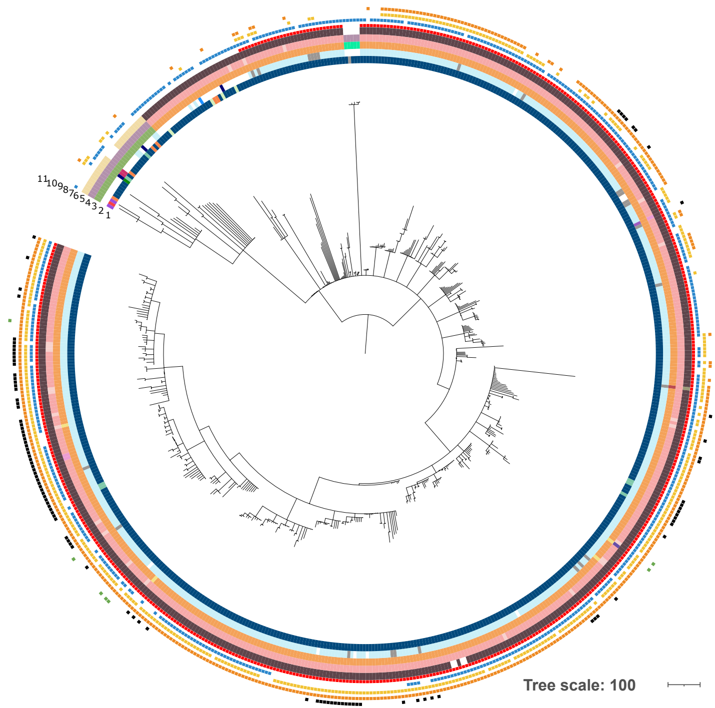


Figure 6.4: Phylogenetic analysis of an international collection of hospital-associated, animal-associated, community-associated (healthy individuals), food-associated, and environmental *K. pneumoniae* ST101. A maximum likelihood phylogeny was generated from whole genome alignment of 596 *K. pneumoniae* ST101 using the Gubbins algorithm. The right panel shows the pattern of predicted recombinations. Red bars show polymorphic sites suggesting horizontal sequence transfer. Each row relates to an isolate in the phylogeny and each column represents a base in the reference genome (IT0132A). N/S: not specified, NICU: neonatal intensive care unit.

6.3.4 Analysis of the sequence data revealed that livestock-associated strains were genetically distinct from hospital-associated strains

To determine genetic relatedness, a study and strain-specific scheme was developed. A total of 4427 loci were identified from ST101 isolates in the whole genome. 223 loci were deleted because they did not contribute to the core genome, leaving 4202 loci for comparison among different ST101 clone origins (**Figure 6.5**). Overall, the gene-by-gene approach mirrored clustering based on K- and O-locus with the livestock-associated ST101 *K. pneumoniae* strains. The Belgian broiler strain (1101124) had 78 more and 164 fewer alleles than the strain KPSW02 from Thailand and Belgian pig (1101433) strains, respectively. The two pig strains had 86 core polymorphisms between them. The community-associated strain SB5560 from Madagascar was most similar to the livestock-derived strains, with a 95, 173, and 181 allelic distance to the pig strain from Thailand, Belgian broiler strain, and Belgian pig strain, respectively. The Swedish strain 08EU827 obtained from a feces sample of an ICU patient was the closest clinically relevant strain, with 211, 289, and 297 allelic variants in KPSW02, 1101124, and 1101433, respectively. The core genome of food strain F0025 was similar to Vietnamese, Swedish, and Canadian hospital-associated strains (less than 20 allelic differences) (**Table S6.2**). Hospital-associated isolates did not cluster by region nor by the time of their isolation (**Figure 6.4** and **Supplementary Figure 6.4**).



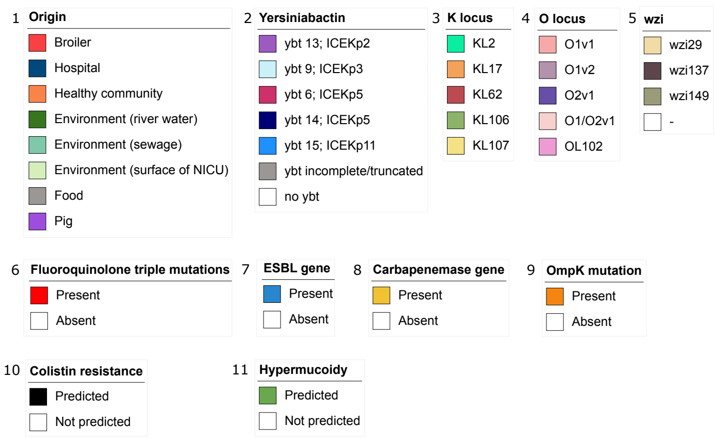


Figure 6.5: Minimum spanning tree of hospital-associated, community-associated (healthy individuals), livestock-associated and environmental *K. pneumoniae* ST101. Figure was generated by iTOL using cgMLST profile data. Rings 1 and 2 picture the origin of the sequence and the presence of yersiniabactin. Rings 3,4 and 5 indicate K locus, O locus and wzi type. The presence of resistance determinants including fluoroquinolone triple mutations, presence of an ESBL gene, presence of a carbapenemase gene, mutations in OmpK (OmpK35 and OmpK36) and mutations or genes predicted to be involved in colistin resistance are annotated in rings 6,7,8,9 and 10. Predicted hypermucoidy based on the presence of *rmpA/rmpA2* is highlighted in ring 11. ICE: integrative and conjugative element, NICU: neonatal intensive care unit.

6.4 Discussion

K. pneumoniae ST101 is an emerging high-risk opportunistic pathogen which has been reported mostly in hospital-outbreak settings in several countries (11–16). A broad collection of antibiotic resistance genes, including carbapenemase genes, is making ST101 highly adapted to the hospital environment (8). Indeed, a recent study showed that the nosocomial transmission of carbapenem-resistant *K. pneumoniae* substantially impacts the epidemiology of these clones in Europe (10). Although the detection of high-risk *K. pneumoniae* clones in animals remains scarce (22–24), the spread of (resistant) bacteria between One-Health compartments exists (10). Hence, the occurrence of important nosocomial clones in animals may cause a reason for concern. In this study, we detected two *K. pneumoniae* ST101 in livestock animals (broiler chicken (n=1) and weaned pig (n=1)) in Belgium and eight nosocomial strains from Spanish and Italian hospitals. In addition, we analyzed an international collection of publicly available ST101 strains (n=586). In this study, we provide insights into the genetic diversity of *K. pneumoniae* ST101 from the hospital (n=574), healthy individuals in the community (n=6), the environment (n=12), food (n=1) and livestock (n=3).

MGE are an important source of variation between *K. pneumoniae* ST101 of different origin. The *K. pneumoniae* ST101 genome showed to be highly stable apart from the occurrence of a few recombination events outside of the MGE regions. The chromosomal insertion of self-transmissible ICE*Kp* elements in the clinical strains constitutes the main genomic difference between the *K. pneumoniae* chromosomes of animal and clinical origin in this study. This MGE was absent in the small number of available livestock-associated (n=3), food (n=1) and community-associated sequences (n=6) of *K. pneumoniae* ST101 but provided many of the clinical strains (87%) with an advantage for the adaptation within the human host as it contains the virulence determinant yersiniabactin. This siderophore system scavenges iron from the host transport proteins and enhances the ability to survive and replicate within the host (29). In contrast to other siderophores, yersiniabactin also avoids the inflammatory response of the host (30)(31).

Yersiniabactin is, therefore, a key bacterial virulence factor and is significantly associated with invasive infections (19). We detected the ICE*Kp* element in hospital-associated strains from invasive infections as well as in commensal strains isolated from rectal or throat samples, most probably reflecting that the majority of the clinical sequences were deposited in the database in the context of difficult to treat (MDR) infections and/or linked to hospital outbreaks. This mobile cluster of genes showed genetic diversity between clinical strains as we detected four ICE*Kp* variants with different YbST which is probably indicative for long-term maintenance of ICE*Kp* in this lineage (19). The absence of yersiniabactin in some hospital-associated strains, livestock and community strains might be a consequence of the high-energy costs from the polyketide hybrid molecules and the ICE*Kp* cargo genes (19). Similarly, the absence of a fitness advantage of ICE*Kp* in the animal host might explain the absence of this ICE*Kp* in livestock strains. On the other hand, if the absence of the ICE*Kp* element in animal strains is due to the ecological barrier from the physical separation of bacterial populations in distinct host niches (32), high-pathogenicity and invasive strains could arise after the introduction of *ybt* in the animal strain background (19). The introduction of the ICE*Kp* in an asparagine-tRNA, an integration hotspot for genomic islands (33), as observed in the hospital-associated strains might occur in the chromosome of animal strains over time. For the mobilization to recipient cells, the ICE*Kp* contains a *virB* operon. In livestock strains, the *virB* operon was detected on plasmids conferring antibiotic resistance. This T4SS for genetic exchange may thus potentially act as an important contributor to genome plasticity and bacterial fitness via conjugation.

In the study collection of 596 sequences, hypervirulent clinical clones carrying a combination of core pathogenicity factors (K1 and K2 capsules; O1 and O2 LPS) with accessory virulence factors such as *rmpA/rmpA2* (n= 7) for hypermucoidy and *iuc* for aerobactin siderophore synthesis (n= 17) (2) were detected. Convergence of resistance and virulence was not detected in isolates from animals, animal products and in healthy

carriers in the community. These non-clinical isolates showed low virulence capacity and no carbapenemase production.

Of all hospital-associated strains, 97% showed ESBL-or carbapenemase production. When this is combined with resistance to fluoroquinolones, only limited treatment options remain (17). Indeed, triple mutations causing fluoroquinolone resistance (ParC S80I, GyrA S83Y and GyrA D87A/G/N) were present in 91% of the hospital-associated strains and are known to be associated with a fitness advantage in high-risk MDR clones (34). Currently, the risk of acquiring MDR Enterobacteriaceae is linked to antibiotic selective pressure, contaminated drinking water and lack of hygiene (35). Colistin use in the Belgian pig farm was reflected by high-level colistin resistance in the pig strain, highlighting the importance of antibiotic selective pressure and the need to restrict antibiotic use in livestock. The public health risk posed by this opportunistic pathogen, taking into account its genotypic and phenotypic antibiotic resistance profile as well as the lack of critical high-virulence traits such as yersiniabactin in livestock-associated *K. pneumoniae* ST101 in this study, appears to be minor compared to hospital-associated strains.

However, there are some limitations to our research that must be addressed. The first limitation is the small number of livestock-associated *K. pneumoniae* ST101 strains available for analysis. Second, because the data was collected from a variety of sources, it does not capture precisely how prevalent *K. pneumoniae* ST101 is in livestock herds. The publicly available sequence data revealed large geographic and temporal variations in sampling (location, date of sample collection, and geographic regions), and it frequently failed to mention the clinical and epidemiological contexts in which the isolates were obtained and the sequences deposited. Presumably, most of the ST101 sequences were used to characterize MDR bacteria in a nosocomial or endemic environment, which is most likely why the prevalence of resistance and aggressiveness genes in our study was influenced. Third, the influence of the community on the introduction of these crucial nosocomial clones in livestock animals or vice versa is still

unknown. As a result of these restrictions, raising WGS data from livestock-associated and community-associated strains is essential to detect the presence of these pathogens and their resistance and virulence genes. Our study, nevertheless, offers important information on *K. pneumoniae* ST101 resistance and virulence properties from a variety of origins, suggesting lower antibiotic resistance and the lack of high-virulence features such as yersiniabactin in livestock-associated, community-associated, and food-associated *K. pneumoniae* ST101 compared to hospital-associated strains. Future research should focus on the detection of clones in the community, in the hospital, and in livestock enclosures, employing a One-Health approach within a well-structured prospective study with representative sampling (geographic and temporal) in different sectors and settings.

6.5 Materials and methods

6.5.1 Strain collection and characterization

A total of 10 *K. pneumoniae* ST101 strains were collected within two point-prevalence surveillance studies of antibiotic-resistant *Enterobacteriaceae* in hospitals as well as in livestock. Strain (1101124) was isolated from feces of a broiler chicken in October 2017 and strain (1101433) was isolated from the feces of a weaned pig in February 2018 at two different farms in Belgium within the framework of the i-4-1-Health study (36). Human strains were collected within the Resistance in Gram-Negative Organisms: Studying Intervention Strategies (RGNOSIS) study (ClinicalTrials.gov NCT02208154; EU-FP7, RGNOSIS). Three hospital-associated *K. pneumoniae* strains were collected from one single patient at one hospital in Italy in October 2014. The first two strains were recovered on the same day from an endotracheal aspirate (IT0132A) and from one rectal sample (IT0132R1). The third strain (IT0132R2) was collected three weeks later from a rectal sample. In addition, five strains were collected from screening specimens (throat and rectal samples) of five different patients at three different hospitals in Spain between April 2015 and August 2016 (BCR0495, BCR0504, BCR0133, FE1669, PS1684E). Further, all

K. pneumoniae ST101 sequences originating from different sources (human including sequences from hospital-associated infections (n=566) and healthy, asymptomatic carriers in the community (n=6), animal (n=1), food (n=1) and the environment including river water (n=1), hospital sewage (n=7) and a surface at a NICU ward (n=4)) available on NCBI and A Global Platform for Genomic Surveillance: Pathogenwatch on 27 September 2021 (n= 586) were added for comparison analysis (**Supplementary Table 7.1**). All sequences were added to the analysis to minimize bias because of selection.

6.5.2 Antibiotic susceptibility testing

MIC of ampicillin, cefotaxime, ceftriaxone, ceftazidime, ceftazidime-avibactam, imipenem, ciprofloxacin, norfloxacin, amikacin, gentamicin, tobramycin and trimethoprim-sulfamethoxazole were determined by a quantitative gradient diffusion method using ETEST® (bioMérieux, Marcy l'Etoile, France) for ten study strains from humans (n=8) and from livestock (n=2). For colistin, MIC were determined using broth microdilution according to the ISO 20776-1 standard using 96-well polystyrene microplate (ref. 82.1582.001, Sarstedt, Nümbrecht, Germany). Results were interpreted according to EUCAST clinical breakpoints (v 10.0, 2020).

6.5.3 DNA extraction and whole genome sequencing

All strains (n= 10) from this study were selected for short-read sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Briefly, for short-read sequencing, a single colony was inoculated in 4 mL Mueller-Hinton broth and incubated overnight at 37°C. Genomic DNA was extracted using the MasterPure Complete DNA & RNA Purification kit (Epicentre, USA) and purified with DNA Clean & Concentrator TM-10 Kit (Zymo Research, USA). DNA concentrations and quality were measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using the Nextera XT sample preparation kit followed by 2x 250 bp paired end sequencing using MiSeq (Illumina Inc, USA).

Five *K. pneumoniae* ST101 strains from livestock animals (n=2) and clinical isolates (n=3) were selected for long-read sequencing on PacBio Sequel I (Pacific Biosciences, CA, USA). For long-read sequencing, high-molecular-weight DNA was isolated from fresh overnight cultures. Briefly, a single bacterial colony was inoculated in 10 mL Mueller Hinton broth and incubated overnight at 37°C under. DNA was extracted using the MagAttract HMW DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA concentrations and quality were measured using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing libraries were prepared using the SMRTbell Express Template Prep kit 2.0 (Pacific BioSciences) and whole-genome sequencing was performed on the PacBio Sequel I using the Sequel Sequencing kit 3.0 (Pacific BioSciences). The sequences were submitted in NCBI under BioProject PRJNA685961.

6.5.4 Sequence analysis and genetic characterization

Assembly of long-read sequencing data was performed with HGAP using default parameters, included in SMRT Link v8.0.0 (Pacific Biosciences). Short-read sequencing data was assembled using SPAdes (v3.13.0) (37). Assembly quality was assessed using Quast (v5.0.2) (38). Publicly available sequences of *K. pneumoniae* ST101 (n=586) were downloaded from NCBI and from Pathogenwatch (**Supplementary Table 7.1**). Subsequent analysis was performed using BacPipe (v1.2.6) (39), including the PubMLST database (40), ResFinder (41) and PlasmidFinder (42) databases. The assembled short-read and long-read genomes were annotated using Prokka (v1.12) (43) integrated in BacPipe. For the long-read sequences, insight in the accessory genome was obtained using web-based tools: PHAge Search Tool Enhanced Release (PHASTER) for identification of prophage regions (44), IslandViewer using the IslandPath-DIMOB prediction method was used to identify genomic islands (45) and ISFinder predicted the presence of IS elements (46). Recombinant whole genome sequences were identified using Gubbins (Genealogies Unbiased By recomBinations In Nucleotide Sequences) (47).

Multiple alignment of genomes was done using Mauve (48). All sequenced isolates were screened for *in silico* K locus and O typing and presence of resistance and virulence determinants using Kleborate (49). Chromosomal insertion of ICE*Kp* structures was determined by the flanking direct 17 bp repeats 'CCAGTCAGAGGAGCCAA' and ICE*Kp* variants were determined using Kleborate (19) (49).
Statistical analysis was performed using a two-sample t-test assuming unequal variances. Genome wide comparison was done using cgMLST. For cgMLST, a gene-by-gene approach was used by generating a study-specific scheme and analyzing cgMLST based allelic loci distance using ChewBBACA (50). Microreact was used to visualize allelic loci distances among isolates (https://microreact.org/project/j1fyqBYfCiKPZLa4qjotDe/ad1fbbb1).

6.6 Addendum

6.6.1 Acknowledgements

We are grateful to the collaborators in the participating hospitals and farms for their contribution to the collection of the microbiological and epidemiological data.

6.6.2 Contribution to authorship

Conceptualization, B.B.X, Y.G., H.G.; data collection and writing: S.D.K, J.P.R.R, S.G.R, B.B.X; writing—original draft preparation, S.D.K; writing—review and editing, S.D.K, J.P.R.R, S.G.R, B.B.X, Y.G.; Project administration: CL; supervision, B.B.X, H.G.; All authors have read and agreed to the published version of the manuscript.

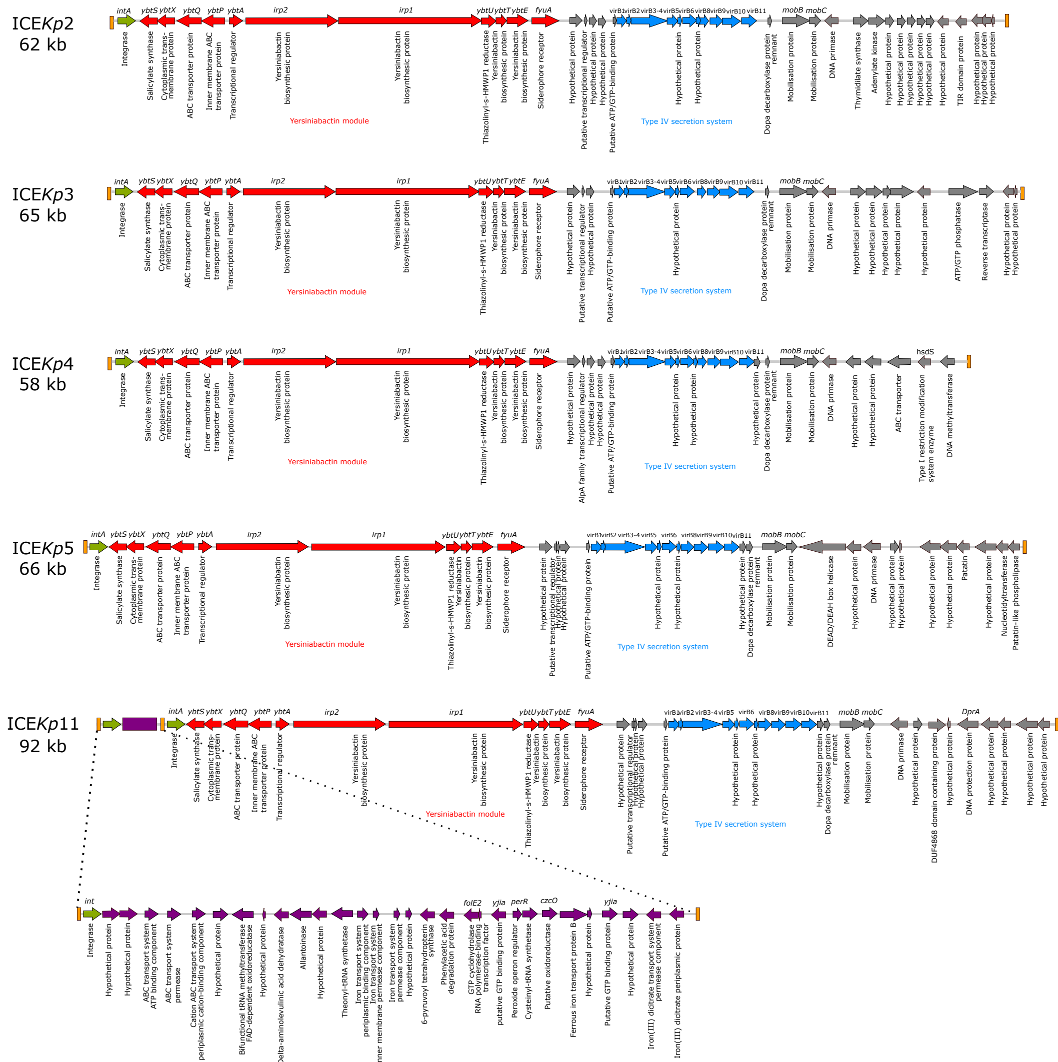
6.6.3 Funding

The i-4-1-Health project was financed by the Interreg V Flanders-The Netherlands program, the cross-border cooperation program with financial support from the European Regional Development Fund (ERDF) (0215). Additional financial support was received from the Dutch Ministry of Health, Welfare and Sport (325911), the Dutch Ministry of

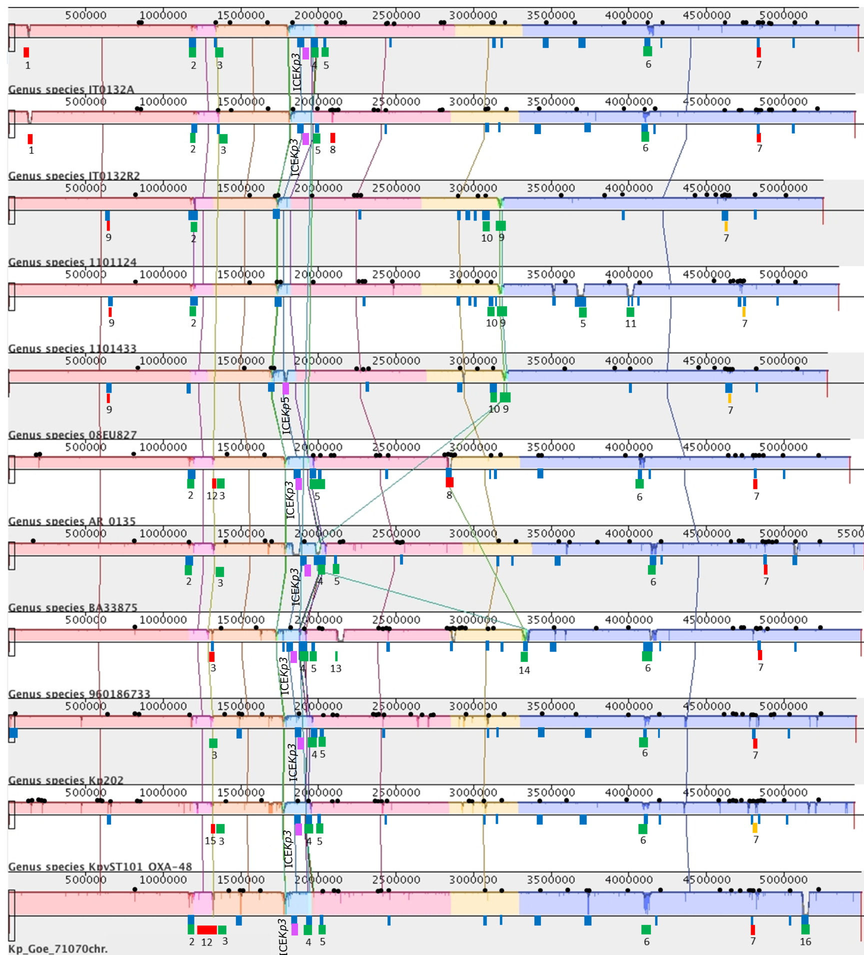
Economic Affairs (DGNR-RRE/14191181), the Province of Noord-Brabant (PROJ-00715/PROJ-01018/PROJ-00758), the Belgian Department of Agriculture and Fisheries (no reference), the Province of Antwerp (1564470690117/1564470610014) and the Province of East-Flanders (E01/subsidie/VLNL/i-4-1-Health). Selective and non-selective agar plates and ETEST strips were provided by bioMérieux (Marcy l'Etoile, France); FecalSwabs® and tryptic soy broths were provided by Copan Italy (Brescia, Italy). The authors are free to publish the results from the project without interference from the funding bodies, bioMérieux or Copan Italy. The clinical strains collected from RGNOSIS and the project was supported by funding from the European Community (R-GNOSIS FP7/2007–2013 under Grant Agreement no. 282512).

6.6.4 Supplementary information

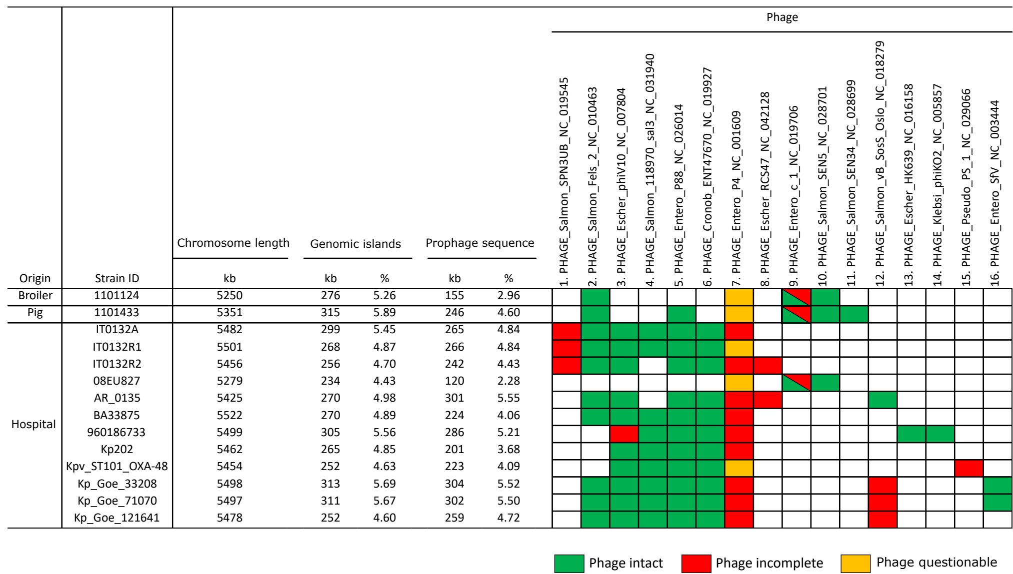
Supplementary Table 6.1: List of strains and available metadata used for analysis. Available online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.838207/full#supplementary-material.



Supplementary Figure 6.1: ICE*Kp* structures detected in *Klebsiella pneumoniae* ST101. ICE*Kp* elements are flanked by direct 17 bp repeats 'CCAGTCAGAGGAGCCAA' (orange blocks).

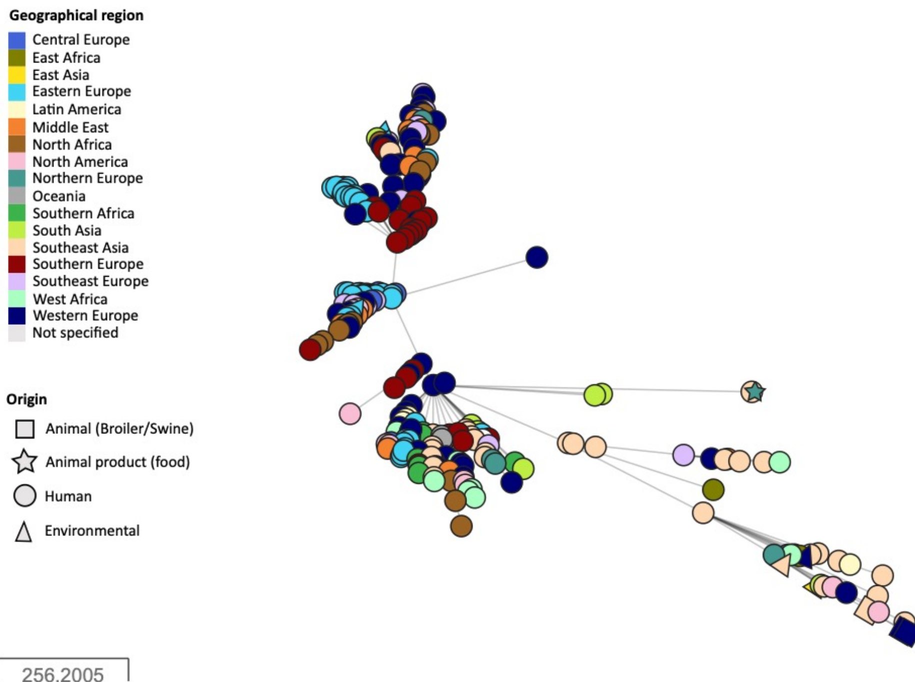


Supplementary Figure 6.2: Alignment of *K. pneumoniae* ST101 chromosomes using long-read sequencing data and Mauve. Black dots represent IS elements, purple bars represent the ICE*Kp* elements, blue bars show genomic islands detected using the IslandViewer IslandPath-DIMOB prediction method. Red bars show incomplete phages, green bars show intact phages and orange bars indicate questionable phages as determined by the PHASTER software. Numbers 1-16 indicate the identified phage: 1) Phage_Salmon_SPN3UB_NC_019545, 2) Phage_Salmon_Fels_2_NC_010463, 3) Phage_Escher_phiV10_NC_007804, 4) Phage_Salmon_118970_sal3_NC_031940, 5) Phage_Entero_P88_NC_026014, 6) Phage_Cronob_ENT47670_NC_019927, 7) Phage_Entero_P4_C_001609, 8) Phage_Escher_RCS47_NC_042128, 9) Phage_Entero_c_1_NC_ 019706, 10) Phage_Salmon_SEN5_NC_028701, 11) Phage_Salmon_SEN34_NC_028699, 12) Phage_Salmon_vB_SosS_Oslo_NC_018279, 13) Phage_Escher_HK639_NC_016158, 14) Phage_Klebsi_phiKO2_NC_005857, 15) Phage_Pseudo_PS_1_NC_029066, 16) Phage_Entero_SfV_NC_003444. ICE: integrative and conjugative element.



Origin	Strain ID	Chromosome length	Genomic islands		Prophage sequence	
		kb	kb	%	kb	%
Broiler	1101124	5250	276	5.26	155	2.96
Pig	1101433	5351	315	5.89	246	4.60
Hospital	IT0132A	5482	299	5.45	265	4.84
	IT0132R1	5501	268	4.87	266	4.84
	IT0132R2	5456	256	4.70	242	4.43
	08EU827	5279	234	4.43	120	2.28
	AR_0135	5425	270	4.98	301	5.55
	BA33875	5522	270	4.89	224	4.06
	960186733	5499	305	5.56	286	5.21
	Kp202	5462	265	4.85	201	3.68
	Kpv_ST101_OXA-48	5454	252	4.63	223	4.09
	Kp_Goe_33208	5498	313	5.69	304	5.52
	Kp_Goe_71070	5497	311	5.67	302	5.50
	Kp_Goe_121641	5478	252	4.60	259	4.72

Supplementary Figure 6.3: Chromosome length and length and percentage of genomic islands and prophage sequences in *K. pneumoniae* ST101 of livestock and hospital origin based on long-read sequencing data. Heatmap shows phage sequences (intact, incomplete or questionable) detected in each genome. kb: kilobases.



Supplementary Figure 6.4: Minimum spanning tree of an international collection of *K. pneumoniae* ST101 of human, animal and environmental origin based on cgMLST profile data. Interactive phylogeny is available at https://microreact.org/project/j1fyqBYfCiKPZLa4qjotDe/ad1fbbb1.

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CHAPTER 7
Discussion and future perspectives

7.1 Main findings and general discussion

Since the introduction of antibiotics, antibiotic resistance has emerged worldwide. Common bacterial infections are becoming increasingly hard to treat with available antibiotics. Effective data-driven action to stop the emergence and spread of AMR is needed to protect the use of antimicrobials now and in the future. Gaining insights into the antibiotic use and the carriage of antibiotic-resistant bacteria in healthy individuals in the community, patients in healthcare settings and livestock, will allow for the implementation of control strategies that can lead to improved patient care as well as public and animal health.

AMR is a One Health issue since human and animal health are interconnected and bacteria are not restricted by geographic borders. The use of both genotypic and phenotypic techniques are needed (i) to gain insights in the baseline levels of antibiotic resistance in One Health sectors involved, (ii) to identify potential pathways that lead to the spread of antibiotic resistance and, eventually, (iii) to implement control measures. This thesis contributed to an estimation of the prevalence of antibiotic resistance and an improved understanding of the resistance mechanisms and spread of antibiotic resistance in healthy humans, patients and animals.

Chapter 3 showed remarkable differences on the quantity of antibiotic use and presence of antibiotic resistance at the level of the farm in two neighboring countries with different antibiotic policies. The percentage of samples positive for ESBL-producing *E. coli* was notably higher in Belgium compared to the Netherlands, however the percentage of ciprofloxacin-resistant *E. coli* was high in broilers in both countries. The percentage of antibiotic-resistant *E. coli* varied greatly between and within farms. These variations

could not be explained upfront by prior antibiotic use (TI with one year lag). Although, similar studies have shown association between antibiotic use and resistance (1,2), our study was not powered to demonstrate these links as longitudinal data on antibiotic use as well as duration, specific dosing and administration routes of antibiotic treatments were not available.

In **chapter 4**, we present a detailed analysis of the genetic characteristics and complex epidemiology of ESBL-producing and ciprofloxacin-resistant *E. coli* from these Belgian broiler and pig farms to identify the potential pathways that may lead to the presence and spread of antibiotic resistance. In addition, we examined genome-wide associations of genetic markers with phenotype. The study showed that livestock is a reservoir for a large variety of AMR genes, virulence genes and plasmids. The complex epidemiology with diverse combinations of ESBL genes, ST types, antibiotic resistance and virulence profiles makes it difficult to translate these findings to policy recommendations on human health. However, the pandemic multidrug-resistant clone ST131 commonly associated with human infections was not detected and $bla_{\mathrm{CTX\ M\text{-}15}}$ was rarely found. Instead, CTX-M-1 predominates in ESBL-producing *E. coli* in Belgian food-producing animals. The study showed that the IncI1-I(alpha) plasmid is a major plasmid type contributing to the spread of ESBLs in Belgian farms. PMQR genes were found in a remarkable low number of isolates and play a limited role in the occurrence of ciprofloxacin-resistant *E. coli* in Belgian farms. Instead, triple mutations in *gyrA* and *parC* were significantly associated with high-level fluoroquinolone resistance. Plasmids co-harboring PMQR and ESBL genes were also detected. We showed multiple genetically related bacteria in different animals of the same farm and of distinct farms suggesting a common reservoir or transmission of resistant bacteria. Our findings reveal a multifaceted landscape of transmission pathways where the spread of these resistances involves both dissemination of resistant clones and horizontal transmission of plasmids.

In **chapter 5**, we describe the presence and spread of colistin-resistant Enterobacterales in One Health sectors examined in Belgium and the Netherlands. Colistin-resistant bacteria were present in hospitalized patients, residents in long-term care facilities, children attending day care centres, broilers and pigs. Occurrences varied per sector and significant associations were detected between colistin use and resistance in pig farms. Colistin resistance was caused by a variety of chromosomal mutations and *mcr*-genes. *E. coli* harboring *mcr*-1 and *mcr-2* genes on IncX4 plasmids and *mcr-5* genes on IncFII (29) plasmids were shown to circulate within pig farms, while *mcr-9* and *mcr-10* were detected in human-associated *Enterobacter* isolates. Clonally related isolates were detected in different patients, healthy individuals and livestock animals of the same site suggesting local transmission. Highly related *K. pneumoniae* ST45 and *E. coli* ST10 were found between different sampling sites, suggesting that these clones have the potential to spread colistin resistance through the human population or suggesting acquisition from exposure to a common (food) source. Animal-to-human transmission or vice versa was not detected. Colistin resistance was detected in hypervirulent *K. pneumoniae* and various *E. coli* pathotypes from human and animal sectors, indicating that these commensal bacteria may have pathogenic potential. In general, resistance to critically important antibiotics (fluoroquinolones, extended-spectrum cephalosporins, aminoglycosides and carbapenems) was low, however, colistin-resistance was also detected in international high-risk clones, such as *E. coli* ST131 and ST1193 harboring a high number of virulence genes and showing resistance to critically important antibiotics.

Chapter 6 highlights the resistance and virulence properties of *K. pneumoniae* ST101 from various One Health sectors involved. *K. pneumoniae* ST101 is an emerging high-risk pathogen which is highly adapted to the hospital environment and is causing outbreaks in several countries. We detected two *K. pneumoniae* ST101 in two Belgian farms. Detection of these high-risk clones in animals remains scarce, hence the occurrence of these important nosocomial clones in animals may cause a reason for concern. However, phylogenetic analysis showed that the livestock-associated strains were genetically distant from clinical strains. The hospital-associated and non-clinical isolates

displayed clear differences in phenotype (MIC values) and genotype (resistance, virulence, plasmid content and prophage sequences). The absence of ICE*Kp* carrying the yersiniabactin siderophore in livestock-associated strains indicated a lower virulence capacity compared to hospital-associated strains. These mobile genetic elements seem to be an important source of variation between *K. pneumoniae* ST101 of different origin which otherwise shows to have a highly stable genome with few recombination events outside the prophage-containing regions. This data suggests lower antibiotic resistance and the lack of high-virulence features such as yersiniabactin in livestock-associated, community-associated, and food-associated *K. pneumoniae* ST101 compared to hospital-associated strains.

Our studies have several limitations. Our studies lack extensive epidemiological and clinical data, leaving gaps in our understanding of pathogen transmission. Furthermore, the lack of universal genomic cut-off values for determining whether different isolates were transmitted presents a significant challenge. Finding the most appropriate genetic distance threshold for identifying genetically related or unrelated isolates is an ongoing challenge. It is essential to continue working towards developing methodologies that will help us overcome these challenges and improve our understanding of transmission routes of strains. The absence of relevant metadata can be a significant obstacle when attempting to confirm potential dissemination among different sectors, such as hospitals and hospital wards. When confronted with these challenges, strict thresholds for clonal relatedness based on literature were applied. Moreover, it is important to note that sequences from public databases may not always provide the clinical and epidemiological contexts in which the isolates were obtained leading to limitations in interpretation. It is essential to prioritize comprehensive and thorough data collection to improve our understanding of the spread of bacterial isolates. Better integration of population structure data, additional data derived from analysis of whole genome sequencing and contextual meta-data is urgently needed. This level of integration can provide a more complete picture which would help to identify causal variants and their effect on complex traits such as antibiotic

resistance. In our studies, a selection bias was introduced by selectively choosing farms with a higher-than-average total antibiotic use compared to the national benchmark value in the countries. This bias may have skewed our results and impacted our overall findings. In addition, our research did not take the antibiotic use and resistance in veal calves into account, despite the earlier reported concerns around selection pressure and resistance in this sector (3,4). Despite these limitations, our research contributed to advancing our knowledge of AMR and its spread.

Although the environmental sector was not included in this work, this study corroborates the objectives of the European Union One Health Action Plan and the WHO global action plan against AMR (5,6). Through the enhancement of detection and epidemiological surveillance of resistant micro-organisms, we provide valuable information for guiding control strategies against AMR. As we continue to face the growing threat of AMR, a multidisciplinary approach is crucial to combat this global public health threat.

As we work towards developing better strategies to prevent the spread of diseases and AMR, the One Health approach involving both veterinary and human medicine, will remain an essential tool in the hands of scientists and medical professionals to monitor the emergence of pathogens in our communities, hospitals and livestock environments. More specifically, a combination of phenotypic and genotypic techniques is invaluable in identifying the sources of antibiotic resistance and the extent of transmission of resistant bacteria or their resistance genes.

WGS uncovers reservoirs of AMR genes in the animal and human domains and should be utilized to properly study the complex interactions between the different examined ‘One Health’ sectors. In this thesis, WGS is an essential tool for investigating the clonal spread at regional levels, provide insights into the AMR mechanisms, monitoring the emergence of resistance in high-risk clones and tracking the convergence of AMR and hypervirulence. The valuable insights into the circulation of plasmids and mobile genetic elements related to the spread of ESBL and *mcr* genes highlight the importance of understanding the transmission dynamics of resistant bacteria and their mobile elements in farms, long-term care facilities, hospitals and day care centres. By utilizing WGS,

specific measures and recommendations for future research can be provided to help mitigate the spread of antibiotic resistance.

7.2 Practical implications

7.2.1 Practical implications for livestock production: the need for prudent antibiotic use and infection prevention measures

High prevalences of antibiotic resistance in food-producing animals is a potential transmission route to humans via the food chain, which in turn can lead to increased resistance in the clinical settings. We investigated the antibiotic use, the occurrence of antibiotic resistance in broilers and pigs as well as the genetic background and epidemiology of these resistant bacteria. While essential to monitor, antibiotic use and resistance in cattle (3,4) was not included in our analysis.

Since the restriction of critically important antibiotics to human health care for veterinary use, such as carbapenems, third-and fourth-generation cephalosporins and fluoroquinolones, was implemented in the Netherlands in 2013 (7) and in Belgium in 2016 (8), the sales of these antibiotics in veterinary medicine fell sharply (9–11). Yet, the high occurrences of several important antibiotic resistance mechanisms in broiler and pig farms found in our study indicate that actions to reduce antibiotic resistance in this sector are further needed. In addition, fluoroquinolones and third-and fourth-generation cephalosporins were used mainly in broiler farms, while colistin was used in the majority of the studied pig farms. While no significant associations between the use of fluoroquinolones and third-and fourth-generation cephalosporins and the presence of fluoroquinolone-resistant and ESBL-producing *E. coli* could be found in this study, the colistin use in pig farms was associated with colistin resistance at farm level. Taken together, the study provides opportunities to create awareness among farmers, veterinarians, and stakeholders of the alarming rates of antibiotic resistance. To reduce

the antibiotic resistance in farms, the prudent use of antibiotics and effective biosecurity protocols at farm level are crucial. Through education and awareness, the results of the i-4-1-Health project could lay the foundation for a more sustainable and effective agricultural industry. Within this project, coaching of the farmers to create awareness on antibiotic resistance and improve antimicrobial stewardship measures could change the attitudes of farmers regarding antibiotic use, improve biosecurity levels and reduce the antibiotic use in these farms (12). By promoting best practices in biosecurity, farmers can reduce the need for antibiotic use and minimize the risk of resistant infections. This proactive approach to antibiotic resistance is an important step towards preserving the efficacy of these life-saving medications. With colistin being a critically important antimicrobial for human (13) and veterinary medicine (14) and with the emergence of plasmid-borne *mcr* genes, the use of colistin in animals was tightened by the European legislative framework. Since 2020, colistin can now only be used in veterinary medicine in Europe after physical examination, diagnosis, antimicrobial susceptibility testing and if no other antibiotics are effective. Also, the critically important third-and fourth-generation cephalosporins and fluoroquinolones are restricted by the same criteria (15). This continuous effort to reduce the use of critically important antibiotics in livestock has the potential to lower resistance rates in animals (16). Moreover, field-generated research of local relevance can help guide antimicrobial use choices of veterinarians based on scientific evidence rather than personal experience (17).

In addition to the reduced and appropriate antibiotic use, prevention of infections is crucial. Preventive measures can be a combination of tools such as improving biosecurity and hygiene, appropriate nutrition, and feed supplements such as probiotics, vaccination, healthy parent stock and regular veterinary visits for the monitoring of animal health and welfare (18,19). The continued circulation of closely related plasmids and isolates within the farms emphasizes the need for effective biosecurity measures, such as increased farm hygiene and quality of drinking water. However, other factors, such as antibiotics used earlier in the production chain and existing resistance in the farm environment, must also be considered when investigating the presence of antibiotic-resistant bacteria. This was

demonstrated by the surprisingly high prevalences of ciprofloxacin-resistant *E. coli* in (Belgian) broiler farms considering the limited use of fluoroquinolones in these farms since the restriction of critically important antibiotics for human medicine in livestock (7,8). Knowing the genetic background of fluoroquinolone resistance in *E. coli* from livestock, which is mainly caused by chromosomal mutations in *gyrA* and *parC* rather than plasmid-mediated resistance genes, the circulation of plasmids seems to play a limited role in the presence of fluoroquinolone resistance in *E. coli*. The causes of these high prevalences of fluoroquinolone resistance in the broiler production chain require further investigation.

Our findings shed light on the vital importance of delving into plasmid circulation in order to combat the spread of ESBL genes. These genes tend to spread via plasmids, particularly the IncI1-I(alpha) plasmid. Notably, we found plasmids in which ESBL and PMQR genes were co-localized. Furthermore, we observed that on farms, IncX4 plasmids that harbored *mcr-1.1* or *mcr-2.1* were consistently present over time. These findings highlight the pressing need for research exploring plasmid circulation both within and between farms. By employing long-read sequencing, we can gain a more comprehensive understanding of plasmid dynamics and how plasmids move throughout and between farms. With this new information, we can take the next steps towards mitigating the spread of antibiotic resistance genes.

In summary, this study shows that it is critical to curtail the unnecessary use of antibiotics across all levels of the livestock production chain. The use of whole genome sequencing is a valuable tool in identifying and understanding the spread of resistance, both within individual farms and between them. We should continue to prioritize research into this area to develop sustainable practices that promote both animal welfare and antimicrobial stewardship.

7.2.2 Practical implications for clinical settings and the community: the need for tracking of resistance and virulence, faster diagnostics and infection control

The need for last-resort antibiotics in healthcare settings is intensively reported. Although data on resistance in the community is scarce, evidence for carriage of MDR bacteria in the healthy population is increasing (20–23). Indeed, relatively high number of children attending day care centres were carriers of colistin-resistant Enterobacterales. The influence of the community on the introduction of crucial clones in livestock animals or in hospitals is still largely unknown. Raising WGS data from community-associated strains is essential to detect the presence of these pathogens and their resistance and virulence genes in the community. Data suggests that introduction of MDR and/or hypervirulent bacteria from the community is possible. Therefore, extended risk-factor-based screening of selected populations at admission to the hospital seems crucial (23).

Like patients and residents in healthcare settings, the investigated healthy children are prone to the risk of transmission within day care centres. Indeed, clusters of related bacteria were identified within hospitals, day care centres and LTCF suggesting possible dissemination of resistant bacteria between patients, children and elderly present in these facilities. Moreover, genetically related clones of *K. pneumoniae* ST45 and *E. coli* ST10 in different hospitals, day care centres and LTCF suggest that these clones have a high potential to spread colistin resistance through the human population or acquisition of these clones via exposure to a common (food) source.

Willems and colleagues recently reported the increased infection risk in patients colonized with resistant pathogens (24). To enable the timely response to the presence and spread of antibiotic-resistant and/or hypervirulent clones, standardization in laboratory automation and real-time data for the detection of virulence/resistance and detection of outbreaks in real-time (rather than retrospectively) are needed. While PFGE and MLST are typing methods used in conventional epidemiology, WGS is a high-resolution technique for more precise characterization and discrimination of bacterial isolates (25). The generation and analysis of big data from WGS is becoming cheaper which will enhance its implementation into clinical practice. Whilst still using the gold

standard of culturing, sequencing should be standardized and accredited allowing it to be used for faster diagnostics and real-time outbreak detection. For example, nanopore sequencing could become useful for genomic epidemiology in the clinical setting due to its flexibility in time (6-24h) and batch size (outbreaks with low or high number of isolates). Using the nanopore sequencing technology, antibiotic resistance genes and plasmids can be identified after 4h, after which artificial intelligence and machine learning for predictions of antibiotic resistance from genomic data can guide empirical antibiotic treatments to restrict the antibiotic spectrum and to overcome treatment failure by targeted therapy. The understanding of the genetic background of antibiotic resistance and virulence using genome-wide association studies will allow for the determination of phenotype-genotype correlations needed for such applications. Secondly, standardized protocols for data generation and analysis allows for precise and accurate genotyping and SNP analysis for multi-centre outbreaks (26). Evidently, for the interpretation of results, WGS data should be linked with phenotypic and epidemiological data. Data from phenotypic and genotypic tools could be integrated within tools for infection prevention and control measures. For example, the Infection Risk Scan (IRIS) uses objective measurements to guide AMR control strategies: patient comorbidities, (appropriate) use of indwelling medical devices, (appropriate) use of antimicrobial therapy, rectal carriage of antibiotic-resistant bacteria and their clonal relatedness, environmental contamination, hand hygiene performance, personal hygiene of healthcare workers and the presence of infection prevention preconditions. By combining data from the patient, the clinical setting and WGS, such tools can identify targets for the improvement of infection control and antimicrobial use (27). Alternatively, FTIR is a rapid typing tool based on comparison of infrared light absorption patterns of bacterial polysaccharides. FTIR proved to be a low-cost typing technique with a short turn-around time (3-4h) and could be an alternative method for the quick identification of nosocomial outbreaks, while WGS is performed for cluster confirmation and genomic characterization (28).

Besides detection of the presence and transmission of antibiotic resistance, a holistic approach employing infection prevention strategies and care bundles is needed to address

different causes of infection (such as contact transmission, lack of hand hygiene and environmental contamination, inappropriate use of antibiotics, inappropriate use of indwelling medical devices) and the stages of infection (prevention, detection and control) (27,29).

In summary, tracking and surveillance of virulence and resistance, faster diagnostics and better infection control and hygiene both in the healthcare setting and in the community are important AMR control strategies.

7.2.3 Practical implications in a One Health context: occasional spillover and the role of plasmids in the cross-ecological spread of resistance

AMR is one of the global health problems involving the transfer of bacteria and genes across various sectors (agriculture, livestock, humans, environment) and is therefore best investigated using a One Health approach. Determining the pathways of transmission of antibiotic resistance is critical to understand and manage AMR. Integrating the knowledge from different sectors facilitates the development of prevention and management strategies as well as a coordinated cross-sectoral timely response to reduce the risk of disease emergence and spread. Thus, AMR surveillance in involved One Health sectors provides a better understanding of the emergence and spread of antibiotic resistance in different settings and geographic areas.

The antibiotic selection pressure in humans and animals makes these the most important reservoirs for resistance evolution. As shown by our studies, livestock is a complex reservoir for a large variety of AMR genes, virulence genes and plasmids. International clones of *E. coli* (ESBL-producing or fluoroquinolone-resistant *E. coli* ST69, ST117, ST23, ST58, ST648, ST744 and colistin resistant *E. coli* ST10, ST38, ST405 and ST648) and *K. pneumoniae* (colistin-resistant ST15, ST101 and ST147) were identified in livestock. However, it is difficult to assess the risk that the presence of this variety of resistance genes and widespread clones in livestock poses to human health. Importantly,

the pandemic multidrug-resistant clone *E. coli* ST131 and $bla_{CTX\text{-}M\text{-}15}$ commonly associated with human infections were rarely detected in Belgian and Dutch pigs and broilers. Humans are the main reservoir of *E. coli* ST131 and the limited number of reports of ST131 *E. coli* in animals implies occasional spillover from the human sector. A study of Bonnet and colleagues showed that different ST131 lineages are linked to different hosts using AMR and virulence factor networks. Avian ST131 formed a separate cluster of invasive strains responsible for severe infections in avian species and rarely also in humans. The link between host, ST131 population structure and virulence factor content showed that virulence factors are the major factors of host colonization (30). Similarly, in our study on *K. pneumoniae* ST101, we detected distinct features of AMR and virulence among *K. pneumoniae* ST101 of human, animal and environmental origin. Comparing high-risk lineages of human and non-human origin could identify potential risks for human health care. The ICE*Kp* element harboring the yersiniabactin siderophore was identified as a key virulence factor in hospital-associated isolates. On the other hand, isolates from animals, animal products and the healthy community showed low virulence capacity (no yersiniabactin and colibactin) and no carbapenemase production. Moreover, convergence of virulence and resistance in *K. pneumoniae* ST101 was seen solely in hospital-associated strains. The lower resistance and virulence levels in non-hospital associated *K. pneumoniae* ST101 imply a lower public health risk compared to hospital-associated strains. Together, these findings might indicate a low risk of cross-transmission in long-term carriage of animal isolates in humans and vice versa. Indeed, inter-host transmission of colistin-resistant *E. coli* between humans and animals was also not detected in our study. The presence of resistant bacteria seems to reflect the antibiotic selection pressure in each sector rather than transmission of resistant isolates between One Health domains examined.

On the other hand, plasmids can act as cross-ecological sources of resistance weakening the boundaries of resistance. Many intestinal bacteria are carriers of mobile genetic elements (plasmids, integrative conjugative elements, insertion sequences, transposons)

that can facilitate the acquisition of genes and their transfer to pathogens. The transfer of mobile antibiotic resistance genes across micro-organisms, across hosts and across sectors allows the spread of these genes in various habitats. Understanding the prevalence of these mobile genetic elements (e.g. plasmids) in different niches can guide targeted strategies against the spread of antibiotic resistance genes. For example, ESBL genes on plasmids, extensively present in Belgian livestock, could be disseminated across niches. Near-identical plasmid backbones carrying diverse accessory functions such as resistance genes are shared across species and niches suggesting relevant inter-niche transfer of antibiotic resistance genes via plasmids (31). In a recent study by Lin and colleagues, transmission proportions of antibiotic resistance genes between biomes with dissimilar characteristics were very low indicating ecological boundaries. However, in the same study *mcr-9* was found in food, human gut, human skin, fermentation and bioreactors suggesting the potential transmission of this antibiotic resistance gene (32). The wider context of antibiotic resistance genes emergence and dissemination via plasmid sharing requires further investigation using a One Health approach.

7.3 Future outlook

Several knowledge gaps remain concerning antibiotic use, resistance, and the spread of multidrug resistant clones. Research on (i) the resistance in the environment, (ii) the role of antibiotic use and presence of resistance within the entire livestock production chain and the farm environment, (iii) identification of factors important for the colonization of specific hosts and disease-causing properties of bacteria, (iv) research on plasmid transmission and reduction of antibiotic resistance gene transfer, (v) the use of AI to combat AMR, and (vi) identification of successful strategies for the prevention of resistance emergence and spread in all involved One Health sectors is needed.

7.3.1 Resistance in the environment

The inclusion of the environmental sector and antimicrobial residues measurements in One Health research remains limited. Harmonized and standardized methods to understand antibiotic resistance and the connections between the human, animal and environmental microbiota are needed to perform risk assessments and inform control strategies. Antibiotics and resistant bacteria reach the environment via human and animal excretions (urine and feces), through aquaculture or plant production and through waste streams and farm effluents. Exposure via the environment can occur through drinking water, surface water, raw vegetables, and wildlife (33,34). Future studies should not only detect resistance genes, but also their potential for horizontal gene transfer, their compatibility with potential human and animal pathogens and the presence of selective pressure that favors mobilization, because all will have implications for managing risks (35). For example, wastewater might represent an aggregation of antibiotics, disinfectants, metals and nutrients from households, hospitals and factories with a large diversity in micro-organisms making it a potential hotspot for antibiotic resistance gene exchange and possibly an important intervention site in the environmental sector (32). Therefore, sampling of (waste)water or wildlife could be a good starting point as these ecosystems provide a view on the interface between different sectors. Environmental emission of human-and animal-associated bacteria through wastewater streams could provide an opportunity to investigate the abundance and pattern of resistance in a region. For example, raw sewage contains pooled fecal bacteria from a large population which could be monitored complementary to the surveillance of resistance in clinical and agricultural settings via phenotypic analysis of isolates or analysis of antibiotic resistance via shotgun metagenomics (34). Standardized methods could make comparisons across time, regions and sectors possible.

7.3.2 The role of antibiotic use and resistance within the livestock production chain

The use of antibiotics and resistance in the entire livestock production chain should be investigated further. The causes of the observed high prevalences of fluoroquinolone

resistance in *E. coli* from broilers are currently unknown. Further research could focus on the dynamics of resistance spread within the broiler production chain: from breeders to hatcheries and broiler farms. The role of the farm environment on resistance spread should be mapped. Residues of fluoroquinolones in feathers of chickens and broiler breeders could be investigated. Eventually, the risk of the spread of fluoroquinolone resistance from commensal bacteria to zoonotic pathogens, such as *Campylobacter* spp. and *Salmonella* spp. and the risk for human health could be investigated.

7.3.3 Research on plasmid transmission and reduction of antibiotic resistance gene transfer

The spread of antibiotic resistance genes via mobile genetic elements (plasmids) should be further investigated. Focus on the dynamics of mobile genetic elements should be included in genomic surveillance. Long-read sequencing data is needed, especially when investigating transmission and outbreaks. Moreover, understanding the drivers for successful plasmids and their bacterial hosts (e.g. *E. coli* ST131 with the IncF-family plasmids encoding $bla_{\text{CTX-M}}$ ESBL genes), may help predict antibiotic resistance emergence and spread (36). Insights in the within-host emergence and evolution of plasmids in hospitalized or healthy humans and animals and the spread of these plasmids between sectors can help identify targets to interfere with this emergence and spread. One such technique is plasmid curing, which involves the removal of the plasmids carrying resistance genes. For example, the adaptive immune system of bacteria, CRISPR-Cas, has been developed into a gene-editing tool for the prevention and control of antibiotic spread. By designing guide-RNA guiding the CRISPR-Cas system to target drug-resistant genes, the CRISPR-Cas system can effectively remove these genes from the resistant bacteria. Additionally, the presence of CRISPR systems in bacteria may interfere with the bacteria acquiring drug-resistant plasmids and maintain sensitivity (37). Although this research is still in its infancy, these new techniques hold great promise especially for resistance that spreads via plasmids such as beta-lactamase genes.

7.3.4 Identification of factors for colonization of specific hosts and disease-causing traits of bacteria

Research on the bacterial characteristics important in the colonization of specific hosts and their disease-causing properties is needed. Research on the features of host interactions and adaptation of Gram-negative bacteria might identify whether or not barriers for the direct transmission of these bacteria across sectors exist. For example, livestock-associated methicillin-resistant *Staphylococcus aureus* (MRSA) evolved independently from common hospital-associated and community-associated MRSA each with host-specific features such as separate *S. aureus* clonal complexes and associated *spa* types (38). However, strains of community-acquired MRSA can share genes with both livestock-associated and hospital-associated MRSA and some clones are present in different sectors, blurring the dissimilarity between strains. MRSA is now carried by humans and animals and can be transmitted between these different hosts (39). For this purpose, WGS data of livestock-associated isolates and isolates from the healthy community need to be collected. Together, this might provide further insights into the potential risks involved in the colonization by MDR and/or virulent Gram-negative isolates in animals and humans. Moreover, this research could also identify potential targets for vaccine development.

7.3.5 The use of artificial intelligence to overcome antimicrobial resistance challenges

AI can be used to control AMR by analyzing genomic data to identify new resistance mechanisms, to predict drug resistance patterns based on sequence and structural information, to identify potential drug targets by analyzing protein-protein interactions, to predict effective antibiotic (combination) therapies and to design new antibiotics and vaccines (40). Using algorithms to identify hidden patterns and to make predictions has a tremendous potential in diagnostics, personalized medicine, in drug development and vaccine design. Although AI has the potential to revolutionize our response to AMR,

several risk and ethical issues must be considered. The success of AI depends on the quality of the data to train AI-based predictive models. Nowadays, public databases are biased towards the sequencing of hospital strains, whereas data on sensitive strains will also be crucial to train models. Moreover, the black box of algorithms might make it impossible to understand the determination of output, transparency is required before an algorithm can be used for patient care. Also, the privacy and security of patient data needs to be assured. To address these concerns, robust data governance frameworks should be implemented (41).

7.3.6 Identification of successful strategies for the prevention of resistance emergence and spread in One Health sectors

We can learn from successful initiatives and collaborations at the local, national, and international levels that have addressed antibiotic resistance from a One Health perspective — such as joint efforts in human medicine, veterinary medicine, agriculture, and environmental sciences. Identifying best practices and guidelines that can be adopted or adapted to different settings to prevent and control the development and spread of antibiotic-resistant bacteria are crucial. It is likely that reinforcing antimicrobial stewardship programs and the development of novel antibiotics will not be sufficient to combat the increasing AMR. Although new antibiotics against top priority organisms (e.g. carbapenem-resistant Enterobacteriaceae) are urgently needed, the pipeline of new antibiotics with activity against MDR Gram-negative species is limited. Approaches that will result in reduced antibiotic selective pressure, and hence antibiotic resistance, are: antibody-based therapies, immune stimulation, probiotics, phage therapies, vaccines (42,43), and the use of CRISPR-Cas and RNA interference to remove antibiotic resistance. Other approaches to reduce the selection pressure of antibiotics in human and veterinary medicine are antimicrobial stewardship methods such as appropriate empirical therapy guided by local guidelines based on local epidemiology, optimal dosing, appropriate treatment duration and regular review of the antimicrobial therapy (44). In animals, metaphylactic group treatments can be reduced by implementing alternatives

such as vaccinations, improved biosecurity and improved herd health management (45). Combination therapies of existing drugs (e.g. colistin and tigecycline with or without meropenem or double β-lactam therapy) might suppress resistance (46). Also cycling (either at institutional/guideline level by changing antibiotics each month for a specific indication or at prescription level by changing antibiotics each day/week/month) and sequencing (sequentially changing antibiotics every few days) of antibiotics are proposed as approaches to increase the heterogeneity of antimicrobial use and potentially suppress AMR, although more clinical data is needed (46). Finally, rapid diagnostics are needed for fast antibiotic de-escalation to decrease the spectrum of empirical antimicrobial therapy and to reduce the impact on the patients' microbiome and the emergence of AMR (44).

In summary, our regional One Health study could provide a framework for future, more elaborate One Health interventions. To mitigate AMR, a transitioning from a fragmented response to a comprehensive evidence-based public health response is needed. This can be achieved by political commitment, access to fast diagnosis in a lab network, prudent and appropriate antibiotic use, prevention of infections by infection prevention and control as well as vaccinations, and worldwide surveillance and research within a One Health framework.

To close the current gap in knowledge, future research should focus on the detection of a broad spectrum of pathogens in the community, in the hospital, the environment and in livestock enclosures by employing a One-Health approach within a well-structured prospective study with representative sampling (geographic and temporal) in different settings and countries. We should aim to build joint strategic programming and global coordination of research and innovation in the One Health sectors involved. Building transnational systems to support collaborations between European and international initiatives will establish effective information exchange between multiple disciplines (clinicians, veterinarians, pharmacists, food producers, pharmaceutical industry, policy makers and researchers). This will support evidence-based policy making in the One

Health domain. The battle against AMR across disciplines allows working towards positive outcomes for humans, animals and ecosystems and promote long-term health in a holistic manner. Moreover, the One Health approach used in AMR surveillance can be used as a building block for other areas with interactions between humans, animals and the environments such as healthy food, clean energy and air and actions against climate change.

7.4 Conclusion

In conclusion, this work provides an in-depth overview of the multifaceted landscape of different antibiotic resistance mechanisms in Belgium and the Netherlands. We also provide a detailed analysis of the genetic characteristics of human-associated and animal-associated resistant bacteria. We estimated the attribution of various involved One Health sectors to the molecular epidemiology of antibiotic resistance and the extend of transmission of resistant bacteria and their resistance genes. This work can serve as an example for multisectoral national and regional plans to collect data on antimicrobial use and resistance to guide policy and reduce resistance rates. Future research and collaborations should focus on the detection of resistant clones and plasmids in the community, hospitals, livestock and the environment employing a One Health approach within a well-structured study with representative geographic and temporal sampling.

7.5 References

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Acknowledgements- Dankwoord

Dit werk is het resultaat van een fantastische samenwerking. De expertise die werd gedeeld heeft deze thesis gevormd, én heeft ook mezelf doen ontplooien als wetenschapper en als mens. Daarom wil ik graag iedereen bedanken die klaarstond de voorbije zes jaar.

Allereerst wil ik mijn promotor **Prof. Dr. Herman Goossens** bedanken voor de kansen die ik kreeg. Bedankt voor uw 'no-nonsense' stijl, het enthousiasme, het vertrouwen en de begeleiding tijdens deze onvergetelijke jaren.
Next, I would like to thank **Prof. Dr. Surbhi Malhotra-Kumar** for the guidance and support. Bedankt **Prof. Dr. Ir. Sandra Van Puyvelde** voor uw goede raad en positivisme. Thank you, **Prof. Dr. Youri Glupczynski** for your scientific contribution and advice.

I want to acknowledge the members of my **doctoral committee**; Prof. Dr. Barbara Michiels, Prof. Dr. Geert Vandeweyer, Prof. Dr. Timothy Walsh and Prof. Dr. Boudewijn Catry for the valuable comments and investing your time and efforts in reviewing this thesis.

Bedankt aan alle **collega's van het i-4-1-Health project** voor de wetenschappelijke input, samenwerking en leerrijke discussies. Bedankt aan de medewerkers van het labo microbiologie van het Universitair Ziekenhuis Antwerpen voor de aangename samenwerking, met name **Stefanie** en **Natascha** voor jullie enthousiasme.
I was very lucky to work with a great team of (former) colleagues at the Lab of Medical Microbiology. To all the current and former PhD office members **An, Pien, Sarah, Thomas, Matilda, Michael, Mathias, Minh, Juan Pablo, Jelle, Sam, Lara, Enya** and **Glingston** thank you for the fantastic office vibes, parties, dinners, festivals, etc. Thanks to **Joyce, Amber, Liesbet, Katherine, Leen, Tuba, Tomi, Rohit, Biljana, Qiang, Daniele, Melanie, Esther, Ferran, Greet, Annelies, Anke, Jimmy, Ines, Ann, Safia** and **Leandra** for your kindness. Bedankt **Jasmine** om me onder je vleugels te nemen bij de start van dit avontuur. **Liesbeth, Anouk, Sabine, Gert, Stalin, Kitty, Mandy K., Mandy A., Sofie, Gill** en **Kevin,** geen enkele vraag was te veel voor jullie. Bedankt voor de enorme hulp tijdens alle experimenten en practica, maar ook voor de leuke lunchpauzes en uitstapjes. Ik koester alle leuke momenten.
A special thanks goes to **Britto and Christine** for the encouragements and always being there to help.

Tenslotte wil ik mijn familie bedanken om altijd voor me klaar te staan. **Mijn ouders, grootouders en zus** voor hun onvoorwaardelijke liefde, steun en bemoedigende woorden en **Mathieu** om alles vanop de eerste rij mee te maken en mijn leven mooier te maken.

Hartelijk dank!

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